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HIGH ENVIRONMENTAL SALINITY INDUCES MEMORY ENHANCEMENT AND INCREASES LEVELS OF BRAIN ANGIOTENSIN-LIKE PEPTIDES IN THE CRAB *CHASMAGNATHUS GRANULATUS*

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Summary

Previous work on the brackish-water crab *Chasmagnathus granulatus* demonstrated that an endogenous peptide similar to angiotensin II plays a significant role in enhancing long-term memory that involves an association between context and an iterative danger stimulus (context-signal memory). The present results show that this memory enhancement could be produced by moving crabs from brackish water to sea water (33.0‰) and keeping them there for at least 4 days. The possibility that such a facilitatory effect is due to osmotic stress is ruled out. Coincidentally, the level of angiotensin-II-like peptides in crab brain, measured by radioimmunoassay, increases with the length of exposure to sea water, reaching a significantly different level at the fourth day. The presence of angiotensin-II-like immunoreactive material in neural structures of the supraoesophageal and eyestalk ganglia was confirmed by immunohistochemical analysis. The results are interpreted as supporting the hypothesis that exposure to water of high salinity is an external cue triggering a process mediated by angiotensins that leads to enhanced memory in these crabs.

Key words: memory, angiotensin, water salinity, osmoregulation, invertebrate, crab, *Chasmagnathus granulatus*.

Introduction

There is an accumulation of evidence to support the view that angiotensin plays a role in the acquisition and recall of newly learned tasks (for reviews, see Wright and Harding, 1994, 1997). A disrupting effect on memory was initially attributed to centrally administered angiotensin II (ANGII) (e.g. Melo and Graeff, 1975), but more recent and extensive work has documented memory enhancement in animals treated with ANGII or biologically active fragments of ANGII (e.g. angiotensin IV) using diverse active and passive avoidance learning paradigms (e.g. Braszko et al., 1988, 1991, 1998; Wright et al., 1993; Winnicka and Wisniewski, 1999).

For several years, research on the role of angiotensins in processes involving learning was performed only with mammals, mainly humans and rodents, although the presence of components of the renin–angiotensin system has been demonstrated in the central nervous system of invertebrates. A complete renin–angiotensin system regulating fluid homeostasis has been described in the leeches *Theromyzon tessulatom* and *Erpobdella octoculata* (Laurent et al., 1995; Salzet et al., 1995), angiotensinogen-like epitopes have been found in the central nervous system of *Aplysia californica* (Gonzalez et al., 1995) and angiotensin converting enzyme (ACE)-like activity has been detected in the blue crab *Callinectes sapidus* (Smiley and Doig, 1994) and the housefly *Musca domestica* (Lamango et al., 1996). A cDNA that codes for a protein with high similarity to mammalian ACE (Cornell et al., 1995) has been cloned in *Drosophila melanogaster*, and ACE-like immunoreactivity has been found in the central nervous system and neurohaemal organs of several insects (Schoofs et al., 1998).

These observations suggested that it would be worthwhile to explore the mnemonic properties of angiotensin an invertebrate. A conserved functional role for angiotensin would be in line with several other instances where neuropeptides have retained the same functional role throughout the evolutionary process. These include, for example, the function of endogenous opioids in the modulation of food intake (e.g. Morley, 1995; Wong et al., 1991), the role of insulin and insulin-like growth factors in glucose metabolism (e.g. Floyd et al., 1999; Richardson et al., 1997) and the function of cholecystokinin as a satiety factor (e.g. Zimmering et al., 1988; Morley, 1995).

To study the mnemonic properties of angiotensin in an invertebrate, we chose a model of memory in the crab *Chasmagnathus granulatus* on the basis of the fact that the presentation of a danger stimulus (a screen passing overhead) elicits an escape reaction that declines after a few trials and this decrement persists for a long period. This robust long-term memory is mediated by an association between the danger stimulus and the environmental cue (Tomsic et al., 1998) and the paradigm has been termed context-signal memory (CSM); extensive studies aimed at recognizing subserving mechanisms have been performed (e.g. Tomsic et al., 1991; Romano et al., 1996; Beron de Astrada and Maldonado, 1999; Freudenthal et al., 1998). Retention of CSM for at least 5 days can be ensured by 15 screen presentations lasting 9 s, given 3 min apart (strong training protocol), but not by fewer than 11 training trials (weak training protocol) (Maldonado et al., 1997).

Our research on the role of angiotensins in this memory model (Delorenzi et al., 1995, 1996, 1997; Delorenzi and Maldonado, 1999) has demonstrated that robust CSM is obtained when crabs are injected with human ANGII (50 pmol) even after a weak training protocol, i.e. angiotensin allows an otherwise insufficient amount of training to induce CSM. This memory-enhancing effect of ANGII is dose-dependent, reversible by saralasin (5 pmol), i.e. by a specific ANGII receptor antagonist, and vanishes when the peptide is given 1 h after weak training. Moreover, CSM is impaired by saralasin (5 pmol) administered before or after strong training, but there is no amnesic effect when the antagonist is given 1 h later. Thus, it was concluded that the acquisition and/or consolidation of CSM are modulated by an endogenous peptide similar to ANGII. In addition, both ACE-like activity and ANGII-like immunoreactivity have been demonstrated in the nervous tissue and gills of the crab *Chasmagnathus granulatus* (Delorenzi et al., 1996).

What environmental change triggers a process that is mediated by endogenous angiotensins and yields an enhancement of the memory capabilities of the crab similar to that induced by exogenous ANGII? In searching for an answer to this question, a change in medium salinity seemed a reasonable possibility to be explored. In fact, the classical function of both central and circulating angiotensins in vertebrates (Fitzsimons, 1998), confirmed for invertebrates (Laurent et al., 1995; Salzet et al., 1995), is that of fluid homeostasis, i.e. angiotensins mediate a physiological response to a disturbance in the water balance akin to that produced by a change in medium salinity. *Chasmagnathus granulatus* usually lives in brackish water (10.0–14.0 ‰) but is a euryhaline animal that proves to be a powerful hypo-hyperosmotic regulator when confronted with a decrease or an increase in water salinity (Schmitt and Santos, 1993; D'Incao et al., 1992). The present study, therefore, explored the possibility that a change in medium salinity brings about an increase in the level of angiotensin-like peptide and an improvement in CSM in *Chasmagnathus granulatus*.

