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Alteration of c-Fos mRNA in the accessory lobe of crayfish is associated with a conditioned-cocaine induced reward

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ABSTRACT

The major molecular and anatomical substrates of drug related reward in mammals have received considerable attention. In contrast, molecular mechanisms and specific neuroanatomical targets of drug associated reward in invertebrate models of drug addiction have gone largely unexplored. With a modular nervous system amenable to molecular techniques, crayfish offer a novel system for simultaneously exploring molecular mechanism and neuroanatomical targets of cocaine-induced reward in an invertebrate system. We aimed to determine whether novelty in a cocaine-paired stimulus is accompanied by changes in c-Fos mRNA in the accessory lobe of crayfish. The first set of experiments revealed that cocaine-conditioned animals demonstrated reward in a drug-paired compartment in contrast to saline-conditioned animals. Following the expression of reward, we designed a second set of experiments to determine context-specificity of the cocaine-conditioned novelty effect in altering c-Fos mRNA expression in the accessory lobe of cocaine treated crayfish. This is the first report that characterized context-specific alteration of c-Fos mRNA expression in the accessory lobe of crayfish during drug-induced reward.

1. Introduction

Addictive drugs are able to seize control over behavior 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. The activation of major substrates or circuits of reward by addictive drugs can to a large extent be more consistent and powerful than activation triggered by natural contexts. The major substrates of reward including molecular/cellular mechanism and specific anatomical targets or pathways have been investigated extensively in mammalian models of drug addiction (Berridge and Robinson, 1998; Chocyk et al., 2006; Hope et al., 2006; Ikemoto and Panksepp, 1999; Kalivas and McFarland, 2003; Kalivas and Volkow, 2005).

Work in invertebrates also contributed novel molecular mechanisms of drug addiction. For instance, an integral role for the development of behavioral sensitization to cocaine has been demonstrated for the trace amine tyramine (TA) in *Drosophilia* (McClung and Hirsh, 1998), where circadian gene regulation, TA biosynthesis, and behavioral sensitization are tightly coupled (Andretic et al., 1999; Kaun et al., 2011). These studies reveal a surprising similarity in the molecular mechanism between flies and mammals in the rewarding effects of cocaine, suggesting evolutionarily conserved molecular brain mechanisms for drug effects that are shared across the phylogenetic pedigree. Furthermore, evidence for conserved monoamine re-uptake mechanisms in invertebrates (Porzgen et al., 2001) suggests the existence of the requisite sites of action for testing drug-sensitive reward in invertebrates. Although there are many similarities in the neurochemical and molecular mechanism of drug addiction in mammals and invertebrates, the precise neuroanatomic targets of drug associated reward are yet to be explored in invertebrate systems.

Flies and other invertebrates, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, have been helpful for analyzing the unconditional changes that result from drug exposure, but not for the study of active reward during drug addiction (Wolf and Heberlein, 2003). Rewarding properties for psychostimulants and morphine have been demonstrated in crayfish (Nathaniel et al., 2009, 2010; Panksepp and Huber, 2004b). Thus, exploring the molecular mechanism and neuroanatomical targets of drug associated reward in an invertebrate system (crayfish) that demonstrates drug-sensitive reward will contribute an evolutionary and comparative framework to our knowledge of natural reward as a significant life-supporting process.

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This study of drug addiction in crayfish is being made possible by decades of advances in crustacean neuroscience. Studies that explored transmission at the neuromuscular junction (Furshpan and Potter, 1959), the role of glutamate and GABA as excitatory and inhibitory neurotransmitters (Iverson et al., 1971; Kravitz et al., 1963; Otsuka et al., 1967), the neural coordination of escape (Herberholz et al., 2004), the complex modulation of stomatogastric networks (Norris et al., 1994) and the biasing effects in behaviors (Kravitz, 1988) revealed that crayfish offer an excellent model to study the specific neural circuitry and neurochemistry of behavior. Work in behavioral pharmacology of aminergic modulation (Benton et al., 1997; Huber and Delago, 1998; Panksepp and Huber, 2004a; Panksepp et al., 2003; Tricarico and Gherardia, 2007) provided an understanding of how the amine substrates foster behavioral responses to drugs within motivational contexts and affective states of crayfish.

The amine system in crayfish features fewer than 1000 neurons, including 30–35 dopamine neurons localized within the brain and nerve cord of crayfish (Furshpan and Potter, 1959; Tierney et al., 2003). These neurons have large somata of about 100 μm in diameter with wide projections of their axons (Tierney et al., 2003). Mounting evidence demonstrates that the well-characterized crayfish nervous system with its large, recognizable, accessible neuronal elements is uniquely amenable to molecular techniques. For this reason, the crayfish model provides an opportunity to simultaneously explore the molecular mechanism and the neuroanatomical target of reward in a system with a capability for drug-sensitive reward.

Mapping of the crayfish and lobster brain (Beltz, 1999; Polanska et al., 2007; Sullivan and Beltz, 2001a; Tierney et al., 2003) revealed that crayfish are among a handful of crustaceans with a well-developed accessory lobe. The unique neuronal projections of the accessory lobe (Fig. 1) suggest a functional significance of the accessory lobe in processing high order environmental stimuli. This tempts a speculation for the hypothesis that the accessory lobe might be a crucial player in the neural circuitry for reward-related choices of stimuli in the environment. The rationale is hinged on the anatomical ability of the accessory lobe to integrate a range of environmental stimuli (visual, tactile, olfactory; Sullivan and Beltz, 2005b), and these stimuli are known to be directly linked with reward-associated choices of specific objects or environments during drug-induced reward.

The c-Fos protein or mRNA has been used to test for an activation of brain regions by drugs associated with reward in many studies in mammals (Perrotti et al., 2004; Velázquez-Sánchez et al., 2009; Watanabe et al., 2009; Yamada et al., 2007; Zavala et al., 2007; Xu, 2008; Zawilska, 2003). C-Fos is an immediate early gene (IEG) that transduces signals from cell surface receptors into modifications in gene expression (Hyman et al., 1993; Morgan and Urran, 1991). The IEGs are considered to reflect neuronal activities due to their function in controlling gene transcription via their protein forms that bind to regulatory sites on DNA (Beckmann and Wilce, 1997; Hoffman and Lyo, 2002; Ons et al., 2010; Slattery et al., 2005). Induction of c-Fos protein or its mRNA can give insight into the molecular alterations associated with drug-initiated reward. This is because it signals the onset of long-term alterations in cellular phenotype, and provides insight into the brain regions affected by psychostimulants (Slattery et al., 2005). Our rationale for using c-Fos mRNA to explore the role of the accessory lobe in conditioned-elicited cocaine reward is because in mammals, c-Fos mRNA or protein profile has proven useful for identifying brain regions and pathways for reward, including the nucleus accumbens, and a number of cortical and subcortical areas (Marchant et al., 2010; Slattery et al., 2005; Teegarden et al., 2008; Velázquez-Sánchez et al., 2008). These studies show that c-Fos profiling might help to elucidate brain regions that are activated by a psychostimulant to induce motoric

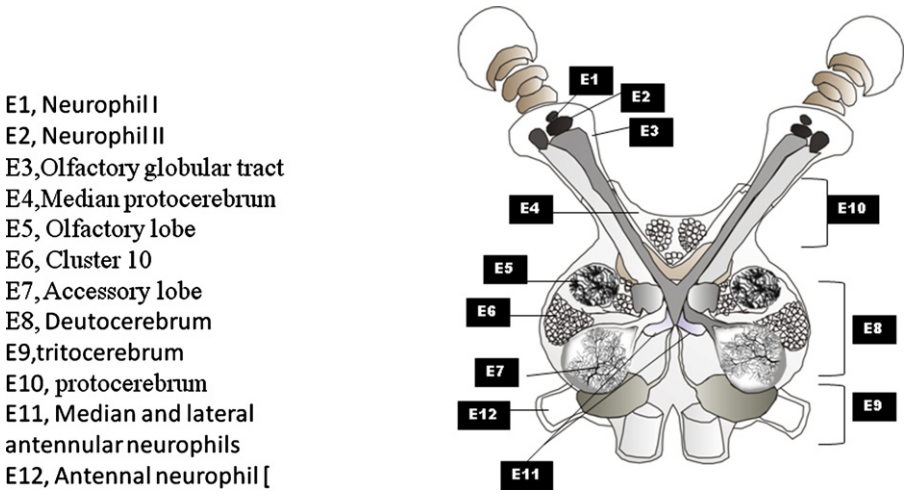

Fig. 1. Schematic illustration of the crayfish brain showing the unique anatomical projections of the accessory lobe. The accessory lobes are subdivided into an outer cortex and an inner medulla (Polanska et al., 2007). Cortex and medulla differ concerning the types of input that they receive, and in their connection pattern to the lateral protocerebrum as mediated by the projection neurons. They do not receive primary afferent fibers from the olfactory or any other sensory modalities. Instead they receive their inputs through the axons in a deutocerebral commisure from dopamine neurons that send massive projections over the accessory lobe (Sullivan and Beltz, 2001a; Tierney et al., 2003). Also, a pair of large serotonergic neurons branch unilaterally in the accessory lobe of each side, and the brain interneurons also converge with serotonin giant cells in the accessory lobe glomeruli (Sandeman et al., 1995). The deutocerebral interneurons provide tactile and visual sensitivity and target the medulla, whereas the cortex receives mainly a chemosensory input from cluster (9) local olfactory interneurons (Sullivan and Beltz, 2005b). There are at least four different types of architectural types of interneuronal inputs into the accessory lobe that receive information from different parts of the brain including the central body, triotocerebrum, and protocerebrum (Polanska et al., 2007). The interneuronal inputs are distributed throughout the central layer of the accessory lobe, such that the interneuronal inputs that relay information from the central layer project to regions of the medial or lateral layers. The Intrinsic interneurons mediate local processing within restricted regions of the medial or lateral layers, while the projection neurons integrate input across all regions of the medial or lateral layers. The output is sent to distinct areas of the lateral protocerebrum in the hemiellipsoid body, such that neuropil II of the contralateral hemiellipsoid body receives direct input from both accessory lobes, whereas neuropil I receives direct input only from the ipsilateral accessory lobe. The accessory lobe also receives olfactory inputs by means of a separate group of local interneurons that branch in both the olfactory lobe and the ipsilateral accessory lobe. Therefore, in contrast to the olfactory lobe, which receives only olfactory inputs, the accessory lobe receives higher-order multimodal inputs of visual, tactile and olfactory stimuli. The unique neuronal connections and the multimodal inputs provide evidence of important functional significance of the accessory lobes, such that the global processing of multimodal higher-order multimodal inputs of visual, tactile and olfactory stimuli might take place within the accessory lobe.

