Research report

Effects of a single and repeated morphine treatment on conditioned and unconditioned behavioral sensitization in Crayfish

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ABSTRACT

Recent neuroethological work suggests that drug-sensitive reward in Crayfish represents a useful new model system for the study of drug dependence. Monoamine re-uptake mechanisms, which are conserved across vertebrate and invertebrate taxa, offer sites of action for testing drug-induced behavioral sensitization. The present study explored drug-associated behavioral sensitization in Crayfish by concurrently mapping measures of locomotion and rewarding properties of morphine. Behavioral effects of mammalian drugs of abuse are thought to depend on the patterns of drug regimens, and are similar across vertebrates. In this study, we determined whether behavioral sensitization induced by single and repeated morphine treatments extend to invertebrates. The first set of experiments indicated that intracirculatory infusions of single or repeated doses of morphine (2.5 μg/g, 5.0 μg/g and 10.0 μg/g) result in persistent and comparable locomotory sensitization even 5 days following the infusion. In the second experiment, we explored the short and long-term rewarding effects of a single or repeated morphine drug regimen using the conditioned place preference (CPP) experiment. Morphine-induced CPP also persisted for a drug free period of 5 days, indicating that this amount of time was not sufficient to disrupt the established CPP between morphine and context-dependent cues in Crayfish. Results from our study indicate that a single dose of morphine was sufficient to induce long-term behavioral sensitization in Crayfish, and that such effect is comparable to the effect of repeated morphine regimes. Behavioral sensitization studies in Crayfish thus contribute an evolutionary, comparative context to our understanding of the natural variation of reward as an important life-sustaining process.

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1. Introduction

Several lines of evidence indicate that drug-induced sensitization is associated with a continued and enduring amplification of positive reinforcement effects following drug intake [1–4]. The degree of drug-induced behavioral sensitization depends on the precise patterns of drug regimes [1]. For instance, repeated drug intake separated by long intervals is thought to be more effective in inducing sensitization, when compared to a chronic dosage regime involving either high and/or escalating dosage with short intervals [2–4]. Repeated, intermittent, or chronic exposure to amphetamine cause unrelenting sensitization by enhancing locomotion in rats, with marginal intensification after 3 days of treatment and profound effects after one week of treatment [5,6]. Repeated treatments of rats with morphine [7] and cocaine [8]

also induce long-lasting behavioral sensitization. Even a rat's single exposure to cocaine [2,9–12], amphetamine [3] and morphine [13] produces enduring sensitization. The aforementioned studies in mammals provide a wealth of information about the consequences of a repeated and a single drug exposure regime on behavioral sensitization. Taken together, the findings across vertebrates indicate that repeated and single drug treatments can comparably induce behavioral sensitization. Whether the comparable effects of single and repeated drug regimes can be extended to an invertebrate model of drug addiction had yet to be explored. We tested this issue in the current study in wild caught population of Crayfish exposed to repeated or single morphine regime.

Aiming to unravel major scientific issues using a simpler system approach can be traced back to the genetic analysis of yeast, bacteria or fruitflies in a search for fundamental mechanisms controlling gene expression and growth in multicellular systems. There is no doubt, there are many scientific issues that are yet to be fully resolved, and most of them lie within the field of neuroscience. Precisely, how neurons and neural circuit give rise to behavior, and how experience and the external environment affect these interactions are central issues in drug addiction research. Crayfish with

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relatively highly modular, neural and neuromodulatory systems offer an intriguing model system to explore the neurobiological and behavioral mechanisms that are involved in drug-induced behavioral sensitization. This work may effectively complement studies in mammals.

It is well known that the compulsive components of addiction hinge on motivational subcortical neural circuits, with anatomical, neurochemical and motivational similarities shared across all vertebrates and even extends to invertebrates [14,15], such as crustaceans. For instance, the central nervous system of crustaceans contains neuromodulatory systems, which contain the same monoamines that in vertebrates are targets of drugs such as cocaine and morphine. The aminergic system in Crayfish is mapped on less than 1000 large and accessible neurons [16–18] that contain about 30–35 dopamine neurons located in the brain and nerve cord of Crayfish [19]. To date, all integral elements underlying addictive behaviors, are mapped on the dopamine neurcohemical system that promotes drug-associated reinforcement [37]. The dopamine is a neurochemical signal that is conserved and shared across all mammals and non mammalian species especially in invertebrates such as Bees [38], arthropods [39] and in Crayfish [18]. Crayfish with highly stereotype behavioral patterns offer an opportunity to characterize proximate neurochemical mechanisms and fundamental neurobiological changes that underlie reward to amphetamine, cocaine [20] and morphine [37] in our previous studies.

Using Crayfish in the current study, we determined whether behavioral sensitization evoked by a single and repeated drug pretreatment regimes, which are thought to represent the same neurobiochemical behavioral sensitization effect in vertebrates [21] can be observed in an invertebrate model of drug addiction. First, we characterized the effects of a single and repeated morphine exposure on locomotion behavior in Crayfish using an open field test. We evaluated locomotion performances to determine the effect of immediate morphine treatment. Five days later, we re-assessed locomotory performance to determine the presence of long-lasting effect of morphine on behavioral sensitization. In the second experiment, we used a place preference conditioning procedure that paired a bolus of morphine with the unconditioned stimulus (UCS) of a textured background environment to explore the rewarding effect of single and repeated morphine regimes in Crayfish. This allowed us to measure the strength of the association of the textured environmental cues with morphine. This article presents the immediate as well as the long-term conditioned and unconditioned behavioral changes in Crayfish that accompanied single and repeated treatments with morphine.

2. Materials and methods

2.1. Animals

Intact, intermolt male Crayfish (*Orconectes rusticus*) were used for all the experiments in this study (body weights 12.5–28.6 g). Animals were wild-caught from the Portage River near Bowling Green, OH. Individuals were maintained in the laboratory in individual plastic containers on large flow-through holding trays. Water was pumped up from a large container where it was continuously filtered and aerated. Temperature was maintained at $20 \pm 1\,°C$. The animals were housed under 16:8 light/dark cycle and were fed twice a week with small pieces of tuna.

2.2. Apparatus

For the unconditioning experiment, we constructed a rectangular aquarium made from Plexiglas ($2.2\,m \times 0.9\,m \times 0.75\,m$). The walls of the aquarium were translucent. The tank received continuous flow of aerated water. Lighting for video recording was provided by four strip lamps with 20 W florescent bulbs at the sides of the aquarium. A digital camera (Sony DCR-VX1000) was mounted above and provided viewing angle sufficient to cover the entire aquarium. For the conditioning test, we used the same aquarium ($2.2\,m \times 0.9\,m \times 0.75\,m$), and divided it into two compartments with the floor covered by a hard and a soft-texture respectively. The hard-textured environment consisted of thick and smooth tiles, while the soft-texture environment was created by lining the floor of the aquarium compartment with soft felt material.

2.3. Surgical protocol

Crayfish were buried in crushed ice for about 20 min in preparation for surgery. During surgery, an incision was drilled in the caudal 1/3 of the dorsal carapace, lateral of the midline to avoid damaging the underlying heart. A 15 mm section of deactivated, fine-bore, fused silica capillary (Agilent, i.d. = 250 μm) was implanted into the pericardial sinus, about 3 mm deep, and stiffened with cyanoacrylate glue. Following successful surgery, animals were returned to their plastic holding containers overnight for recovery.

2.4. Injection protocols

Deactivated, fine-bore, fused silica needle (Agilent, i.d. = 100 μm) was connected to the implanted cannula with a short section of Tygon microbore tubing (Fisher Scientific, i.d. = 250 μm). A microdialysis swivel (intech, 375/25p) prevented the canula from becoming tangled. The void volume of the cannula was filled to assure immediate delivery when the microdialysis pump (CMA Model 102, CMA Microdialysis Inc., North Chelmsford, MA, USA) was used to deliver different doses (2.5 μg 5.0 μg and 10.0 μg/g of the animal body weight) of morphine sulphate (Sigma, St Louis USA) into the pericardial system of Crayfish. 125 mM saline was used as a control. We administered drug delivered directly into the pericardial system which in crustaceans, also serves as a primary and effective neurochemical site for endogenous monoamine release [35].