Materials and methods

Animals

Adult male crabs of the species *Chasmagnathus granulatus* Dana, 2.6–2.9 cm across the carapace and weighing 17±0.4 g (mean ± S.E.M., N=60), were collected from the rias (narrow coastal inlets) of San Clemente del Tuyú, Argentina. In the laboratory, they were kept in collective tanks (consisting of plastic containers (35 cm×48 cm×27 cm), slightly tilted lengthwise and filled with 1 l of suitably diluted sea water so that the water was 2 cm deep at the lower end) to a density of 20–25 crabs per tank. Water in the tanks and other containers used during experiments was prepared with hw-Marinex (Winex-Germany) (the salinity in the collective tanks was approximately 12.0 ‰, pH 7.4–7.6). The holding room containing the tanks was maintained on a 12 h:12 h light:dark cycle (lights on from 07:00 to 19:00 h). The animals were not fed, and their water was changed every 2 days. The temperature of both the holding and experimental rooms was maintained between 22 and 24 °C. Experiments were started 2–3 days after the arrival of the animals over a period from January to June. Each crab was used in one experiment only.

Experiments on context-signal memory

Apparatus

The apparatus has been described in detail elsewhere (Maldonado et al., 1997). Briefly, the experimental unit was the actometer: a bowl-shaped plastic container with a steep concave wall and a circular central flat floor 10 cm in diameter and covered to a depth of 0.5 cm with water. The crab was placed in the container, which was suspended by strings from an upper framework and illuminated with a 10 W lamp placed 30 cm above the animal. An opaque, motor-operated rectangular screen (25.0 cm×7.5 cm) was moved horizontally over the head of the animal, cyclically from left to right and *vice versa*. A cycle of movement lasted nearly 4 s. Screen displacements provoked a running response by the crab, causing the container to vibrate. A stylus was glued centrally to the bottom of the container and connected to a piezoelectric transducer. Container vibrations induced electrical signals that were amplified, integrated during the recording time (9 s) and translated into numerical units ranging from 0 to 3000, before being processed by a computer. The scores were directly proportional to the amplitude and number of vibrations recorded for a period of 9 s. The experimental room contained 40 actometers, separated from each other by partitions. A computer was employed to program the number of trials, trial duration and intertrial interval and to monitor experimental events.

Experimental procedure and design

A trial consisted of two successive cycles of screen movement. Since each cycle lasted nearly 4 s, the total trial time was approximately 9 s. Each crab was subjected to a training session and a testing session, separated by a 24 h

intersession interval. Crabs were housed individually during the entire intersession interval in plastic containers (individual rest tanks) filled to 0.5 cm depth with water and kept inside dimly lit drawers. Each experiment included two pairs of groups, each pair consisting of a trained group (TR) and a control group (CT) with the same number of crabs per group. During the training session, TR crabs were given seven trials separated by 171 s, while CT crabs were kept in the actometers without training. During the testing session, all the crabs, i.e. both CT and TR, received two trials separated by 171 s. Before the first training trial or the first test trial, there was a 10 min adaptation time in the actometers. The activity of TR crabs was recorded throughout each trial with screen presentation, during both training and testing, and for periods of 9 s separated by 171 s during adaptation periods, i.e. without screen presentation. The activity of CT crabs was recorded for periods of 9 s separated by 171 s during the training session and both adaptation periods (i.e. without screen presentation) and during each trial time at testing (with screen presentation). Each group consisted of 30–40 animals.

Drugs and injection procedure

Crustacean saline solution (Hoeger and Florey, 1989) was used as a vehicle. Saline or angiotensin II (ANGII) solution (50 pmol per crab; 50 µl) was injected through the right side of the dorsal cephalothoracic-abdominal membrane by means of a syringe fitted with a sleeve to control the depth of penetration to 4 mm, thus ensuring that the injected solution was released in the pericardial sac. Human angiotensin II was purchased from Sigma Co.

Analysis of results

Context-signal memory retention was assessed by focusing the data analysis on testing scores. A trained group (TR) that has undergone a training session 24 h previously is considered to show memory retention at testing when its response level is significantly lower than that of the respective control group (CT). Rescorla (1988) convincingly argued in favour of using this sort of analysis instead of a paired training/testing comparison, stressing the need to distinguish between the time of input (training session) and the time of assessment (testing session).

In all previous experiments in our laboratory, a significant difference between CT and TR (*t*-test, P=0.05) was disclosed at testing 24 h after 20 or more crabs were given 15 or more training trials separated by 171 s (strong training protocol). In contrast, no CT–TR difference was found when fewer than 11 trials were given (weak training protocol). In behavioural experiments in the pesent paper (see Tables 2 and 4), crabs received weak training, so the response level of the TR crabs was expected to be similar to that of the CT crabs, and the results were therefore analyzed using *a priori* planned comparisons, weighed analysis of variance (ANOVA) with P=0.05 (Rosenthal and Rosnow, 1985). The planned comparisons included two types of contrasts: between control groups and between groups of each CT–TR pair.