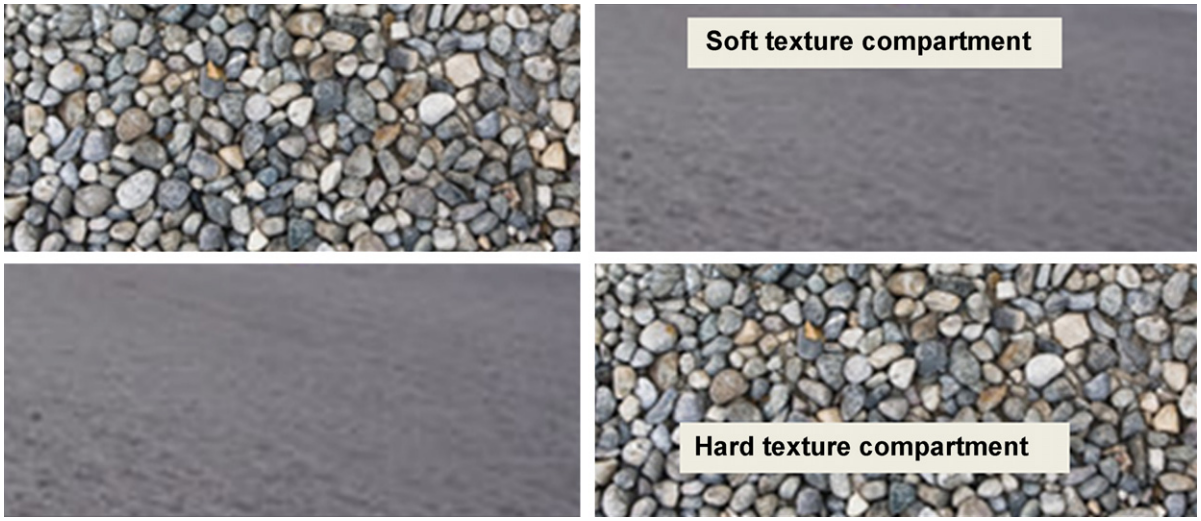

Fig. 2. The experimental aquarium was divided into two zones. The first zone comprised of four white walls with a hard floor covered with crushed concrete gravel that composed of unconsolidated rock fragments of size classes roughly between 5 and 10 mm. The second zone comprised of four white walls and a floor covered with soft sand. The sand covered the whole floor of the aquarium, such that the animals could not detect edges. The differences between the two compartments consisted of soft and hard textural cues.

and motivational alterations. The expression of c-Fos mRNA has already been used for cellular detection and mapping brain structures associated with circadian activities in the brain of crayfish (Granados-Dominguez et al., 2005). In the current study, we used it as a marker for the mapping of the functional anatomy of accessory lobe in conditioned-elicited cocaine reward.

Using the conditioned place preference (CPP) test paradigm, our first set of experiments determined whether cocaine-conditioned crayfish demonstrated reward in a cocaine-paired hard or soft tactile environment. The experiment determined whether cocaine-associated contextual cues can exert an influence on the behavior of crayfish through associative pairing between the cocaine and a novel tactile environment. We designed the second sets of experiments to determine the effect of a cocaine-conditioned specific novelty cue in the alteration of the basal expression of c-Fos mRNA in the accessory lobe. A context-specific expression of c-Fos mRNA in the accessory lobe of drug treated crayfish would suggest the presence of molecular and neuroanatomical reward substrate in an invertebrate model of drug addiction.

2. Materials and methods

2.1. Animals

We used adult intermolt male crayfish (*Orconectes rusticus*; 8–25.0 g body weight) that possessed full intact appendages for this experiment. The specimens were collected from the river. Individuals were sorted and housed in a big tank of freshly filtered/aerated water at $20 \pm 1\,^{\circ}C$ in a 16:8 h light/dark circle. Crayfish were fed 1–2 times per week with tuna.

2.2. Apparatus

The conditioning apparatus for this experiment consisted of a rectangular opaque white Plexiglas aquarium. The dimensions of the apparatus are 220 mm × 90 mm × 75 mm (length, width and height). Four strip lamps with 20 W florescent bulbs were mounted at the sides of the aquarium to provide continuous lighting during the experiments. Water flowed gently into and out of the arena through tubes at each end of the aquarium, thus the aquarium received a softly continuous flow of filtered and aerated water which did not affect the movement of crayfish. Crayfish were not biased to move toward the flow side. A digital Carl Zeiss Sony DCR-VX1000-NTSC camera with 40× optical zooming was mounted on the ceiling to cover the entire aquarium. A removable Plexiglas barrier separated the aquarium into two zones (Fig. 2), one comprising four white walls with a hard floor covered with crushed concrete gravel that composed of unconsolidated rock fragments of size classes roughly between 5 and 10 mm. The other zone comprised of four white walls and a floor covered with soft sand. The materials covered the entire floor of the aquarium, so that the animals could not detect edges. The differences between the two compartments consisted of soft and hard textural cues.

In this experiment, we used the place conditioning test to examine the rewarding effects of cocaine, while changes in locomotor activity levels were examined in an open-field unconditioned test to assess behavioral sensitization by measuring the locomotion independently. We took into consideration that utilizing the conditioning trials to assess the locomotor responses can result in the measurement of conditioned responses (not sensitization; Martin-Iverson and Reimer, 1996). For this reason, we measured the locomotor activities of the animals using an open field paradigm, keeping in mind that sensitization and conditioned place preference are generally considered separate phenomena (Seymour and Wagner, 2005). For the unconditioning experiment, we constructed a rectangular aquarium made from the opaque Plexiglas (150 mm × 90 mm × 75 mm; length, width and height). Aerated water was gently and continuously passed through the tank. The four strip lamps of 20 W florescent bulbs mounted at the sides provided lighting for video recording of behavioral activities of the animals.

2.3. Surgical protocol

We anesthetized the animals by chilling them in crushed ice for about 20 min in preparation for surgery. Using the tip of the injection needle, we created an incision in the caudal 1/3 of the dorsal carapace, lateral of the midline to avoid damaging the heart blood vessels. Since the hematopoietic system of crayfish is interiorly located, we focused our surgery on the dorsal carapace of the second abdominal segment. This approach provided significant success in our surgeries without damaging the heart. Following successful surgery, a 15 mm section of deactivated, fine-bore, fused silica capillary (Agilent, i.d. = 250 μm) was implanted into

the pericardial sinus, about 3.0 mm deep (to prevent piercing the heart and destroying the hemaotopic system), and stiffened with crazy glue. After successful surgery, animals were returned to their plastic holding containers overnight for recovery.

2.4. Drug injections

A microdialysis swivel (intech, 375/25p,CMA Model 102, CMA Microdialysis Inc., North Chelmsford, MA, USA) was used to systematically inject cocaine (HCl (FW: 339.8; Sigma, St. Louis: C 5776) at 3.0 μg/g, 6.0 μg/g and 12.0 μg/g of the animal body weight into the pericardial system of crayfish. The different doses referred to the free base concentrations, and the cocaine was prepared in 125 mM saline. Different doses of cocaine were injected directly into the pericardial system which serves as a primary neurochemical site for endogenous monoamine release (Panksepp and Huber, 2004b; Tierney et al., 2003). Injections of 125 mM saline served as control. During injection protocol, we connected the deactivated, fine-bore, fused silica needle (Agilent, i.d. = 100 μm) to the implanted cannula with a short segment of Tygon microbore tubing (Fisher Scientific, i.d. = 250 μm). The injection was administered into the second abdominal segment in a lateral position to the nerve cord (Fig. 3A and B). The syringe remained in place for approximately 15 s to avoid leakage from the point of injection.

2.5. Behavioral analysis

Initial experiments were focused on characterizing the general behavioral effects of cocaine administration. We tracked the behavioral activities using a custom-designed video tracking system that processes a single video frames at 320 ms from a camera (Sony DCR-VX1000). Conditioned and unconditioned behavioral displays were analyzed using the Any-maze software (Stoelting Co. USA). All behaviors were observed through a camera projected to a computer monitor, such that the animals were not disturbed in the enclosed arena. Our experimental area was enclosed, such that no observer was visible to the animal.

