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REGULAR ARTICLE

# Neuroanatomical distribution of angiotensin-II-like neuropeptide within the central nervous system of the crab *Chasmagnathus*; physiological changes triggered by water deprivation

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**Abstract** The angiotensins constitute a neuropeptidergic system that emerged early in evolution. Their classical osmoregulatory and dipsogenic functions and their mnemonic actions have been demonstrated both in vertebrates and in some invertebrates. Previously, we have shown that, in the euryhaline and semiterrestrial crab *Chasmagnathus granulatus*, water deprivation correlates with an increased level of brain angiotensin-II-like neuropeptide/s (ANGII-like) and improves memory processes through ANGII receptors. We have proposed that the release of brain angiotensins in response to water shortages is an ancient mechanism for coordinating various functions that, together, enable organisms to tolerate this environmental change. Here, we have evaluated the physiological changes in ANGII-like levels in diverse structures of the central nervous system of these animals during water deprivation. The neuroanatomical distribution of ANGII-like is described in the optic lobes and brain of *Chasmagnathus granulatus* and the physiological changes in ANGII-like distribution in various brain neuropils is evaluated after water deprivation. Our results indicate that ANGII-like is widely distributed, especially in the medial protocerebrum. After 2 h of water deprivation, ANGII-like immunoreactivity increases in the central body and decreases in the olfactory neuropil and, after 6 h of water deprivation, is markedly reduced in several brain areas. Although further experiments are needed to establish that the angiotensinergic system is involved in the balance of body fluids in this crab, our results suggest that ANGII regulates several functions during water shortages.

**Keywords** Memory · Osmoregulation · Neuropeptide · Evolution · *Chasmagnathus granulatus* (Crustacea)

**List of abbreviations**

| | |
|---|---|
| AMPN | anterior medial protocerebral neuropils |
| ANGII | angiotensin II |

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| | |
|---|---|
| ANGII-ir | angiotensin-II-like immunoreactivity |
| AnN | antenna II neuropil |
| AOI | area of interest |
| BLG2 | bistratified lobula giant 2 |
| CA | cerebral artery |
| CB | central body |
| CEC | circumesophageal connectives |
| CNS | central nervous system |
| HB | hemi-ellipsoid body |
| LAN | lateral antenna I neuropil |
| LMT | lateromedial tangential layer |
| MAN | median antenna I neuropil |
| MLG1 | monostratified lobula giant 1 |
| MT | medulla terminalis |
| OGT | olfactory globular tract |
| ON | olfactory neuropil |
| PDH | pigment-dispersing hormone |
| PDH-ir | pigment-dispersing hormone immunoreactivity |
| PMPN | posterior medial protocerebral neuropils |
| PT | protocerebral tract |
| Tn | tegumentary neuropil |

## Introduction

The ability to cope with changes in environmental salinity is one of the most important adaptations for the survival of decapod crustaceans; nevertheless, little is known regarding the nature of neuroendocrine factors that control their hydromineral metabolism. To our knowledge, the crustacean hyperglycemic hormone is the only neuropeptide known to be involved in osmoregulation in crustaceans (Spanings-Pierrot et al. 2000; Morris 2001; for a review, see Serrano et al. 2003). Even though angiotensins constitute a neuropeptidergic system that emerged early in evolution (Laurent et al. 1995; Nishimura 2001; Salzet et al. 2001), a comparison of amino acid sequences has shown that some residues (such as Arg2, Tyr4, His6, Pro7, Phe8 and Leu10) are well conserved among vertebrates. On the other hand, other residues are highly variable and differ even among species of the same class (Takei et al. 2004; Watanabe et al. 2009). The only angiotensin sequenced in invertebrates to date and whose structure is identical to that of humans is angiotensin I of leeches (Salzet et al. 1993; Laurent et al. 1995). Evidence for the classical osmoregulatory and dipsogenic functions of the angiotensins has been provided in some invertebrate species (Makra and Prior 1985; Fitzsimons 1998; Takei et al. 2004; for a review, see Salzet et al. 2001; Satou et al. 2005a, 2005b). However, to the best of our knowledge, the structure of angiotensin II (ANGII) has not been identified in arthropods as yet.

Our earlier results in the euryhaline and semiterrestrial crab *Chasmagnathus* have led us to propose that ANGII is a coordinator of several physiological functions. *Chasmagnathus*, which is a potent osmoregulator, is subjected to daily fluctuations in water availability because of both foraging behaviour and tidal cycles and can tolerate air exposure for several hours (Schmitt and Santos 1993). We have previously shown that water shortage, an ethologically plausible scenario, results in increased brain ANGII-like immunoreactivity (ANGII-ir) and an ANGII-mediated improvement in memory (Delorenzi et al. 1995, 1996, 1997, 2000; Delorenzi and Maldonado 1999; Frenkel et al. 2002, 2005a, 2005b). Specifically, we have shown that, when crabs are water-deprived for 2 h, an increase in both brain ANGII-ir, as evaluated by radioimmunoassay, and haemolymph sodium occurs, returning to basal values 6 h later (Schmitt and Santos 1993; Frenkel et al. 2002). In order to test a plausible neuronal source for brain ANGII-ir, we have evaluated the presence of ANGII-like-immunoreactive material in the central nervous system (CNS) by immunohistochemistry and found ANGII-like-immunoreactive material in some neuron somata and in neuronal processes of the brain and optic lobes, two polyclonal antisera to human ANGII producing similar patterns of immunolabelling (Delorenzi et al. 2000). However, this analysis is insufficient to describe the neuroanatomical distribution of the ANGII-like neuropeptide in the optic lobes and brain. Therefore, one of the goals of the present study has been to analyse in detail the distribution of ANGII-like-ir in *Chasmagnathus* brain and optic lobes.

In decapod crustaceans, the optic lobes comprise the lamina, medulla, lobula and lateral protocerebrum (Fig. 1a). The most distal of the three optic neuropils is the lamina, whereas the most proximal is the lobula, which receives retinotopic information from the medulla via the second optic chiasm (Sztarker et al. 2005). The medulla terminalis (MT) and the hemi-ellipsoid body (HB) constitute the lateral protocerebrum (Derby and Blaustein 1988; Sandeman et al. 1992). In most malacostracan crustaceans, the lateral protocerebrum is displaced away from the medial brain into the eyestalks and is connected to the medial protocerebrum via the protocerebral tract (PT). The lateral protocerebrum receives output information from the olfactory neuropil (ON; Sullivan and Beltz 2001a, 2001b; Mellon 2007). The brain (Fig. 1b) in decapod crustaceans contains the medial, deuto- and tritocerebrum. The medial protocerebrum can be subdivided into the anterior (AMPN) and posterior (PMPN) medial protocerebral neuropils. The central body (CB) is an unpaired neuropil that extends across the midline and is embedded between them. The deutocerebrum contains two conspicuous paired structures, viz. the ONs, which receive afferent chemosensory