2.5. Behavioral analysis

A custom-designed video tracking system was used to provide a detailed analysis of the spatial activities of Crayfish. The tracking system obtained single video frames ever 300 ms from a camera (Sony DCR-VX1000) mounted above the tank. The video signal was streamed to a video digitizer board on a Apple Power PC Macintosh (81001/100AV) computer. The location of Crayfish was obtained using a freeware, Java-based application (available at http://iEthology.com/).

2.6. Statistical analysis

We determined the pre-conditioning and CPP test outcomes by analyzing the time spent in each compartment. A direct comparison of time spent between the soft or hard texture was analyzed using the Student's t-test. To characterize morphine-induced unconditioned behavioral sensitization, locomotor performances were obtained for each 15 min interval within the 60 min test session. A 3×4 mixed-model ANOVA compared between-group variance between different doses of morphine (2.5 μg/g, 5.0 μg/g and 10.0 μg/g), and individual time intervals to assess pre- and 5 day post-treatment of CPP-induced rewarding effect of morphine. A statistically significant effect was followed by post-hoc pair-wise comparisons. All our analyses were done using the SPSS version 15.0 (Prentice Hall, USA). Analyses specific to each experiment are outlined in the appropriate result section. In addition, specific behavior patterns of Crayfish following the drug-induced behavioral sensitization are described.

2.7. Experimental design

2.7.1. Experiment I: unconditioned locomotion test

Unconditioned tests were conducted a day after surgery. Each Crayfish was injected with one of several doses of morphine (2.5 μg/g 5.0 μg/g and 10.0 μg/g). The Crayfish was placed into the aquarium and injected with morphine over 5 min followed by continued tracking without infusion for another 60 min.

2.7.2. Unconditioned spatial movement patterns and surface preference

Spatial activities of Crayfish were assessed inside the test aquarium. We placed individual Crayfish in the aquarium for 2 consecutive days and their spatial characteristics were monitored for 60 min each day between 10.00 and 11.00 am. Four strip lamps with 20 W florescent bulbs were mounted at the sides of the aquarium to provide illumination. The amount of time spent in each compartment was monitored and used to measure the Crayfish's natural preference for soft- or hard-textured surfaces.

2.7.3. Experiment II: Morphine-induced CPP

During the place conditioning test, 28 Crayfish were randomly assigned to one of 4 groups ($n = 7$ per group) using a two by two factorial design for all combinations of soft vs. texture and control vs. morphine (2.5 μg/g 5.0 μg/g and 10.0 μg/g doses). Crayfish received random injections of morphine in the hard and soft-texture compartments. For the control group, Crayfish received 125 mM saline injections in both the hard and soft-texture compartments. The conditioning session commenced when a Crayfish was connected to the infusion cannula and placed in the separated hard or soft-texture compartment. The separation was done using a removable Plexiglas divider. After placing the Crayfish into the aquarium, morphine injection lasted for 5 min for the session. Thereafter, Crayfish were allowed to move freely for another 25 min. For the single drug regime, prior to drug administration, Crayfish were injected with saline followed by 20 min of saline to establish baseline locomotor activity. The animals were left to move freely and not injected again

before the baseline measurements were taken. The conditioning for the repeated doses of morphine injection was performed using an unbiased, balanced protocol consisting of 5 consecutive days, such that each crayfish received two conditioning sessions per day (1 drug, 1 vehicle), in random order and separated by 9 hours. On the other hand, crayfish in the control group received 2 vehicle injections per day. Behavioral testing was carried out on day 6, during which the Plexiglas divider was removed and the Crayfish was placed at the center of the aquarium with free access to both sides for 60 min. Data were collected between 10.00 and 11.00 am. The amount of time spent in each compartment was recorded to assess individual conditioned preferences. No injection was administered on the day of the preference test, in order to maintain consistent procedures used during the preliminary baseline test for the individual's spatial activities. In summary, we used conditioned place preference procedure approach to examine the rewarding effects of morphine in Crayfish, by pairing morphine the unconditioned stimulus with two contrasting tactile environments to test the rewarding properties of morphine in Crayfish. We gave the animal an opportunity to choose to enter and explore either environment, and the time spent in either environment was considered an index of the reinforcing value of the drug. The animal's choice to spend more time in either the soft or hard compartment was assumed to be an expression of the positive reinforcing experience within that compartment. In this context, our CPP behavioral test associates drug consumption and memorized environment that we in turn, used to assess the rewarding properties of morphine by determining the sensitization to the rewarding effects of morphine-induced by pretreatment regimens. Our hypothesis is that the single or repeated pairing of the two compartment with morphine will lead to an established CPP, and this will provide measures of the incentive or motivational properties of morphine in Crayfish. Schematic representation of the experimental design used in the present study is presented in Fig. 1.

3. Results

3.1. Single dose and repeated injections of morphine produce enduring effects on locomotor responses in Crayfish

Regardless of the dose, intra-circulatory injection of a single or repeated morphine results in enduring enhancement of locomotion when compared with saline injections (Fig. 2A–C). Following a single dose of morphine, locomotion enhancement was apparent during the entire challenge period of 60 min. The morphine-induced locomotion enhancement was not significantly different in the first 45 min following injections of 2.5 μg/g, 5.0 μg/g or 10.0 μg/g as a single dose of morphine. For the repeated dose regimen, locomotion was not significantly different in the first 45 min for the 2.5 μg/g dose of morphine. However, following 5.0 μg/g and 10.0 μg/g injections, locomotion was higher during the first 30 min and declined progressively during the last 30 min of the test session. Interestingly, over the last 15 min of the test session, locomotion significantly declined in both the single and repeated drug regimes. Means of locomotor activity (Fig. 2A–C) were significantly higher during repeated drug regime than a single dose treatments (2.5 μg/g; [$F_{(7,71)} = 33.47$, $P < 0.01$], 5.0 μg/g [$F_{(7,71)} = 69.18$, $P < 0.01$] and 10.0 μg/g [$F_{(7,71)} = 116.74$, $P < 0.01$].
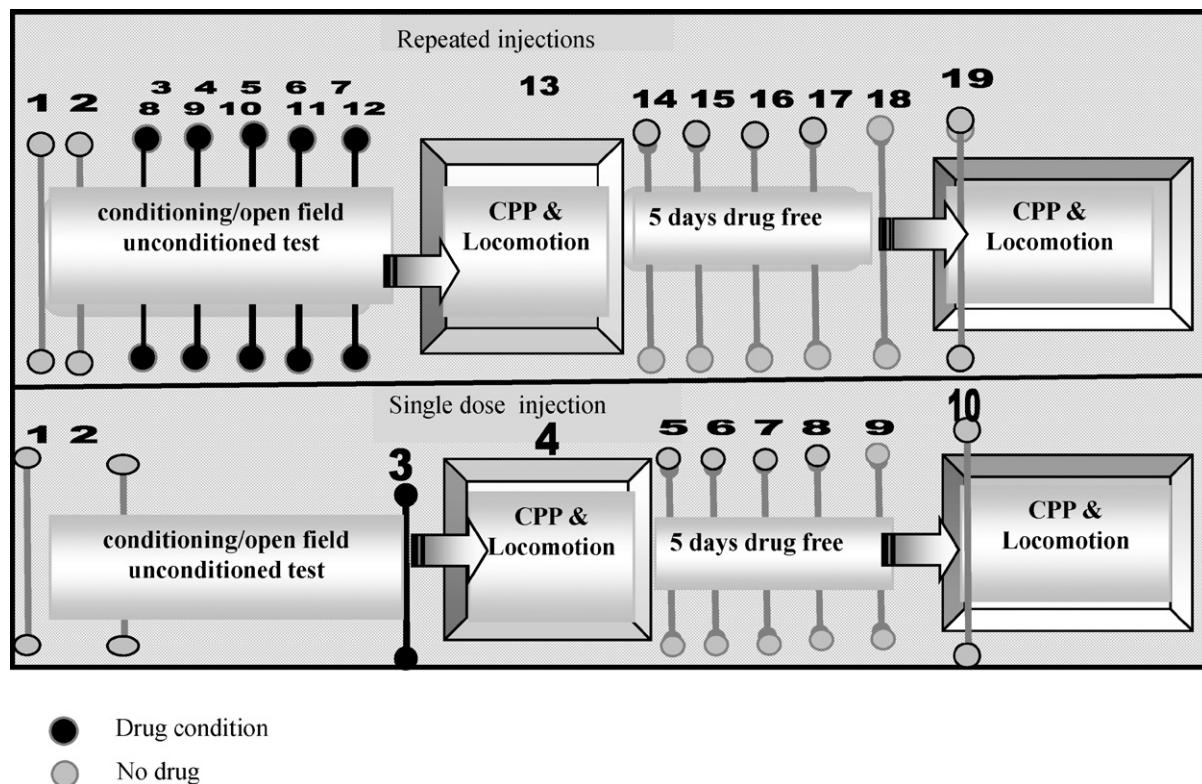

