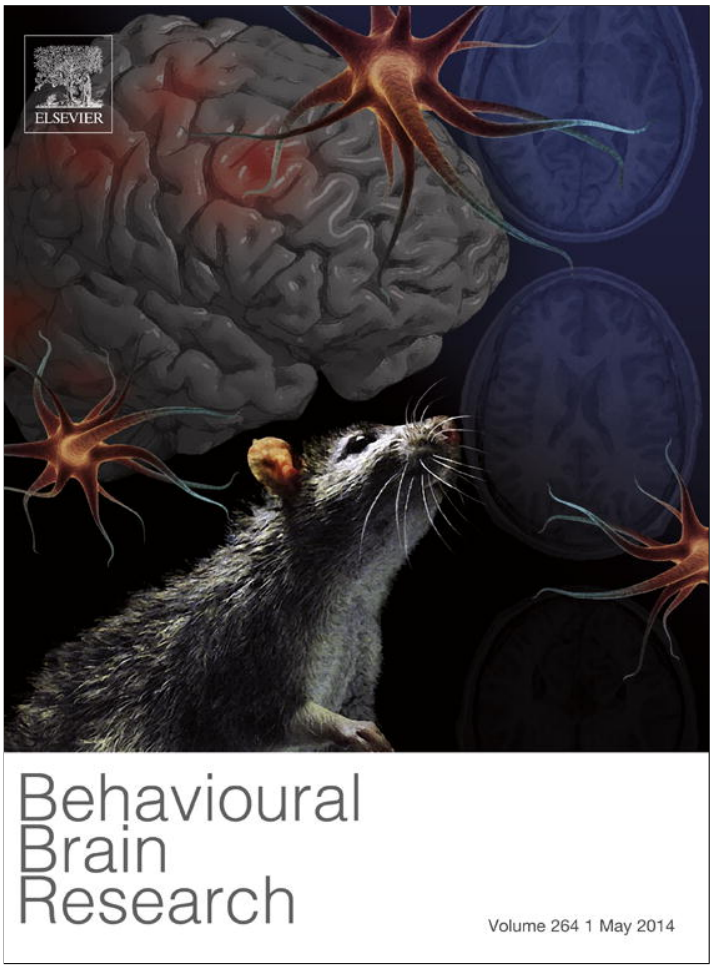

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Research report

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.
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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

on a stereotaxic frame (David Kopf Instruments, Tujunga, CA). The coordinates were calculated from the intersection point of the three lines seen in the head exoskeleton. The coordinates were as follows (in mm): −1 antero-posterior, ±0 lateral and −3 ventral [9] to target either of the accessory lobe. We penetrated about 0.3 mm deep to target the accessory lobe in the deutocerebrum of the crayfish brain. We focused on the accessory lobe because a previous study [5] implicated the accessory lobe as a neuroanatomical reward substrate in the brain of crayfish. A 26.5 gauge needle was used to gently create an incision, and a fused silica cannula was fixed in place and reinforced with superglue as bonding material. Following successful surgery, animals were allowed to recover overnight in their holding containers.

2.4. Behavioral experiment 2

All animals consistently showed exploratory behavioral patterns in the aquarium with gravel background during the two days of testing. For this reason, we used the 9 animals in Group 1 of experiment 1. Three days after successful surgery, animals received injections of 0.2, 0.6, and 1 mg/kg doses of morphine into the brain. This experiment was designed to characterize changes in exploratory behavior following infusion of different doses of morphine. We connected 0.5 m of deactivated, fine-bore, fused silica needle material (Agilent, i.d. = 100 μm) to the crayfish brain with Tygon microbore tubing (Fisher Scientific, i.d. = 250 μm). The injection cannula was indirectly connected to a microdialysis pump (CMA/102) via a microdialysis swivel mounted above the experimental arena. To insure that morphine or saline was delivered immediately when the pump was turned on, we primed the cannula to fill its void volume. Animals were divided into two groups. The experimental group comprised of crayfish [$n = 27$; $n = 9$ per each dose of morphine (0.2, 0.6 and 1.0 mg/kg)] used in the experimental group. A vehicle-injected (125 mM saline) group for each dose of drug (0.2; $n = 9$, 0.6; $n = 9$ and 1.0; $n = 9$) served as control. We placed crayfish of different dose–response groups in a white Plexiglas aquarium (2.50 m × 1.00 m × 0.85 mm) with a gravel substrate, left undisturbed for a habituation phase of 2 h, and videotaped for 40 min. A range of doses for morphine (0.2, 0.6 and 1 mg/kg of body weight) were administered directly into the brain for 5 days, while a vehicle-injected (125 mM saline) group served as control. The protocol was such that each crayfish received two morphine sessions (2 morphine injections) per day, separated by 9 h for 5 days (see Fig. 1). Crayfish in the control group received 2 vehicle injections per day. The total injection volume for experiment 2 was adjusted to not exceed 1/50 of the estimated hemolymph volume for each animal [6].

