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# Picrotoxin but not bicuculline partially abolishes the cardio-inhibitory responses induced by visual stimulation in the crab *Neohelice granulata*

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## HIGHLIGHTS

- Cardio-inhibitory responses (CIR) are triggered by visual danger stimuli.
- GABA may be mediating the extrinsic regulation of CIR.
- Picrotoxin but not bicuculline partially abolished CIR.
- CIR are probably under an autonomic-like control.

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## ABSTRACT

Conspicuous and sustained heart arrests, revealed as an increase in the magnitude of cardiac interbeat intervals, are elicited in the crab *Neohelice granulata* upon the presentation of a visual danger stimulus (VDS). Aiming to study the regulation of cardio-inhibitory responses (CIR) in vivo, we investigated whether GABA mediates the extrinsic regulation of the cardiac activity. We examined the possibility of abolishing CIR by injecting the GABAergic antagonists picrotoxin and bicuculline, right before sensory stimulation. Picrotoxin partially abolished the reversible cardiac arrests induced by VDS, whereas bicuculline showed no effects. These results suggest that the rapid responses of the cardiac system of the crab *Neohelice* to environmental disturbances, reminiscent of an autonomic-like regulation associated with fear, flight or fight, may be extrinsically regulated by the GABAergic system.



## 1. Introduction

Sudden environmental stimuli of different modalities (visual, vibratory, chemical, electrical or tactile) may temporarily reduce (bradycardia) or interrupt the heart rhythm, inducing reversible cardiac arrests [15]. These cardio-inhibitory responses (CIR) occur in many animal groups, such as mollusks [30,56], crustaceans [14,23], fish [3,4,28], amphibians [33], birds [11], and mammals [50]. CIR have also been reported in the crab *Neohelice granulata* as an index of sensory perception and as evidence of a neuroautonomic response [7,8,25]. Several attempts have been made to explain the possible functional role of this transient cardiac inhibition in response to external stimuli because its prevalence across invertebrates and vertebrates leads to the assumption that it has a universal adaptive function. The main explanations provided include the following: a) decelerative heart rate (HR) changes have been identified as indexes of emotion in rabbits as well as in humans [16,35]; b) cardiac inhibition has been associated with attentional phenomena in vertebrates [22,31,44] and, accordingly, with the orienting response in invertebrates [48]; c) CIR have been described as a component of the "death-feigning behavior" in decapod crustaceans [41] and of the "concealing behavior" in vertebrates [51]; d) a reflexive drop in the HR may be caused by blood redistribution from vegetative to locomotor muscles in preparation for flight [34]; and e) CIR may represent an example of the so-called "startle-induced bradycardia" of many animal species to rapid and intense sensory stimuli [7,23,24,32]. However, the mechanisms underlying CIR are still unknown.

The basic contraction rhythm of the decapod crustacean heart emerges from the bursting discharges of the nine-cell cardiac ganglion, which is regulated by extrinsic nerves arising from the central nervous system [1]. Each nerve contains excitatory and inhibitory axons. In isolated hearts, stimulation of the excitatory axons speeds the contraction rate, whereas stimulation of the inhibitory units slows down

*Abbreviations:* BHR, basal heart rate; BIC, bicuculline; CIR, cardio-inhibitory response; DMSO, dimethyl sulfoxide; ECG, electrocardiogram; GABA, γ-aminobutyric acid; GABAi, GABA-like immunoreactivity; HR, heart rate; IHR, instant heart rate; MUS, muscimol; PTX, picrotoxin; SAL, saline solution; VDS, visual danger stimulus.

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or stops the heart [19,39,58]. En passant recordings from these nerves in semi-intact animals have revealed periodic increases in inhibitory nerve firing rates correlated with bradycardia [18,62]. The activity of these nerves has also been characterized during sensory-induced modification of the HR, and three sensory modalities (stretch, chemical and tactile) are known to mediate inhibitory cardiac reflexes [18]. In the lobster *Homarus americanus*, the successful cutting of dorsal nerves was confirmed by the loss of the brief period of bradycardia known as startle response usually triggered by sensory stimulation [24].

As for peripheral inhibition, γ-amino-butyric acid (GABA) is a candidate for inhibitory transmission. In support of an early study [47], voltage-clamping of the large cells of the cardiac ganglion of *Homarus* indicated that GABA application increases conductance to $Cl^-$ [29]. In several other Malacostraca, innervations of the ganglionic motor neurons by extrinsic cardioregulatory fibers have been extensively studied and a considerable amount of evidence indicates that GABA is involved in these synapses in isopods [54], stomatopods [2] and decapods [61]. The application of either GABA or its agonist muscimol (MUS) mimics the effects of the cardioregulatory fibers [12,40,60,61], and both effects are blocked by specific antagonists such as picrotoxin [2,47,61]. More recently, a study of the cardiac ganglion of the Caribbean spiny lobster *Panulirus argus* revealed GABA-like immunoreactivity (GABAi) originated from a single bilateral pair of fibers that entered the heart via two dorsal nerves [17]. These formed extensive processes in the neuropil throughout the ganglion, including a pericellular network around large cell somata.