Fig. 1. Schematic representation of the experimental design used in the present study. Testing for initial preference was carried out in days 1 and 2 for the conditioning test. Conditioning and unconditioning testings were carried out in different experimental arena. For the conditioning test for the repeated drug treatment, the conditioning consists of 10 alternate days (3–12) of drug and saline injections using the unbiased balanced protocol. Crayfish were treated for 10 consecutive days with alternate injections of morphine 2.5 μg/g, 5.0 μg/g and 10.0 μg/g doses. After conditioning, animals were immediately confined to the conditioning compartment for 25 min. On day 13, the partition separating the compartment was removed, and Crayfish were placed at the center and allowed to move freely for 60 min in a drug free state to test for the expression of CPP. Following conditioning and the initial CPP test, each Crayfish was allowed for a drug free period of 5 days without morphine during this period. Thereafter, Crayfish were given a test for CPP on day 19 for the final test for CPP, also on day 20. The same protocol was used for the unconditioned repeated testing. For the single dose treatment, Crayfish received drug on day 3, after the initial preference in a drug free state for days 1 and 2. Following conditioning, CPP was done on day 4 followed by 5 days of drug free and the final CPP test was done on day 10. The same protocol was adapted for the unconditioned testing for locomotion behavioral testing. Precisely, the Crayfish was allowed to move freely in the open field test 60 min each for 2 days in a drug free state. On the third day, the animal received saline and allowed to move for 20 min to establish a baseline locomotion. Thereafter, the animal was injected with morphine over 5 min followed by continued tracking without infusion for another 60 min for the single dose. For the repeated dose of the unconditioned locomotion testing, each animal received morphine injections for 5 consecutive days.
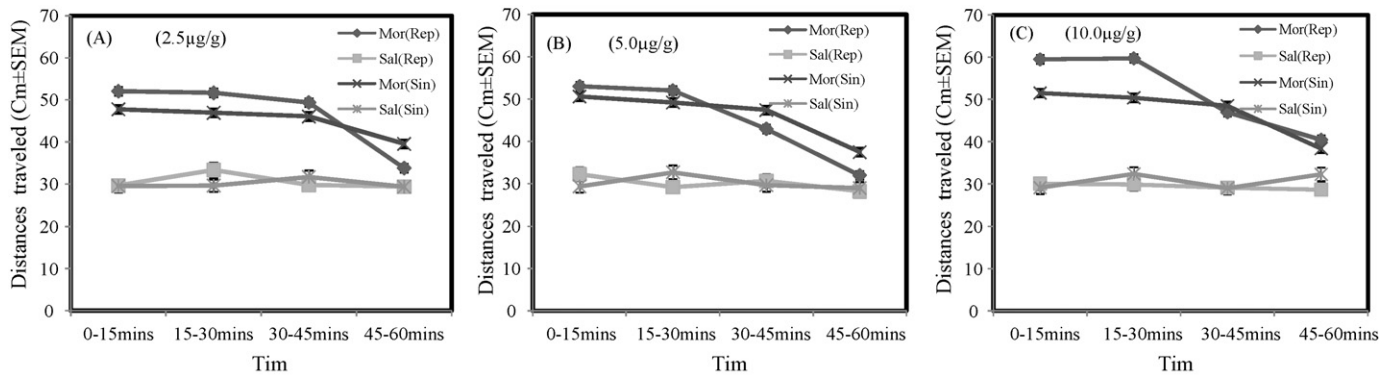
Fig. 2. (A–C) Locomotor responses of Crayfish pretreated with 2.5 μg/g, 5.0 μg/g and 10.0 μg/g doses of morphine or saline injections. Crayfish were randomly assigned into four groups for the single and repeated doses of morphine treatments ($n = 9$ per group): Repeated treatment with morphine (Mor Rep); Repeated treatment with saline (Sal Rep; control); Single dose treatment with morphine (Mor Sin) and the single dose treatment with saline (Sal Sin; control). Data are expressed as mean traveled distances (cm) ± SEM per 15 min interval. ANOVA results are as follows: for repeated 2.5 μg/g pretreatment, there was a significant reduction in locomotion in the last 15 min post injection [$F_{(3,35)} = 36.32$, $P < 0.0001$]. The effect of time was also significant in the 5.0 μg/g [$F_{(3,35)} = 90.70$, $P < 0.0001$] and 10.0 μg/g [$F_{(3,35)} = 91.04$, $P < 0.0001$] doses of morphine respectively. For the single dose treatment, the effect of time was significant at 2.5 μg/g [$F_{(3,35)} = 54.17$, $P < 0.0001$], 5.0 μg/g [$F_{(3,35)} = 60.20$, $P < 0.0001$], and 10.0 μg/g [$F_{(3,35)} = 19.37$, $P < 0.0001$].

3.2. Single dose and repeated injections of morphine produce comparable long-term effects on locomotion behavior

The long-term effect of a single and repeated drug treatment on locomotor activity is presented in Fig. 3A–C. Five days after treatment, morphine-pre-exposed Crayfish to a single or repeated drug treatment were still sensitized to locomotor effect of the different doses of morphine. For the repeated drug regime, the facilitating effect of morphine on locomotion was significant at 2.5 μg/g [$F_{(3,35)} = 7.68$, $P < 0.001$], 5.0 μg/g [$F_{(3,35)} = 17.21$, $P < 0.001$] and 10.0 μg/g [$F_{(3,35)} = 58.81$, $P < 0.001$] when compared with saline condition. A single dose of morphine consistently enhanced locomotion of Crayfish 5 days after drug administration. The enhancement effect was significant at 2.5 μg/g [$F_{(3,35)} = 7.85$, $P < 0.001$], 5.0 μg/g [$F_{(3,35)} = 15.74$, $P < 0.001$] and 10.0 μg/g [$F_{(3,35)} = 47.78$, $P < 0.001$] respectively. Pretreatments with saline were not associated with significant effects on the locomotion of Crayfish injected again with saline.