2.6. Cocaine-induced CPP

We assigned our experimental animals into three groups ($n = 9$ per group) including control, hard-texture/cocaine and soft-texture/cocaine groups, such that the hard-texture/cocaine or soft-texture/cocaine group received 3.0 μg/g, 6.0 μg/g or 12.0 μg/g dose of cocaine during conditioning. The control group received saline injection during conditioning. Our experimental design consisted of three phases: pre-exposures that explore the spatial activities of crayfish, the conditioning and the CPP test. In the pre-exposure test, we explored the spatial characteristics of crayfish locomotion within the test aquarium by placing individual crayfish ($n = 9$) in the test aquarium for 2 consecutive days for 60 min. We measured their movement and spatial activities in the aquarium, using the video tracking system. A detailed description of the experimental design used in the current study is presented in Fig. 4.

During the conditioning trials, we attached the injection cannula to the tubing, and directly connected it to the crayfish. The animal was gently placed in the experimental aquarium, followed by cocaine injection for the first 5 min of the 30 min session. Conditioning sessions were conducted twice per day. Each animal was restricted to one side of the CPP apparatus for 30 min during a morning session, and confined to the opposite side of the apparatus for 30 min during the afternoon session. We used the biased CPP design by pairing the unconditioned stimulus (US) with the initially non-preferred side of the apparatus. We adapted this approach because previous studies (Thiel et al., 2008, 2009) revealed that though reward-CPP is established regardless of whether a biased or unbiased design is used, the biased design approach has an advantage of greater sensitivity in detecting varying degrees of preference shifts (Thiel et al., 2009).

All possible pairwise counterbalanced combinations of environment and drugs were tested during conditioning. For instance, the starting side for the first conditioning session was counterbalanced, such that the hard-texture/cocaine group was exposed first to their initially hard-texture non-preferred side immediately following drug injection, and the control group of crayfish was exposed to their initially soft-texture preferred side immediately following saline injections. The animals received the opposite of these conditions during the afternoon session. Likewise, the soft-texture/cocaine group was first exposed to the soft-texture preferred environment following cocaine injections followed by saline injections. The control group was exposed to the hard-texture non-preferred environment. Also, the animals received the opposite of these conditions during the afternoon session. The control group consisted of crayfish that received vehicle infusions in both the hard-texture and soft-texture environments. A vehicle treated animal received 2 saline injections each day. Conditioning sessions were conducted at the same time each day. Each animal received cocaine injections for 5 successive days. A conditioning animal received two injections per day; 1 drug and 1 vehicle injection per day. Morning and afternoon sessions were separated by 6 h (10.00 am and 4 pm) to allow for sufficient cocaine clearance from the hemolymph.

After discontinuing the repeated drug administration, we measured the conditioned preference. For the CPP test, we removed the Plexiglas barrier and placed each crayfish at the center of the aquarium. We allowed the animal free access to both the hard and soft-texture compartments for 60 min, thus maintaining the same protocol that we used when measuring the spatial activities of crayfish in an unconditioned environment. We recorded the amount of time spent in each environment to measure the individual unconditioned preference. We used increased time spent in the paired environment as a measure of preference for the specific stimulus, whereas a decrease in time spent indicated an aversion (conditioned place aversion).

In this experiment, we used the conditioned place preference procedure approach to examine the rewarding effects of cocaine in crayfish by pairing cocaine as the unconditioned stimulus with two contrasting tactile environments. We gave the animal an opportunity to choose to enter and explore either environment, and the time spent in either environment was considered as an index of the reinforcing value of cocaine. The animal's choice to spend more time in either the soft or hard compartment was considered an expression of the positive reinforcing experience within that compartment. Our CPP test associated cocaine consumption and the memorized environment that we in turn, used to assess the rewarding properties of cocaine in crayfish.

2.7. Cocaine-induced unconditioned locomotion test

For the unconditioned locomotion test, we used a different group of crayfish, and each crayfish was injected with one of the three doses of cocaine (3.0 μg/g, 6.0 μg/g or 12.0 μg/g, $n = 9$ for each dose) a day after the surgery. The crayfish was placed into the aquarium and injected with cocaine over 5 min followed by continued tracking without infusion for another 60 min. In this experiment, we used the open field experiment to measure the locomotion (distances traveled) of crayfish during cocaine or saline injection.

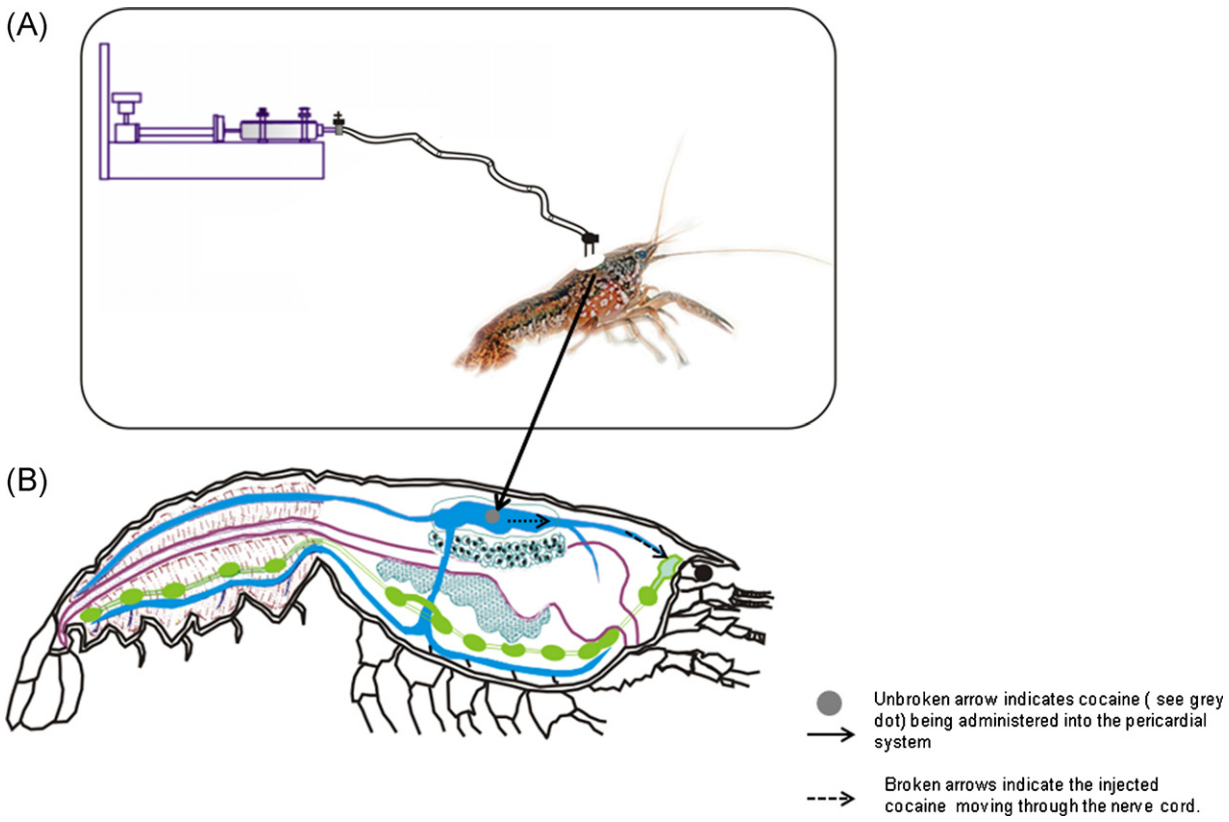

Fig. 3. Injections of cocaine into the pericardial system of crayfish. During cocaine injection, 0.5 m of deactivated, fine-bore, fused silica needle (Agilent, i.d. = 100 μm) was coupled to a crayfish with implanted cannula in the pericardial system (A). The coupling was done using Tygon microbore tubing (Fisher Scientific, i.d. = 250 μm). In turn, the tubing was connected to a microdialysis swivel (Intech, 375/25p). We injected the different doses of cocaine (3.0 μg/g, 6.0 μg/g and 12.0 μg/g; indicated by the grey dot, pointed to by the black unbroken arrow) directly into the pericardial sinus magnified in (B). We injected into the pericardial system because the pericardial organs of crustaceans are primary sites of monoamine release, and any manipulations of amines at that site (see grey dot) are delivered to the nerve cord and to the brain (black broken arrows indicate the movement of the injected drug through the nerve cord).