In our previous work in the crab *Neohelice*, immunohistochemical techniques have allowed us to identify GABA-like immunoreactive processes entering the heart, which later branched in slender and varicose fibers forming a network around the somas of large neurons of the cardiac ganglion. These results strengthen the idea that GABA has a role in the peripheral inhibition of the cardiac system [59].

Only one study, done in the Nile tilapia (*Oreochromis niloticus*), examined *in vivo* the effect of several drugs on reversible cardiac arrests to visual stimuli. In this teleost fish, stressful conditions like handling the animal outside the water or a nociceptive stimulus reduced cardiac interbeat interval, while a subanesthetic dose of barbiturate (pentobarbital sodium, Nembutal, 5 mg $kg^{-1}$) inhibited reversible cardiac arrests induced by a moving shadow stimulus [28]. In the same work, diazepam injections (1.0 and 2.0 mg $kg^{-1}$) abolished the reduction in magnitude of reversible cardiac arrests in a dose-dependent manner. These data suggest that the magnitude of cardiac interbeat intervals could be modulated by a benzodiazepine.

In order to determine whether GABA mediates CIR induced by visual stimuli in the crab *N. granulata*, in the present work we examined *in vivo* the possibility of abolishing CIR by injecting $GABA_A$ antagonists right before sensory stimulation.

## 2. Materials and methods

### 2.1. Animals

Animals were adult male *N. granulata* crabs (previously *Chasmagnathus granulatus*, Crustacea, Grapsidae, Dana, 1851), 2.7–3.0 wide from side to side, weighing approximately 17 g, collected in the *rías* (narrow coastal inlets) of San Clemente del Tuyú, Buenos Aires province, Argentina (36° 21′ S, 56° 43′ W). Once in the laboratory, animals were lodged in collective tanks (35 cm × 48 cm × 27 cm) filled to a depth of 2 cm with diluted seawater to a density of 20 randomly picked individuals. Water used in tanks and other containers during experiments was prepared with *Red Sea Salt* (Red Sea Fish Pharm. Ltd.), salinity 1.0–1.4%, and pH 7.4–7.6. Animals were maintained within a range of 22–24 °C and a 12-h light/dark cycle (lights on from 07:00 h to 19:00 h). Experiments were run between 8:00 h and 17:00 h and performed within the first two weeks after animals' arrival. Each crab was used in only one experiment. All experiments were conducted 2 or 3 days after the initial wiring of the animals (see below). During the recovery period, crabs were kept in individual tanks and fed daily with rabbit food pellets (Nutrients, Argentina). After the experiments the animals were returned to the field and released 30 km away from capture area to avoid recapture. Experimental procedures were in compliance with Argentine laws for Care and Use of Laboratory Animals.

Animals were restrained by immobilizing the crabs prior to the experiment, enclosing them in close-fitting thick elastic bands with the legs positioned in an anterior position and slightly under their bodies. This procedure allowed us to stabilize the electrocardiogram (ECG), making it easier to quantify HR.

### 2.2. Cardiac response: recording procedure

A small jack with two metallic pins, where electrodes were soldered, was cemented with instant adhesive to the dorsal carapace in a position anterior to the heart. The electrodes were made of pure silver wire (0.25 mm in diameter, Vega & Camji S.A., Argentina) previously cut in 25-mm-long sections. The free ends of both wires were inserted in holes drilled in the cardiac region of the dorsal carapace placed to span the heart in a rostral–caudal arrangement and separated 4–5 mm from each other. The electrodes easily pierced the hypodermis and were cemented in place with instant adhesive (Fig. 1A). All recording experiments were conducted 2–3 days after wiring of the animals to allow them to recover from stressful handling, which has been shown to alter HR for a few days [38,57]. Prior to each experiment, each crab was lodged in an actometer: a bowl-shaped opaque container with a steep 12-cm-high concave wall (23 cm top diameter and 9 cm floor diameter) covered with marine water to a depth of 0.5 cm and illuminated with a 10 W lamp placed 30 cm above the animal. A plug connected to an impedance converter (UFI, model 2991, California, USA) was slotted in each jack cemented on the animal to monitor HR. The impedance converter measured the changes in the resistance between two electrodes, caused by hemolymph movement associated with each heart contraction

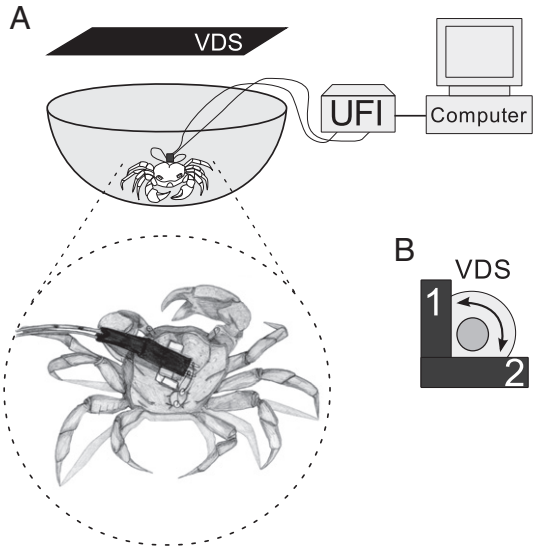


**Fig. 1.** Experimental set-up. A) Scheme of the set-up used for recording the HR during the presentation of the VDS. The crab was lodged in the actometer (a bowl-shaped opaque container), and connected to the impedance detector (UFI) by means of a jack cemented on the dorsal surface of the carapace. The impedance output was sent to a computer to monitor the HR. A detailed drawing of the crab shows the placement of the jack. B) A view from above: the VDS is a motor-operated rectangular opaque screen placed 6 cm above the actometer and moved horizontally from position 1 to position 2 (1 cycle). One VDS presentation lasted 5 s and consisted of two successive cycles of screen movement.