Apart from locomotion behavior, Crayfish consistently displayed different behavioral patterns throughout testing (Fig. 4A–I). Regardless of dose or regime of drug intake, the Crayfish persistently explored the corners of the aquarium using the antennae to survey its immediate surroundings. Sometimes Crayfish displayed full or truncated tail flipping. During 'full' tail flips, the uropods were maximally projected throughout active flexion, such that the first 3 pairs of pereopods were projected forward. During 'truncated' tail-flips, abdominal segments 4–6 and the uropods were not extended. The pereopods either moved actively or trail passively. Following approach to the aquarium corner, Crayfish exhibit grooming during which, they consistently used the third maxilliped to cleanse the second antennae while the antenule was used to brush over the lateral antennules. Other notable behavioral patterns included a series of stereotypic movement of mouthparts and some form of mild tremor of the leg or continuous and aggressive extensions of the cheliped, especially at higher doses of 5.0 μg/g or 10.0 μg/g of morphine. In some instances the animals would extend the three pairs of legs laterally to display series of stereotypic movements of the limbs at the same spot without any walking. At all drug doses and towards the end of 60 min recording time (between 10.00 and 11.00 am), Crayfish showed immobility during which, the walking appendages became inactive, at this point the animal does not move in space. Although we did not quantify the aforementioned behaviors in Crayfish, they were conspicuous in the first day of test of pre-exposure. The intensity decreased after the 5-days post exposure treatment analysis, but such behavioral displays were not salient during saline infusions.
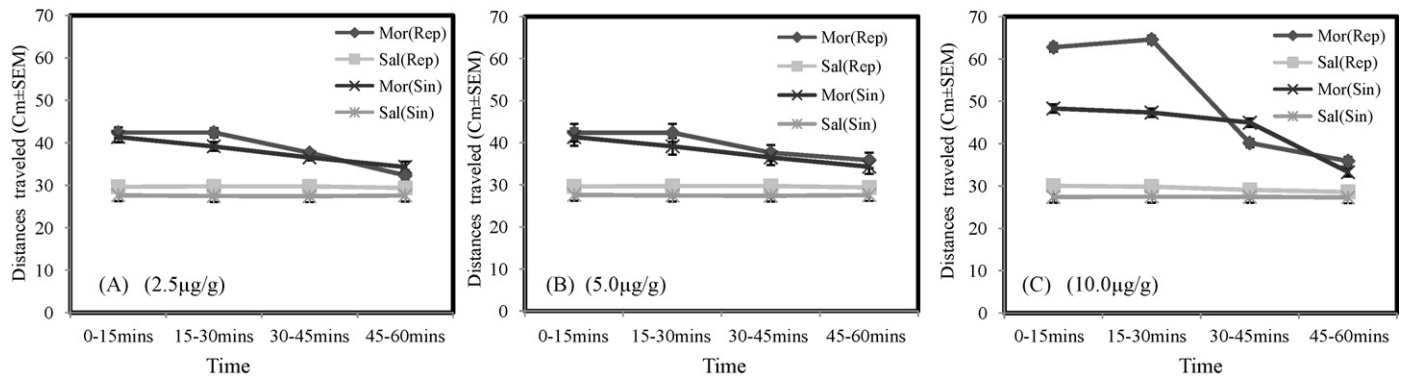

Fig. 3. (A–C) Locomotor responses of Crayfish 5 days post-treatment with 2.5 μg/g, 5.0 μg/g and 10.0 μg/g doses of morphine or saline for the single and repeated drug regimes. Repeated treatment with morphine (Mor Rep); Repeated treatment with saline (Sal Rep; control); Single treatment with morphine (Mor Sin) and single treatment with saline (Sal sin; control). Data are expressed as means of traveled distances (cm) ± SEM per 15 min interval. In the 5 days post morphine repeated treatments, ANOVA results indicate significant effect of time in the expression of locomotion at 2.5 μg/g [$F_{(3,35)} = 7.68$, $P < 0.001$], 5.0 μg/g [$F_{(3,35)} = 17.21$, $P < 0.001$] and 10.0 μg/g [$F_{(3,35)} = 56.81$, $P < 0.001$]. Five days post morphine repeated treatments with morphine resulted in significant effect of time interval in the expression of locomotion at 2.5 μg/g [$F_{(3,35)} = 7.86$, $P < 0.0001$], 5.0 μg/g [$F_{(3,35)} = 15.74$, $P < 0.0001$], and 10.0 μg/g [$F_{(3,35)} = 47.78$, $P < 0.0001$].
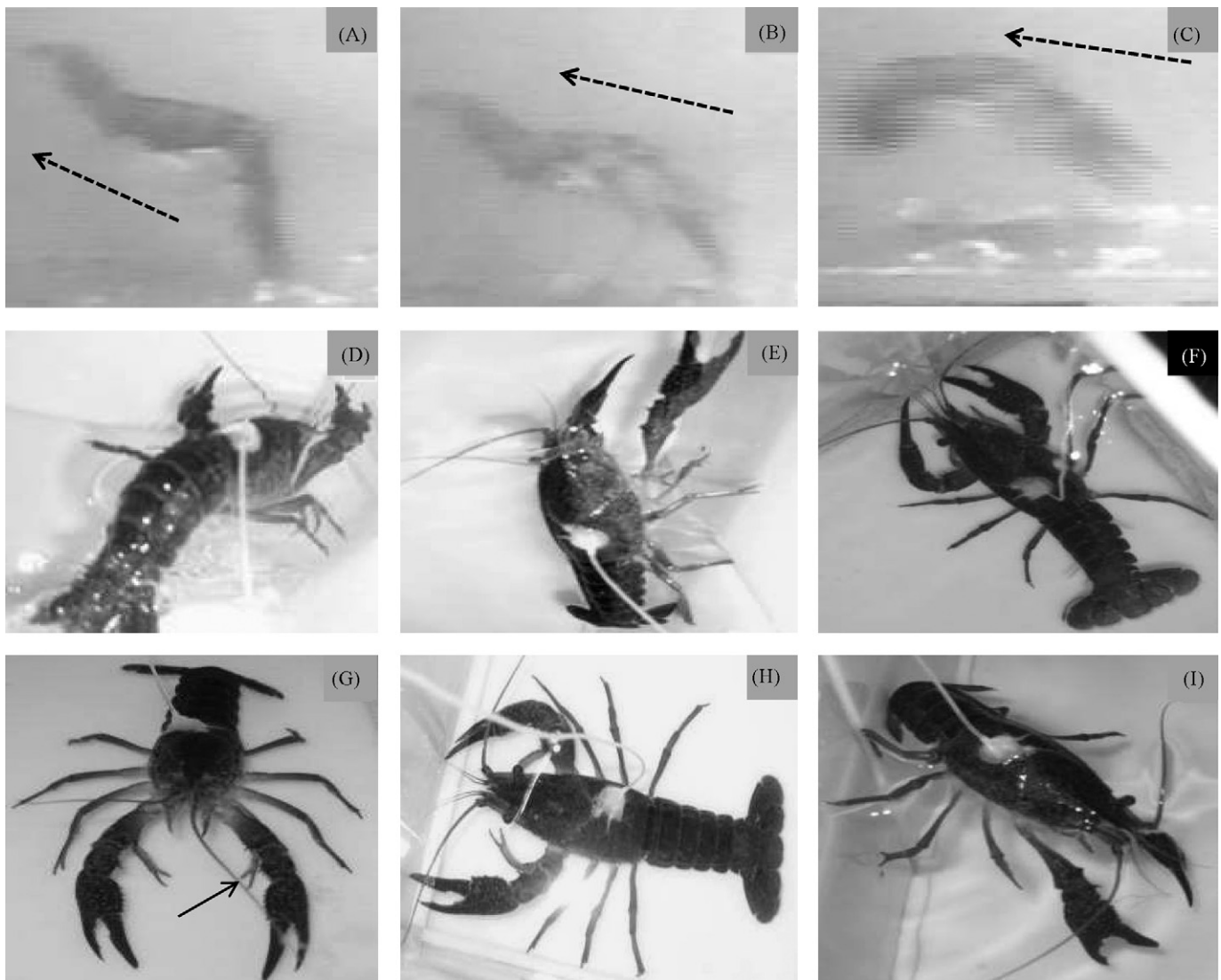
Fig. 4. (A–I) The different behavioral patterns of Crayfish following a single or repeated dose of morphine injections. Fig. 3A–C shows a Crayfish displaying full tailfliping. The direction of full tailfliping following drug injection is shown by the dotted arrow. During each complete full tailflip circle, the Crayfish would extend the uropods maximally throughout active flexion, such that the first three pairs of pereopods were projected forward, and the animal will translate backwards as shown by the direction of the arrow. Sometimes the Crayfish would display aggressive extensions of the cheliped (D) resulting in complex postural changes as shown in Fig. 3E. Crayfish persistently explored the corners of the aquarium using the antennae to survey their immediate surroundings (F), and would frequently use the third maxilipped to cleanse the second antennae (G; see arrow). In many instances, the three pairs of legs are extended laterally and the animal displayed series of stereotypic movements of the limbs at the same spot without any walking (H). At all drug doses and towards the 60 min of recording time, Crayfish moved to one corner of the aquarium and remain immobile during which, the walking appendages became inactive, at this point the animal does not move in space (I).