2.5. Behavioral experiment 3

Animals in the experimental groups received saline injections instead of morphine for another 5 days. Changes in exploratory behavioral patterns and observed atypical behaviors characteristics of withdrawal signs were characterized following 5 days prolonged elimination of morphine injections in crayfish.

2.6. Removal of brain and reconstruction of the position of the cannulae

At the end of the experiments, individuals were injected with cresyl violet dye and decapitated to reconstruct the position of the implanted cannulae. Crayfish were deeply anaesthetized in crushed ice for 10 min. The implanted cannulae were gently removed. The brains were dissected by mounting the crayfish on a sylgard dish dorsally, such that the ventral side was up. Animals were pinned to the dish with very thin pins of 0.1 mm diameter and 10 mm long. All appendages that project from the head region were carefully removed with fine forceps. The cuticle plate was removed to expose the brain, and the lateral brain connections were carefully dissected including the eyestalks. The nerve cord was cut deep anteriorly so that a significant part of the central nervous system was exposed. The fresh wet brain was gently removed, stored in 4% paraformaldehyde for 24 h, and then placed in 20% sucrose solution before sectioning. Brain sections were mounted on slides viewed and identified on a microscope. Successful location of the tip of cannulae at the accessory lobe was confirmed via cresyl violet dye staining. We used animals with clear cresyl violet dye stained brains indicating the location of the tip of the cannullae on the accessory lobe for statistical analyses.

2.7. Behavioral analysis

We analyzed different aspects of exploratory patterns in morphine-treated animals using a custom-designed video tracking system. Our tracking system processes a single video frames at 320ms from a camera (Sony DCR-VX1000) that we mounted

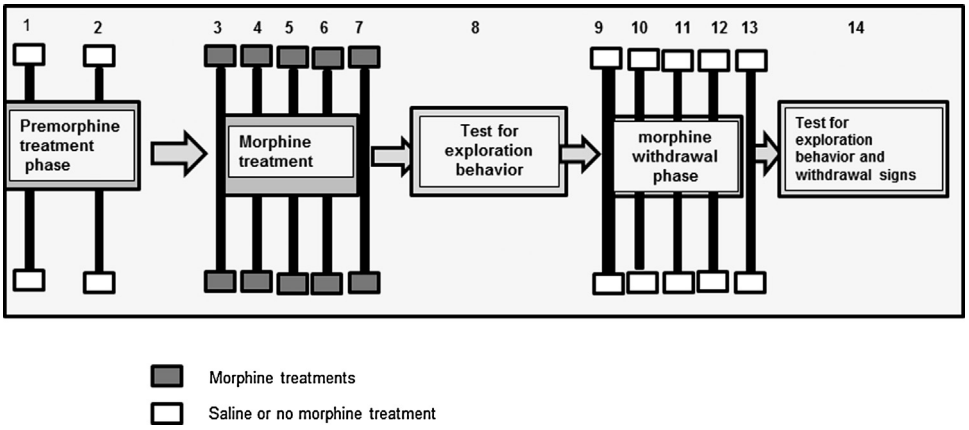

Fig. 1. Schematic representation of the experimental design used in the present study. Testing for initial exploration of the experimental aquaria was carried out in days 1 and 2. The chronic morphine treatment consisted of 5 days (3–7) of morphine injections. Each crayfish received two morphine sessions (2 morphine injections) per day separated by 9 h for 5 days. On day 8, the effect of morphine on exploratory behavior was tested. Crayfish were placed inside the aquarium and allowed to move freely in a drug free state to test for the expression of exploratory behaviors. Following conditioning and the initial test, each crayfish was given 2 saline injections per day separated by 9 h for 5 days (days 9–13). Crayfish did not receive morphine during this period. Thereafter, crayfish were tested for exploratory motor patterns and evidence for withdrawal signs (day 14).