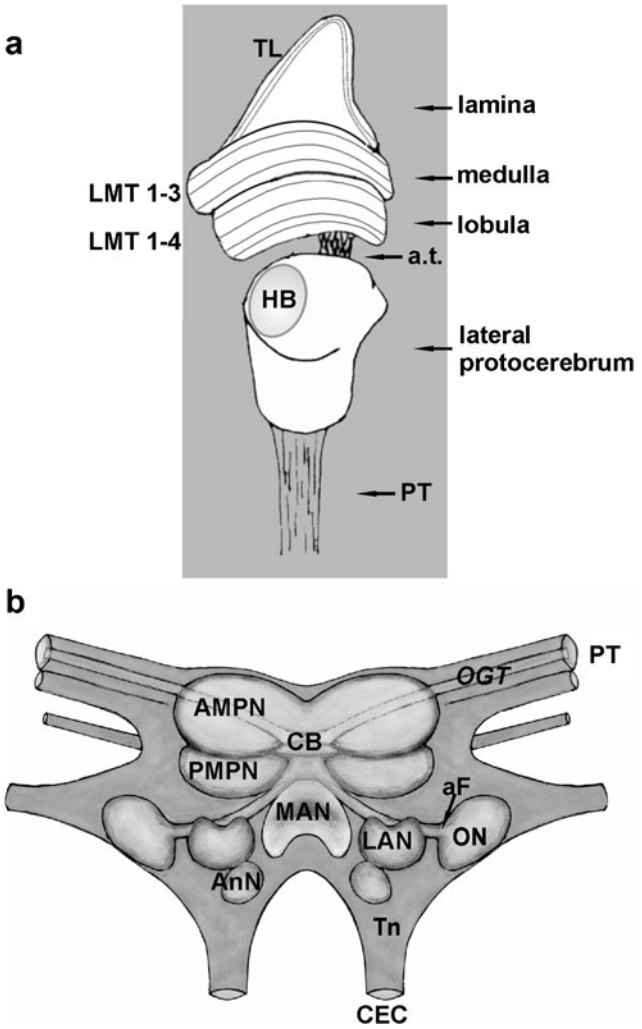



**Fig. 1** Representations illustrating the (**a**) optic lobes (adapted from Berón de Astrada and Tomsic 2002; *grey lines* tangential layers), and (**b**) brain neuropils (*aF* anterior foramen, *AMPN* anterior medial protocerebral neuropils, *AnN* antenna II neuropil, *a.t.* axonal tract, *CB* central body, *CEC* circumesophageal connectives, *HB* hemi-ellipsoid body, *LAN* lateral antennal neuropil, *LMT1–3* lateromedial tangential layers 1–3, *LMT1–4* lateromedial tangential layers 1–4, *MAN* medial antennal neuropil, *OGT* olfactory globular tract, *ON* olfactory neuropil, *PMPN* posterior medial protocerebral neuropils, *PT* protocerebral tract, *TL* tangential layers [distal and proximal], *Tn* tegumentary neuropil)

input from the olfactory receptor neurons of antenna I (Sandeman and Mellon 2002; McKinzie et al. 2003; Schachtner et al. 2005; Sullivan and Beltz 2005; Mellon 2007). The median antenna I neuropil (MAN) extends across the brain posterior to the protocerebrum. The lateral antenna I neuropil (LAN) is known to receive afferents from the mechanoreceptors of antenna I (Sandeman et al. 1992). The tritocerebrum contains the antenna II (AnN) and tegumentary (Tn) neuropils. The former receives mechanosensory afferents from antenna II and contains the motoneurons that control its movements (Tautz 1987; Sandeman et al. 1992). The Tns process inputs from mechanoreceptors and other sensillae of the dorsal carapace (Sandeman et al. 1992). Sandeman et al. (1992) have recognized 17 different clusters of cell bodies associated with the decapod brain; these clusters are numbered 1–17 from anterior to posterior.

The second goal of our study has been to test the hypothesis that water deprivation affects the ANGII-ir distribution in the various neuropils of the brain. To this end, we first describe, with a murine monoclonal antibody, the detailed ANGII-ir distribution in the brain and optic lobes by using a whole-mount immunohistochemistry approach. We then evaluate the physiological changes in ANGII-like distribution in various brain neuropils when animals are water-deprived.

## Materials and methods

*Animals* Adult male intertidal crabs (*Chasmagnathus granulatus* Dana, 1851), 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 (30cm×45cm×20cm) filled to a depth of 0.5 cm 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 (lights on: 0700–1900 hours). Water was changed every 2 days. The temperature of the holding and experimental rooms was kept between 22°C and 24°C. Experiments were carried out within the first 2 weeks after the arrival of the animals. The 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.

*Water deprivation experiment* Two groups of crabs were water-deprived for 2 or 6 h. These times were chosen because water deprivation for 2 h, but not 6 h, is known to facilitate memory in *Chasmagnathus* (Frenkel et al. 2002, 2005a, 2005b). A non-water-deprived group was also included as a control. After treatment, animals were anaesthetized by immersion for 5 min in an ice-salt water mix and their nervous system was dissected in 4% paraformaldehyde (PFA) in 0.2 M phosphate buffer pH 7.4.

*Immunohistochemistry* The isolated brains and optic lobes were fixed overnight in 4% PFA at 4°C. For whole-mount immunohistochemistry, the tissues were washed for 4 h in several changes of phosphate-buffered saline (PBS) at room

temperature and in one change of PBS-1% Triton X-100 (PBS-Tx) for 30 min and then blocked with 2% normal goat serum and 5% low-fat milk overnight at 4°C. A monoclonal antibody against ANGII, clone 4B3 (Imboden et al. 2009), was used at a concentration of 0.3 μg/ml in buffer solution. This antibody was generated against synthetic human ANGII (Imboden et al. 2009). The specificity for the monoclonal antibody against ANGII has been previously documented in rats (Frei et al. 2001). The primary antibody was incubated in 2% normal goat serum for 48 h at 4°C. Subsequently, tissues were washed in several changes of PBS for 4 h at room temperature and incubated in a secondary Alexa Fluor 488 (Molecular Probes, USA)-labelled goat anti-mouse antibody at a dilution of 1:1000 overnight at 4°C. Tissues were washed in PBS-Tx, dehydrated in increasing ethanol concentrations in PBS and coverslipped with methyl salicylate (Sigma, USA). Pre-absorption (Imboden et al. 2009) was performed by using a batch procedure with the synthetic ANGII peptide, which was covalently linked through its N-terminus to CH-Sepharose 4B. The gel was loaded into a glass column and the monoclonal ANGII-antibody solution effluent was used for immunocytochemical preabsorption controls. These effluent controls, performed on *Chasmagnathus* brain and optic ganglia, showed absolutely no staining. Further controls were carried out with the secondary antibody without the primary antibody. All controls performed in the CNS of *Chasmagnathus* showed absolutely no staining.

*Specificity of the antibody* The specificity of the monoclonal antibody against human ANGII (4B3) is well documented elsewhere. This antibody produces the same staining as a polyclonal antibody against ANGII in rat adrenal glands. The 4B3 antibody is a protein-G-purified murine monoclonal antibody (Mab-Trap G II column, Amersham Sciences) generated against a synthetic human ANGII peptide. For immunization of the mice, ANGII peptide was cross-linked with glutaraldehyde to keyhole limpet haemocyanin. In a dot-blot assay (Imboden et al. 2009), the monoclonal antibody 4B3 (against ANGII) showed total cross-reactivity with ANGIII (2–8), ANG(3–8), ANG(4–8) and ANG(5–8), but not with human angiotensinogen, ANGI(1–10) or ANG(1–7). Moreover, 4B3 did not cross-react with neuropeptide Y, oxytocin or vasopressin (Frei et al. 2001; Imboden et al. 2009). In addition, the staining pattern in the present work matched our preliminary basic description with two different polyclonal antibodies and cryostat sections of crab brain (Delorenzi et al. 2000). Since this previous study was performed with two polyclonal antibodies against human ANGII, we decided to make use of a monoclonal antibody in the present investigation. Specifically, we chose 4B3 because it has been widely tested and has shown 100% reactivity with ANGII to ANGII 5–8. ANGII 5–8 contains a relatively well-conserved portion of ANGII (Takei et al. 2004). In addition, we selected 4B3 because it was raised against human ANGII and the only angiotensin sequenced in an invertebrate (a leech) is identical to human ANGI (Salzet et al. 2001). Remarkably, all our behavioural and pharmacological studies demonstrated the mnesic effects of the human angiotensins used as agonists (ANGII and ANGIV) and those of saralasin and Dival as antagonists of the system.

Western blot analyses of *Chasmagnathus* brain extracts were performed in order to determine whether proteins of a molecular weight higher than that of the angiotensin peptide (the predicted size of a putative *Chasmagnathus* ANGII is close to 1 kDa) were immunoreactive; we were unable to find any high molecular weight band with this antibody (see Fig. S4 in Supplementary Material).

*Nonspecific fluorescence* No immunolabelling was detected when we used pre-immune serum, the preabsorption solution or the secondary antibody alone in *Chasmagnathus* brain and optic ganglia. However, some cluster areas (5–11) showed autofluorescence. Consequently, they were not analysed and are not included in this report.