[37,38,46]. The output from the impedance leads was sent to the analog-to-digital converter of a computer data acquisition and analysis system (Fig. 1A).

### 2.3. Environmental disturbance

To test the crabs' perception of a particular environmental alteration, a moving visual danger stimulus (VDS) was used (Fig. 1B). Between 20 and 30 restrained crabs were tested in each experimental condition.

#### 2.3.1. Visual danger stimulus (VDS)

Each VDS consisted in moving horizontally from left to right, and vice versa, an opaque rectangular screen (25 cm × 7.5 cm) positioned 6 cm above the actometer. Each VDS lasted 5 s and consisted of two successive cycles of screen movement (Fig. 1B, Fig. 2B).

### 2.4. Drugs and injection procedures

Crustacean saline solution (SAL) [26] or dimethyl sulfoxide (DMSO) was used as vehicles, while picrotoxin (PTX) or bicuculline (BIC) was used as drugs. A volume of 50 μl PTX or SAL, or 10 μl BIC or DMSO per animal was given through the right side of the dorsal cephalothoracic–abdominal membrane, using a syringe fitted with a sleeve to control depth of penetration to 4 mm, thus ensuring that the injected solution was released in the pericardial sac. For the injection procedure, each crab was kept outside the actometer for approximately 20s and then returned.

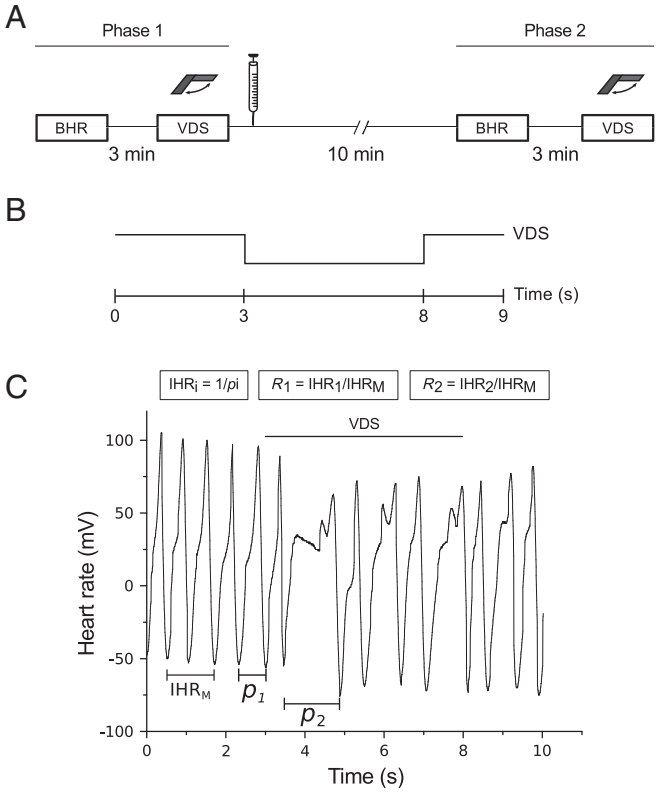


**Fig. 2.** Experimental design and ECG analysis. A) The experimental protocol consisted of two phases where each block represented a 9-s recording interval. In phase 1, during BHR block, the IHR was recorded in the absence of stimuli, and 3 min later, during VDS block, the IHR was recorded during stimulation. Immediately after, drugs or vehicles were administered followed by a 10-min delay. In phase 2, BHR and VDS blocks were repeated. B) Time course of stimulus presentation during the VDS block. The 5-s VDS was presented after a 3-s delay. C) A representative ECG recording of cardiac activity. The duration of the cardiac event or period ($p$) was measured and used to calculate the IHR as the inverse of the period ($IHR_i = 1/p_i$). The solid line stands for the VDS presentation. $IHR_M$ is the mean instant heart rate. $IHR_M$, $IHR_i$, $R_1$ and $R_2$ are defined in the text.

A 50-μl injection containing 5.5 μg, 10 μg and 15.9 μg of PTX (Sigma Aldrich, P1675) diluted in crustacean saline solution to a final concentration in hemolymph of 0.24 μg $g^{-1}$, 0.43 μg $g^{-1}$ and 0.69 μg $g^{-1}$ respectively, was injected to drug-treated group of animals in three consecutive experiments. Control animals received a 50-μl SAL injection.

A 10-μl injection containing 25 μg and 33 μg of BIC ((+)-Bicuculline, Fluka Analytical, 14340) diluted in DMSO to a final concentration in hemolymph of 1.21 μg $g^{-1}$, 1.40 μg $g^{-1}$ respectively was injected to experimental animals in two consecutive experiments. Control animals received 10 μl of DMSO.