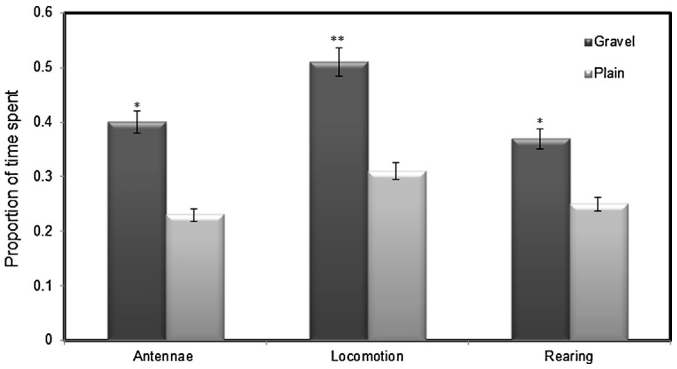

Fig. 2. Effects of different environments (gravel and plain floors) on crayfish exploratory behaviors. Results represent mean percentage of time spent displaying each exploratory motor pattern (*$P < 0.05$, and **$P < 0.01$). Data were collected for 40 minutes.

above the tank to provide a general profile view of the spatial activities of the animals. We streamed the videos to a computer. The spatial activities of crayfish were analyzed using the Any-maze (Stoelting Co. USA), that uses as input the automatically digitized time-series of the animal's location for the visualization, analysis, capturing, tracking and quantification of each specific motion pattern. Since active exploratory behavior in crayfish is indicated by patterns for locomotion, rearing and antenna movements [27–29], we analyzed the movement patterns for the different parameters (locomotion, rearing and antenna) for each animal during exposure to the experimental aquaria. Changes in exploratory behavioral patterns following injection of morphine and saline were identified. All forms of walking/translatory movements were considered as locomotion. Scourging of the antennae back and forth was considered as antennae movements. Crayfish was considered to be rearing when the animal stands on rear pair of walking legs, stereotypically using the posterior region of the abdomen for support. The three pairs of walking legs moved in a pattern comparable to that used for forward locomotion. Rearing occurred most of the time in the corners of the aquarium.

3. Result

In experiment 1, most animals consistently showed exploratory behavioral patterns inside the aquarium with a gravel background during the 2 days of testing. One-way ANOVA revealed significant effects of exposure to a novel environment (gravel) with an increase in three exploratory behaviors -locomotion, rearing and antennae movements ($F[2,26] = 121.92$; $P < 0.05$), with a robust increase in locomotion (Fig. 2). Locomotion, rearing and antennae movements increased ($F[2,26] = 113.23$; $P < 0.05$) following the exposure of crayfish to the plain environment.

In experiment 2, morphine treatments increased exploratory behaviors in crayfish. One-way ANOVA revealed significant effects of morphine treatments for antenna movements ($F[4,41] = 134$; $P < 0.05$), locomotion ($F[4,44] = 142$; $P < 0.05$), and rearing($F[4,44] = 128$; $P < 0.05$). Crayfish consistently displayed active exploratory behaviors in a dose-dependent manner during morphine treatments in the gravel arena (Fig. 3). Compared with saline, and novel environment, post-hoc analysis revealed differences for 0.2 mg/kg (*$P < 0.01$), 0.6 mg/kg (**$P < 0.001$), and 1.0 mg/kg (***$P < 0.0001$) for antennae movements, locomotion and rearing behaviors.

In experiment 3, a morphine-induced increase in exploratory behaviors was rendered in crayfish following repeated saline injections for 5 days, in the previously morphine-paired gravel background arena (Fig. 4). One-way ANOVA revealed significant

Table 1
Behaviors displayed by crayfish following elimination of morphine injections. A markedly different set of atypical behaviors of crayfish that indicate withdrawal-like signs were observed when different doses of morphine injections were terminated, and saline was injected into the head ganglia. Data are mean (±SE) from nine animals.