*Image processing* Fluorescence in tissue samples was visualized by using a confocal microscope (Olympus FV300, Japan) with a 488-nm argon laser, an UplanFl 20× objective (NA 0.5), a dicroic cube SDM 570 to split the acquisition channel and an emission:BA510 (Olympus Optical). For images taken with the 20× objective, the pixel size was 0.69 μm/pixel. Data were recorded by using FluoView Software and saved in a 16-bit TIFF format. Sequential images from optic lobes and brain (approximately 230 μm in z-axis) were deconvoluted (Autodeblur and Autovisualize X, Media Cybernetics, USA) with a quantitative blind point spread function-searching algorithm (3D-Blind Deconvolution, 10 iterations, high noise level). The supplementary material shows plain sequential images from optic lobes and brain obtained by using a 20× objective, corresponding to Figs. 2b, 3b, 4, 5 (Fig. S1, S2, S3); frame brightness and contrast were adjusted by using ImageJ 1.40 g software (NIH, USA http://rsb.info.nih.gov/ij/). The ImageJ 1.40 g software maximum projection function was used for stacks.

*Image analysis* The sequential images obtained after the deconvolution processes were analysed by using algorithms developed with an image analysis program (ImagePro Plus, v6.3, Media Cybernetics, USA). Various areas of interest (AOI) with ANGII-like-ir, viz. PT, medial protocerebrum,

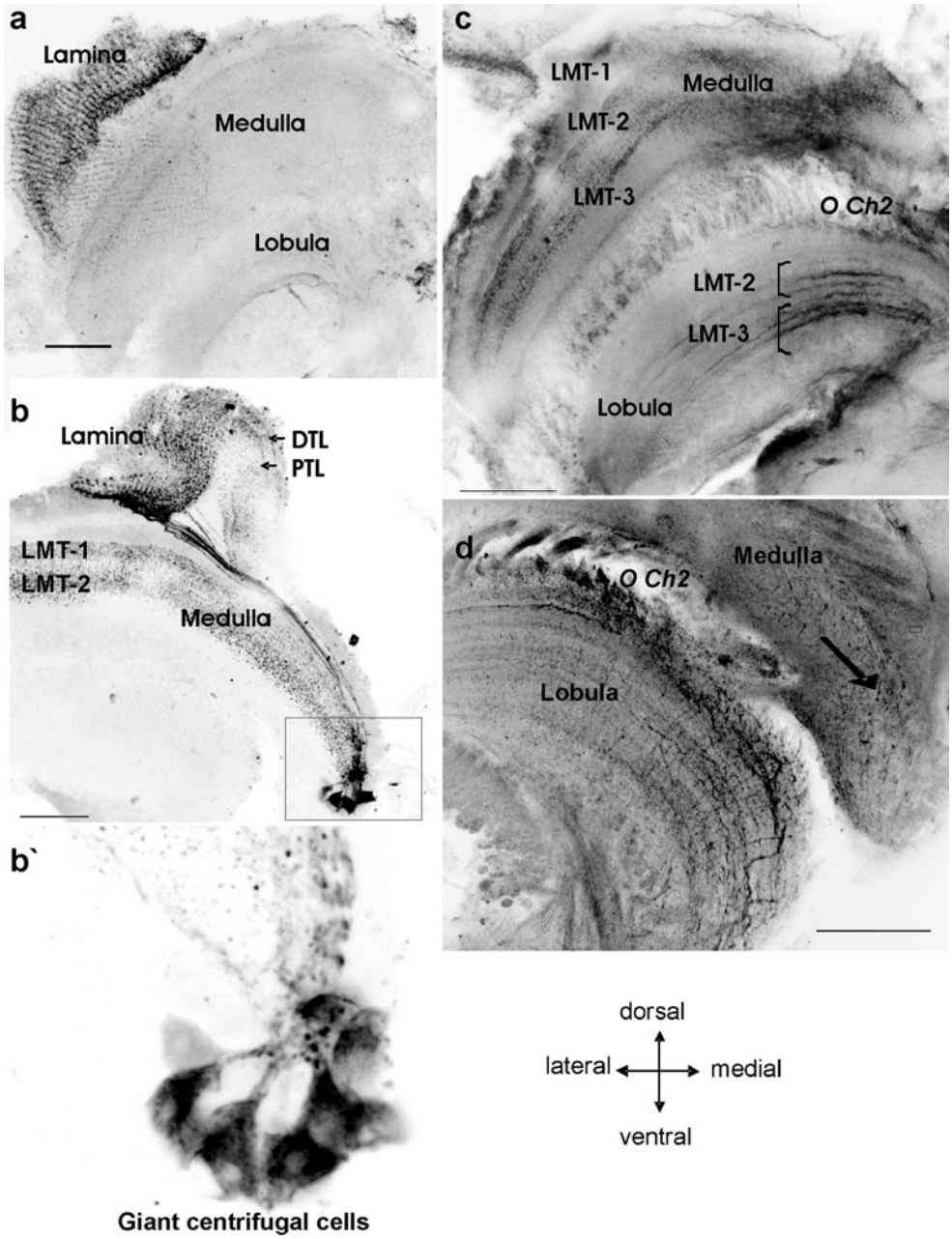



**Fig. 2** **a–d** Immunolocalization of angiotensin-II-like (ANGII) neuropeptides in *Chasmagnathus* optic lobes. **a** Maximum intensity projection view of lamina, medulla and lobula. This tangential view shows the ANGII-immunoreactive fibre arrangement of the lamina. **b** Maximum intensity projection view of the optic lobes from another animal, revealing ANGII-immunoreactive fibres in the proximal (*PTL*) and distal (*DTL*) tangential layers of the lamina. An immunolabelled group of giant centrifugal cells lying in the medial zone of the medulla send their projections to the lamina. The medulla is also labelled by the ANGII antibody; two layers can be distinguished: lateromedial tangential layers 1 (*LMT-1*) and 2 (*LMT-2*). **b'** Higher magnification view of the angiotensinergic giant centrifugal cells. **c**, **d** The medulla shows intense immunoreactivity in the surroundings of lateromedial tangential layers 1, 2 and 3 (**c**, *LMT-1*, *LMT-2*, *LMT-3*). The medulla has some wide-field tangential processes that are ANGII-immunoreactive (**d**, *arrow*). Within the lobula, parallel layers of immunolabelled material are present in the surroundings of lateromedial tangential layers 2 and 3 (**c**), representing branches of the bistratified lobula giant 2 neurons; their axonal tracks appear profusely labelled (see Fig. 3c). The lobula possesses abundant ANGII-immunoreactive fibres, apparently in the surroundings of the lateromedial tangential layers and in the anteroposterior tangential layers. Note optic chiasm 2 (*OCh2*) lying between the medulla and the lobula (**d**). *Bars* 100 μm