### 2.5. Experimental setup

The experimental room had 20 actometers, separated from each other by partitions, and was dimly illuminated. Previous to each experiment, wired animals were carried individually to the experimental room, located in their respective actometer and connected to the impedance converter. A computer was used to program trial sequences and stimuli presentation as well as to monitor experimental events. The computer scanned each actometer in an orderly and consecutive manner during an interval of 9 s in which either a stimulus was presented and HR recorded (stimulation trial) or HR was recorded without stimulation (adaptation period).

### 2.6. Experimental protocol

Each experiment lasted 1 day and involved two phases (before and after drug administration) with an adaptation period and a stimulation trial each. In phase 1, during adaptation period, basal heart rate (BHR) was recorded allowing animals to become familiar to the experimental setup without being disturbed. This adaptation period was followed by a stimulation trial, during which VDS was presented to both groups of animals (control and drug-treated) and HR was recorded (Fig. 2A, phase 1). Immediately after, animals were injected with either drug or SAL/DMSO. After a 10-min delay, during phase 2, VDS was presented again (Fig. 2A, phase 2).

### 2.7. Data analysis

The CIR is a brief and sometimes subtle response which lasts no longer than a few seconds and involves an increase in interbeat interval or period (p) of the cardiac event. Although these alterations bring about changes in overall HR of the animal, we have previously determined that instant heart rate (IHR) is the parameter which best describes the changes generated by sensory stimulation and that a 9-s recording time provides a suitable interval for measuring cardiac responsiveness to sensory stimulation [8]. The cardiac activity was thus recorded for 9 s, during which either the stimulus was presented after a 3-s delay (stimulation trial) or no stimulus was presented, and BHR was recorded (adaptation period). The duration of the cardiac event or period was measured and used to calculate IHR as the inverse of the period ($IHR = 1/p$).

Previous studies in crustaceans have shown that HR can vary widely both between and within individuals and with experimental conditions [23,25]. Thus, we normalized IHR as IHRi/IHRM where IHRi is the IHR of each single event and IHRM is the mean IHR [8].

To control that changes in IHR attributable to stimulation were significantly different from spontaneous changes in IHR, two ratios ($R_1$ and $R_2$) were statistically compared. $R_1$ represents the quotient between $IHR_1$ (the IHR of one event randomly selected before stimulus on-set) and $IHR_M$ ($R_1 = IHR_1/IHR_M$), whereas $R_2$ represents the quotient between the $IHR_2$ (the IHR of one representative event during the cardiac response) and $IHR_M$ ($R_2 = IHR_2/IHR_M$) (Fig. 2C). To assess whether pharmacological treatment had some effect on BHR, the $IHR_M$ during

the 9-s adaptation period was statistically examined comparing the mean IHR before and after drug treatment.

### 2.8. Statistical analysis

Data were analyzed using analysis of variance (ANOVA) with a between-groups (drug treatment) factor and a within-subjects (repeated measures) factor with two levels ($R_1$ and $R_2$) followed by Tukey's post-hoc tests. Differences were considered significant at $p \leq 0.05$. The same analysis was performed to compare the mean IHR before and after drug treatment. Differences were considered significant at $p \leq 0.05$. All data presented in the figures are mean values and corresponding standard error (S.E.M.).

## 3. Results

### 3.1. Cardio-inhibitory response (CIR) to environmental disturbances

Deep heart arrests and sustained bradycardia were elicited upon the presentation of a stimulus considered to be threatening, such as the VDS (Fig. 3, phase 1).