3.3. Pre-exposure test reveals the unconditioned spatial movement patterns and surface preference of Crayfish for the soft or hard compartment in untreated conditions

Measurement of the spatial activity for 60 min between 10.00 and 11.00 am during the first day of the test revealed that Crayfish spent significantly more time in the hard texture compartment than the soft-texture compartment (Fig. 5). Prior to the single dose of morphine treatment test, Crayfish showed a significant preference for the hard texture background in the first day (t-test for $\mu = 50.0\%$; $t_{[6]} = 3.87$, $P = 0.008$), and the preference significantly shifted to the soft-texture compartment in the second day (t-test for $\mu = 50.0\%$; $t_{[6]} = 3.09$, $P < 0.05$). In animals assigned for the repeated dose of morphine treatment test, the preference for the hard texture was significant in the first day (t-test for $\mu = 50.0\%$; $t_{[6]} = 6.81$, $P < 0.001$). The preference shifted to the soft-texture compartment in the second day. However, such preference was not statistically significant (t-test for $\mu = 50.0\%$; $t_{[6]} = 0.27$, $P = 0.79$). The results indi-

cate that the test compartments were truly unbiased in terms of hard or soft-texture compartment preference of untreated Crayfish. The results guided our decision in the use of an unbiased balanced protocol for the drug conditioning for the CPP test.

3.4. Repeated injections of morphine after 5 days and 1 day single injection of morphine are rewarding to Crayfish in a CPP conditioning test

Fig. 6A shows the result of the CPP test for a single regime morphine administration following conditioning. Single doses of 2.5 µg/g, 5.0 µg/g or 10.0 µg/g produced a CPP in the hard texture compartment during monitoring of activity for 60 min. At all morphine doses, Crayfish spent a greater amount of time in the morphine-paired compartment than in the saline-paired compartment. ANOVA for the between-groups variable, dose of morphine (2.5 µg/g, 5.0 µg/g and 10.0 µg/g) and 15 min time interval indicate a significant effect of different morphine doses
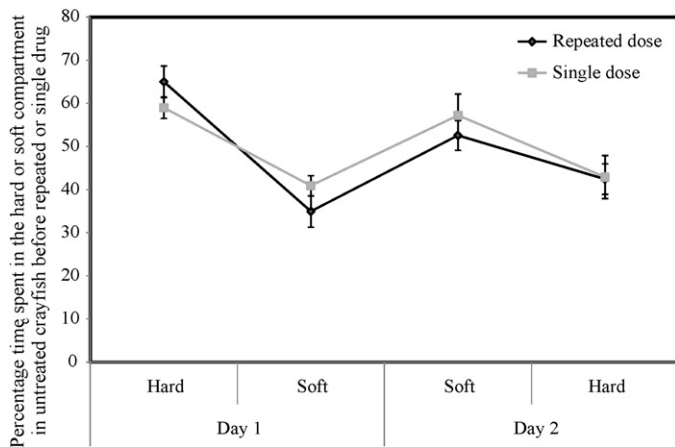
Fig. 5. Percentage time spent during 2 days monitoring of spatial activities prior to injection of morphine in the hard or soft-texture background in Crayfish that later received repeated or single morphine treatments. Prior to a single treatment test, Crayfish showed significant preference for the hard texture background in the first day, (t-test (μ = 50.0%); $t_{[6]}$ = 3.87, P = 0.008) and the preference significantly shifted to the soft compartment in the second day (t-test (μ = 50.0%); $t_{[6]}$ = 3.09, P = 0.021). Prior to repeated morphine treatments, Crayfish showed significant preference for the hard texture in the first day (t-test (μ = 50.0%); $t_{[6]}$ = 6.81, P < 0.0001). The preference shifted to the soft compartment in the second day. However, such preference was not statistically significant (t-test (μ = 50.0%); $t_{[6]}$ = 0.27, P = 0.79).

[$F_{(5,168)}$ = 31.51, P < 0.001], a non significant effect of time interval (0–15 min, 15–30 min, 30–45 min, 45–60 min) in the expression of CPP [$F_{(3,144)}$ = 0.20, P = 0.901], and a non significant interaction between drug and time interval [$F_{(15,167)}$ = 0.65, P = 0.83]. Post-hoc pair-wise comparisons analysis revealed that the significant effect of the drug was attributable to a greater amount of time spent in the morphine-paired compartment (P < 0.05) than in the saline-paired compartment.

Repeated treatments with each dose of 2.5 μg/g, 5.0 μg/g and 10.0 μg/g of morphine for 5 consecutive days (Fig. 6B) produced a CPP in the hard texture compartment. Crayfish spent a greater amount of time in the morphine-paired compartment than in the saline-paired compartment, indicating that morphine is rewarding for Crayfish exposed to 5 days of drug conditioning and tested for CPP. ANOVA found a significant difference in CPP between the drug doses [$F_{(5,168)}$ = 29.67, P < 0.001], a non significant effect of time interval in the expression of CPP [$F_{(3,144)}$ = 0.003, P = 1.03], and a non significant interaction between drug and time [$F_{(15,167)}$ = 0.65, P = 0.47]. Post-hoc pair-wise comparisons analysis revealed no significant difference (P > 0.05) between the means of time spent in the saline-paired conditions.

Paired repeated or single dose of morphine of 2.5 μg/g, 5.0 μg/g and 10.0 μg/g) infusion did not produce a CPP in the soft-texture following monitoring of activity for 60 min (Fig. 7A and B). CPP test was measured at 15 min bins interval. Each CPP test session lasted for 60 min. Following conditioning at all doses, the Crayfish spent a greater amount of time in the saline-paired compartment than in the morphine-paired soft-texture compartment.

Five days after the first CPP test in the single dose drug regime injection, CPP test once again indicated that morphine is rewarding for Crayfish 5 days after the last CPP test (Fig. 8A). Crayfish consistently spent a greater amount of time in the previously single dose morphine-paired compartment than in the saline-paired compartment. ANOVA for between-group variance, doses of morphine and time interval indicate a consistent significant effect of different doses of morphine [$F_{(5,168)}$ = 41.55, P < 0.001] 5 days after it was last administered. This results indicates a long-term sensitization effect of morphine in Crayfish. The effect of time interval was not significant [$F_{(3,144)}$ = 0.25, P = 0.84]. A significant effect of an interaction

between drug and time [$F_{(15,167)}$ = 2.49), P = 0.003] existed when compared with a non significant effect [$F_{(15,167)}$ = 0.65, P = 0.83] of such interaction in the first day of CPP test.

Five days after the first CPP test for the repeated morphine regime, another CPP test was carried out (see result in Fig. 8B). Morphine was rewarding for Crayfish re-tested for CPP 5 days after the first CPP test. ANOVA found a significant difference between the different doses of morphine [$F_{(5,168)}$ = 24.34, P < 0.001], a non significant effect of time interval in the expression of CPP [$F_{(3,144)}$ = 0.25, P = 0.64], and a non significant interaction between drug and time [$F_{(15,167)}$ = 1.22, P = 0.27]. Post-hoc pair-wise comparisons revealed no significant difference between means of the saline-paired conditions.