Behavior	0–10 min	11–20 min	21–30 min	31–40 min
Squirming	5 ± 0.27	4 ± 0.27	0.4 ± 0.12	0 ± 0
Twisting posture	6 ± 0.37	3 ± 0.20	2 ± 0.13	1 ± 0.04
Static posture	2 ± 0.12	4 ± 0.27	1 ± 0.02	2 ± 0.13
Antennae clinging	3 ± 0.24	3 ± 0.23	1 ± -0.03	2 ± 0.16
Inactivity	4 ± 0.29	1 ± 0.12	2 ± 0.18	3 ± 0.29

effects of termination of morphine with a reduction in antennae movements ($F[2,26] = 183.142$; $P < 0.05$), locomotion ($F[2,26] = 127.34$; $P < 0.05$),and rearing ($F[2,26] = 192.67$; $P < 0.05$). In general, crayfish displayed decreased exploratory patterns during withdrawal from morphine, indicating that paring with saline instead of the drug provides measures of exploratory or incentive properties of morphine in crayfish. A markedly different set of atypical behaviors of crayfish that indicate withdrawal-like states were observed when morphine injection was terminated, and saline was injected into the brain (Table 1). Crayfish displayed static postures, during which the animals do not move forward or backward anywhere in the aquarium. The three pairs of walking legs are elevated forward, and the animal stands on hindmost pair of walking legs, resembling the movement pattern similar to that displayed during rearing behavior, only that the animal does not move and remains motionless in the corners of the aquarium. Most of the postural rigidity took place with a slower onset and more rapid disappearance.

A set of atypical behaviors that reflect withdrawal signs in crayfish were displayed following elimination of morphine injections (Table 1). Squirming occurred when the animal displayed a stereotypic unconditioned jerky movement usually accompanied by a decrease in locomotor activity. A twisting posture was characterized by an overall flexion of the abdomen, walking legs, tail, claws that pointed downward and out in front of the thorax, such that the animal is twisted to the right or left. During a static postural display, the animal is localized in a rearing position, the three pairs of walking legs do not move in forward or backward direction and the animal remains static in the corners of the aquarium with occasional movement of the antennae, mouthparts and antennules. Antennae clinging appear to represent an atypical behavior that is contrary to antennae grooming that occurred during morphine injections. In antennae clinging, crayfish used the third maxilleped to grasp the second antennae near their base. The base of the antenna does not move upward and back toward the eyestalk as seen during antennae grooming. The entire length of the antenna was clinged to the maxilleped. Inactivity occurred when most appendages are not active, but mouthparts are occasionally moving in a rhythmical fashion. The observed behavioral occurrences were mostly pronounced during the first 10-min observational period. We did not observe the set of atypical behaviors outside the period following morphine injection. The set of atypical behaviors were displayed following saline injection immediately preceding stage morphine withdrawal phase.

4. Discussion

Three major findings arise from the experiments in this study. First, novel stimuli appear to directly augment exploratory behaviors in crayfish. Second, injections of morphine into the brain of crayfish enhance robust arousal resulting in an increase in locomotion and exploration of the environment. Third, there was a general decrease in exploratory behaviors when morphine injection was terminated, and saline was injected into the brain of crayfish. In