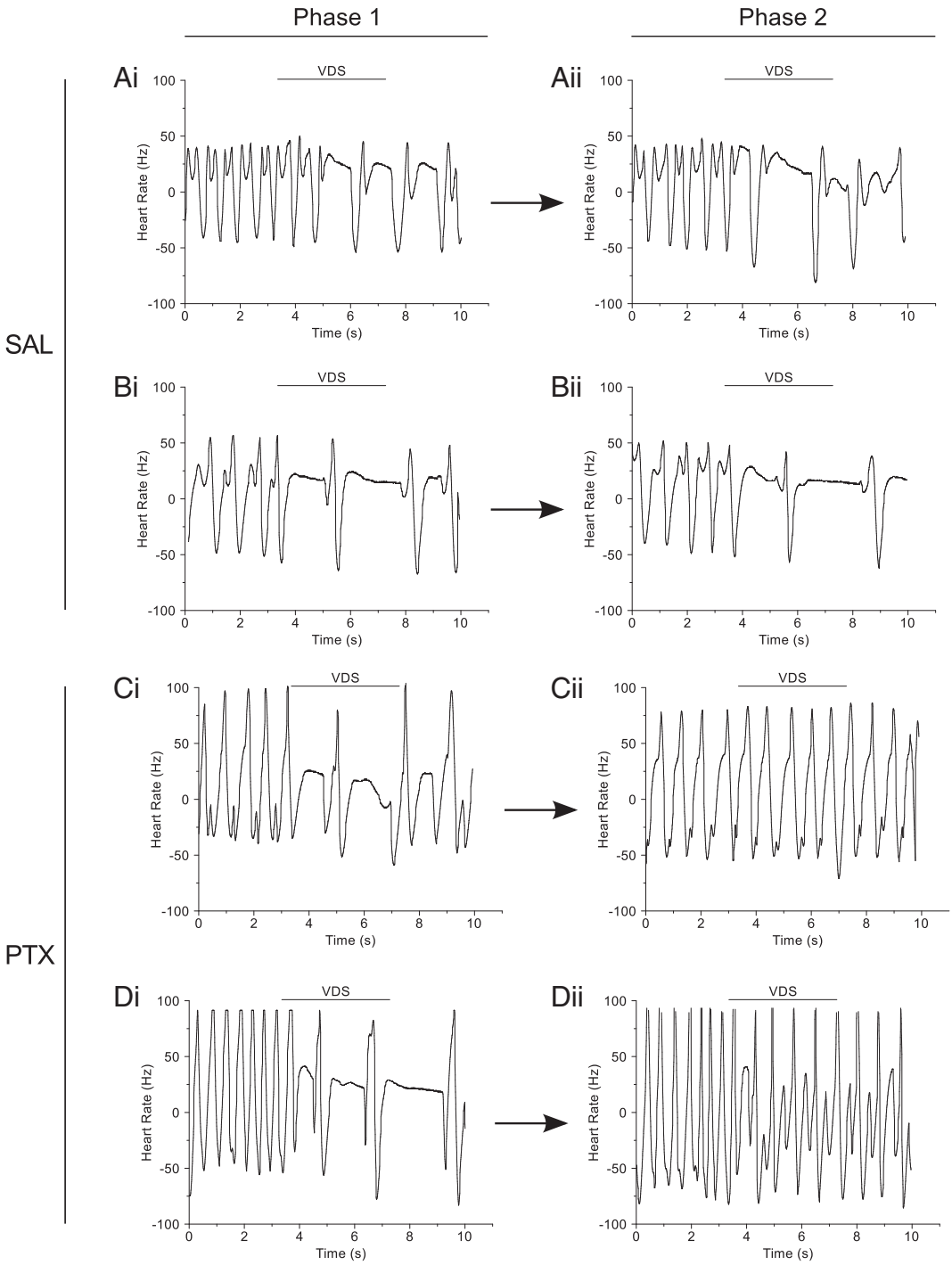


**Fig. 3.** Representative ECG of PTX- and SAL-treated animals upon stimulation. Representative 9-s ECG recordings of four different crabs before (phase 1) and after (phase 2) drug treatment. The CIR persisted in control animals after a SAL injection (Aii and Bii); whereas CIR is clearly reduced in PTX-injected animals (Cii and Dii). The solid lines stand for VDS presentation.

The $IHR_M$ was 2.57 ± 0.91 Hz ($n = 15$), with an interbeat period of 392 ± 138 ms (or 155 beats/min). During CIR, $IHR_M$ decreased to 0.69 ± 0.24 Hz, with an interbeat period of 1339 ± 669 ms (or 46 beats/min). Upon a second presentation of the stimulus to the same animal, a similar profile was obtained, revealing the consistency of the response (Fig. 3Ai–Aii; Bi–Bii).

### 3.2. The GABAergic antagonist picrotoxin partially abolishes the reversible cardiac arrests induced by VDS

Previous studies in the stomatopod *Squilla oratoria* have examined the effects of GABA in isolated hearts [2]. These studies showed that concentrations of GABA > $10^{-6}$ M lead to a dose-dependent decrease in HR and contraction force and that GABAergic antagonist, PTX, at $10^{-4}$ M completely blocked cardiac inhibition. Also, in the hermit crab *Aniculus aniculus* [61], pharmacological tests in inhibitory cardioregulatory nerves have shown that PTX at $5 \times 10^{-5}$ M antagonizes the inhibition of the cardiac ganglion induced by GABA at $5 \times 10^{-5}$ M and by stimulation of cardio-inhibitory axons.

Here, we studied the putative role of GABAergic system in the mediation of CIR in vivo. To determine whether GABA mediates the cardio-inhibitory response induced in *Neohelice* by sensory stimulation, we investigated the possibility of abolishing CIR by injecting a $GABA_A$ antagonist right before sensory stimulation. Previous to injection, and upon stimulation, animals of both experimental and control groups showed cardiac arrests or bradycardia that determined an important reduction in $IHR_M$. After administration of either SAL or PTX to control and experimental groups, respectively, animals were stimulated again with VDS. Fig. 3 shows four representative ECG recordings of two different animals before and after injection of either SAL or PTX. Although animals showed important response variability, CIR response persisted in control animals after SAL injection (Fig. 3Aii, Bii) and was clearly reduced in drug-injected animals (Fig. 3Cii, Dii).

The statistical analysis (ANOVA) performed on these data to analyze phase 1 revealed a main effect of factor $R$ [$F(1,38) = 169$, $p < 0.0001$]. There was neither Group effect ($F(1,38) = 3.89$, $p > 0.05$) nor Group × factor $R$ interaction ($F(1,38) = 0.316$, $p > 0.5$). This indicates that the response of both groups upon stimulus presentation was indistinguishable. This result was expected taking into account that at this moment (before injection) the treatment assigned to both groups was identical (Fig. 4A).