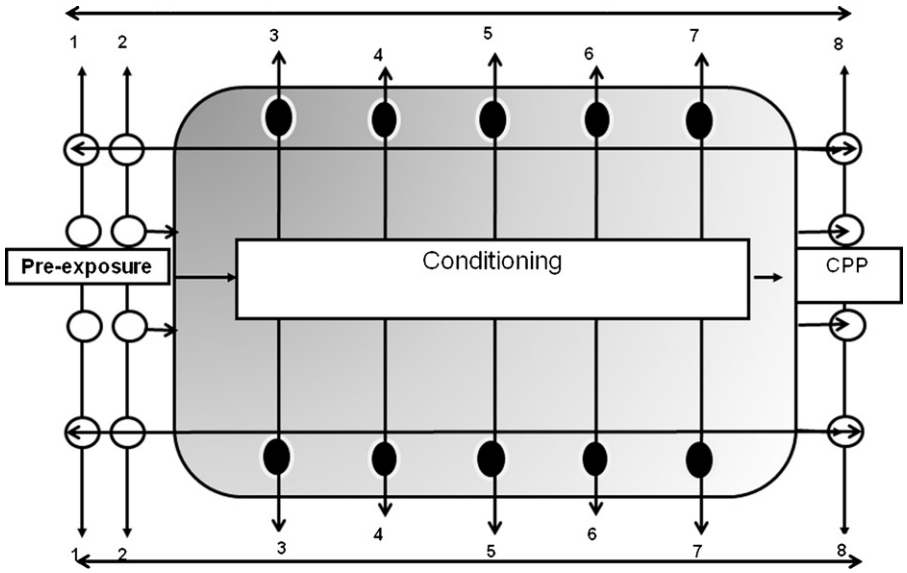

Fig. 4. Schematic illustration of the experimental design used in the current study. Our experimental design comprised of three phases: pre-exposures that explore the spatial activities of crayfish, the conditioning and the CPP test. In the pre-exposure test, we explored the spatial characteristics of crayfish within the test aquarium by placing individual crayfish ($n = 9$) in the test aquarium for 2 consecutive days for 60 min. We measured their movement and spatial activities to test for the initial preference in days 1 and 2. The conditioning consisted of 5 alternate days (3–7) of drug and saline injections. We used the unbiased balanced approach. For the conditioning experiment, crayfish received cocaine injections in both the hard-texture and the soft-texture environment in a counterbalanced biased approach experimental design (see details in Section 2). We injected cocaine (3.0 μg/g, 6.0 μg/g and 12.0 μg/g) alternately for 5 consecutive days. Following conditioning, we confined the animals to the conditioning compartment for 30 min. The partition separating the compartments was removed, and on day 8, crayfish were placed at the center and allowed to move freely for 60 min. No injections were given on day 8 of the preference test, thus maintaining the same procedure as that used during the preliminary baseline test of exploring the spatial activities of crayfish. The crayfish was allowed free access to the entire aquarium for 60 min. The amount of time spent in each compartment was recorded to assess individual unconditioned preferences.

2.8. Removal of Brain and extraction of tissues from the accessory lobes

After the CPP and unconditioned test, crayfish were deeply anaesthetized in crushed ice for 10 min. The implanted cannula was gently removed, and the crayfish was mounted on a Sylgard dish dorsally, such that the ventral side was up. We pinned the animal 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. We removed the cuticle plate 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. We gently mounted the fresh wet brain on slide. Without covering it with a slide cover, we viewed and identified the accessory lobe on a microscope and carefully used a thin scapel to extract nervous tissue from both accessory lobes of the crayfish brain. The extracted tissue was transferred to the fixing medium using a thin curvy forcep. Following the successful extraction of the accessory lobes tissues, we fixed the tissues samples in RNA*later* solution of c-Fos mRNA and subsequently tested for c-Fos mRNA 30 min after extraction from the brain taking into account that c-Fos mRNA is rapidly induced, within 30–60 min and c-Fos protein after 90–120 min, such that both mRNA and protein expression return to baseline after 6 h (Zangenehpour and Chaudhuri, 2002).

2.9. c-Fos mRNA extraction from the accessory lobes of crayfish brain, Isolation and RT-PCR analysis

We analyzed the expression of c-Fos mRNA in the brains of animals tested for reward ($n = 9$) and locomotion ($n = 9$) for the 3.0 μg/g or 12.0 μg/g doses of cocaine regime. We did not analyze for animals with 6.0 μg/g because some animals died after drug treatments and CPP test. UltraClean Tissue and Cells RNA isolation kit (MoBio Laboratories Inc. Carlsbad, CA) were used to homogenize the accessory lobes and RNA extraction. Crayfish brains were removed from RNA*later* solution and homogenized with sterile plastic pestle in 300 μl of TR1 solution. RNA extraction was done strictly according to manufacturer's protocol. Total RNA was amplified and cDNA was made using oligo dT_{20} primer (Invitrogen) and Supercript III RT-PCR (Invitrogen) following the protocol provided in the kit. Two primers, 2c-fos Forward: 5′-GCAGGCAGCAGC TAA ATG and 2c-fos Reverse: 5′-GCA CAGGGGCTCAAAGTC, specifically designed for crayfish were used. Amplifications were performed in a mix reaction containing 1× PCR buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl, pH 8.8, 0.01% Tween-20], 0.2 mM of each d(NTP)s, 2 mM of$MgCl_2$, 0.05 U/μl of Taq DNA polymerase (EuroClone), and the corresponding oligonucleotides (forward and reverse), 1 lM for c-Fos and 0.4 lM for GAPDH. The cycling parameters of c-Fos amplifications were as follows: 95 °C 30 s, 60 °C 30 s, 72 °C 30 s, 30 cycles, giving a 353-bp fragment. For GAPDH, they were; 95 °C 30 s, 60 °C 30 s, 72 °C 30 s, 25 cycles, giving a 376-bp fragment. We used PCR analysis of GAPDH to control cDNA quantities used as templates for PCR assays. PCR products were electrophoresed on 1.2% agarose gels, stained with ethidium bromide (Sigma), and visualized under UV light. We confirmed the specificity by the presence of a single band of the expected size. Densitrometric analysis was performed using the AIS Imaging System software (Ontario, Canada). Results were shown as mean ± S.E.M. of four different experiments.

2.9.1. Statistical analysis

All statistical analyses were done using the SPSS version 11 (SPSS Science, Chicago, IL). Data for the pre-conditioning and CPP test outcomes were analyzed by determining the time spent in each compartment. A direct comparison of time spent between the soft and hard-texture cues were analyzed using Student's *t*-test. The CPP data were analyzed at 15 min time bins to determine the dose and time course expression of a cocaine-induced CPP. Further analysis was done using the One-way ANOVA with repeated measures that compares the percentage of time-spent intervals, and assessed the pre-treatment of CPP-induced rewarding effect of cocaine. We used a 3 × 4 mixed model ANOVA to compare between-group variance different doses of cocaine (3.0 μg/g, 6.0 μg/g and 12.0 μg/g), and individual time intervals. This analysis assessed the pre- and 5 day post-treatment of CPP-induced rewarding effect of cocaine. For the densitometric analysis, we used Student's *t*-test for the direct comparison of the effect of cocaine on c-Fos mRNA expression in the conditioned and unconditioned test. One-way ANOVA was used to determine the effect of cocaine on the c-Fos mRNA expression in the conditioned or unconditioned test. Bonferoni Post hoc mean comparisons were additionally used to compare the percentage of time spent in the compartment. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Movement and spatial activities of crayfish without cocaine treatment

We have previously demonstrated that repeated intracirculatory infusions of 2.5 μg/g, 5.0 μg/g and 10.0 μg/g doses of morphine over 5 days serve as a reward when paired with a distinct visual or tactile environment (Nathaniel et al., 2009; Dziopa et al., 2011), and stimulate unconditional behavioral responses in crayfish (Nathaniel et al., 2010). A previous study (Panksepp and Huber, 2004b) reveals that crayfish is sensitive to incentive properties of the conditioned stimuli when paired with lower doses of cocaine. The aforementioned studies encouraged us to examine context-specificity of the cocaine-conditioned novelty effect in altering c-Fos mRNA expression in the accessory lobe of cocaine treated crayfish.

To examine the unconditioned preference for the soft or hard-texture environment, we analyzed the spatial activities of crayfish without cocaine treatment in the soft and hard-texture environment ($n = 9$). The results revealed that crayfish showed preference for the soft-texture environment during the repeated measures of the activities for 60 min in each day of the test (Fig. 5). This contradicts our hypothesis that crayfish will spend an equal amount of time in each of the tactile environments. In the first day, crayfish spent 62.24% ± 2.8 (S.E.M.) of its time in the soft-texture environment, and 38.59% ± 2.7 (S.E.M.) in the hard-texture environment. The preference for the soft-texture environment was significant (*t*-test (=50.0%); $t[_8] = 4.25$, $P = 0.002$). During the second day of testing, the crayfish maintained its preference for the soft-texture environment (59.04% ± 4.0), while 40.63% ± 3.9 (S.E.M.) of its time was spent inside the hard-texture environment. The preference for the soft-texture environment was again statistically significant (*t*-test (=50.0%); $t[_8] = 2.31$, $P = 0.049$). These results indicate a crayfish's baseline preference for the unconditioned soft-texture environment. Because there was a preference for the soft compartment in the baseline studies, we did not use any of the animals with inadequate expression of clear choice preference for the soft environment.