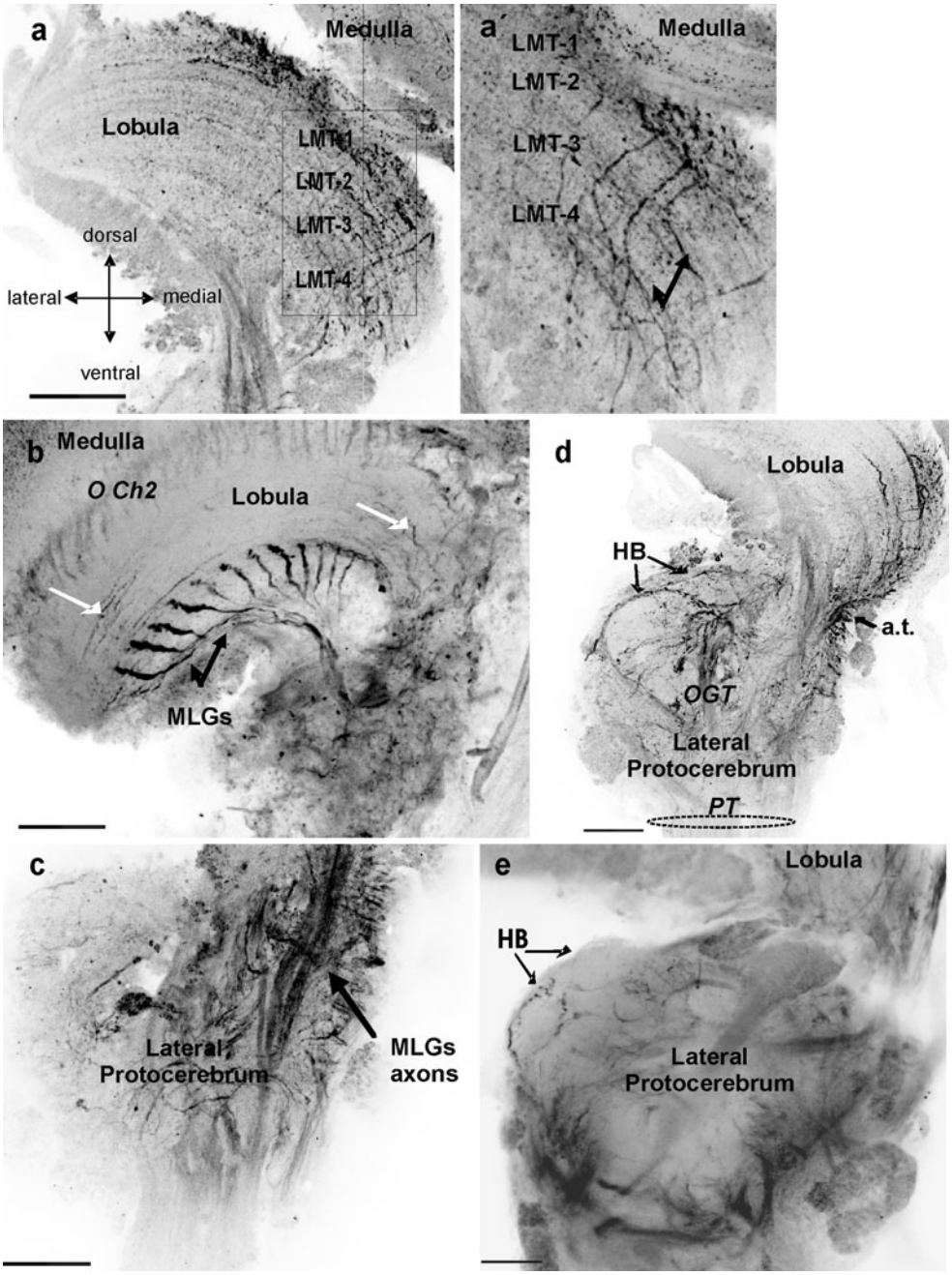



**Fig. 3** Immunolocalization of ANGII in *Chasmagnathus* optic lobes along antero-posterior axis. **a** The lobula presents abundant ANGII-immunoreactive fibres in a parallel arrangement. They are situated more medially than laterally and are in the surroundings of the lateromedial tangential layers 1–4 (*LMT-1* to *LMT-4*). Some additional fibres run perpendicular to the former, presumably in the anteroposterior tangential layers. The stack represents approximately 20 μm confocal images, showing the lobula. **a'** Higher magnification of the lobula; ANGII-immunoreactive elements in tangential processes (*black arrows*). **b** Thirteen axons of the monostratified lobula giant neurons (*MLGs*) are labelled (*black arrows*); note the ANGII-immunoreactive fibres in tangential processes (*white arrows*). **c** In the lateral protocerebrum, the thick tract corresponding to the monostratified lobula giant neurons can be distinguished. **d** A tract corresponding to the bistratified lobula giant neurons is revealed (*a.t.*). A thick stained tract, probably the olfactory globular tract (*OGT*), bifurcates in two oval structures that apparently constitute the two halves of the hemi-ellipsoid body (*HB*). **e** The last maximum intensity projection view shows a labelling pattern similar to that of **d**. The stacks represent approximately 20 μm confocal images (*OCh2* optic chiasm 2, *PT* protocerebral tract). *Bars* 100 μm

CB, cerebral artery (CA), ON, olfactory globular tract (OGT) and tritocerebrum) were established for all the analysed images. The total area of each AOI was recorded for every sequence by using the Count/Size function of the Measure menu of the image analysis program. The highest and the lowest immunohistochemical staining intensities found among all the analysed images were also recorded as a range in order to establish the limits of the staining that the image analysis program was able to detect. The area occupied by the immunostaining inside their corresponding AOI was then calculated by using the Count/Size function of the Measure menu of the image analysis program. The fraction of stained area (immunohistochemically positively stained structures) was calculated by using the following formula: fraction of staining = (positively stained area within the AOI/AOI area). Data were exported into a spreadsheet in order to perform the statistical analysis.

*Statistical analysis* All scores (immunopositive area/analysed area) are represented as means±SE. We analysed the data by using STATISTICA 7, by performing a one-way analysis of variance (ANOVA), and by post-hoc analysis (LSD test).

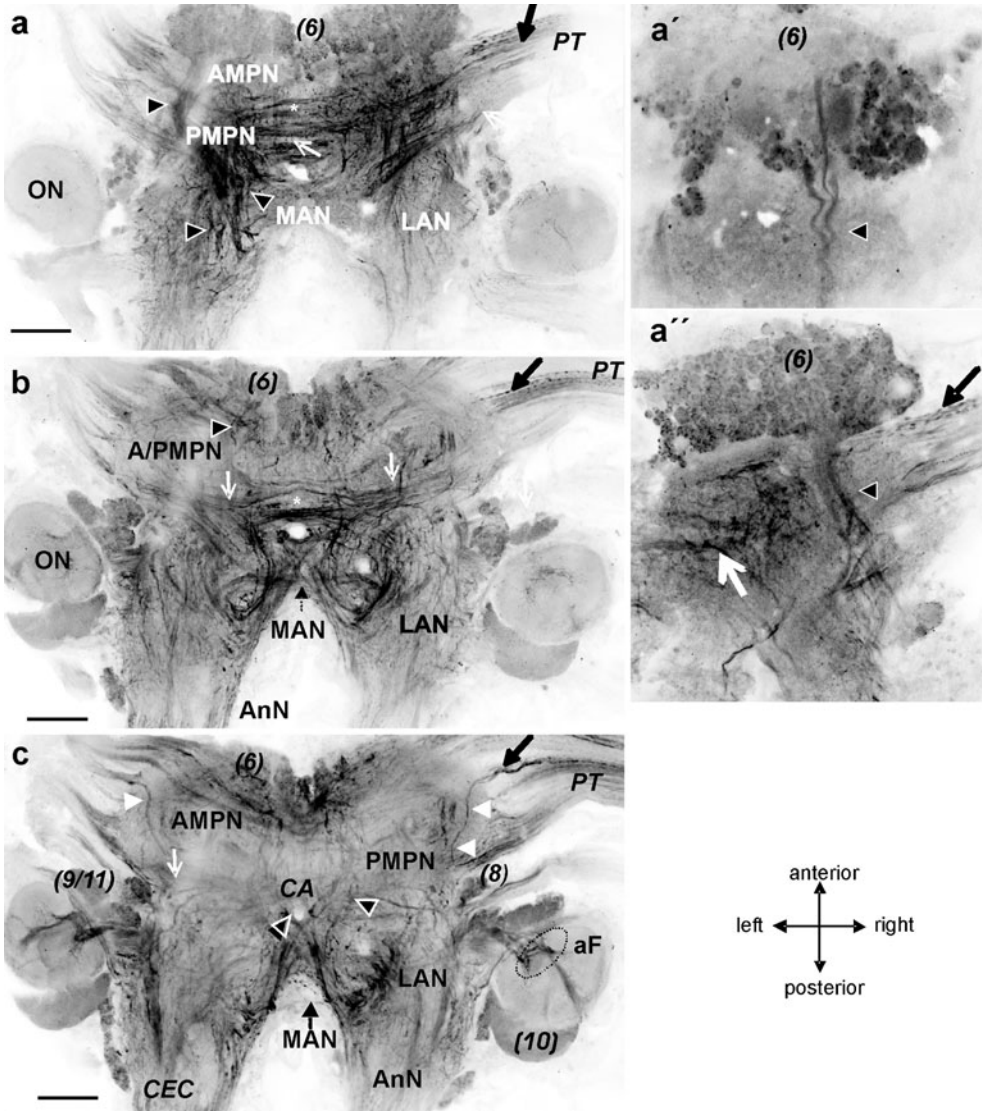



**Fig. 4** Immunolocalization of ANGII in the *Chasmagnathus* brain along the dorsoventral axis. **a** The dorsalmost maximum intensity projection shows neuronal process leaving cluster (6) (*[6]*) in the antero-posterior axis that bifurcates in the lateral (*LAN*) and medial (*MAN*) antennal neuropils (*black arrowheads*). Numerous additional ANGII-immunoreactive fibres run in the left-right axis (*white arrows*) and appear at the level (*black arrow*) of the protocerebral tract (*PT*), branching at both sides of the anterior (*AMPN*) and posterior (*PMPN*) medial protocerebral neuropils and crossing the midline (*star*). **a'**, **a"** Higher magnification showing stained neuronal projections from somata of cluster (6) (*black arrowhead*) and protocerebral tract (*black arrow*) to the anterior medial protocerebrum neuropil. Some other ANGII-immunoreactive fibres also cross the midline (*white arrow*). **b** A less dorsal view showing fibres originated in cluster (6) running across the anterior and posterior medial protocerebrum neuropils to the medial antenna neuropil and, possibly, to the antenna II neuropil (*AnN*). **c** This almost middle maximum intensity projection view shows labelled fibres arriving from the protocerebral tract (*black arrow*) running through the medial protocerebrum and the lateral antenna l and antenna II neuropils, leaving the brain through the posterior connectives. Other immunoreactive fibres, originating in cluster (6), go through the medial protocerebrum, crossover near the central artery (*black arrowhead*) and descend through the medial antennal neuropil close to the medial side of the connectives. ANGII-immunoreactive fibres originating in clusters (9/11) reach the olfactory neuropil via the anterior foramen (*aF*). *Numbers* denote cell somata clusters (*CA* cerebral artery, *CEC* circumesophageal connectives, *ON* olfactory neuropil). *Bars* 100 μm