When animals were stimulated 10 min after injection, those that received 0.24 μg $g^{-1}$ of PTX showed a partial abolishment of heart arrests, although CIR was still detected when compared with its own baseline. However, the inhibitory response of drug-treated animals was significantly lower than that of controls. The statistical analysis performed on these data to analyze phase 2 revealed significant effects of Group [$F(1,38) = 26$, $p < 0.0001$], factor $R$ [$F(1,38) = 178$, $p < 0.001$] and Group × factor $R$ interaction, [$F(1,38) = 20$, $p < 0.001$]. A post hoc Tukey's test on these data allowed us to conclude that although significant differences between $R_1$ and $R_2$ were still found within each group ($p < 0.001$), the comparison between the $R_2$ index of both groups was also significant ($p < 0.001$). These results indicate that although IHR was significantly reduced upon VDS presentation in both groups, in animals injected with PTX, CIR was reduced in comparison to that observed in control animals (Fig. 4B).

PTX was further investigated in higher concentrations (0.43 μg $g^{-1}$ and 0.69 μg $g^{-1}$) to achieve complete abolishment of CIR. However, both concentrations showed undesired effects on animals after the experiment. Several animals suffered autotomy and between 5 and 10% of the animals died. Thus, we did not include the data of these groups.

### 3.3. Effect of PTX on the basal heart rate

The effect of PTX could have been targeted to heart arrests or resulted in a generalized increase in BHR instead. The latter could have hindered heart arrests, making it difficult to detect them. Representative ECG recordings in Fig. 3Cii and Dii show that after injection and upon stimulation with VDS, conspicuous reversible arrests are no longer visible, suggesting that PTX could antagonize them specifically (Fig. 3Cii), while a mild bradycardia was observed in some animals (Fig. 3Dii). Thus, we focused our analysis on the examination of the adaptation period, i.e., the 9-s recording interval (BHR) introduced previous to stimulation of phases 1 and 2 of the experimental session (see Fig. 2A). This allowed us to accurately assess whether the drug had a generalized effect on IHR or if it was specifically and mainly targeted to cardiac heart arrests. For this analysis, the mean value of IHR was compared between both groups before and after drug administration (Fig. 5). The statistical analysis (ANOVA) of these data revealed a main effect of time before versus after injection [$F(1, 38) = 70$, $p < 0.0001$], but no Group effect or Group × factor $R$ interaction ($F < 1.5$, $p > 0.05$). So, the generalized decrease observed in interbeat period after injection in both groups was probably a result of handling stress and not a drug effect.

### 3.4. Bicuculline does not antagonize CIR induced by VDS

Previous studies in *Neohelice* using BIC showed facilitatory effects over different memory processes [10]. However, PTX did not show comparable effects over the same processes (personal communication).

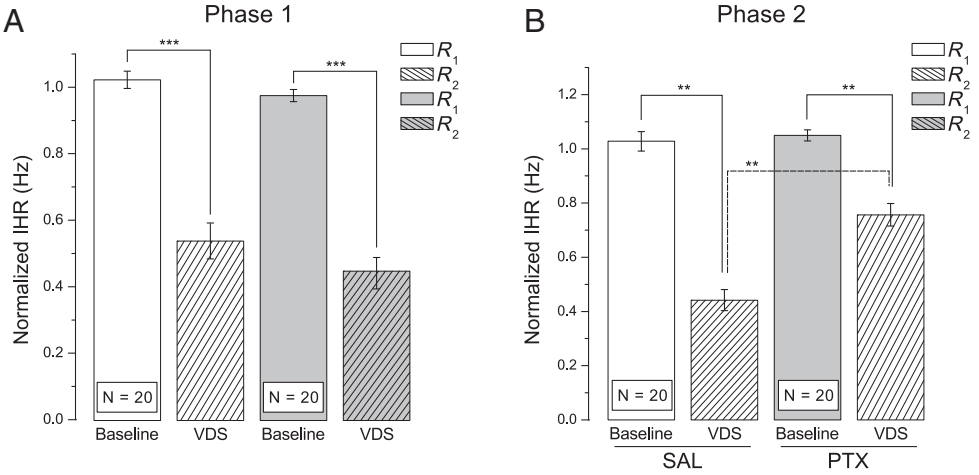


**Fig. 4.** PTX partially abolishes CIR. A) In phase 1 (before drug administration), the VDS triggered a CIR which involved a significant reduction of the IHR (***, $p \leq 0.0001$). The response was similar in both groups since up to this moment the treatment assigned was identical. B) In phase 2, injection of PTX (0.24 μg $g^{-1}$) significantly abolished the VDS-triggered CIR in comparison to the SAL-treated animals (**, $p \leq 0.001$). IHR values are normalized and presented with their corresponding S.E.M.

Thus, we decided to explore the effect of this classical $GABA_A$ antagonist on the extrinsic inhibitory regulation of the cardiac system.