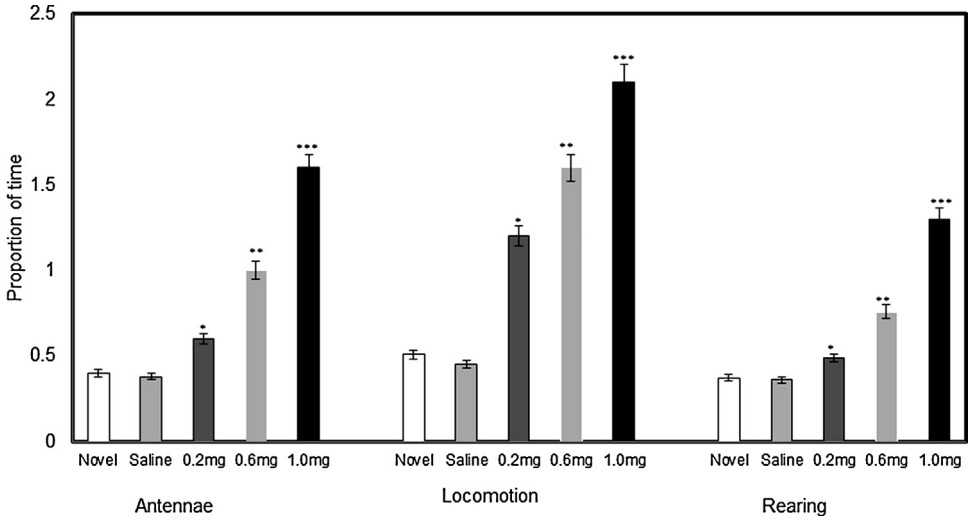

Fig. 3. Effects of different doses of brain injections of morphine on crayfish exploratory behaviors. Results are presented as mean percentage of time spent displaying each exploratory behavioral category. Post-hoc analysis revealed differences for 0.2 mg/kg (*$P < 0.01$), 0.6 mg/kg (**$P < 0.001$), and 1.0 mg/kg (***$P < 0.0001$) for antennae movements, locomotion and rearing behaviors of morphine injections compared to behavior in the novel environment following saline injections ($P < 0.05$).

addition, elimination of morphine resulted in a series of atypical behavioral changes that indicate withdrawal signs in crayfish.

Regardless of dose, morphine increased locomotion in crayfish when paired with a novel environment. A morphine-induced increase in locomotion were significantly established at 0.2 mg, 0.6 mg and 1.0 mg doses, and was rendered in crayfish following repeated saline injections for 5 days in the previously morphine-paired gravel background arena. This finding indicates that paring with saline without the drug provides measures of the incentive or motivational properties of morphine in crayfish. Therefore, the reduction in exploratory behavior in the absence of response-contingent drug availability probably led to the observed decline in the significance of the drug-paired stimuli in crayfish. The fact that locomotion was still elevated in the withdrawal animals, and even higher than the control group suggest that priming of morphine injection can re-instate the initially established increase locomotion at all doses, indicating that the observed morphine-induced locomotion is unrelenting, and that with time, it can be restored by morphine following extinction in an invertebrate model just like in mammals. Exploration of the environment as demonstrated by patterns for locomotion, rearing and antenna movements increased in crayfish that were tested in the gravel environment, compared to crayfish that we tested in the plain background environment. Our findings indicate that novel stimuli can directly activate brain networks to promote exploratory behaviors that are typically used to search for natural rewards. It is possible that these appetitive motivational conditions may be engaged by a psychostimulant such as amphetamine [9] and morphine–a depressant to enhance exploration of the novel gravel experimental arena. Exploratory behaviors that are usually used by crayfish to seek natural rewards include locomotion, rearing, and antenna movements [9,27]. Administration of different doses of morphine may seemed to have aroused the appetitive conditions related to the effect of morphine within the crayfish head ganglia to enhance