Radioimmunoassay

The radioimmunoassay (RIA) method, developed in a previous study to determine levels of immunoreactivity in *Chasmagnathus granulatus* (Delorenzi et al., 1996), is described briefly here. Animals were anaesthetized by cooling, and the tissues were rapidly removed. Samples of haemolymph from other crabs were drawn from the blood sinus at the base of the fourth or fifth pair of pereiopods between 12:00 and 14:00 h. Separate pools of 10 supraoesophageal ganglia, four thoracic ganglia or five samples of blood (250 µl) were placed in 2 mol l^{-1} acetic acid, boiled for 20 min, homogenized and centrifuged at 8000 ***g*** for 20 min. The pellet was used for protein determination using the method devised by Lowry et al. (1951). The supernatants were lyophilized, and the residues were dissolved in radioimmunoassay buffer (0.05 mol l^{-1} NaH_2PO_4, 0.1 % bovine serum albumin, 0.3 mmol l^{-1} sodium azide, 1 µmol l^{-1} aprotinin, 0.1 mmol l^{-1} EDTA, 0.1 nmol l^{-1} phenylmethylsulphonyl amide, pH 7.4). Anti-angiotensin II antibody was raised in New Zealand white rabbits immunized with human ANGII coupled to bovine serum albumin, using a glutaraldehyde reaction (25 % w/v) (Becker et al., 1976). ^{125}I-labelled ANGII was prepared using the chloramine-T method (Greenwood et al., 1963), using ^{125}I (629 GBq mg^{-1}, DuPont) and purified by high-performance liquid chromatography (Waters Ass., Milford, MA, USA). The minimum detectable amount of ANGII was 6 pg. The intra-assay coefficient of variation was 6 %, and the interassay coefficient of variation was 15 %. The antibody used in this RIA had less than 1 % cross-reactivity with human thyrotropin-releasing hormone, vasopressin and angiotensin I. All the reagents described in this section were purchased from Sigma Co. unless indicated otherwise.

Immunohistochemistry

Brains and optic lobes were rapidly dissected as above and fixed overnight in 4 % paraformaldehyde in 0.1 mol l^{-1} phosphate-buffered saline (PBS). After buffer washes and a 24 h immersion in 30 % sucrose (in buffer), the tissues were frozen on the stage of a Leitz cryostat. Serial cryostat sections were cut at a thickness of 20–25 µm and mounted on chrome-alum-coated slides. Two antisera to angiotensin II were used for immunolabelling: one obtained from Peninsula (Belmont, CA, USA) and the one described above. Both antisera were applied at dilutions ranging for 1:500 to 1:2000 in a dilution buffer consisting of 0.01 mol l^{-1} PBS with 0.5 % bovine serum albumin, 2 % horse normal serum and 0.25 % Triton X-100. Sections were incubated in antiserum for 48 h at 4 °C. Antibody binding was visualised with the avidin:biotinylated enzyme complex method using a commercial kit (Vectastain ABC Elite; Vector Laboratories, Burlingame, CA, USA) with peroxidase as the marker and diaminobenzidine as the chromogen. As a control for specificity, we preincubated each of the two angiotensin antisera with angiotensin II (Peninsula) (50 nmol ml^{-1} in diluted antiserum) overnight with gentle agitation at 4 °C. These preabsorbed antisera were then used for immunocytochemistry, as outlined above. In a second

control, primary antiserum (anti-angiotensin) was omitted from the protocol. Micrographs were obtained using a Zeiss Axioplan II microscope with a chilled integrated colour CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). Images were edited in Adobe Photoshop 4.0.

Exposure to water of different salinities

Since *Chasmagnathus granulatus* is a semiterrestrial crab that lives in brackish water (10.0–14.0 ‰) in intertidal zones, a salinity of approximately 12.0 ‰ was considered standard and was chosen for the collective tanks. During experiments, crabs were exposed to more closely controlled conditions of salinity (2.5, 12.0 or 33.0 ‰). For this purpose, no more than 20 animals were placed in exposure tanks consisting of roofed untilted containers (50 cm×45 cm×27 cm) larger than the collective tanks, with 2 l of water, renewed daily, and salinity controlled by an optical densitometer. Water depth in exposure tanks was approximately 0.5–1.0 cm so that, although these semiterrestrial crabs were not completely submerged, the water level reached the Milne-Edwards aperture, i.e. the opening of the branchial cavity.

Results

Exposure to high salinity for 6 days increases the level of brain angiotensin

Three groups of 50 crabs each were placed for 6 days in exposure tanks with water of different salinity (2.5, 12.0 and 33.0 ‰) (Table 1). For one group, the salinity was nearly the same (12.0 ‰) as that of the collective tanks, while for each of the other two groups, the new salinity in the exposure tanks was higher (33.0 ‰) or lower (2.5 ‰) than that of the collective tanks. After 6 days in exposure tanks, the level of angiotensin-II-like immunoreactivity in the blood, brain and thoracic ganglion of *Chasmagnathus granulatus* was estimated by radioimmunoassay. The rationale for choosing such a period of exposure is that the initial effects of a change in medium salinity (e.g. oscillations in blood osmolarity or blood glucose concentration) vanish after 6–7 days (Nery and Santos, 1993; Luquet et al., 1992; A. Delorenzi, unpublished observations).

Table 1. *Experimental protocol used in experiments to determine the effects of salinity on angiotensin-II-like immunoreactivity in the central nervous system and blood of the crab* Chasmagnathus granulatus

Collective tank salinity (‰)	Exposure tank salinity (‰)						RIA
12.0	33.0	33.0	33.0	33.0	33.0	33.0	*
	12.0	12.0	12.0	12.0	12.0	12.0	*
	2.5	2.5	2.5	2.5	2.5	2.5	*
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	

*Radioimmunoassay (RIA) was performed on pools of 10 brains (N=5), four thoracic ganglia (N=5) and five pooled samples of blood (N=5).

The results are given in Fig. 1, which shows that the level of ANGII-like immunoreactivity in the brain, after 6 days of exposure, was significantly higher for crabs exposed to 33.0 ‰ salinity than for those exposed to 2.5 or 12.0 ‰ salinity (one-way ANOVA, $F_{3,13}$=5.53; P<0.05; Duncan test, P<0.05). There were no significant differences between groups in the amount of ANGII-like immunoreactivity in the blood or thoracic ganglion.

Exposure to high salinity for 6 days enhances context-signal memory

Consistent with the above results and with the reproducible finding that exogenous ANGII given immediately after weak training enhances CSM (Delorenzi and Maldonado, 1999; Delorenzi et al., 1996), a not unreasonable prediction is that high salinity enhances memory. To test this, two experiments were performed.