Before drug administration, both groups of animals showed a conspicuous CIR upon stimulation with VDS. The ANOVA performed to analyze phase 1 revealed a main effect of time before versus after injection [$F(1, 30) = 408$, $p < 0.0001$], but no Group effect or Group × factor *R* interaction ($F < 1$, $p > 0.05$). This indicates that the response of both groups of animals to the stimulus was very similar. This result was expected taking into account that at this moment (before injection) the treatment assigned to both groups was identical (Fig. 6A).

However, after treatment, the GABAergic competitive antagonist BIC did not reveal a compensatory effect on CIR (Fig. 6B). In phase 2, a main effect of time before versus after injection [$F(1, 30) = 176$, $p < 0.0001$], but no Group effect or Group × factor *R* interaction ($F < 1$, $p > 0.05$) was observed. No statistical differences were observed when drug-treated animals were compared with saline-treated crabs. We further assessed the drug effect in a higher concentration (1.40 μg $g^{-1}$ per animal) but found no abolishment of CIR (data not shown).

## 4. Discussion

Although temporary reductions (bradycardia) or rapid interruptions of heart rate (arrests) induced by changes in the animals' environment have been widely reported, here we investigated for the first time the mechanisms underlying this phenomenon by *in vivo* examination in *Neohelice* crabs. Our results confirm previous evidence showing that the magnitude of cardiac interbeat interval could be modulated by specific pharmacological treatment [28].

### 4.1. Role of GABA in the extrinsic control of cardiac activity

Previous reports have revealed that diazepam abolishes stress-induced reduction in the magnitude of reversible cardiac arrests [28]. The fact that benzodiazepines act on $GABA_A$ receptors, facilitating the inhibitory action of GABA, suggests that this neurotransmitter may be involved in CIR regulation. Our results explicitly addressed this point and further strengthened it.

From the experiments with PTX, a non-competitive $GABA_A$ antagonist, we can conclude that CIR was partially reduced since no reduction was observed in control animals upon stimulation. Furthermore, PTX did not show a generalized effect on BHR, given that GABAergic regulation upon sensory stimulation seemed to result mainly from a phasic action on the cardiac system specifically addressed to reversible cardiac arrests. Taken together, these results suggest that GABA may be mediating CIR although BHR was not completely restored.

However, CIR may result from a balance between excitatory and inhibitory tones. A reduction of excitatory stimulation might be required together with the inhibitory input from the GABAergic system. The fact that we did not obtain a complete abolishment of CIR, although we investigated PTX in higher concentrations (0.43 μg $g^{-1}$ and 0.69 μg $g^{-1}$), strengthens this hypothesis. By using PTX at similar doses, other authors completely blocked the cardiac inhibition induced by stimulation of cardio-inhibitory fibers [2]. However, these studies were performed in isolated hearts. Nevertheless, other arguments besides dose concentration should be considered. In the first place, we cannot rule out the fact that other agents may be simultaneously acting considering that the cardiac ganglion is multiply modulated by biogenic amines, neurotransmitters or other peptides [13]. Secondly, the time of injection used in this work was based on previous studies in *Neohelice* of our laboratory [10]. Nevertheless, we cannot discard the possibility that 10 min was not sufficient to reach the levels in hemolymph strictly needed to completely antagonize CIR.

Regarding the results obtained with BIC, we found no effect of this $GABA_A$ competitive antagonist on CIR. We may have missed the time window of action of this drug. However, the time of injection was planned taking into account a previous study where this time window and dose were assessed in the same animal model [10]. In this work, the authors studied the role of the GABAergic system in different stages of memory formation and reported that MUS, a $GABA_A$ agonist, impaired and BIC improved consolidation and reconsolidation processes. However, non-competitive antagonist PTX showed no effect on memory processes (personal communication). In several invertebrate species, GABA-operated chloride channels exhibited an agonist profile resembling that of the mammalian $GABA_A$ receptor, but BIC and PTX failed to block GABA receptors of *Ascaris* muscles [27,55] and of *Limulus* heart [6]. It has also been found that many central neurons in insects are insensitive to BIC [5,36,45]. Moreover, according to the response to BIC and PTX ascending interneurons of the crayfish terminal abdominal ganglion have been classified into two types: PTX-sensitive and PTX-insensitive [42].

Based on these data, we propose the existence of two sub-populations of $GABA_A$-like receptors in *Neohelice*: a central sub-population, which is sensitive to MUS and BIC but insensitive to PTX, and a more peripheral sub-population, which is sensitive to PTX but insensitive to BIC. Therefore, our results suggest that BIC-sensitive $GABA_A$-like receptors may not be involved in the extrinsic control of cardiac activity.

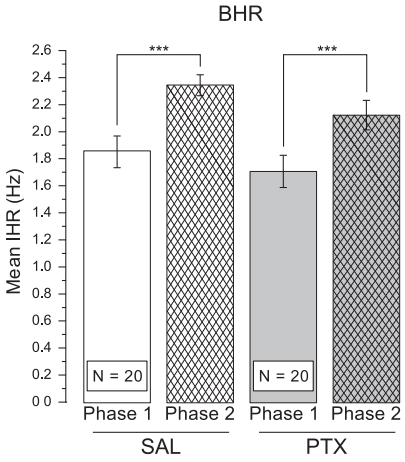


**Fig. 5.** PTX effects over the BHR. Analysis of the BHR block, prior to VDS presentation in phases 1 and 2. A decrease in the interbeat period was observed after injection in both groups (***, $p \leq 0.0001$). Mean IHR values are presented with their corresponding S.E.M.