## Results

### Optic lobes: lamina, medulla, lobula

The lamina is composed of a plexiform layer and two layers formed by tangential processes: the distal tangential layer and the proximal tangential layer. These layers are mainly composed of centrifugal tangential cells extending out from the medulla (Sztarker et al. 2005). We found strong ANGII-ir in this neuropil, including both the distal tangential layer and the proximal tangential layer (Figs. 2a, b, S1, S2). The long ANGII-immunoreactive fibres are shown to be the

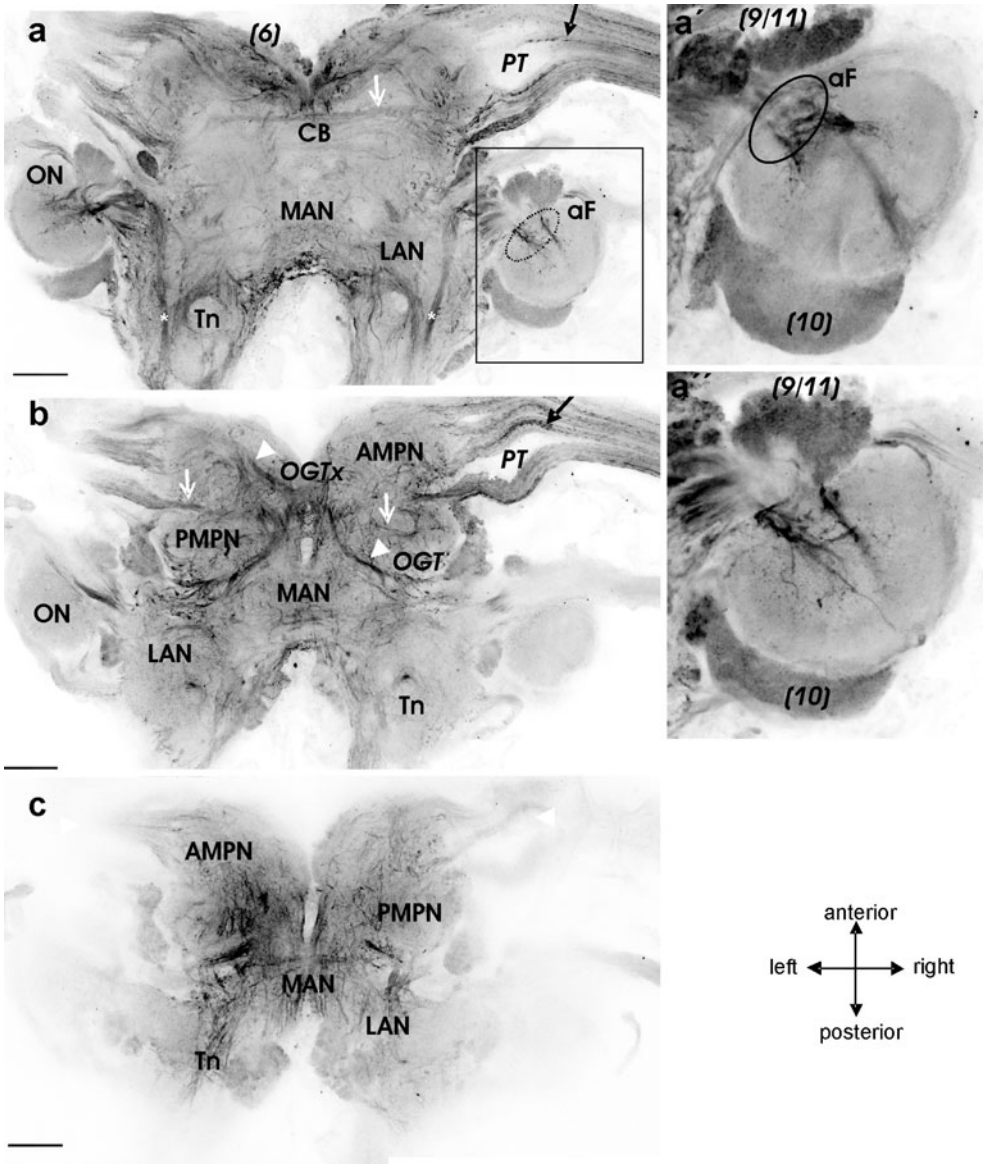



**Fig. 5** Immunolocalization of ANGII in *Chasmagnathus* brain in the dorsoventral axis. **a** The central maximum intensity projection shows some punctuated ANGII immunoreactivity (ANGII-ir) at the anterior side (*white arrow*) of the central body (*CB*). At the level of the deutocerebrum, note the bilateral, strongly labelled fibres that probably originate at cluster (9/11) and that branch in the olfactory neuropil running across the tritocerebrum (*stars*). **a'**, **a"** Higher magnification of the right olfactory neuropil showing neuronal ANGII-ir originating in somata of clusters (9/11) (*[9/11]*), which arrives there through the anterior foramen (*aF*). These projections seem to innervate the glomeruli at their medial side. **b** The olfactory globular tract (*OGT*) shows an important immunoreactive bundle coming from the protocerebral tract, crossing over the midline and forming a chiasm (*OGTx*) at the medial protocerebrum. An ANGII-immunoreactive network lies both at the medial protocerebrum (*white arrows*) and at the medial antennal neuropil. **c** This ventral maximum intensity projection shows labelled fibres shaping a widespread network connecting the brain. *Numbers* denote cell somata clusters (*AMPN* anterior medial protocerebral neuropil, *LAN* lateral antennal neuropil, *MAN* medial antennal neuropil, *ON* olfactory neuropil, *PMPN* posterior medial protocerebral neuropil, *PT* protocerebral tract, *Tn* tegumentary neuropil). *Bars* 100 μm

linking fibres of the giant centrifugal cells from the medulla, which are distinctly stained (Figs. 2b, b′, S1). In the medulla, most tangential neurons extend their processes either along the anteroposterior or lateromedial axis, defining the lateromedial tangential (LMT) layers 1–3 and anteroposterior tangential layers (Sztarker et al. 2005). Figure 2b, d demonstrates the distinct ANGII-immunoreactive fibres in the medulla; these seem to be present all over the retinotopic columns. Figure 2c also reveals the presence of ANGII-ir in the LMT1–3 layers. The lobula receives retinotopic information from the medulla via the second optic chiasm, which presents distinguishable ANGII-ir (Fig. 2d). Tangential processes oriented lateromedially define four tangential layers,

LMT1–4. We have found ANGII-ir in the four layers (Figs. 2c, 3a, a′). Previous studies involving the use of intracellular recording and staining have shown that LMT-2 and LMT-3 layers comprise the bistratified dendritic tree of wide-field motion-sensitive neurons termed bistratified lobula giant 2 (BLG2). The BLG2 dendrites converge towards the medial side of the neuropil into a thicker single axonal trunk that can be followed towards the midbrain (Berón de Astrada and Tomsic 2002; Sztarker et al. 2005; Medan et al. 2007). Both the layers and the single axonal track (a.t. in Fig. 3d) of this neuron are strongly ANGII-ir (Figs. 2c, 3d). Strata of other tangential processes also compose the lobula; we have also repeatedly found ANGII-immunoreactive elements in these processes (Fig. 3a′, b). An ensemble of previously well-characterized groups of giant monostratified neurons named MLG1 (Sztarker et al. 2005; Medan et al. 2007) often present strong ANGII-ir (Figs. 3b, S2) and can be followed towards the lateral protocerebrum (Fig. 3c).