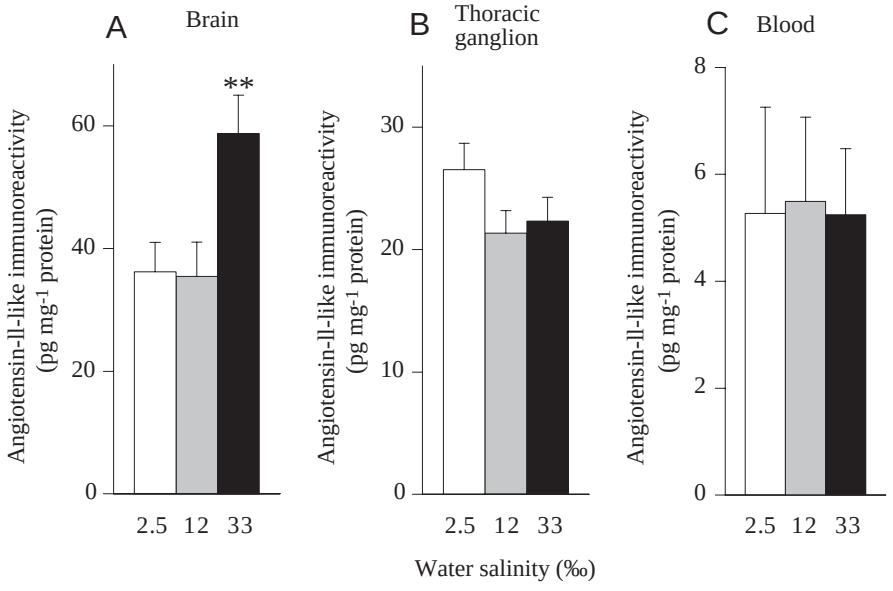

Fig. 1. Levels of angiotensin-II-like immunoreactivity after 6 days of exposure to different salinity (2.5, 12.0 and 33.0 ‰) (A) in pooled samples of 10 supraoesophageal ganglia (brains) (five pools), (B) in pooled samples of four thoracic ganglia (five pools) and (C) in four pooled samples of blood (250 µl) (four pools). Angiotensin-II-like immunoreactivity was evaluated by radioimmunoassay. Duncan test (33.0 ‰ *versus* 12.0 ‰ or *versus* 2.5 ‰): **P<0.01. Values are means + S.E.M. The experimental protocol is explained in Table 1.

Table 2. *Experimental protocol used in experiments to determine the effect of exposure to different salinities on context-signal memory in* Chasmagnathus granulatus

		Exposure tanks		Actometers		Rest tanks	Actometers
	CT–TR pairs	Exposure (days)	Salinity (‰)	Training: number of trials*	Injection	Intersession interval (h)	Testing: number of trials
Experiment 1	CT–TR1	6	12	7	Saline	24	2
	CT–TR2	6	12	7	ANGII	24	2
Experiment 2	CT–TR1	6	2.5	7	–	24	2
	CT–TR2	6	12	7	–	24	2
	CT–TR3	6	33	7	–	24	2

*CT crabs were not presented with the screen during the training session, but their activity was recorded during each trial.
CT, control group; TR, training group; ANGII, animals injected with angiotensin II (50 pmol per animal).

In one experiment (Table 2, experiment 1), two groups of CT–TR pairs were kept for 6 days in exposure tanks, then given a training session in actometers (seven trials for TR crabs), placed in individual rest tanks for 24 h and transferred back to actometers for testing (two trials for CT and TR crabs). During all these phases, the water had the standard salinity (12.0 ‰). Immediately after the last training trial, one CT–TR pair (CT–TR1) was injected with saline solution and the other pair (CT–TR2) with ANGII (50 pmol). Results corresponding to the two-trial test showed a significant difference between groups for the ANGII pair, but not for the saline pair (Fig. 2A). Planned comparisons disclosed a significant difference between ANGII groups ($F_{1,96}$=5.2, P<0.05) but no differences between saline groups or between control groups. Thus, the enhancing effect of angiotensins on CSM, previously demonstrated in crabs kept for 2 days in collective tanks with brackish water (Delorenzi et al., 1996), also occurs after a long stay in exposure tanks where the salinity is controlled at 12.0 ‰.

In the second experiment (Table 2, experiment 2), three groups of CT–TR pairs were each kept at a different salinity, 2.5, 12.0 or 33.0 ‰ (CT–TR1, 2 and 3 respectively), during all phases, i.e. during the 6 day stay in the exposure tanks, the training session, the 24 h rest interval and the two-trial testing. No injections were given to these groups. No difference was observed in levels of activity during pre-training (i.e. for the 10 min period in the actometers before training, without presentation of the danger stimulus) or in the level of the escape response during the seven training trials (data not shown), indicating that differences in salinity have no effect on performance. However, results corresponding to the two-trial testing session, conducted 24 h