### 4.2. Putative autonomic-like control of CIR

Rapid responses in the cardiac system to environmental disturbances in crustaceans are similar to fear, flight or fight responses mediated by the autonomic nervous system [46]. The mechanism underlying this autonomic-like control system of HR changes is insufficiently understood despite the efforts made in this direction. Studies in several fish species have reported the abolishment of CIR by section of branchial or vagal cardiac nerves as well as by atropine injection [3,9,43].

Additionally, systemic injection of cholinergic antagonist atropine and the β-adrenergic antagonist propranolol in *O. niloticus* has shown that a double cardiac autonomic control is present in this teleost fish, but that reversible cardiac arrests resulting from external disturbances such as visual stimulation are peripherally mediated by muscarinic receptors because they were abolished by atropine but not by propranolol [28].

Also, in crustaceans, an autonomic-like control system for rapid cardiac and respiratory regulations to be primed for 'fight or flight' when the need arises has been proposed both by us and by others [7,25,37,38,46,49]. The associated neural and humoral control of body function by the autonomic nervous system has likely evolved to regulate basic survival strategies in vertebrates as well as in invertebrates. The

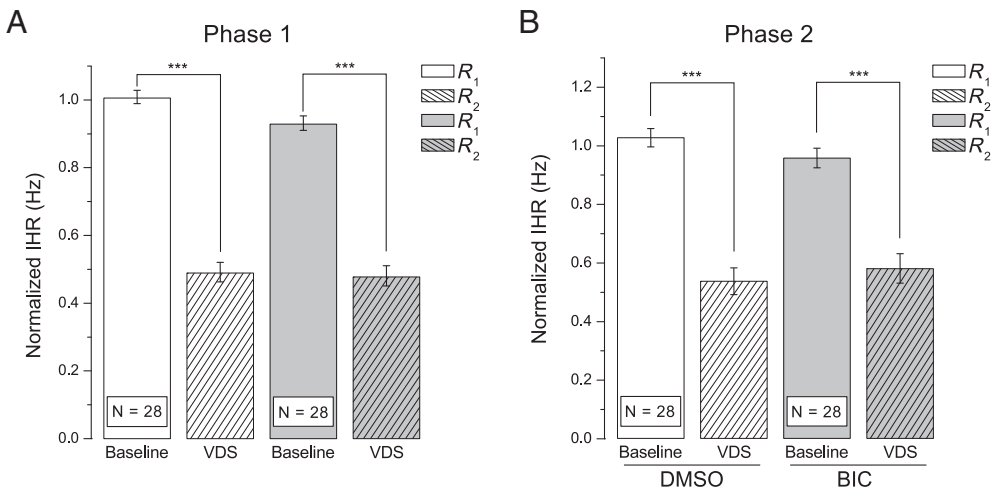


**Fig. 6.** BIC effects over the CIR. A) In phase 1, the VDS triggered a CIR which involved a significant reduction of the IHR (***, $p \leq 0.0001$). The response was similar in both groups since up to this moment the treatment assigned was identical. IHR values are normalized and presented with their corresponding S.E.M. B) In phase 2, after injection of BIC (1.21 μg $g^{-1}$), no drug effect was observed. CIR abolishment is not apparent in BIC-treated animals in comparison to the DMSO-treated animals.

neural control of cardiac and ventilatory systems of decapod crustaceans and vertebrates is similar. The cardiac ganglion, which is in close physical association with the heart, is under continuous control by inhibitory and acceleratory neurons located in the thoracic ganglion. Regarding neurotransmission, it is well established that the parasympathetic nervous system of vertebrates uses chiefly acetylcholine (ACh) and that the sympathetic releases norepinephrine, which activates adrenergic receptors on the peripheral target tissues. In contrast, the transmitters used at most of the chemical synapses of the crustacean cardiac system remain unknown. The innervation of ganglionic motor neurons by extrinsic inhibitory cardioregulatory fibers has been most intensively studied, and considerable evidence indicates that GABA mediates inhibition at these synapses [17]. While less is known about the excitatory cardioregulatory fibers, some evidence is consistent with the involvement of acetylcholine [21,53,54], glutamate [2] and dopamine [20,61] in this role in certain species [2,20,53]. In addition to the direct neural control of the cardiac and ventilatory generators, crustaceans possess a highly elaborated system of the so-called neurohemal organs, specialized structures in which nerve terminals come into contact with the circulatory system. These structures may be analogous to the adrenal glands of vertebrates, since both are designed for rapid release of substances, which leads to alterations in sensory and motor functions, thus altering animal behavior [52].

The idea of an autonomic control of CIR modulating the magnitude of cardiac interbeat intervals is further strengthened by present findings suggesting GABAergic involvement in extrinsic control of cardiac activity and by evidence of the presence of GABAi in the ganglionic trunk of *Neohelice* [59].

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