### Lateral protocerebrum: MT and HB

Because the spatial arrangement of the lateral protocerebrum is complicated in crabs, we have been unable to reveal the true limits between the HB and MT in *Chasmagnathus*. Within the putative HB, we have found a loose network of ANGII-immunoreactive fibres mainly oriented towards the lateromedial axis (Fig. 3d, e). A dense ANGII-immunoreactive tract that innervates this structure appears to be the OGT within the PT (Fig. 3d). With regard to the MT, a bundle of ANGII-immunoreactive fibres crosses it from the lobula to the central brain. Some of these fibres are the axons of the fourteen MLG1 neurons (Fig. 3c). A dense and intricate, but not uniform, network of labelled fibres innervates the whole MT (Fig. 3c–e).

### Medial protocerebrum

The medial protocerebrum contains the most distinctly ANGII-like-immunolabelled neuropils within the brain. The ANGII-like staining pattern of the PT resembles a "rosary string" (Figs. 4a, a″, b, c, 5a, b) extending between the two halves of the AMPN and crossing the midline (Figs. 4a, a″, b, c, 5a, b). ANGII-immunoreactive fibres, coming presumably from the PT, cross the PMPN along the lateromedial axis less densely. Together, the fibres traverse the right and left sides of the brain (Fig. 4a). We have also found some fibres that descend from the PT and that run to the deuto- and tritocerebrum (Figs. 4c, 5b). Moreover, a bundle of intensely immunolabelled fibres is present in both the AMPN and PMPN presumably emerging from cluster (6), crossing the medial protocerebrum in the anteroposterior axis (Fig. 4a, a′, a″, b, c). Numerous fluorescent cell somata are present in the protocerebral cell cluster (6) but we have been unable to determine whether the fluorescence is specific. For this reason, we have not included them in this report. Two symmetric bundles of ANGII-immunoreactive fibres descend from cluster (6), run in the middle of the medial protocerebrum and cross to the contralateral side immediately below the CA; the fibres continue descending along the tritocerebrum close to the medial side of the circumesophageal connectives (CEC; Fig. 4a′, a″, b, c). Several thick parallel immunolabelled commissural fibres accompany the CB, mainly at its anterior side (Fig. 5a). Slightly ventral to the CB lies the OGT, which contains numerous immunolabelled fibres (Fig. 5b).

### Deutocerebrum

ANGII-ir appears as fibres that run surrounding the ONs mainly in the medial side (Figs. 4c, 5a, a′, a″). Two groups of labelled fibres that might have originated in cells from clusters (9/11), which house local olfactory interneurons, are shown in Figs. 4c, 5a, a″. One enters the ON through the anterior foramen and forms arborizations within the glomeruli. The other group goes through the lateral side of the LAN and reaches the AnN in the tritocerebrum (Fig. 5a–c, Fig. S3). Many cell somata from clusters (9/11) and (10) appear labelled but, as in cluster (6), we have been unable to determine whether there is specific ANGII-ir. The axons of cluster (10) projection neurons form a thick fibre bundle, the OGT, which leaves the ON in a medial direction. We have found abundant ANGII-immunoreactive fibres all along the OGT (Fig. 5b). The MAN also contains thick ANGII-immunoreactive fibre bundles that cross its posterior area and project to the medial side of the CEC (Figs. 4b, c, 5b, c). A thick bundle of ANGII-immunoreactive fibres appears to originate in cluster (6) and reaches the MAN (Fig. 4a). The LAN, in *Chasmagnathus*, seems to be moderately immunoreactive to ANGII (Figs. 4a–c). In true crabs, the accessory lobes have been hypothesized to be reduced to a relict structure (Sandeman et al. 1992; Helluy et al. 1995) and, in *Chasmagnathus*, they are unnoticeable under the present experimental conditions (Figs. 4, 5).

### Tritocerebrum

The AnN is innervated by an array of ANGII-immunoreactive fibres that lie parallel to the long axis of this neuropil (Fig. 4c). The Tn is also labelled (Fig. 5). Furthermore, a loose network of immunolabelled fibres (Fig. 4c) stretches from the MAN along the medial sides of both CEC.

### ANGII-like distribution after water deprivation

We assessed whether water deprivation elicited changes in the pattern of brain ANGII-ir. Previous results had shown that 2 h of water deprivation resulted in improved memory attributable to an increase in brain ANGII levels (Frenkel et al. 2002, 2005a, 2005b). Here, two groups of crabs ($n$=6 per group) were water-deprived for either 2 h or 6 h (2-h-deprived and 6-h-deprived groups); the control group remained in water (non-deprived group). In order to evaluate the dynamic changes in brain angiotensins, we defined seven AOIs to be studied: PT, medial protocerebrum, CB, CA, ON, OGT and tritocerebrum (Fig. 6). The statistical analysis of ANGII-ir quantification by using a one-way ANOVA for each AOI showed a significant effect for the treatment in the medial protocerebrum [$F_{(2,\ 15)}$=3.88; $P$<0.05], CB [$F_{(2,\ 15)}$=8.95; $P$<0.005], CA [$F_{(2,\ 14)}$=5.95; $P$<0.05], ON [$F_{(2,\ 15)}$=4.34; $P$<0.05] and OGT [$F_{(2,\ 14)}$=4.05; $P$<0.05]. For each AOI, when the ANOVA was significant, we performed a Fisher-LSD post-hoc comparison. In general, we found three types of differences: 1) a decrease when compared with the control group, 2) an increase when compared with the control group and 3) a decrease when comparing 2 h vs. 6 h of water deprivation. In the medial protocerebrum and the CA, we discerned a significant decrease in ANGII-ir after 6 h of water deprivation (both $P$<0.05), whereas in the ON, ANGII-ir significantly decreased after 2 h and 6 h of water deprivation (all $P$<0.05). In addition, we also noted a significant increase in ANGII-ir after 2 h of water deprivation in the CB ($P$<0.05); this returned to base levels after 6 h ($P$>0.1). Simple parallel controls that lacked the primary antibody showed an index of less than $5\times10^{-5}$. In order to control the specificity of the effect of 6-h water deprivation on ANGII-ir in various areas of the brain, we performed immunolabelling for another neuropeptide, viz. pigment-dispersing hormone (PDH), previously cloned and described in the brain of several crustaceans (Dircksen et al. 1987). The PDH-ir of a 6-h-deprived group and a control group (n=4 per group) was evaluated. The general PDH-ir distribution in the brain closely resembled that shown by Hsu et al. (2008) in *Cancer productus*. No changes were found in the index of PDH-ir (immunopositive area/analysed area) for the 6-h water deprivation group vs. non-deprived crabs (Fig. S5, Supplementary Material).