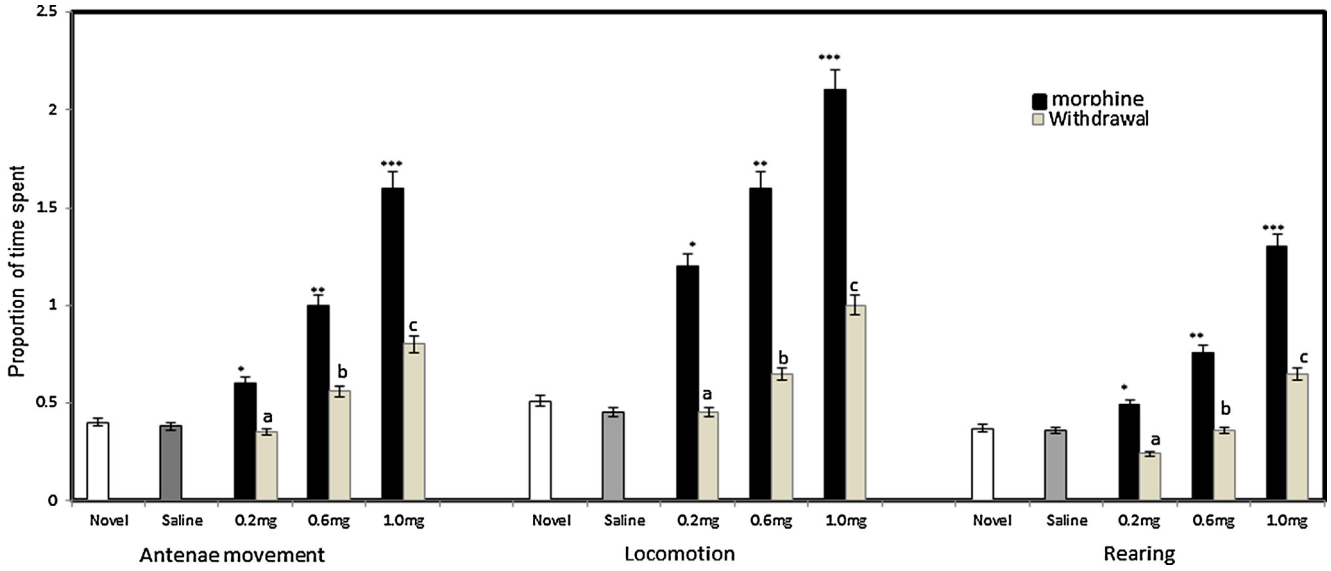

Fig. 4. Effects of repeated saline injections for 5 days following chronic morphine injections on crayfish exploratory behaviors. Results are presented as mean percentage of time. Post-hoc analysis revealed differences for 0.2 mg/kg ($^{a}P < 0.01$), 0.6 mg/kg ($^{b}P < 0.001$), and 1.0 mg/kg ($^{c}P < 0.0001$) for antennae movements, locomotion and rearing behaviors. Morphine injections increased antennae movements, locomotion and rearing behaviors for 0.2 mg/kg (*$P < 0.01$), 0.6 mg/kg (**$P < 0.001$), and 1.0 mg/kg (***$P < 0.0001$) doses.

the observed increase in rearing, locomotion and antennae movements. In this context, we argue that activation of the major substrates of exploration by morphine can be more powerful than activation triggered by natural contexts.

An extensive range of behaviors is known to be directly linked with a drug-induced effect, and these include exploration and approach. These behaviors tend to indicate an appetitive motivational state shown when animals seek natural rewards such as food, water, sexual stimuli, and a safe environment [1,2,9]. In crayfish, an active exploratory behavior is shown by patterns for locomotion and antenna movements, facilitated by tactile and olfactory cues [27,28], identified primarily via antennae and antennules, and sent to the olfactory lobe of the brain [29]. The olfactory lobe of crayfish is modulated by serotonin and dopamine transmission [29], and represents a site for the rewarding action of cocaine [5], and conceivably other drugs as well. The presence of drug sensitive circuits in the head ganglia of crayfish suggest the presence of an evolutionarily conserved adaptation that facilitates exploratory and approach behavioral patterns via increased incentive salience of environmental stimuli or increase exploratory motor patterns [9]. These unique behavioral patterns were as well suppressed through a decreased incentive salience of environmental stimuli resulting in the observed withdrawal signs in crayfish.

Interpretations of crayfish-morphine withdrawal signs may have evolutionary explanations. The accessory lobe is a structural component thought to contribute to the neural circuit associated with the rewarding value of a particular stimulus or exploration of the spatially complex environment that crayfish live [5]. Although the neurochemistries and neuroanatomies of exploration and approach brain circuits in crayfish are yet to be fully investigated in the existing animal models of drug addiction, all neurochemical processes of drug-induced reward process are generally hinged on the dopaminergic system [1,30]. Dopamine functions have been associated with regulating motivational affective responses [31], reward related stimuli [32], learning process [33], and in the Pavlovian mechanism of cues attractiveness. Dopamine is a neurochemical signal that is conserved and shared across all mammals and non-mammalian species [34,35]. In addition, it is a monoaminergic system present in the crayfish's head ganglion. It is suggested to facilitate exploration and approach motor patterns in crayfish [36], and in the interaction with a wide range of conditions required for survival [9]. Dopamine is thought to modulate the survival circuits [36], by stimulating appropriate underlying neural networks [1,9]. Since the dopamine system modulates neural functions at several levels to bring about coordinated exploratory responses to environmental challenges [33], the dopamine system may as well alter the activity of neural decision making centers. Such effect will promote the occurrence of an adaptive behavior such as exploration, and the acquired affective incentive value for cues associated with natural and drug rewards [37]. Because, the dopaminergic pathways serve as a key modulator of exploratory patterns via an increase in cAMP induced by dopamine agonist [38], it is possible that elimination of drug intake affected the display of exploratory patterns to elicit withdrawal due to the inability of saline injections to stimulate the relevant underlying networks for exploration [1,31]. Training by repeated pairings of the novel environment with saline led to a reduction in already established exploratory patterns, indicating that withdrawal symptoms in crayfish just like in mammals [39] can be elicited not only by drug-associated environmental cues but also by drug-associated pharmacological cue. The reduction in drug-induced exploratory motor patterns in the absence of response-contingent drug availability probably led to the observed marked different set of atypical behaviors that are characteristics of crayfish withdrawal signs. The inhibition of such a pharmacological effect attenuates behavioral sensitization in crayfish. We observed that abstinence-induced withdrawal produced some behavioral changes opposite to the morphine-induced effect in crayfish. For instance, squirming, inactivity or static postures occurred during withdrawal, whereas an increase in locomotion and rearing in the corners of the aquarium occurred in a dose dependent manner following repeated morphine injections. Although, twisting postural displays were observed when morphine injections were eliminated, morphine injections at a higher dose (1.0 mg/kg) produced twisting postural displays. This observation supports the possibility of an evolutionarily conserved adaptation that is salient via an increase and during a decrease in the incentive salient environmental stimuli.

