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Both heat shock and water deprivation trigger Hsp70 expression in the olfactory lobe of the crab *Chasmagnathus granulatus*

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ABSTRACT

Heat-shock proteins (Hsp) are synthesized in the central nervous system in response to traumas but also after physical exercise and psychophysiological stress. Therefore, an increase in Hsp expression is a good marker of changes in metabolic activity. In the crab *Chasmagnathus*, a powerful memory paradigm has been established. Memory modulation is possible by water shortage. The brain areas activated by either training protocols and/or water-deprivation are still unknown. Hsp expression might be a marker to sensing the increase in metabolic activity in crab *Chasmagnathus* brain neuropils engaged in the physiological responses triggered by water deprivation and cognitive processing. Here, we observed an increase in brain Hsp of 70 kDa (Hsp70) expression after a heat-shock treatment. Additionally, immunohistochemistry analysis revealed that, under basal conditions, some glomeruli of the olfactory lobes showed Hsp70 immunoreactivity in an on–off manner. Both a hot environment and water deprivation increased the number of glomeruli expressing Hsp70. This marker of neuropil's activity might turn out to be a powerful tool to test whether crustacean olfactory lobes not only process olfactory information but also integrate multimodal signals.

Chasmagnathus is a euryhaline and semi-terrestrial crab that inhabits brackish water and is usually confronted with water-deprivation [34]. A powerful memory paradigm based on a change in its defensive strategy against a visual danger stimulus has been established [20,25]. In this crab, long-term memory is mediated by NF-κB transcription factor activation [29], the cAMP signalling pathway [19] and is regulated by muscarinic and glutamatergic mechanisms [19]. Several treatments modulate this memory; both high environmental salinity acclimatization and water deprivation are real-life episodes that facilitate memory through angiotensin II (ANGII) receptors [6,9–11]. Specifically, 2 h of water deprivation, which transiently increases blood sodium concentration and brain ANGII, enhances memory consolidation, retrieval and reconsolidation [9–11]. Although some of the neuronal substrates that support memory have been shown in optic ganglia [40], the brain areas activated by either training protocols and/or water-shortages are still unknown. Therefore, we want to develop a neuronal marker for detecting brain areas engaged in physiological and behavioral responses.

Heat-shock proteins (Hsp) are conserved through evolution and play a central role in the molecular defence machinery against stress [7,23]. Despite being constitutive polypeptides, they are also inducible by many kinds of stressors in different cell types [7,37]; the degree of expression depends on metabolic activities. Hsp70 were found in postsynaptic structures [17,38]. In *Drosophila* the major 70 kDa heat-shock proteins (Hsp70) are strongly induced by high temperatures and play a key role in protecting synaptic structures during heat shock [16]. Brain Hsp70 are also induced after physical exercise [2] and psychophysiological stress [8,12,15,24]; these evidences indicate that Hsp70 expression is a good marker of metabolic activity changes, including brain areas engaged in cognitive processing [1,27].

In crustaceans, heat shock [28,35] and osmotic and environmental [4,5,39] stress significantly increase Hsp expression, in muscles and hepatopancreas. Moreover, Hsp70 was involved in the defence strategy of *Daphnia magna* to cope with predator stress [24,26]. Here we test whether Hsp70 expression is a good marker of metabolic activity changes in *Chasmagnathus*' brain. The results demonstrated that Hsp70 is an effective marker of some brain areas activated by both a hot environment and water shortage; Hsp immunopositive (Hsp+) areas seem to disclose neuropils engaged in the physiological responses triggered during these experimental circumstances.

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Adult male *Chasmagnathus granulatus* intertidal crabs, 2.7–3.0 cm across the carapace, weighing 17 ± 0.2 g, were collected from narrow coastal inlets of San Clemente del Tuyú, Buenos Aires Province, Argentina. In the laboratory, they were lodged in collective plastic tanks (30 cm × 45 cm × 20 cm) filled to 0.5 cm deep with diluted (12‰, pH 7.4–7.6) marine water (Kent Sea salt, USA) at a density of 20 crabs per tank. The holding room was kept on a 12 h light:12 h dark cycle (light on 07:00–19:00 h). Water was changed every 2 days. Temperature of holding and experimental rooms was kept between 22 °C and 24 °C. Experiments were carried out within the first 2 weeks after animal's arrival. Each crab was used in one experiment. Experiments were carried out year-round. All research reported was conducted in accordance with an equivalent to the European Community Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize the number of animals used.

Experiment 1: Two groups of 20 crabs were split; one remained submerged in hot water 30 °C for 3 h and at 24 °C for 1 h (30 °C Group), the other under control conditions 24 °C for 4 h (24 °C Group). There were two reasons for choosing this protocol: (a) previous results demonstrated that Hsp expression increases 4–6 h after several treatments [8,24] and (b) when measuring the effects of heat shock, animals were enabled to recovery at the acclimation temperature for 1 h [35]. Crabs were then anesthetized and brains were removed and processed for western blot analysis.

Experiment 2: Two groups of crabs were split; a group was heat-shocked in a similar way but in even hotter water: 35 °C for 3 h and at 24 °C for 1 h (35 °C Group). Control group (24 °C) remained at 24 °C for 4 h. Brains were dissected and immunohistochemistry was performed. The terminology used for identifying different anatomical structures in *Chasmagnathus* brain was taken from Sandeman et al. [31].

Experiment 3: Two groups of crabs were split: one was water-deprived for 2 h (w.d. Group) and then kept in water for the next 2 h because Hsp expression increases 4–6 h after several treatments [8,24]. Moreover, this protocol resembles the one for heat-treatments: water-deprived animals recover their previous condition before brain dissection. Two hours of water deprivation facilitates memory in *Chasmagnathus* [9–11]. The control group was not water-deprived (n.d. Group).