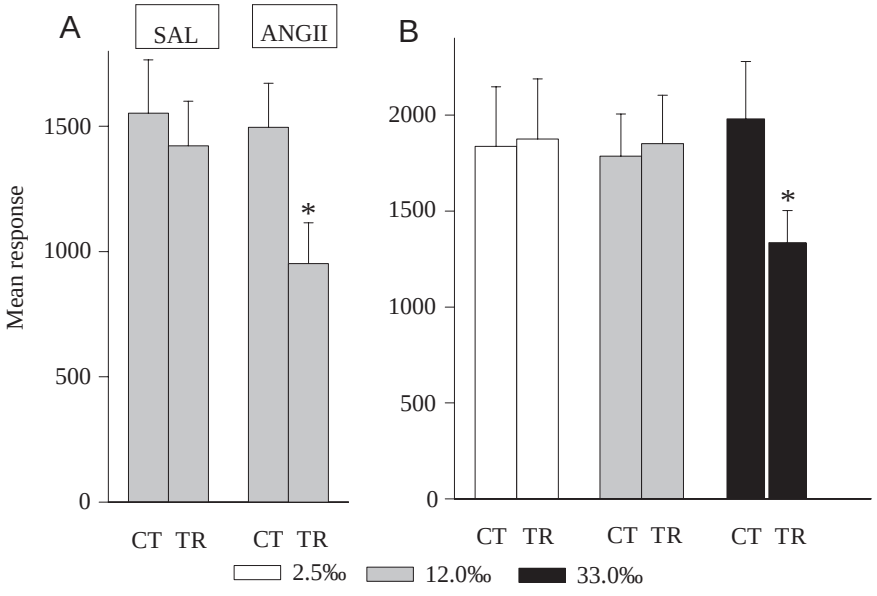

Fig. 2. (A) Facilitatory effect of a post-training injection of angiotensin II on context-signal memory. CT, control group; TR, trained group. Two pairs of CT–TR groups (25 crabs per group) were kept at a salinity of 12.0 ‰ for 6 days and then given a weak training session (seven trials for TR groups), a 24 h intersession interval and two test trials. In one group of CT–TR pairs, animals were injected with saline vehicle (SAL) and in the other with angiotensin II (ANGII), 50 pmol per animal. The ordinate shows the mean accumulative scores of the escape response (see Materials and methods) for the two testing trials (two-trial block) 24 h after the weak training. Planned comparisons are made between the CT and TR groups of each pair and between control groups: *P<0.05. Values are means + S.E.M. The experimental protocol is shown in Table 2 (experiment 1). (B) Facilitatory effect of keeping crabs at a salinity of 33.0 ‰ for 6 days on context-signal memory. Three pairs of CT–TR groups (32 crabs per group) were kept at different salinities (2.5, 12.0 and 33.0 ‰) for 6 days; all were then subjected to a weak training session (seven trials for TR groups), a 24 h intersession interval and two test trials. Other details are as in A. The experimental protocol is shown in Table 2 (experiment 2).

Table 3. *Experimental protocol used in experiments to determine the effects of the time of exposure to high salinity on angiotensin-II-like immunoreactivity in the supraoesophageal ganglia of the crab* Chasmagnathus granulatus

Collective tanks salinity (‰)	Salinity in the exposure tanks (‰)				
	Day 1	Day 2	Day 3	Day 4	RIA
12.0	33.0	33.0	33.0	33.0	*
	12.0	12.0	33.0	33.0	*
	12.0	12.0	12.0	12→33 (1 h)	*
	12.0	12.0	12.0	12.0	*

*Radioimmunoassay (RIA) was performed on pools of 10 brains (N=4).

after training, disclosed differences between CT and TR crabs in the groups kept at 33.0‰ (planned comparisons: $F_{1,186}$=4.5, P<0.05) but no difference between CT and TR crabs at salinities of 2.5 and 12.0‰ and no differences between controls (Fig. 2B). A robust retention of memory was also obtained when a salinity of 45.0‰ instead of 33.0‰ was used (data not shown).

These results shown that an insufficient amount of training (i.e. seven training trials) can yield CSM either if the crabs are given a post-training injection of angiotensin or if they are kept for 6 days at a salinity approximately three times higher than 12.0‰ which, in turn, is correlated with an endogenous increase in brain angiotensin levels.

Levels of brain angiotensin increase with the duration of exposure to high salinity

To explore levels of brain angiotensin in animals exposed to high salinity for periods considered too short to allow full recovery of normal haemolymph osmolarity (less than 6 days), four groups of 50 crabs were exposed to high salinities for different times (Table 3). For one group, salinity was 33.0‰ for 4 days after transfer from the collective tanks (brackish water); for a second group, salinity was 12.0‰ for 2 days and then 33.0‰ for the next 2 days; for a third group, salinity was 12.0‰ for 4 days and raised to 33.0‰ for the last hour of the fourth day, while the fourth group was kept at a salinity of 12.0‰ for the whole 4 days. At the end of 4 days in the exposure tanks, the level of angiotensin-II-like immunoreactivity was estimated in separate pools of 10 supraoesophageal ganglia (four pools for each group) by radioimmunoassay.

The results (Fig. 3) show an increase in the level of ANGII-like immunoreactivity in the brain as the total length of exposure to high salinity (33.0‰) increases. A one-way ANOVA showed a significant main effect (F=3.56, P=0.047). Only the group that had been kept for 4 days in 33.0‰ showed a level of ANGII-like immunoreactivity significantly greater than that of the animals kept for 4 days in 12.0‰ (P<0.05; Tukey honest significant difference test).

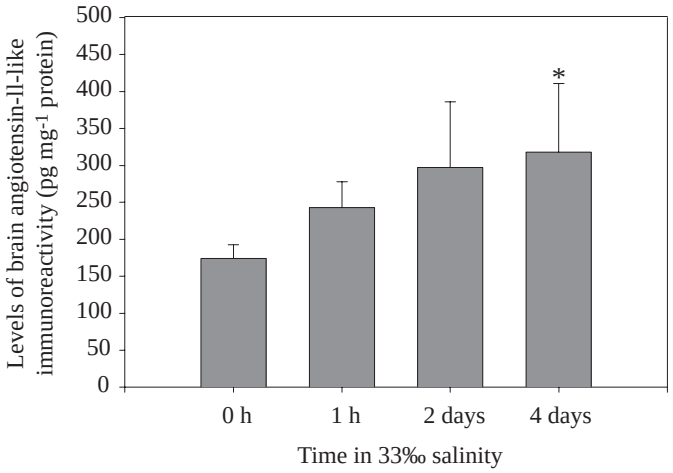

Fig. 3. Levels of brain angiotensin-II-like immunoreactivity in the supraoesophageal ganglia of crabs kept at a salinity of 33.0‰ for different times (0 h, 1 h, 2 days or 4 days). Angiotensin-II-like immunoreactivity was evaluated by radioimmunoassay (RIA) in pools of 10 supraoesophageal ganglia (brains) (four pools). Values are means + S.E.M. Tukey test of multiple comparisons: *P<0.05. The experimental protocol is shown in Table 3.