3.2. Repeated injections of cocaine produce a context specific reward in the hard-texture environment to crayfish during a CPP conditioning test

For the hard-texture/cocaine group, conditioning resulted in a robust preference for the drug-paired compartment, such that crayfish spent a greater amount of time in the cocaine-paired hard-texture environment than in the saline-paired compartment

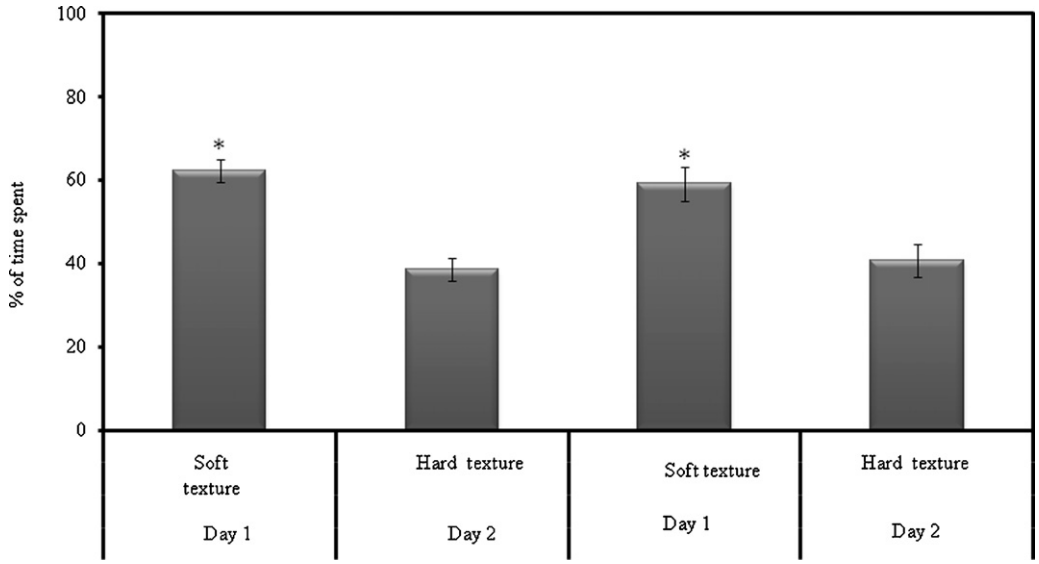

Fig. 5. The activities of crayfish without cocaine treatments in the soft and hard compartments. Our hypotheses was that crayfish will spend equal amount of time in each of the tactile environments. On the contrary, crayfish showed preference for the soft compartment during the repeated measures of the activities for 60 min each day. For instance, in the first day, the preference for the soft-texture environment was significant (*t*-test (=50.0%); $t[_8] = 4.25$, $P = 0.002$). During the second day of testing, crayfish maintained its preference for the soft-texture environment, and the preference for the soft-texture environment was again statistically significant (*t*-test (=50.0%); $t[8] = 2.31$, $P = 0.049$). These results indicate a crayfish's baseline preference for the natural, unconditioned preference for the soft-texture environment.

(Fig. 6). The result reveals that five days of repeated injections of 3.0 μg/g produces 52.68% ± 0.37 (S.E.M.), while 6.0 μg/g and 12.0 μg/g promote spatial activity of 59.35% ± 0.30 (S.E.M.) and 65.16% ± 0.6 respectively of time preference for the hard-texture environment [$F(5,40) = 763$, $P < 0.001$] during monitoring of activity for 60 min.

For the soft-texture/cocaine group, crayfish spent a greater amount of time in the saline-paired compartment (3.0 μg/g; 57.92% ± 0.23 S.E.M., 6.0 μg/g; 59.07 ± 0.37 S.E.M., 12.0 μg/g; 60.56% ± 0.39 S.E.M.) than in the cocaine-paired soft-texture compartment 3.0 μg/g; 42.53% ± 0.13 S.E.M., 6.0 μg/g; 41.69 ± 0.15 S.E.M., 12.0 μg/g; 39.69% ± 0.18 S.E.M.), indicating that vehicle treated-crayfish exhibited a natural preference for the soft-texture compartment (Fig. 7). ANOVA found a significant preference for the saline-paired compartment [$F(5,40) = 757$, $P < 0.001$]. Post hoc pair-wise comparisons analysis revealed no significant difference ($P > 0.05$) between the means of time spent in the cocaine-paired conditions, and saline-paired conditions.

A 3 × 4 model ANOVA for the between-groups variable, dose of cocaine (3.0 μg/g, 6.0 μg/g or 12.0 μg/g) and 15 min time interval reveals a significant effect [$F(2,24) = 682.39$, $P < 0.001$] of different doses of cocaine, a significant effect of time interval (0–15 min, 15–30 min, 30–45 min, 45–60 min) in the expression of CPP [$F(3,72) = 642.59$, $P < 0.001$], and a significant interaction

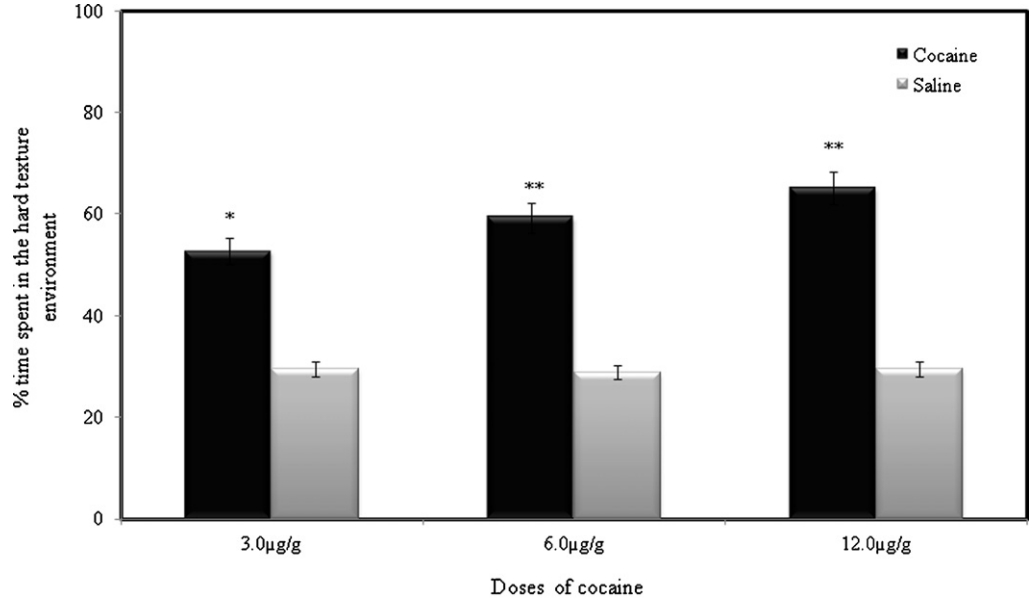

Fig. 6. Repeated infusions of cocaine-induced CPP in crayfish ($n = 9$) in the hard-texture environment. Five days of repeated injections of 3.0 μg/g produces significant time preference for the hard-texture environment during monitoring of the activity for 60 min. At all cocaine doses (3.0 μg/g, 6.0 μg/g and 12.0 μg/g) for the hard-texture/cocaine pairing, crayfish significantly spent a greater amount of time in the cocaine-paired hard-texture environment [$F(5,40) = 763$, $P < 0.001$] than in the saline-paired compartment. The effect of 6.0 μg/g and 12.0 μg/g induced-place preference were not significantly different (**$P < 0.05$), but significantly higher than the effect of 3.0 μg/g (*$P < 0.05$) dose of cocaine.

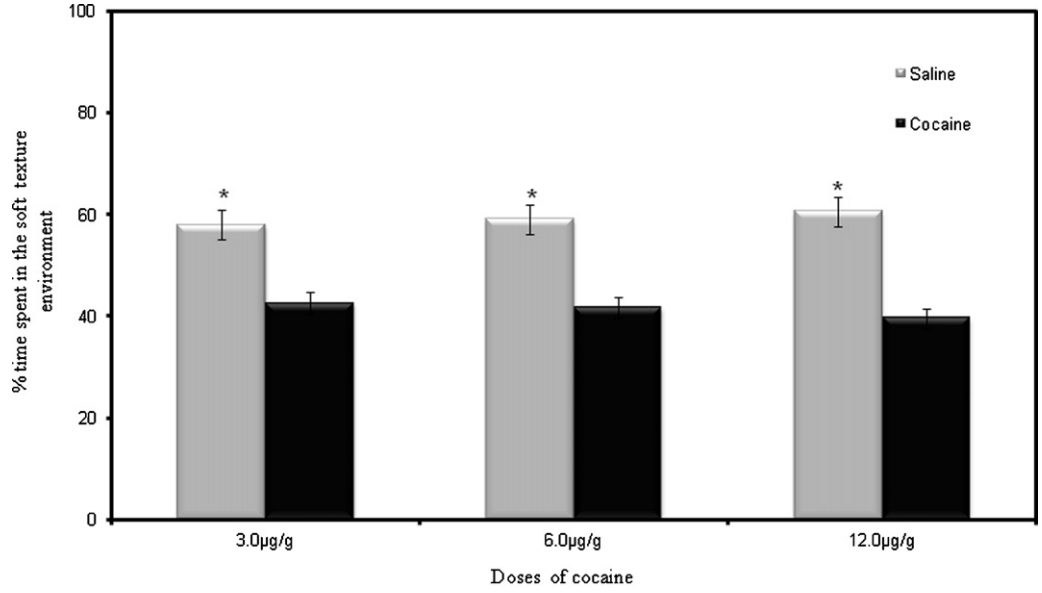

Fig. 7. Repeated infusions of cocaine in crayfish ($n = 9$) for the soft-texture environment during CPP test. Paired repeated injections of 3.0 μg/g, 6.0 μg/g and 12.0 μg/g doses of cocaine did not produce a CPP in the soft-texture, such that crayfish spent a greater amount of time in the saline-paired compartment than in the cocaine-paired soft-texture compartment ($F(5,40) = 534$, $P < 0.005$].

between drug and time [$F(6,72) = 55.17$, $P < 0.001$; Fig. 8A–C]. Post hoc pair-wise comparison revealed no significant ($P > 0.05$) difference between means of the saline-paired conditions. At 6.0 μg/g and 12.0 μg/g doses, cocaine-induced CPP became increasingly stronger as the test session progressed, such that conditioning effects became very obvious in the second phase (30–45 min and 45–60 min) of the test session ($P < 0.05$).