An important discussion relevant to our current results and drug addiction in crayfish is the type of opioid receptors in crustaceans, and the targets of morphine in the brain of crayfish. It is important to point out that the enkephalins (Leu-enk and Met-enk) are two pentapeptides with pharmacological properties similar to narcotic drugs. The endogenous pentapeptide enkephalins are known to bind to the mu and delta opioid receptors, with a slightly higher affinity for delta opioid receptors [40]. Delta opioid receptors activated by enkephalins play a key role in the physiological control of emotion- and motivation-related behaviors [40,41]. The endogenous opioid system is also known to have a great influence on the dopaminergic system. Blockade of the dopaminergic system in D2 receptor knock-out mice triggers an increase in enkephalin supporting the important physiological relationship between both systems [41]. The occurrences of enkephalins have been demonstrated in different species of crustaceans. A study by Mancillas et al. [42] identified enkephalins in the eyestalks of the lobster *Panulirus interruptus*. The author detected immunoreactive Leu-enk material in the medulla interna and axons of the third chiasm. In the sinus gland, and the neurohaemal organ of crustaceans, enkephalin-like material was also demonstrated in *Carcinus maenas* [43,44]. In addition, enkephalin positive fractions were isolated in *C. maenas* [45]. Luschen et al. [46] characterized Leu-enk and Met-enk in the thoracic ganglion of *C. maenas*. The detection of immunoreactive Leu-enk and Met-enk in the sinus gland of the fresh water crab [47], and the demonstration of Met-enk in the brain and eyestalk extracts of *Gecarcinus lateras* [48] are further demonstrations of the occurrence of enkephalins in crustaceans.

In mammals, the biochemical mechanisms that underlie withdrawal – a behavioral manifestation of the development of physical dependence, are not very well understood. Withdrawal is thought to represent a physiological drive that triggers 'craving' during drug abuse [49], and represents a standard experimental indicator of the development of physical dependence in mammals [50–52]. Although the relationship between the morphine withdrawal in crayfish and abuse of morphine by humans cannot be established in this study, however, the functional and evolutionary conservation of neural circuits of reward seeking >is a feature of survival; it is found from insects to humans. It is possible that physical dependence in mammals is obscured by compensatory processes, but readily revealed in lower animals such as crayfish. The crayfish nervous system has nothing to brag about when compared with the billions of neurons in the brain of humans. What the nervous system of crayfish lacks in complexity, it makes this up in a manner that makes crayfish a unique animal model in drug addiction research. Crayfish offered reduced complexity in comparison to any vertebrate model currently used in drug addiction research. It is particularly suited to a search for and detailed exploration of the inner workings of drug addiction research at its key anatomical and neural circuit. In the current study, we have shown that crayfish with a large, recognizable accessible neurons rooted within a well-designed anatomy represent a novel model >that can be used for the pursuit of cross-species insights into the mechanism of drug addiction that effectively complement investigations of mammalian systems.

5. Conclusion

The central nervous system of crayfish contains modulatory systems based on the same monoamines, such as dopamine, which is the natural rewarding system that in mammals are targets of drugs. The characterization of key control processes and the maintenance of morphine sensitive reward and withdrawal in crayfish, indicates that the proximate mechanism underlying drug addiction, just like in any mammalian model of drug addiction, resides in the neuromodulatory regulators of the reward circuits of crayfish

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