## Discussion

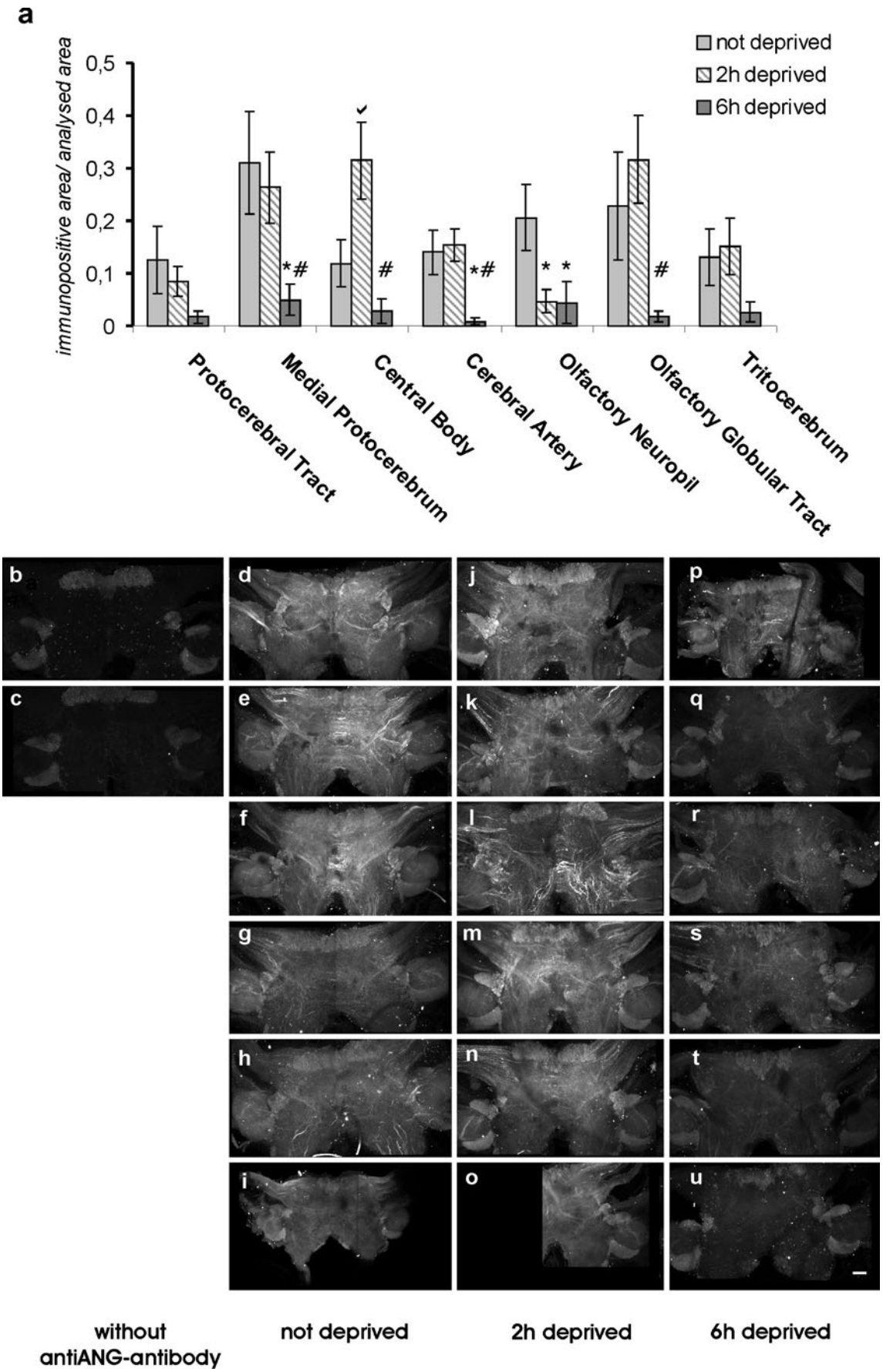
The analysis of the pattern of labelling for ANGII-ir within the adult brain of the crab *Chasmagnathus granulatus* reveals that it comprises neuronal processes in several neuropils of the CNS and that a water-shortage episode changes this ANGII-ir distribution.

**Fig. 6** Changes in ANGII-like immunoreactivity in *Chasmagnathus* brain following 2 h and 6 h of water deprivation. **a** Graph showing changes in ANGII-ir content in various areas. Each *column* represents the mean±SE of the ANGII-ir content calculated as immunopositive area/analysed area, for seven areas after crabs were either not deprived or water-deprived for 2 h and for 6 h. Statistical comparisons by one-way ANOVA for each area of interest ($n$=6 per group): *$P$<0.05, significantly decreased when compared with the non-deprived group; $^{\surd}P$<0.05, significantly increased when compared with the non-deprived group; $^{\#}P$<0.05 significantly decreased when compared with the 2-h deprived group. **b–u** Stacks of confocal images of the brain used for measuring ANGII-ir content, including a control group without the anti-ANGII antibody ($n$=2). *Bar* 100 μm

### Neuroanatomical distribution of ANGII-like neuropeptide within CNS of *Chasmagnathus*

The pattern of labelling for ANGII-ir in the nervous system of *Chasmagnathus* resembles that of some neuronal processes previously described for numerous neurotransmitters and neuropeptides in crustaceans, including various families, such as the enkephalins (Fingerman et al. 1985), substance P (Sandeman et al. 1990; Schmidt and Ache 1994; Schmidt 1997), crustacean-SIF amide (Polanska et al. 2007), serotonin (Sandeman et al. 1988) and CCH (Ollivaux et al. 2002). In the lamina, medulla and lobula, we have found strong ANGII-ir in the tangential layers of the lamina and in some medullary centrifugal cells in the medulla and lobula. The finding that these structures are almost completely devoted to the processing of visual information (York and Wiersma 1975; Sztarker et al. 2005; Glantz and Miller 2009; Sztarker et al. 2009) suggests an important role of this ANGII-like neuropeptide/s in this process, as has been suggested for several neuropeptides described in this neuropil (e.g. Polanska et al. 2007). We have also found that both the bistratified dendritic trees and the thicker single axonal trunk of the widefield motion-sensitive BLG2 neurons (Tomsic et al. 2003; Sztarker et al. 2005; Medan et al. 2007) are angiotensinergic. In addition, the MLG1 neurons have been identified as expressing ANGII-like peptide/s. Several pieces of evidence suggest that the lobula is not only a visual-processing neuropil. The lobula is also often described as being a "higher-order centre" and has been found to be involved in learning and memory processes (Tomsic et al. 2003; Sztarker and Tomsic 2004, 2008; Medan et al. 2007).

As in other crabs, in the lateral protocerebrum of *Chasmagnathus*, the HBs are hardly distinguishable from the adjacent MT. We have discerned a dense ANGII-immunoreactive bundle that innervates the assumed HBs and a network of ANGII-immunoreactive fibres within this structure. In addition, a dense and intricate, but not uniform, network of labelled fibres innervates the whole MT. The lateral protocerebrum is connected to the optic lobes and


a
immunopositive area/ analysed area
0,5
0,4
0,3
0,2
0,1
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not deprived
2h deprived
6h deprived
*#
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#
Protocerebral Tract
Medial Protocerebrum
Central Body
Cerebral Artery
Olfactory Neuropil
Olfactory Globular Tract
Tritocerebrum
b
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without antiANG-antibody
not deprived
2h deprived
6h deprived

receives inputs of higher-order neurons from the visual pathway, ONs and HB of the contralateral eyestalk; this paired structure, which is profusely innervated by angiotensinergic projections, is regarded as the centre of higher-order integration (Strausfeld et al. 1998; Sandeman and Mellon 2002; McKinzie et al. 2003; Schachtner et al. 2005; Sullivan and Beltz 2005).

The medial protocerebrum contains the most intensely ANGII-like immunolabelled neuropils in the brain of non-water-deprived animals (Fig. 6). The medial protocerebrum contains the anterior medial protocerebral neuropils (AMPN), the CB and the protocerebral bridge (Sandeman et al. 1992; Utting et al. 2000). We have found ANGII-immunoreactive projections that innervate the AMPN, whose ANGII-ir decreases after 6-h water deprivation. The CB receives a strong visual input from several brain neuropils, which suggests that it has a higher-order integration function (Utting et al. 2000). We have detected thick parallel immunolabelled commissural fibres accompanying this neuropil. This immunostaining pattern, which is stronger along the anterior edge, is similar to the substance-P-like-ir found in lobsters (Langworthy et al. 1997).

Classically, the deutocerebrum is thought to be a region that processes sensory inputs and motor outputs from (and to) the antenna (Mellon 2007). The lateral and medial antenna I neuropils (LAN and MAN) receive a variety of sensory inputs, including mechanosensory information, non-olfactory chemosensory and statocyst inputs. They also contain processes of motoneurons controlling movements of antenna I (for a review, see Schachtner et al. 2005). Dorsally, we have found a distinctly angiotensinergic bundle originating in cluster (6) neurons; this branches at the level of the MAN and LAN. Medially, both neuropils appear to be crossed by an angiotensin network. The ON processes the primary chemosensory inputs received from antenna I. Nevertheless, the ON has been shown to be involved in the multimodal integration of chemical and mechanical inputs (Mellon 2007). The ON is composed of several types of extrinsic (olfactory receptor and centrifugal projection) and intrinsic (local interneurons and ascending projections) neurons. Angiotensinergic neurons in the ON seem to belong to both interneurons and projection neurons with their axons leaving the deutocerebrum through the OGT. The ANGII-immunoreactive fibres show a pattern similar to that of substance-P-immunoreactive centrifugal neurons or core local interneurons (Schachtner et al. 2005). The OGT links multimodal processing areas in the lateral protocerebrum with the ONs and contains numerous angiotensinergic fibres.