Animals were anesthetized by immersion in ice-salted water mix for 5 min. For each sample 30 central brains (supraesophageal ganglia) were dissected, pooled in 1 ml buffered crab saline solution (pH 7.6) in ice and then centrifuged for 20 s at 1000 × *g*, at 4 °C. For total protein extracts, the ganglions were homogenized in phosphate-buffered saline (PBS 0.1 M, pH 7.4, and protease inhibitors: pepstatin A, 1 μg/ml; leupeptin, 10 μg/ml; aprotinin, 10 μg/ml; PMSF, 0.5 mM). The homogenate was centrifuged at 12 000 × *g* for 5 min at 4 °C and the supernatant stored at −20 °C. Then, 30 μg of proteins were electrophoresed in 10% SDS-polyacrylamide gel electrophoresis (PAGE) at 150 V for 1 h and then electroblotted to PVDF membrane (BIO-RAD, USA) at 100 V, 350 mA for 45 min. Immunodetection was performed using a monoclonal mouse anti-Hsp70/Hsc70 antibody (StressGen, Biotechnologies Corp. SPA822, B509489) at 1/1000 dilution followed by an anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology) at 1/5000 dilution. Detection was carried out with a Luminol chemiluminescence kit (Santa Cruz Biotechnology), exposing the membranes to XAR-5 film (Kodak). The amount of protein present in the samples was determined by the Bradford method and by checking the protein pattern in the Ponceau red staining solution-labelled membrane. Images were digitized using a scanner for negatives (Umax PowerLook III). Protein size was determined by its relative mobility (Rf), and correlating it with the molecular weight of standard proteins, RPN800 Rainbow Molecular Weigh Marker (Amersham Bioscience).

Crab supraesophageal and optic ganglia were dissected as described previously [6] and fixed overnight in 4% paraformaldehyde in 0.1 M PBS. Whole-mount immunohistochemistry: after four buffer washes (30 min/each), tissues were immersed in PBS −1% TritonX-100 (PBS-Tx) for 30 min, then blocked with 2% goat normal serum (GNS) and 5% low-fat milk overnight at 4 °C. The primary anti-Hsp70 antibody was used for tissue immunolabeling at a dilution of 1:500 in 2% GNS for 48 h at 4 °C. Antibody binding was visualized incubating the tissues with an Alexa Fluor 488 (Molecular Probes, USA) labelled goat anti-mouse antibody at a dilution of 1:1000 overnight at 4 °C. Tissues were kept in PBS-Tx overnight, dehydrated in increasing ethanol concentrations in PBS, and coverslipped with methyl salicylate (Sigma, USA).

Fluorescence was visualized with a confocal Olympus FV300 microscope with a 488-nm argon laser, an UplanFl 20× objective (NA 0.5) or an UplanFL 10× (NA 0.3), a dicroic SDM 570 to split acquisition channel, emission: BA 510 (Olympus Optical Co.). For images taken with 20× the pixel size was 0.69 μm/pixel. Data were recorded with FluoView Software and saved in a 16-bit TIFF format. Sequential images from the complete olfactory lobe (OL) (approximately 230 μm in *z*-axis) at 20× with zoom 2× magnification were deconvoluted (Autodeblur & Autovisualize X-Media Cybernetics, USA) with a quantitative blind point spread function searching algorithm (3D-Blind Deconvolution, 10 iterations, high noise level). Every 35-μm section (in *z*-axis) of the original image was separated as an individual stack using the appropriate tool of a image analysis software (ImagePro Plus v6.2, Media Cybernetics). This size was selected based on an initial evaluation of the average size of randomly selected individual glomeruli. As the last two sequential (3D) images were very noisy, they were discarded for further processing. The other four sequences were reduced into four single (2D) images using the Extended Depth of Field function of the program. It was then possible to determine the limit of each glomerulus given that they were slightly auto-fluorescent.

The first experiment was designed to reveal Hsp70 in *Chasmagnathus*' brain. It was repeatedly demonstrated that Hsp70 expression is increased after 4 h of stress onset [4,24,26]. Fig. 1A shows a representative western blot from animals submerged in hot water (30 °C Group) and under control conditions (24 °C Group). The 30 °C Group showed a prominent ≈70 kDa specific band and the 24 °C Group showed no specific marks. Therefore, crabs exposed to heat shock expressed a 70-kDa protein that was recognized by an anti-Hsp70 antibody. This result validates the use of this antibody in the following experiments.

The next aim was studying Hsp localization. We expected a widespread Hsp-immunoreactivity (Hsp-ir) increase in the central nervous system but the labelling appeared with low constancy in several supraesophageal ganglia and optic lobe neuropils. However, Hsp-ir was remarkable in several glomeruli of the olfactory lobe. Fig. 1B shows a general view of a representative brain where some glomeruli are labelled; as described elsewhere [38], labelling appeared as clustered small rounded spots (roundness index: 1.22). Moreover, Fig. 1C (1–4) show that Hsp-ir is evident in some glomeruli but not in others. As Hsp-ir was aggregated and the glomeruli limits were much defined, it was possible to count Hsp+ glomeruli (Fig. 1C4 shows eight Hsp+ glomeruli). This methodology was used for every OL analyzed. We found a significant increase in the number of Hsp+ glomeruli for 35 °C Group (14.0 ± 1.8) vs. 24 °C Group (8.6 ± 1.2) [*T*-Student = 0.010] (Fig. 2A and B).

The next experiment was designed to study brain Hsp expression after water deprivation. Again, we found a diffuse Hsp-ir among animals in several neuropils but conspicuous and punctuated Hsp-ir was specifically found in some OL glomeruli. We found a