

Cellular Localization of a Renin-like Enzyme in Leeches

Michel Salzet & Martine Verger-Bocquet

Laboratoire de Neuroimmunologie des Annélides, ESA CNRS 8017, SN3, IFR 17 INSERM, Université des Sciences et Technologies de Lille. 59650 Villeneuve d'Ascq, France.

Correspondence to: Professor Michel Salzet, Ph.D.,
Directeur, Lab. Neuroimmunologie des Annélides
Membre de l'Institut Universitaire de France
ESA CNRS 8017, SN3; IFR17 INSERM
Cité Scientifique, 59650 Villeneuve d'Ascq, France;
TEL: +33 3 2043 7277; FAX : 33 3 2004 1130
E-MAIL: michel.salzet@univ-lille1.fr

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Abstract

OBJECTIVES: The purpose of this study was to localize in leeches the renin-like enzyme previously characterized as well as the leech angiotensin-converting like enzyme (ACE).

METHODS: Immunocytochemical as well as whole mount experiments were performed with an antibody raised against a fragment of the leech renin-like enzyme (VLWAAEKTQLDTGSS) and with anti-leech ACE.

RESULTS: Anti-leech renin stains the vascular pole of the glomerulus and the afferent arteriole of the rat kidney. Immunostaining of leech sections revealed labeling in neurons and glial cells of the central nervous system (CNS), immunocytes and the nephridial canal, canaliculi and the periphery of the ciliated funnel, as well as the epithelium lining nephridia. Co-localization between antibodies raised against this fragment and a fragment of leech angiotensin-converting enzyme was demonstrated in neurons and glial cells of the leech CNS, as in vertebrates

MAIN FINDING: Leech renin is localized in leeches like in vertebrate in the excretory system and in the nervous system.

CONCLUSIONS: Our findings suggest the presence of a renin-angiotensin system involved in osmoregulation in leeches.

Abbreviations:

CNS: central nervous system;
 TDP: angiotensinogen tetradecapeptide
 PFAPD: plasmodium falciparum aspartic proteinase

Introduction

Renin, which originates in the kidney, plays an important role in the maintenance and regulation of blood pressure in vertebrates [1]. Studies on human kidneys show that renin is first synthesized in an inactive form with a high molecular mass of 55 kDa, and is then converted into active renin with a lower molecular mass of 44 kDa [2, 3]. This enzyme belongs to the family of aspartyl proteases, including cathepsin D, pepsin, chymosin and penicillopepsin, which all contain aspartate residues in their catalytic site [3]. Moreover, renin has 35% sequence homology with these aspartyl proteases [1]. This homology is most marked in the region near the two aspartate residues involved in the catalytic site [2]. The common inhibitor for these aspartyl proteases is pepstatin A [4, 5]. Immunocytochemical studies on renin in the kidney have demonstrated that in adults the molecule is localized in the juxtaglomerular apparatus and the afferent arteriole [1, 6].

The existence of renin has also been well demonstrated in some non-mammalian vertebrates, such as fish [7,8] and the snake *Bothrops jararaca* [9], confirming that this molecule is well conserved throughout vertebrate evolution. This molecule has been well investigated in vertebrates, but data for invertebrates are scarce, with the exception of the Hirudinae [10–18]. We have purified an aspartyl protease from the heads of the leech *Theromyzon tessulatum*. This 32 kDa enzyme was purified to homogeneity after four steps of purification, including gel permeation and anion exchange chromatography followed by reverse-phase HPLC. The enzyme hydrolyses the Leu¹⁰-Leu¹¹ bond of a synthetic porcine angiotensinogen tetradecapeptide (TDP) at pH 7.0 and 37°C, yielding angiotensin I (AngI) and the Leu¹¹-Val¹²-Tyr¹³-Ser¹⁴ peptide as products. It has a specific activity of 1.35 pM AI min⁻¹mg⁻¹ (Km: 22 μM; Kcat: 2.7). The hydrolysis of angiotensinogen is 90% inhibited by pepstatin A (IC₅₀ = 4.6 μM), consistent with renin-like activity [19, 11]. The first 124 amino acid residues of the N-terminal part of the purified S-pyridylethylated leech renin have 26.5–35.5% sequence homology with those of mammals [11], while residues 20–81 exhibit 80% sequence homology with region 175–232 in mammals. This highly conserved region, which is also found in all aspartyl proteases, possesses the aspartyl catalytic residue (DTGSS) [11]. A renin-like enzyme has also been characterized for insects [20,21]. In the German cockroach, *Blattella germanica*, a protein of 36 kDa belonging to the aspartyl

protease family has been cloned [20]. This molecule exhibits 30.6% sequence identity with mammalian renin, and 35% identity with the leech renin-like enzyme [20]. Moreover, in the mosquito *Aedes aegypti*, an aspartyl protease of 35 kDa has been located in lysosomes, and is concentrated in the insect's fat body, digestive organs, in the intestinal tract and in the Malpighian vessels (the major excretory organs in insects) [21]. These aspartyl proteases may therefore be involved in osmoregulation. The aim of this paper was to localize this renin-like enzyme in the central nervous system (CNS) and nephridia of leeches, using a specific anti-leech renin antibody.