3.3. Five days of repeated injections of cocaine enhance locomotion in crayfish

We lost four of the animals in the 6.0 μg/g group, thus, we analyzed locomotion activities of animals that received 3.0 μg/g and 12.0 μg/g doses of cocaine (Fig. 9). Repeated intracirculatory injections of cocaine for five days significantly [$F(19,219) = 11.17$, $P < 0.001$] increased locomotion behavior of crayfish. *t*-Test paired-sample analysis revealed a significant difference in the effect of 12.0 μg/g (mean 1.68 m ± 0.38) (*t*-test; [10] = 0.18, $P = 0.091$) in increasing locomotion when compared to the effect of 3.0 μg/g (mean 1.49 m ± 0.35).

3.4. Cocaine pre-exposure produces a context-specific c-Fos mRNA expression in the accessory lobe of crayfish brain

To determine whether the repeated injection of cocaine that produced a context specific reward in the hard-texture environment is associated with the enhancement of c-Fos mRNA expression in the accessory lobe, we used PCR to analyze the expression of c-Fos mRNA in the brain of crayfish that showed reward to cocaine and during the unconditioned treatments. Densitometric analysis revealed that a sensitization pretreatment schedule induced significant changes of c-Fos mRNA expression in the accessory lobe at 3.0 μg/g and 12.0 μg/g doses of cocaine [$F(3,23) = 162$, $P < 0.001$] in the open field test when compared with the control animals following measurement of locomotor activity (Fig. 10A). The levels of c-Fos mRNA remained low in the control group, and were relatively constant during the open field unconditioned experiments. Bonferoni post hoc test revealed that the effect of 3.0 μg/g and 12.0 μg/g doses of cocaine seems to have the same magnitude of effect ($^*P < 0.05$; 12.0 μg/g, $^*P < 0.05$), meaning that there is no statistically significant evidence that their mean values differ.

PCR analysis also revealed that the mRNA levels of c-Fos in the accessory lobe increased in response to conditioning test after 5 days of cocaine treatments (Fig. 10B). Densitometric analysis revealed significant effect of cocaine injections on c-Fos mRNA [$F(3,27) = 924$, $P < 0.001$] when compared with the control group. Specifically, c-Fos mRNA was induced maximally at a high dose of cocaine (12.0 μg/g; $^*P < 0.05$) when compared with a low dose of 3.0 μg/g ($^{**}P < 0.05$).

We used the Student *t*-test to compare the effect of cocaine in inducing c-Fos mRNA expression in the conditioned and unconditioned tests. *t*-Test paired sample analysis revealed that at 3.0 μg/g, the effect of cocaine in increasing c-Fos mRNA was higher for the conditioned test (*t*-test (=50.0%); $t[_5] = 4.29$, $P < 0.001$) when compared with the unconditioned test. Similarly, 12.0 μg/g dose of cocaine significantly induced more c-Fos mRNA in the conditioning test (Student's *t*-test (=50.0%); $t[_5] = 5.18$, $P < 0.001$) than the unconditioned test. Taken together, our results indicate that the repeated injection of cocaine that produced a context specific reward in the hard-texture environment is also associated with the enhancement of c-Fos mRNA expression in the accessory lobe of crayfish brain.

4. Discussion

Our present study in crayfish is built upon previous studies (Nathaniel et al., 2009, 2010; Panksepp and Huber, 2004b), that suggest that crayfish could provide a unique comparative model for the study of reward mechanisms in drug treated animals. The current study reveals that 3.0 μg/g, 6.0 μg/g and 12.0 μg/g doses of cocaine are rewarding to crayfish when paired with a distinct tactile environment. This finding supports a previous finding by Panksepp and Huber (2004b), and further strengthens the hypothesis that irrespective of dose, cocaine is rewarding to crayfish when paired with a distinct environmental cue, suggesting the significance or noticeability of a specific visual (Panksepp and Huber, 2004b) or tactile environmental cue as novel to crayfish.

In the current study, such specificity may be related to the perception of the tactile hard-texture environment to be relatively novel when compared to the soft-texture environment of the test

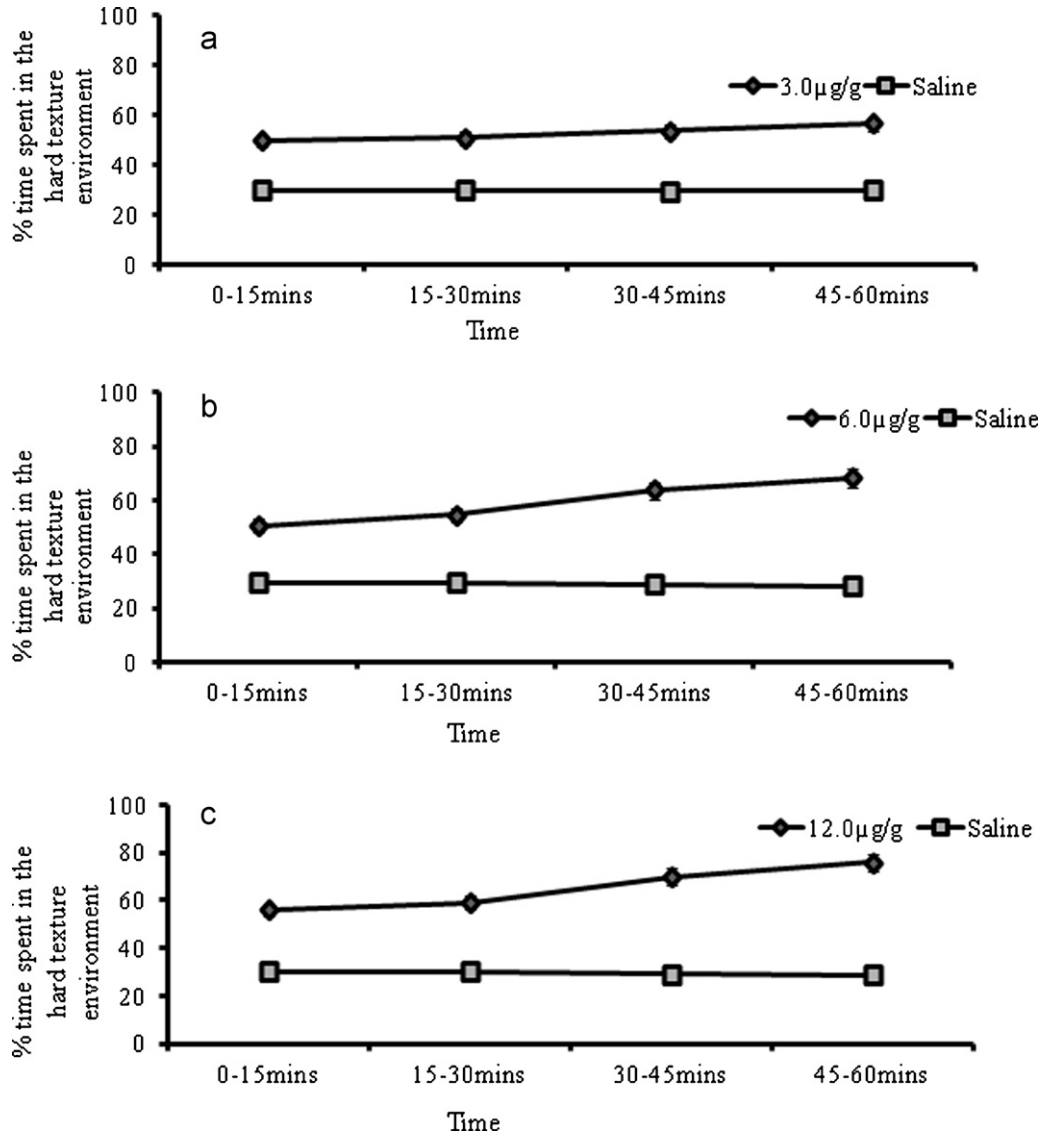

Fig. 8. (A)–(C) Doses and time effect on the expression of CPP during cocaine conditioning for 5 days. Paired repeated doses of cocaine (3.0 μg/g, 6.0 μg/g and 12.0 μg/g) injections produced a CPP in the hard-texture compartment. We measured the CPP test at 15-min bin interval for a 60 min CPP test session. Data were expressed as mean percentage time spent (%) ± S.E.M. ($n = 9$) for each crayfish injected with cocaine or saline. A 3 × 4 model ANOVA for the between-groups variable, dose of cocaine (3.0 μg/g, 6.0 μg/g or 12.0 μg/g), and 15 min time interval indicate a significant effect of different doses of cocaine [$F(2,24) = 682.39$, $P < 0.001$], a significant effect of time interval (0–15 min, 15–30 min, 30–45 min, 45–60 min) in the expression of CPP [$F(3,72) = 642.59$, $P < 0.001$], and a significant interaction between drug and time [$F(6,72) = 55.17$, $P < 0.001$]. Post hoc pair-wise comparisons revealed no significant ($P > 0.05$) difference between means of the saline-paired conditions. Post hoc pair-wise comparison analysis revealed no significant difference ($P > 0.05$) between means of the cocaine-paired conditions at 3.0 μg/g for the 60 min test session. At 6.0 μg/g and 12.0 μg/g doses, cocaine-induced CPP became increasingly stronger as the test session progressed, such that conditioning effects became very obvious in the second phase (30–45 min and 45–60 min) of the test session ($P < 0.05$).

aquarium. In fact, this would not be surprising since crayfish hide under rocks (with hard-texture) for shelter in their natural environments, such as streams or creeks. The specificity in identifying the hard-texture environment may be related to the inherent ability to use tactile cues to find a hard rock for shelter (Alberstadt et al., 1995), especially when they need to withdraw under a rock, waiting for dark, at which time they come out to forage for food. The structural characteristics of a preferred environment or object by crayfish are modulated by the neural mechanism that is associated with tactile response (Baird et al., 2006; Basil and Sandeman, 2000). Crayfish might have used such ability to distinguish between the two contrasting environments provided during the CPP experiments, and the hard-textured rocks may have been perceived as relatively novel compared to the soft nature of the soft sand environment in the aquarium. In this context, novelty is conceivably implicated in the susceptibility of stimulus to a conditioning phenomenon in crayfish.