Context-signal memory enhancement depends on the duration of exposure to high salinity and is not attributable to osmotic stress

The above results suggest a direct functional relationship, mediated by angiotensin II, between high salinity and memory enhancement. It might be argued, however, that stress induced by the abrupt change in salinity might account for the improvement in memory, as proposed several times for other stressful situations in different species (Cahill and McGaugh, 1996). If memory enhancement were attributable to stress, it would be expected that the shorter the exposure period, the more manifest would be the facilitatory effect on memory. To explore this issue, three experiments were carried out (Table 4), each including two groups of CT–TR pairs, one of which was kept at a salinity of 12.0‰ and the other at a salinity of 33.0‰. In experiment 1, animals stayed in the exposure tanks for only 1 h, in experiment 2, for 2 days and in experiment 3, for 4 days. No injections were given in any experiment and, after the different periods of exposure, all groups passed through the same phases: the training session (seven trials), the intersession interval (24 h) and the testing session (two trials), keeping the same salinity.

The results corresponding to the two-trial test are shown in Fig. 4 and indicate that only the group exposed to 33.0‰ for 4 days showed retention. Planned comparisons performed separately for each experiment confirmed this conclusion: the only significant difference in comparisons was that corresponding to the 33.0‰ CT–TR pairs after 4 days of exposure ($F_{1,120}$=4.9, P<0.05). No retention was shown after a 2 day exposure to 33.0‰ salinity or a 1 h exposure, i.e. at a time when the crab is assumed to be under the strongest

Table 4. *Experimental protocol used in experiments to determine the effects of the time of exposure to high salinity on context-signal memory in* Chasmagnathus granulatus

		Exposure tanks		Actometers	Rest tanks	Actometers
	CT–TR pairs	Exposure duration	Salinity (‰)	Training: number of trials*	Intersession interval (h)	Testing: number of trials
Experiment 1	CT–TR1	1 h	12	7	24	2
	CT–TR2	1 h	33	7	24	2
Experiment 2	CT–TR1	2 days	12	7	24	2
	CT–TR2	2 days	33	7	24	2
Experiment 3	CT–TR1	4 days	12	7	24	2
	CT–TR2	4 days	33	7	24	2

*CT crabs were not presented with the screen during the training session, but their activity was recorded during each trial.
CT, control group; TR, training group.

osmotic stress. These findings therefore rule out the possibility that the facilitatory effect is caused by osmotic stress. Instead, they support the view that the effect becomes manifest when the regulatory phase that follows an osmotic shock is complete and, concomitantly, when the level of brain angiotensin-like peptides reaches its highest level (Fig. 3).

Angiotensin-like immunoreactivity in the central nervous system of Chasmagnathus granulatus

Since RIA determinations showed changes in the level of ANGII-like reactivity in the supraoesophageal ganglion, correlating with an increase in water salinity (Fig. 1), it seemed pertinent to complete the present study by testing for the presence of ANGII-like immunoreactive material in the neural structures of the brain. For this purpose, an immunohistochemical analysis was performed on the supraoesophageal ganglion and the optic lobes (located in the eyestalks). Each eyestalk contains four main neuropil regions: the lamina ganglionaris, medulla externa, medulla interna and medulla terminalis, which is an extension of the lateral protocerebrum (Strausfeld and Nässel, 1980; Sandeman et al., 1992). Two antisera to angiotensin II produced very similar patterns of immunolabelling, but no immunolabelling was detected when using antisera preabsorbed with angiotensin II (50 nmol ml^{-1} antiserum) or after omission of the primary antisera. Cell bodies of 10–12 neurons with ANGII-like reactive material were detected at the anterior base of the medulla externa. These labelled cell bodies form extensive processes connecting the medulla externa and the lamina ganglionaris (Fig. 5A–D). Prominent ANGII-like reactive material was also observed in the sinus gland (Fig. 6A,B), indicative of a neurohormonal role for an angiotensin-like peptide. The cellular origin of processes terminating in the

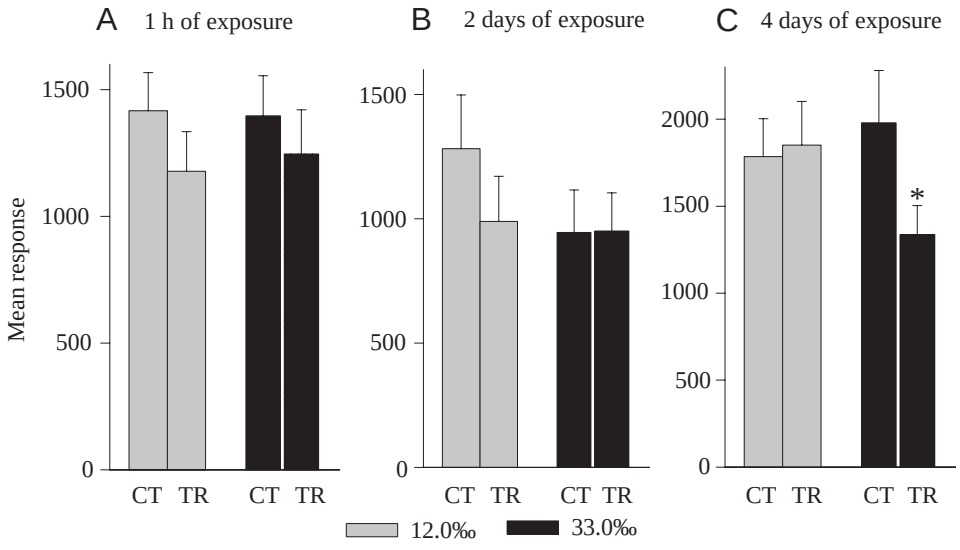

Fig. 4. The effects on context-signal memory of keeping crabs at a salinity of 33.0‰ for different times. CT, control group; TR, trained group. Each experiment included two groups of CT–TR pairs: one kept at a salinity of 12.0‰ and the other at 33.0‰, then given a weak training session (seven trials for TR groups), a 24 h intersession interval and two test trials. (A) Exposure for 1 h (experiment 1, see Table 4). (B) Exposure for 2 days (experiment 2, see Table 4). (C) Exposure for 4 days (experiment 3, see Table 4). The ordinate shows the mean accumulative scores of the escape response (see Materials and methods) for the two testing trials (two-trial block) 24 h after the weak training. Planned comparisons were made between the CT and TR groups of each pair and between the controls of each experiment: *P<0.05. Values are means + S.E.M.