The tritocerebrum comprises nerves of the labrum, the stomatogastic system and the alimentary canal and post-oral commissures. In addition, it is composed of the AnN and a small Tn that processes afferent inputs (mechanoreceptors) and other sensilla from the dorsal carapace (Bullock and Horridge 1965). The AnN contains primary afferent inputs from antenna II and the synaptic fields of motoneurons controlling the movements of the antenna. Similar to the serotoninergic immunostaining in lobsters (Langworthy et al. 1997), most of the angiotensinergic labelling in the AnN appears as longitudinal fibres that link the medial protocerebral neuropils with the ipsi- and contralateral AnN.

## Physiological changes triggered by water deprivation

The study of changes in ANGII-like distribution after water deprivation has shown a significant difference when studying the immunopositive area/analysed area index. We have divided the brain and optic lobes into ten conspicuously different areas that exhibit ANGII-ir. Two lines of thought allow us to conclude that the differences seen between groups are not attributable to global nonspecific changes in the nervous tissue, viz. changes that may affect antibody penetration, a common problem in whole-mount immunohistochemistry (Nässel 1996). First, the parameter measured (positively stained area, see Materials and methods) is independent of the global area intensity. Second, we have performed a control by using an antibody against PDH (Fig. S5), a peptide known to be under strong circadian control. As expected, PDH-ir does not vary among our various experimental groups. Thus, our results strongly indicate that the changes in ANGII-ir expression upon the water deprivation shown here are specific. The analysis of the pattern of labelling within the brain of *Chasmagnathus* reveals that the ANGII-ir is dynamic following a water-shortage episode. The CB is the only area that increases its ANGII-ir level after 2 h and decreases it after 6 h of water deprivation. This area might be the first to trigger the angiotensinergic response that leads to changes in crab behaviour, i.e. the avoidance of water deprivation and memory facilitation (Schmitt and Santos 1993; Delorenzi et al. 2000; Frenkel et al. 2002, 2005a, 2005b). When we have studied the effect of water deprivation on ANGII-ir within the ON, we have noted, in contrast to observations in the other studied areas (except for the CB), a decrease in ANGII-ir 2 h after water shortage. Indeed, this decreased condition remains for 6 h after the water shortage, a result in agreement with our previous findings, which have demonstrated, in the ON, an increased number of glomeruli that express heat-shock protein 70 after 2 h of water deprivation (Frenkel et al. 2008). Some compelling evidence has been presented that the ion-regulatory mechanisms are highly complex in crustaceans. The pericardial organs and sinus gland are important secretory tissues in hydromineral regulation (Morris 2001; Serrano et al. 2003). Experiments with

eyestalk ablation have shown that the sinus gland influences the ion balance (Morris 2001). Our results suggest that other areas are also involved in the complex and multiple actions that allow organisms to cope with water shortages: at least the CB and the ON rapidly respond to a water shortage by changing their ANGII-like levels. Moreover, other areas also respond to water deprivation (by decreasing their ANGII-like levels; Fig. 6). The dynamics in the labelling pattern of ANGII-ir presented here are complementary to previous radioimmunoassay data showing that a water deprivation episode increases the ANGII-ir level at 2 h, which then returns to basal levels at 6 h. A possible explanation is that, at 2 h, the radioimmunoassay is more effective in sensing global differences. The radioimmunoassay has shown that the total amount of ANGII-ir returns to basal levels at 6 h but, in the present study, we have found that the levels do not return to basal values. One possibility is that the parameter evaluated here (positive stained-immunoreactive area/analysed area) decreases because of the complex process of the turnover of these neuropeptides (Wright et al. 2008) after their strong release triggered by the water shortage.

Of particular importance is the possibility that the monoclonal 4B3 antibody used in the present study cross-reacts with other crab peptides. ANGI is not identical in vertebrates; a comparison of the amino acid sequences of ANGI has shown that Arg2, Tyr4, His6, Pro7, Phe8 and Leu10 are well conserved (Watanabe et al. 2009). Although the proposition has been made that the angiotensinergic system emerged early in evolution, the leech is the only invertebrate in which this peptide has been sequenced (Laurent et al. 1995; Nishimura 2001; Salzet et al. 2001). However, classical osmoregulatory actions of angiotensins have also been shown in some invertebrate species (Makra and Prior 1985; Fitzsimons 1998; Takei et al. 2004; for reviews, see Salzet et al. 2001; Satou et al. 2005a, 2005b). In a previous study, which included an immunohistochemistry assay involving polyclonal antibodies against human ANGII and serial sections, the goal was to demonstrate that the ANGII-immunoreactive material was present in the brain. Remarkably, the detailed pattern shown here strongly matches our previous findings with two other polyclonal antibodies against human ANGII (see Fig. S6 reproduced from Delorenzi et al. 2000 and Fig. 2). In addition, the Western blot result performed with the 4B3 antibody makes the possibility of cross-reaction less likely, at least for high molecular weight proteins. However, the neuropeptidomic characterizations made in crustaceans have not revealed angiotensin peptides as yet (e.g. Huybrechts et al. 2003; Ma et al. 2008, 2009a, 2009b; Chen et al. 2009). The peptides described in other crustaceans do not include the sequence recognized by the 4B3 antibody (4B3 shows total cross reactivity with ANGIII (2–8), ANG(3–8), ANG(4–8) and ANG(5–8) (Ile-His-Pro-Phe); Huybrechts et al. 2003; Ma et al. 2008, 2009a, 2009b; Chen et al. 2009). However, we cannot discard the possibility that the 4B3 antibody recognizes something in addition to angiotensins; the present results show a correlation between water deprivation and the amount of ANGII-immunoreactive material in the various brain areas. These changes in the immunoreactive material can be simply interpreted as just an unspecific effect of water deprivation. However, this interpretation does not take into account that we have shown relevant functional, pharmacological and behavioural evidence of angiotensinergic actions in *Chasmagnathus* (Delorenzi et al. 1995, 1996, 1997, 2000; Delorenzi and Maldonado 1999; Frenkel et al. 2002, 2005a, 2005b). As in vertebrates (Braszko et al. 2006; Wright et al. 2008), all the mnesic effects that we have assigned to the endogenous ANGII-like peptide in this crab are always reversed by injecting saralasin, a peptidergic non-selective ANGII receptor antagonist. The improving memory effects of an active metabolite of the angiotensinergic system, ANGIV, are also reversed by the use of the specific peptidergic agonist Dival. Moreover, all the mnesic effects attributed to ANGII during the water-shortage episodes are mimicked by human ANGII and reversed by the antagonist saralasin. Outstandingly, the present results show that the lobula giant neurons, neurons involved in the memory process in *Chasmagnathus* (Tomsic et al. 2003), contain copious ANGII-ir. On the other hand, the amounts of ANGII-ir, as estimated by radioimmune assay, are significant in thoracic ganglia and brain and in gills. Importantly, about 30% of this ANGII-ir coelutes with human ANGII as shown by high-pressure liquid chromatography and radioimmune assay. Moreover, angiotensin-converting enzyme-like activity has been described in diverse tissues of *Chasmagnathus*, namely, in gills and in both thoracic ganglia and brain. Furthermore, the monoclonal 4B3 antibody and other two polyclonal antisera to human ANGII produce similar patterns of immunolabelling in the CNS of *Chasmagnathus* (Delorenzi et al. 1995, 1996, 1997).

Taken together, the previous and present results are in agreement with the view that, when crabs cope with a hydromineral imbalance, the angiotensinergic system is altered and activates coordinated actions, from osmoregulation to behaviour, which, as a whole, enable the animal to survive under this ethologically relevant event (Delorenzi et al. 2000; Frenkel et al. 2002, 2005a, 2005b), although the possibility that the 4B3 antibody recognizes something in addition to angiotensins needs to be considered in order to interpret the present results. Further experiments are needed to establish that the angiotensinergic system plays a role in the balance of body fluids in this crab. The present results are also in agreement with the proposition that the release of brain angiotensins in response to water shortages is an

ancient mechanism for coordinating different functions that, together, enable organisms to cope with this environmental change (Delorenzi et al. 2000; Frenkel et al. 2002, 2005a, 2005b).

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