The central nervous system of crayfish contains neuromodulatory monoamines, including dopamine; this is a target of all drugs of abuse, as cocaine interferes with the reabsorption of dopamine and prevents dopamine from being recycled. This mechanism is observed in both invertebrates and mammals. It contributes to the broad neurochemical process of drug-induced reward that generally hinge on the dopaminergic system (Carr, 1984; Crisp and Mesce, 2006; Ikemoto and Panksepp, 1999; Marinelli et al., 1998; Tassin, 2002). The dopamine hypothesis suggests that the dopamine system regulates motivational-affective responses (Panksepp, 1998; Partridge and Schenk, 1999), reward-related stimuli (Robinson and Berridge, 1993, 2008), and learning processes (Schultz, 1997; Shohamy et al., 2007). In addition, the

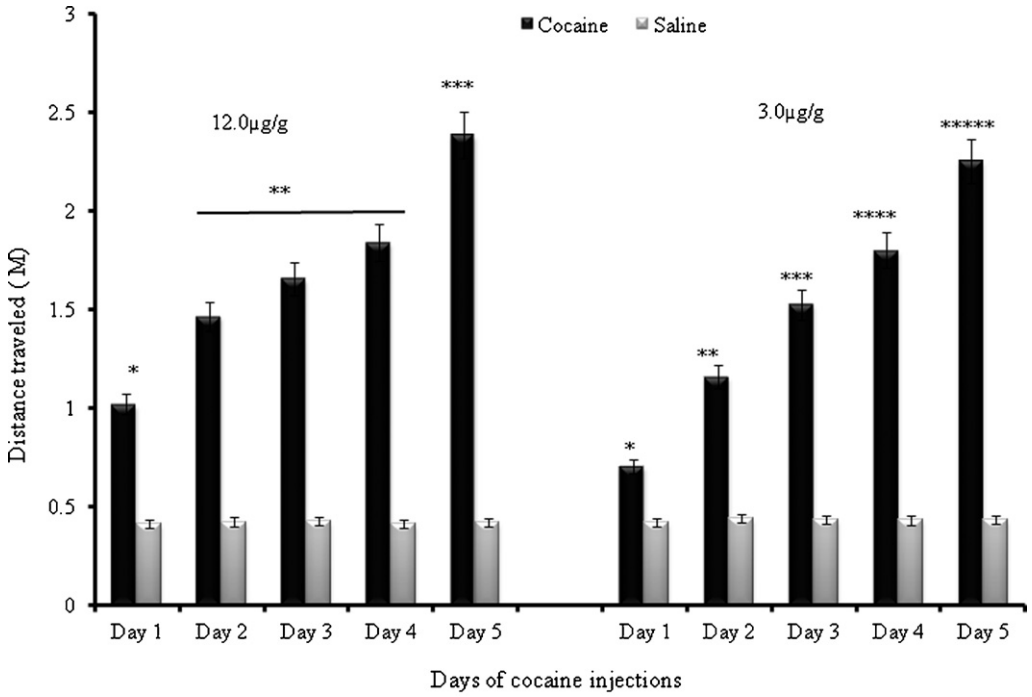

Fig. 9. The effect of 3.0 μ/g or 12.0 μg/g dose of cocaine on locomotion behavior of crayfish. The distances traveled following repeated intracirculatory injections of 12.0 μg/g dose of cocaine for five days was significant [$F(19,219) = 11.17$, $P < 0.001$] with Post hoc test differentiating all days from each other (*, **, ***, $P < 0.05$). 3.0 μg/g dose of cocaine significantly [$F(13,23) = 8.78$, $P < 0.001$] increased locomotion during repeated treatments for five days. Post hoc test differentiates all days from each other (*, **, ***, $P < 0.05$). *t*-Test paired-sample analysis reveals a significance difference in the effect of 12.0 μg/g (mean 1.68 m ± 0.38) (*t*-test; [10] = 0.18, $P = 0.091$) in increasing locomotion when compared to the effect of 3.0 μg/g (mean 1.49 m ± 0.35).

dopamine system is involved in the Pavlovian mechanism of a cue attractiveness that associates motivational salience to drug rewarding. Dopamine is a neurochemical signal that is conserved and shared across all mammals and non mammalian species (Ilona et al., 1999). Interestingly, dopamine-containing neurons in crayfish are accessible and relatively large with about 30–35 somata located in the brain, while 25–30 of the dopamine neurons are located in the suboesophageal ganglion (Tierney et al., 2003). Our current result indicates that previously sensitized crayfish exhibited significantly enhanced conditioned place preference relative to control animals. In this context, the repeated pairing of the two different textural compartments with cocaine presumably led to an established CPP that provided measures of the incentive or motivational properties of cocaine in crayfish. A change in a potential affective state of crayfish and associative learning possibly engineer the formation of a conditioned response to the cocaine-paired hard-texture environment.

Our finding that exposure to a cocaine-paired environment increased c-Fos mRNA expression in the brain of crayfish is in agreement with studies that examined Fos and Fos-related antigens using various animal models of cue-elicited drug reward (Brown et al., 1992a; Franklin and Druhan, 2000a; Ciccocioppo et al., 2001; Miller and Marshall, 2004; Neisewander et al., 2000). Fos is an IEG product that after forming a heterodimer with the products of other IEGs, binds to the AP-1 promoter site in DNA to regulate transcription of various target genes. The basal level of Fos expression is low in neurons, but is transiently increased via second messenger systems following stimulation (Curran et al., 1996, 1985). Our result indicates that cocaine-induced behavioral conditioning was associated with an increase in c-Fos mRNA expression in the accessory lobe of crayfish, suggesting that cocaine-environment association altered gene-regulatory processes.

Studies that investigated the mechanism of environment association alteration of gene-regulatory processes associated with c-Fos mRNA expression in drug treated animals emanated from functional studies in vertebrates (Brown et al., 1992b; Freet et al., 2009; Hotsenpiller et al., 2002; Kufahl et al., 2009; Zavala et al., 2007, 2008). These studies reveal that an increase in neuronal activity triggers the transcription of immediate early genes, including the proto-oncogene c-Fos which, in turn, stimulates the transcription of AP-1-promotor-containing response genes that are responsible for adaptive changes in mature neurons. Several lines of evidence indicate that induction of c-Fos is a significant mechanism in the control of gene expression, and that multiple intracellular messengers might be involved. One extensively studied example is the dopamine-regulated expression of Fos (Hughes and Dragunow, 1995; Yamada et al., 2007). Cocaine is known to indirectly activate dopamine receptors, and the Fos responses elicited by cocaine has been linked to the activation of dopamine D1-like receptors. The activation of D1-like receptors couple with the stimulatory subsets of heterotrimeric proteins to stimulate cytosolic second messengers (Velázquez-Sánchez et al., 2009; Xu, 2008; Yamada et al., 2007; Zavala et al., 2007; Zawilska, 2003).

It is well known that the stimulation of second messengers and differences in the activation threshold for calcium-dependent signaling are important factors that regulate cellular responses to stimulatory signals, including the transcriptional activation of Fos genes (Zawilska, 2003; Xu, 2008). Two major regulators of c-Fos transcription are the phosphorylated forms of mitogen-activated protein kinase (MAPK)[1] and CREB (Yamada et al., 2007). CREB is a Ca^{2+}/cAMP-responsive transcription factor that in its phosphorylated form (pCREB; phosphorylated at Ser^{133}) stimulates transcription of the c-Fos gene by interacting with the CRE transcriptional regulatory component. The MAPK cascade is known to be involved in the activation of c-Fos transcription either by phosphorylating CREB at Ser^{133} or by phosphorylating ternary complex factors that together with serum response factor, bind to the SRE element of the c-Fos promoter (Xu, 2008). Activation of