4. Discussion

In this study, we demonstrate that Crayfish can provide critical insights into the complex interactions of behavioral sensitization and neurochemical changes during drug addiction. It is intriguing that Crayfish, not particularly known for their cognitive abilities, continue to surprise with behavioral phenomena indicating powerful effects for drug-sensitive reward, behavioral sensitization, and drug dependence.

Irrespective of the dose, we found that single or repeated intra-circulatory injections of morphine result in locomotor sensitization. Following 5 days without drug application, sensitization for a single morphine administration on locomotion was still apparent, and strikingly resembled that induced by repeated morphine treatment. Although the magnitude of morphine-induced sensitization on locomotion was reduced 5 days post-treatment, enduring consequences in locomotion appear to be very similar. This finding indicates that a single dose of morphine is sufficient to induce long-term behavioral sensitization in an invertebrate system. The effect of a single dose of morphine in inducing behavioral sensitization has been observed in mammals [7,22,23]. As behavioral sensitization plays a key role in the compulsive facets of addiction, motivational similarities shared by all mammals appear to even extend to invertebrates [14,15,24]. Therefore, the evolutionary homology in neurochemical and behavioral components of drug addiction in mammals and Crayfish suggest that addictive chemical compounds are likely to act on evolutionary conserved neural components for behavioral sensitization beyond those peculiar to mammals.

Single and repeated intra-pericardial infusions of morphine resulted in different postural displays, exemplified by grooming behavior, a series of tail-flipping, movement of mouthparts, continuous exploration of the corners of the aquarium and some form of mild tremor of the leg, especially at the higher doses of morphine. The aforementioned morphine-induced unconditioned behaviors were stereotypic. They were mainly observed in the first day of the pre-exposure test, and the intensity decreased in the 5-days post exposure treatment analysis. In mammals, it has been shown that the enhanced reactivity of acumbens dopaminergic nerve terminals and the sensitized locomotion caused by morphine are functionally connected [3,25]. Several lines of evidence further indicate that behavioral sensitization is associated with functional restructuring in the dopaminergic, glutamatergic and GABAergic projections [2], which are also expressed in Crayfish [26,27]. Since dopamine the major receptor component involved in drug addiction is similarly expressed in vertebrates and invertebrates [24], it is conceivable that the dopaminergic neurotransmission in Crayfish might play a critical role in the locomotor and stereotypic effects of morphine. In this context, there is every likelihood that hyperresponsiveness of the dopaminergic nerve terminal may facilitate the expression of morphine-induced sensitization in Crayfish, such that after 5 days
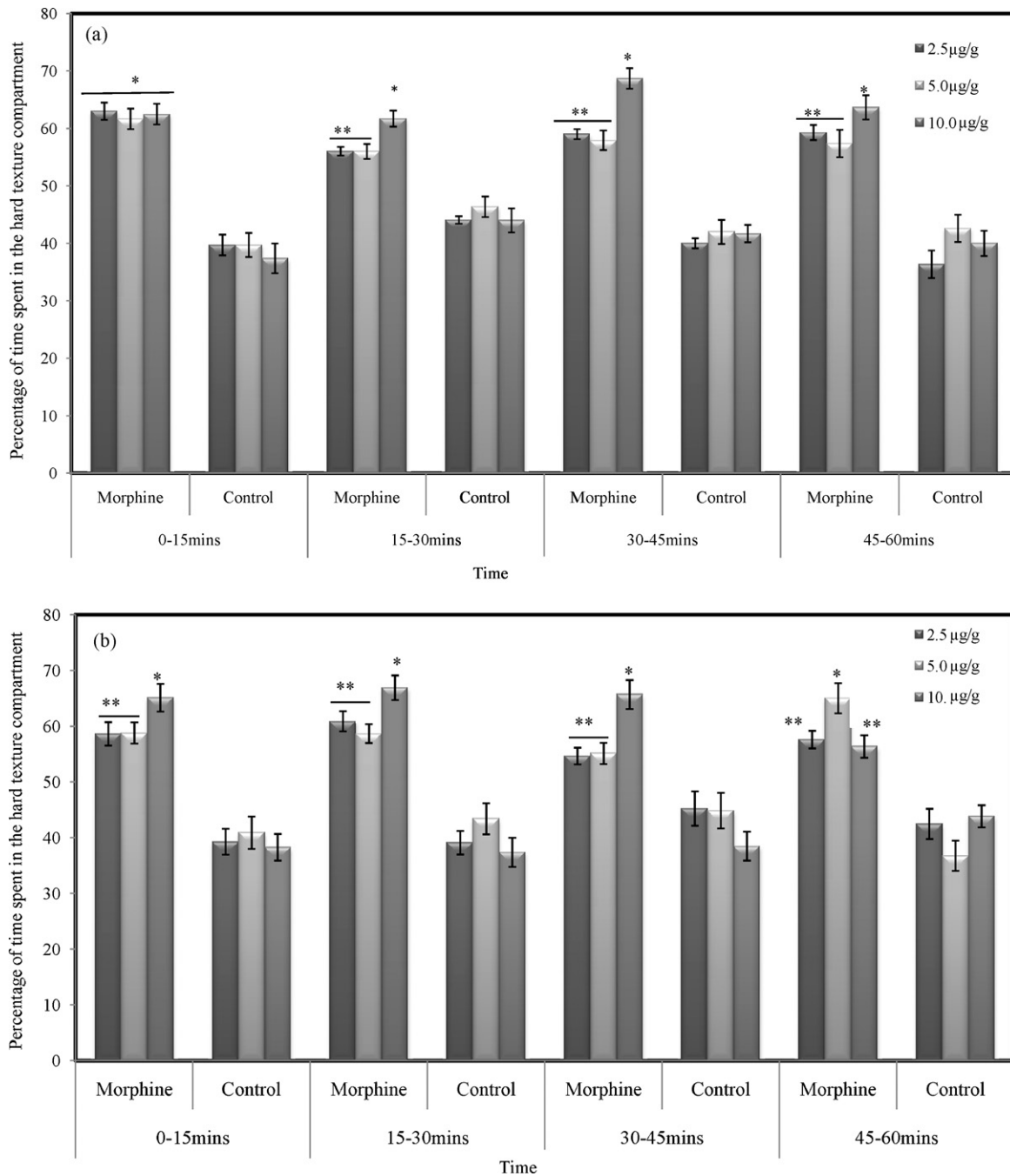
Fig. 6. (A) Effect of dose and time on the expression of CPP. Paired single dose of morphine at 2.5 μg/g, 5.0 μg/g and 10.0 μg/g infusions produced a CPP in the hard texture compartment. CPP test was measured at 15-min bin interval. Each CPP test session lasted for 60 min. Data are expressed as mean percentage time spent (%) ± SEM (n = 7 for each Crayfish injected with morphine or saline). Following conditioning at all doses, the Crayfish spent a greater amount of time in the morphine-paired compartment than in the saline-paired hard texture compartment. A 3 by 4 mixed ANOVA for the between -groups variable, dose of morphine (2.5 μg/g, 5.0 μg/g and 10.0 μg/g), and time interval (15–60 min), indicates a significant effect of the different drug doses [$F_{(5,168)}$ = 31.51, $P < 0.001$], a non significant effect of 15 min time interval in the expression of CPP [$F_{(3,144)}$ = 0.20, $P = 0.901$], and a non significant interaction between drug and time [$F_{(15,167)}$ = 0.65), $P = 0.83$]. Post-hoc pair-wise comparisons analysis revealed no significant difference ($P > 0.05$) between means of the saline-paired conditions. CPP at 10.0 μg/g dose of morphine was significantly higher (*$P < 0.05$) when compared with 2.5 μg/g and 5.0 μg/g (**$P < 0.05$) doses of morphine after the first 15 min of the test of the entire 60 min of the CPP test. (B) Effect of dose and time on the expression of CPP following repeated injections of morphine for 5 consecutive days followed by CPP test. Repeated injections of 2.5 μg/g, 5.0 μg/g and 10.0 μg/g of morphine produced a CPP in the hard texture following monitoring of activity for 60 min. Data are expressed as mean percentage time spent (cm) ± SEM (n = 7for each Crayfish injected with morphine or saline injection). ANOVA found a significant effect of the different morphine doses [$F_{(5,168)}$ = 29.67, $P < 0.001$] in the expression of CPP, a non significant effect [$F_{(3,144)}$ = 0.003, $P = 1.03$] of time interval, and a non significant interaction between drug and time [$F_{(15,167)}$ = 0.65), $P = 0.99$, $P = 0.47$] in the expression of CPP. Post-hoc pair-wise comparisons analysis revealed no significant difference ($P > 0.05$) between means of the saline-paired conditions. Percentage of time spent was significantly higher (*$P < 0.05$) at 10.0 μg/g in the first 45 min of the CPP when compared with 2.5 μg/g and 5.0 μg/g doses of morphine (**$P < 0.05$).