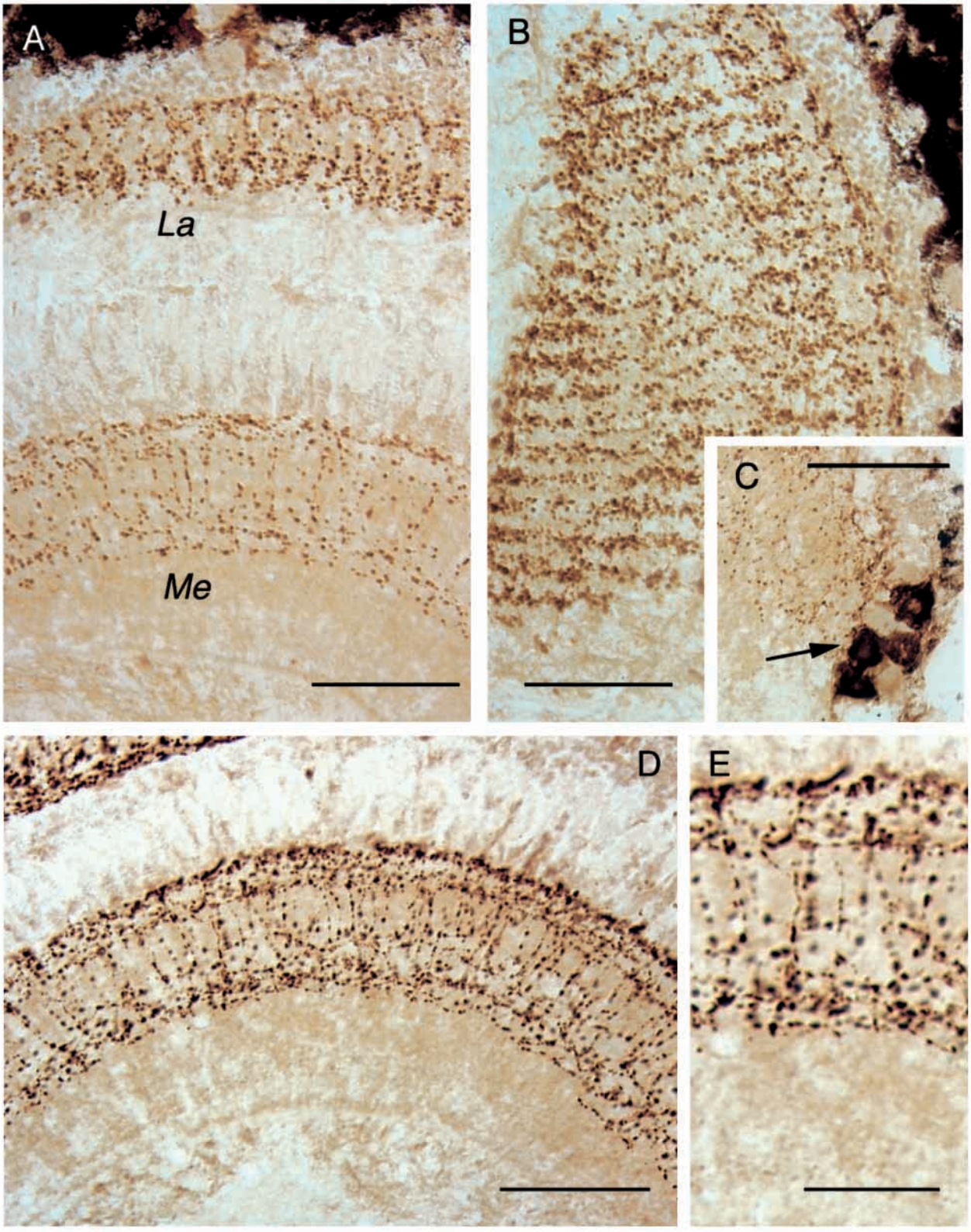

Fig. 5. Micrographs of sections through the optic lobes of *Chasmagnathus granulata* showing angiotensin-II-like immunoreactivity. (A) Labelled neuronal processes in the two outermost neuropils of the visual system. *Me*, medulla externa; *La*, lamina ganglionaris. Scale bar, 100 µm. (B) Labelling of varicose processes in the lamina synaptic neuropil. Scale bar, 100 µm. (C) Cell bodies of neurons with angiotensin-II-like immunoreactive material at the anterior base of the medulla externa. Scale bar, 100 µm. (D) A view of the medulla externa with abundant immunoreactive material in neural processes. Scale bar, 100 µm. (E) Higher magnification of the neuropil of the medulla externa. Scale bar, 50 µm.