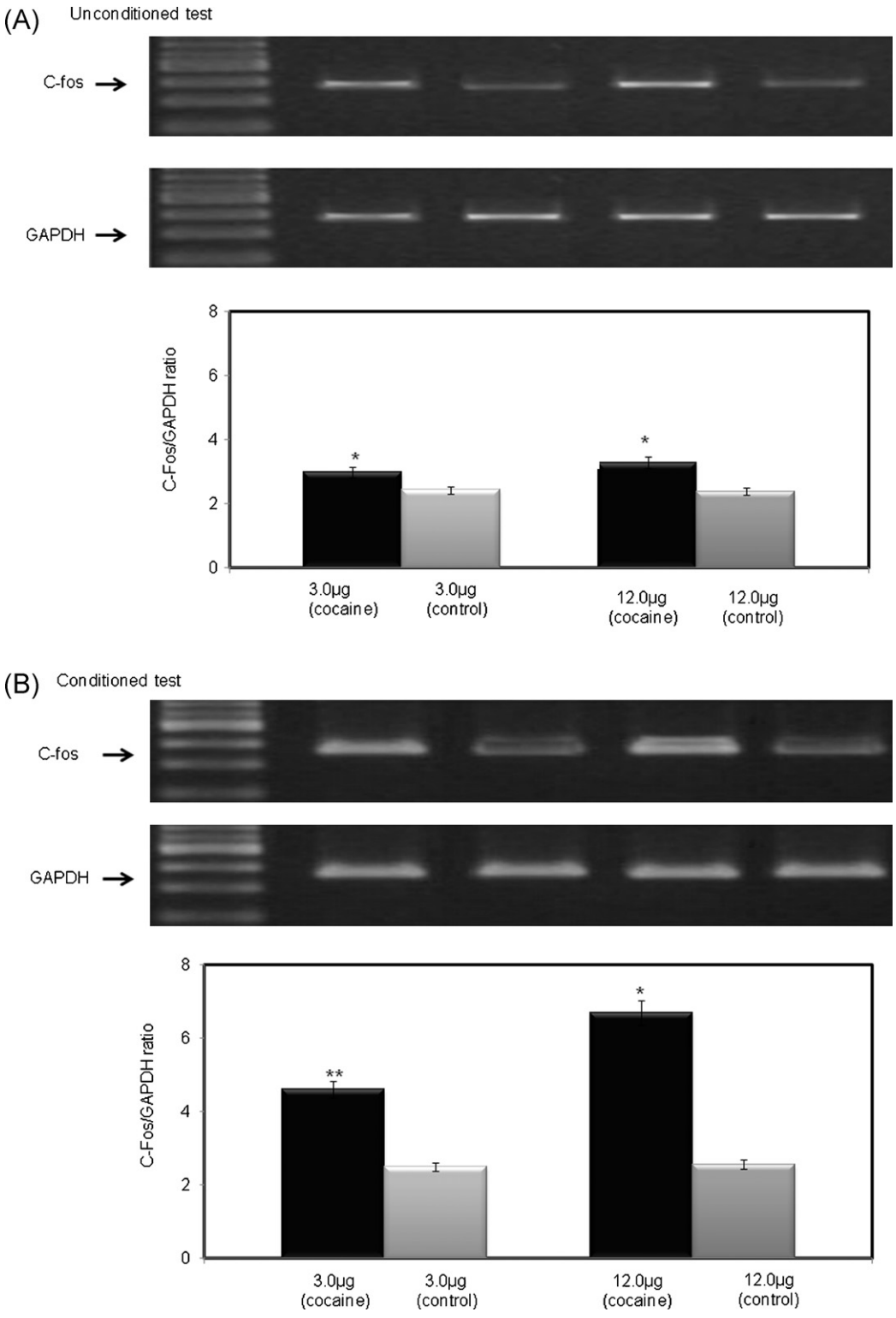

Fig. 10. c-Fos mRNA alterations in cocaine treated animals in conditioned and unconditioned tests. Alterations in *c-Fos* expression in the accessory lobe of the crayfish brain. Data represent mean ± S.E.M. at 35 min following CPP and unconditioning tests after 5 days of cocaine injections ($n = 9$). One-way ANOVA with Bonferoni post hoc test was performed· Student's *t*-test was used compare the effect of cocaine in inducing the c-Fos mRNA expression in the conditioned and unconditioned test c-*Fos* mRNA was measured by quantitative RT-PCR (Top panels) and normalized with GAPDH. Quantification was done by densitometry (Bottom panels). Values are means ± S.E.M. from four separate experiments. Sensitization pretreatment schedule induced noticeable changes in c-Fos mRNA expression patterns in the accessory lobe at 3.0 μg/g and 12.0 μg/g doses of cocaine [$F(3,23) = 162$, $P < 0.001$] when compared with the control animals following measurement of locomotor activity (A). The effect of 3.0 μg/g and 12.0 μg/g doses of cocaine on c-Fos mRNA were not significantly different (3.0 μg/g, $^{*}P < 0.05$; 12.0 μg/g, $^{*}P < 0.05$). Densitometric analysis revealed significant of conditioning effect of cocaine injections [$F(3,27) = 924$, $P < 0.001$] (B) when compared with the control group. c-*Fos* mRNA was induced maximally at a higher dose of cocaine (12.0 μg/g; $^{*}P < 0.05$) when compared with a low dose of 3.0 μg/g ($^{**}P < 0.05$). Student's *t*-test paired sample analyses revealed that at 3.0 μg/g, the effect of cocaine in increasing c-Fos mRNA was higher at the conditioned test (*t*-test (=50.0%); $t[5] = 4.29$, $P < 0.001$) when compared with the unconditioned test. Similarly, 12.0 μg/g dose of cocaine significantly induced more c-Fos mRNA in the conditioning test (*t*-test (=50.0%); $t[5] = 5.18$, $P < 0.001$) than the unconditioned test.

the MAPK cascade can also respond to growth factor stimulation, as well to increased calcium levels via a Ras-dependent pathway (Hotsenpiller et al., 2002).

Several other studies (Guan et al., 2008; McGillis et al., 2002; Schiller et al., 2006) linked the gene-regulatory processes associated with cocaine-induced c-Fos mRNA expression to multiple intracellular messenger pathways that are activated by depolarization and/or calcium entry, including the calcium dependent protein kinases, the protein kinase C pathway, and the cAMP/PKA pathway. Although the mechanism of gene-regulatory processes associated with cocaine-induced c-Fos mRNA expression is yet to be explored in crayfish, it is possible that the activation of the multiple intracellular messengers might be involved in the gene-regulatory processes associated with cocaine-induced c-Fos mRNA expression in the brain of crayfish. In this context, it is likely that injection of cocaine into the brain of crayfish may activate dopamine receptors. The activation of specific dopamine receptors may couple with the stimulatory subsets of heterotrimeric proteins to stimulate multiple intracellular second messengers that might be involved in dopamine-regulated expression of c-Fos gene- expression in the brain of crayfish. Future studies might help to elucidate the role of specific intracellular second messengers.

We found that 12.0 μg/g dose of cocaine significantly induced more c-Fos mRNA in the conditioning test when compared with injections of 3.0 μg/g of cocaine. On the other hand, the effect of both doses (3.0 μg/g and 12.0 μg/g were not significantly different in the unconditioned test. This result suggests that factors, such as dose and the environmental context for the drug administration can influence drug-induced reward and the alteration pattern of gene expression. We argue that the capability of the neural circuitry engaged by drugs to induce specific patterns of gene expression can be determined by the dose of the drug and the environmental context in which they are experienced. This may be connected to the ability of environmental novelty to facilitate drug-induced neuroplasticity. In this context, it is possible that when cocaine was administered to our experimental animals in a novel environment the primary neuropharmacological effects of cocaine on monoamine neurotransmission interacted with the effects of environmental novelty on reward system of crayfish. This interaction probably facilitated the alteration of c-Fos protein in our cocaine-conditioned crayfish, indicating that drug-environment pairing in a specific context is more effective in the alterations of basal c-Fos mRNA expression.

It is noteworthy that studies in vertebrates reveal that cocaine-induced alteration of c-Fos mRNA during reward in several cortical and limbic regions are highly dependent on the environment (Badiani et al., 1998, 1999). Taken together, our finding indicates that systemic cocaine given in advance can increase c-Fos mRNA expression caused by other stimuli at a specific brain target associated with reward in crayfish. Such a stimulus was the novelty effect as seen in the effect of the hard-texture stimulus in inducing reward when compared to the soft-texture stimulus.

We found that there was a significant enhancement of c-Fos mRNA in the accessory lobe, especially when crayfish were paired with a distinct tactile stimulus (hard-texture environment). This result reveals a context-specific expression of c-Fos mRNA in the accessory lobe of cocaine treated crayfish in the conditioning test. Therefore, it is possible that the accessory lobe is a constituent of higher brain structures in the crayfish brain that are associated with high-order selection of choices made in the environment, For instance, choosing a specific tactile or visual stimulus that is associated with reward. In this context, the accessory lobe maybe comparable to higher brain structures in the frontal regions of cerebral cortex of the mammalian system, such as medial prefrontal cortex, anterior cingulate cortex or orbitofrontal cortex that play a major role over choices made in the environment for example, whether to seek a reward.

The accessory lobe is thought to be specialized and closely related to the life style of crayfish that live in semi-permanent burrows in spatially complex environments which they explore in search of food, shelter or conspecifics (Sandeman et al., 1992). Successful adaptive behavior in such complex environments including learning and decision making about food, shelter or conspecifics requires accurate assessment of actions and choices, and contribute to a particular life style. The ability of crayfish's brain to integrate and control such adaptive responses may enhance the search for life-supporting environmental conditions including identifying protective shelters under the rock. In this context, a complete understanding of the facilitator of this adaptive behavior requires that it is eventually mapped onto its respective proximate brain targets. The functional neuroanatomies of conditioning-induced reward circuits in crayfish are not well known. Previous studies (Wachowiak et al., 1996; Sullivan and Beltz, 2005a) linked the accessory lobe to the benthic lifestyle of crayfish. Results from the current study support this idea, and suggest that the accessory lobe may play a major role in the successful adaption to the specific lifestyles of crayfish, especially in contributing to the neural circuit associated with the rewarding value of a particular stimulus or choice in a spatially complex environment that the crayfish live. Thus, our current work provide a basis for speculations or testing several hypotheses about the role of the accessory lobe as part of the brain substrates that are involve in the rewarding value of a particular stimulus. Future studies that examine Fos expression across the central nervous system of the crayfish could identify the circuit that facilitates drug-induced reward in crayfish. In this context, the accessory lobe might contribute to the increasing knowledge of such neural circuit. Representing an invertebrate animal model with proven evidence of rewarding to mammalian drugs of abuse, simplified, modularly organized and experimentally accessible nervous system makes crayfish exceptionally suitable for characterizing the central workings of addiction at its key neuroanatomic location, as elegantly done in the current study.

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