of extended post-treatment, morphine-induced behavioral sensitization was still maintained.

In both the single and repeated morphine treatment regimes, and at all morphine doses, we found that morphine-induced CPP

was established only in the hard texture compartment and not in the soft-texture compartment, indicating that the hard texture environment was novel when compared to the soft-texture environment. In addition, in both the single and repeated morphine
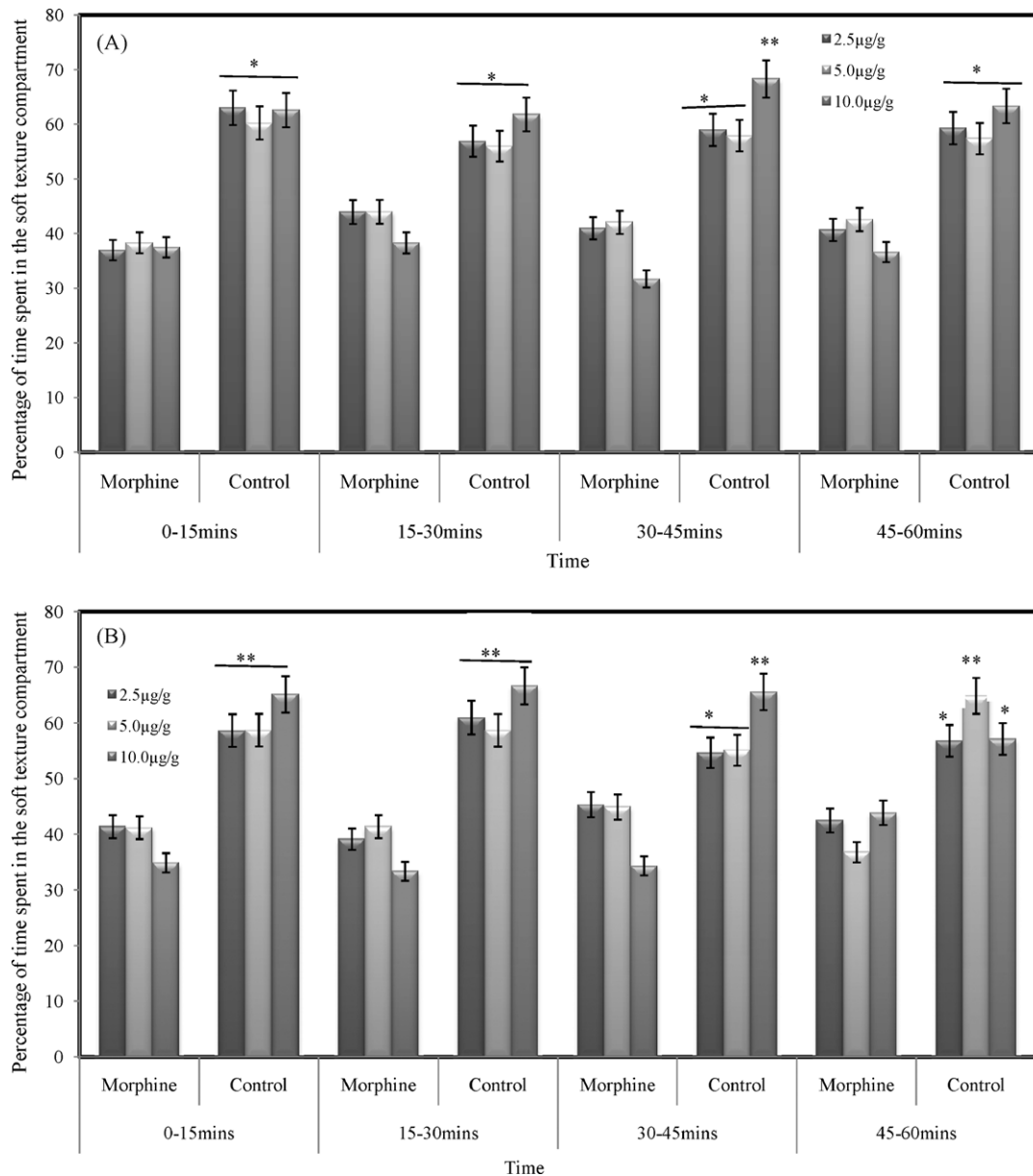
Fig. 7. (A) Paired single dose of morphine of 2.5 μg/g, 5.0 μg/g and 10.0 μg/g) did not produce a CPP in the soft-texture compartment following monitoring of activity for 60 min. CPP test was measured at 15 min bins interval. Each CPP test session lasted for 60 min. Data are expressed as mean percentage time spent ($n = 7$) for each morphine or saline injection. Following conditioning at all doses, the Crayfish spent a greater amount of time in the saline-paired compartment than in the morphine-paired soft-texture compartment. At all doses, the non preference for the soft-texture compartment was significant ($P < 0.05$). (B) Paired repeated dose of morphine of 2.5 μg/g, 5.0 μg/g and 10.0 μg/g) infusion did not produce a CPP in the soft-texture following monitoring of activity for 60 min. CPP test was measured at 15 min bins interval. Each CPP test session lasted for 60 min. Data are expressed as mean percentage time spent ($n = 7$ for each morphine or saline injection. Following conditioning at all doses, the Crayfish spent a greater amount of time in the saline-paired compartment than in the morphine-paired soft-texture compartment. At all doses, the non preference for the soft-texture compartment was significant ($P < 0.05$).

treatments, we found that a morphine-induced CPP could persist for another 5 days without additional exposure to the drug. The fact that the CPP was present 5 days after the last conditioning test indicates that the passage of time is not enough to interrupt the morphine established CPP in Crayfish, meaning that the 5-day drug free period was not adequate to disrupt the established CPP of paring the morphine with contextual-dependent cues. The different doses of morphine when paired with the hard texture compartment seem to have strengthened the expression of behavioral sensitization in Crayfish, even 5 days after the initial test.