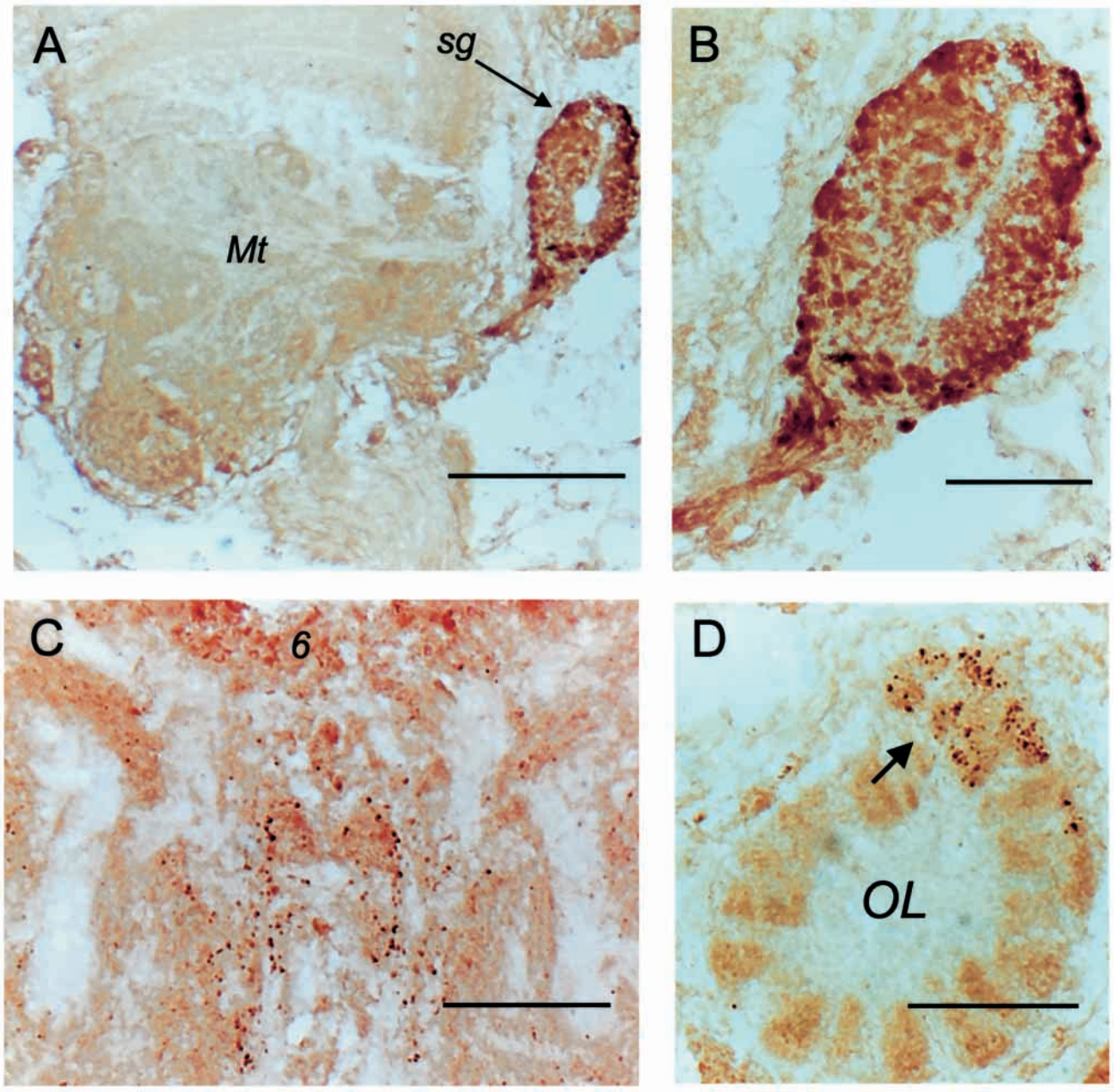

Fig. 6. (A,B) Prominent angiotensin-II-like immunoreactive material in the sinus gland (*sg*). *Mt*, medulla terminalis. Scale bar, 300 μm. (B) The labelled sinus gland at a higher magnification. (C) Immunoreactive neuronal processes in the median protocerebrum (AMPO); *6*, cell cluster 6. (D) Angiotensin-II-like immunoreactive material (arrow) in the columnar neuropils of an olfactory lobe (*OL*). Scale bars in B–D, 100 μm.

sinus gland, however, remains unclear. ANGII-like material in the supraoesophageal ganglion was less conspicuous. Immunostaining of sections with ANGII antiserum showed labelling in neuronal processes in the median protocerebrum (Fig. 6C) and the olfactory lobes (Fig. 6D), but not in the cell bodies.

Discussion

Four main conclusions can be drawn from the present results. First, administration of angiotensin II has a clear-cut facilitatory effect on crab memory (Fig. 2A), a finding consistent with previous work and consistent with the proposal that endogenous angiotensin-like peptides enhance crab memory (Delorenzi and Maldonado, 1999). Second, an increase in water salinity from 12 to 33.0 ‰ induces memory improvement and an increase in the level of angiotensin-like peptides in the brain of *Chasmagnathus granulatus* (Figs 2B, 4). Third, the level of brain angiotensin-like peptides increases with the duration of exposure to a salinity of 33.0 ‰, apparently showing a significant increase above controls levels at 4 days (Fig. 3). No increase in the level of this peptide in

the blood or in the thoracic ganglion was detected. Fourth, memory enhancement induced by exposure to high salinity seems to be accomplished after the crabs have remained in the new salinity for long enough for them to have re-established their initial haemolymph osmolarity (Luquet et al., 1992; A. Delorenzi, unpublished observations). Correlation among these facts suggests that exposure to water of high salinity is an external cue that triggers a process, mediated by angiotensins, that enhances crab memory. This conclusion represents a further step in a line of research that started 4 years ago as an attempt to study the function of angiotensins in the memory of an invertebrate.

The immunohistochemical analysis revealed far more ANGII-like immunoreactive material in the eyestalk ganglia than in the suboesophageal ganglion. The prominent presence of immunoreactive material in the neurohaemal sinus gland together with the determination of haemolymph ANGII-like content by radioimmunoassay (Fig. 1, see Delorenzi et al., 1996) are indicative of a hormonal role for ANGII-like material from that gland. It would be intriguing to use radioimmunoassay to explore whether there is a change in the amount of ANGII-like material in the eyestalk after exposure to different salinities, thus possibly influencing visual cues of memory. In addition, an immunohistochemical analysis of the thoracic ganglion would be appropriate to complete the picture of neuronal ANGII-like immunoreactivity in the crab central nervous system.

No evidence is provided here to support a role for angiotensins in crab osmoregulation. However, the results showing correlation between water salinity and brain angiotensin levels, as well as those indicative of a putative hormonal role for these peptides through their release from the sinus gland, support a function for angiotensins in regulating fluid balance, as in vertebrates and other invertebrates. The presence of salinity-sensing 'hair-peg organs' on crab leg dactyls is well known and it is possible that they play a role in the neuronal transmission of environmental stimuli to the brain (Schmidt, 1989). Thus, crab angiotensins could act both as memory modulators and as osmoregulators, sharing the vertebrate feature of being responsible for a wide spectrum of physiological actions (Yang et al., 1996).

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