Behavioral sensitization to opiates is due to different drug regimes that result in enhancement of behavioral effects of opiate on a long-term effect [28–31]. It then implies that behavioral

changes of Crayfish to morphine could be attributed not only to a direct pharmacological effect of the drug but also to learned associations of the distinct tactile stimuli with the drug rewarding experience. In this context, the changes in Crayfish behavioral output are paralleled by neuroadaptations at various levels (neurochemical and morphological), which predominantly occur in dopamine neuronal system that is involved in reward processing. Such neuroadapative changes in dopaminergic neurons probably play an important role in the observed drug-induced behavioral sensitization in Crayfish. Taken together, our data suggest that a single exposure to morphine is enough to induce long-lasting behavioral sensitization comparable to exposure to repeated drug regimes.
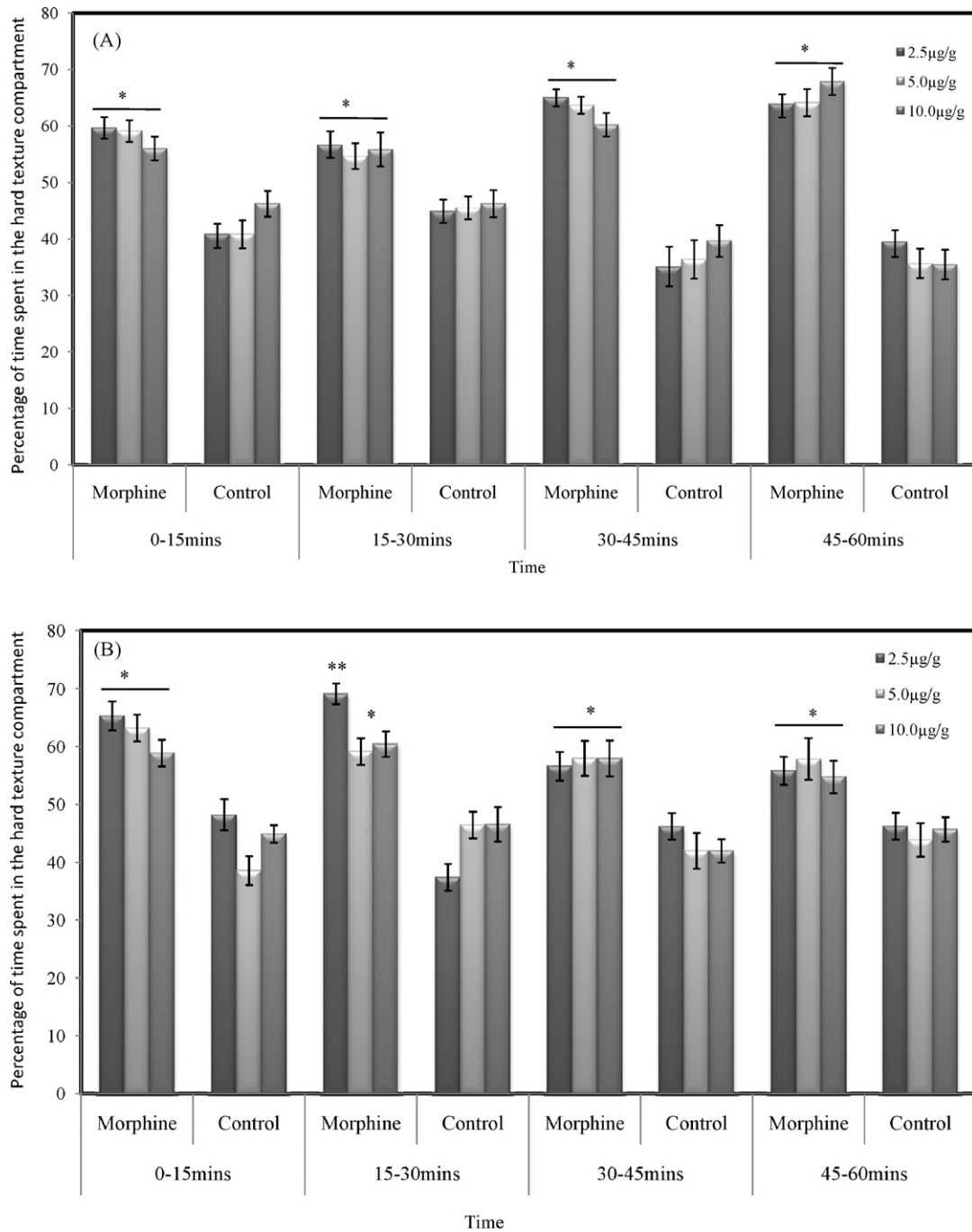
Fig. 8. (A) Effect of dose and time on the expression of CPP, following single injection of morphine (2.5 µg/g, 5.0 µg/g and 10.0 µg/g) followed by CPP test, 5-days after the first CPP test. Data are expressed as mean percentage time spent (cm) ± SEM ($n = 7$ for each morphine or saline injection) per 15 min interval. Five days after the first CPP test, the second CPP test was carried out. ANOVA for the between -groups variable, dose of morphine (2.5 µg/g, 5.0 µg/g and 10.0 µg/g) and time (0–15 min, 15–30 min, 30–45 min and 45–60 min intervals) of CPP test, indicates that morphine is rewarding for Crayfish re-tested for CPP 5 days after the first test. There was a significant effect of different doses of morphine [$F_{(5,168)} = 41.55$, $P < 0.001$], whereas the effect of time was not significant [$F_{(3,144)} = 0.25$, $P = 0.84$)]. The effect of interaction between drug and time was significant [$F_{(15,167)} = 2.49$), $P = 0.003$]. Post-hoc pair-wise comparisons analysis revealed no significant difference ($P > 0.05$) between means of the saline-paired conditions. Percentage of time spent 5 days after the last test for the single injections of 2.5 µg/g, 5.0 µg/g and 10.0 µg/g doses of morphine were not significantly (*$P < 0.05$) different. (B) Effect of dose and time on the expression of CPP 5 days after the first CPP test for the repeated morphine regime. Again, morphine is rewarding for Crayfish re-tested for CPP 5 days after the first CPP test following 5 days of repeated drug administrations. ANOVA found a significant effect of the different doses (2.5 µg/g, 5.0 µg/g and 10.0 µg/g) of morphine [$F_{(5,168)} = 24.34$, $P < 0.001$], a non significant effect of time interval [$F_{(3,144)} = 0.25$, $P = 0.64$], and a non significant interaction between drug and time [$F_{(15,167)} = 1.22$, $P = 1.22$, $P = 0.27$] in the expression of CPP. Post-hoc pair-wise comparisons analysis revealed no significant difference ($P > 0.05$) between means of the saline-paired conditions. Percentage of time spent at 2.5 µg/g, 5.0 µg/g and 10.0 µg/g doses of morphine were not significantly (*$P < 0.05$) different in all doses except during the 15–30 min time interval (2.5 µg/g; **$P < 0.05$, 5.0 µg/g and 10.0 µg/g doses; *$P < 0.05$).

In mammals, it has been proposed that long-lasting neuroadaptations could be controlled by biphasic changes in gene expression [32–34], structural changes at relevant synapses [35], and intermittent stimulation [1,3]. The fact that a single exposure to morphine is enough to induce long-lasting behavioral sensitization comparable to exposure to repeated drug regimes in Crayfish an invertebrate system indicates that long-lasting behavioral sensitization and associated neuroadaptations can be evoked by a

single relevant incentive, just like in mammals. Taken together, our current study, other studies on psychostimulants exposure to Crayfish [35] Crayfish aggression [35], drug seeking behavior in Crayfish [36], suggest that Crayfish system may provide significant new insights into the mechanisms involved in drug addiction that could contribute to the understanding of natural variation of an important life-sustaining process. With a nervous system containing fewer than 1000 individually identifiable monoamine-containing neurons, that could provided the requisite site of action for testing-drug-sensitive reward, Crayfish may provide a simple system model that can considerably reduce the difficulty associated with studying the primary site of action of drugs of abuse. It is true that the adaptive survival of Crayfish are very different from mammals. It is also true that the neurochemical system and behavioral components potentially involved in drug-induced behavioral sensitization and or reward in vertebrates and invertebrates are similar in the general modes of action [40,41], process of activation and inactivation [42], and the major receptor components that initiates behavioral sensitization and reward [18]. Thus, the evolutionary similarity in neurochemical and behavioral components of drug addiction suggest that addictive chemical compounds may potentially act on evolutionary conserved brain components for reward and behavioral sensitization beyond those peculiar to mammals.

Acknowledgements

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