Materials and Methods

Animals.

Mature specimens of the rhynchobdellid leech *T. tessulatum*, reared under laboratory conditions as described by Malecha *et al.* [22], were used in this study.

Adult male Wistar rats (animal use accreditation by the French Ministry of Agriculture N° 04860) were used in this study and maintained under standard care. The animals were anesthetized by an intramuscular injection of Ketamine, and subsequently killed by decapitation. After the kidneys had been removed and dissected longitudinally, a small piece of the outer cortex was removed and placed in fixative.

Leech renin antiserum.

An antiserum (anti-leech renin) directed against the N-terminal fragment (VLWAAEKTQLDTGSSGQ) of leech renin, including the DTGSS sequence, was generated in rabbits using a synthetic peptide coupled to human serum albumin with glutaraldehyde as previously described [10]. Pre-adsorption of the antibody with synthetic peptide (100 μg/ml of pure serum) completely abolished renin-like staining in dot immunoblot assays conducted by the method of Salzet *et al.* [18].

Immunocytochemical procedures.

Both sectioned material and whole mounts were employed.

Whole mounts. Fragments of ventral nerve cords from *T. tessulatum* were treated according to the method of Salzet *et al.* [10]. Primary antibody (anti-leech renin) was diluted at 1:500; FITC-labeled goat anti-rabbit IgG (Sigma, St Louis, MO) was used at a dilution of 1:100. Whole-mounts were examined with a Zeiss Axioskop fluorescence microscope.

Sections. Head parts of *T. tessulatum* or rat kidneys were fixed overnight at 4°C in Bouin-Hollande fixative with saturated 10% HgCl₂. They were then embedded in paraffin wax and serially sectioned at

7 μm . After being dewaxed with toluene, the sections were successively treated with anti-leech renin, diluted 1:200, and with goat anti-rabbit IgG conjugated to horseradish peroxidase as described previously [23]. The specificity of anti-leech renin was tested on consecutive sections mounted on different slides by pre-adsorbing the antiserum overnight at 4°C with the homologous antigen (synthetic peptide from Neosystem, Strasbourg, France) at a concentration of 350 $\mu\text{g/ml}$ of pure antiserum.

Results

Central nervous system localization.

Immunocytochemical studies on rat renal cortex using the anti-leech renin revealed positive and specific staining in the vascular pole of the glomerulus and the afferent arteriole (Figs. 1a, 1b). Other vascular segments of the arterial vascular tree are also stained with the antibody (Fig. 1b). These observations agree with those of Rawashed *et al.* [1,22] for rat and ovine fetal kidneys using a renin-specific antibody, demonstrating the presence of renin in the afferent arteriole and the juxtaglomerular apparatus.

After we had confirmed the specificity of the anti-leech renin antibody on vertebrate tissues, immunocytochemical experiments were performed on leech tissues. Glial cells (Fig. 2a) and neurons (Fig. 2a) in anterior compartments 4,5 and 6 (Figs 2a, 2b; see the scheme in Fig. 2g), as well as median (Figs 3a, 3d), and lateral (Fig. 3c) compartments of the sub-esophageal ganglion (scheme Fig. 2f) and segmental ganglia of the nerve cord (Fig. 3b) were labeled. Adjacent sections treated with anti-leech renin either pre-adsorbed with the homologous antigen (Fig. 3e) or not (Fig. 3d) confirmed the specificity of the staining. Whole mount experiments (Figs 3a–3c) confirmed its cellular localization through the leech brain. These results are consistent with those found in vertebrates, in which both glial cells and neurons synthesize renin enzyme [24]. We speculate that cross talk occurs between these cells for physiological functions such as osmoregulation. Moreover, as neurons and glial cells are immunoreactive to anti-leech renin in compartment 4, which is involved in osmotic control through the release of peptide hormones [13] like angiotensins [10, 14, 15, 18], this sustains our hypothesis.

Co-localization of renin- and ACE-like enzymes.

As we have demonstrated that a renin-like enzyme exists in leeches, we performed co-localization studies with anti-leech renin and anti-leech angiotensin-converting enzyme (ACE) [25]. As shown in Figures 2b and 2c, the same neurons and glial cells are stained with the two antisera. We have speculated that these enzymes could be implicated in angiotensin biosynthesis in the leech CNS [10,14], as in vertebrates, forming a renin–angiotensin-like system.

Urinary excretory tract localization.

Immunocytochemical studies were also performed on the leech nephridia (Fig. 2e), considered the analog of the vertebrate kidney. The nephridial canal, canaliculi (Fig. 2b) and the periphery of the ciliated funnel and lining epithelium of nephridia capsule (Fig. 2d) are recognized by the anti-leech renin. These results agree with those obtained in insects, where an aspartyl protease presenting 30.8% sequence homology with human renin was found in Malpighian vessels [21]. Thus, these enzymes appear to be implicated in osmoregulation in some invertebrates as well as in vertebrates.

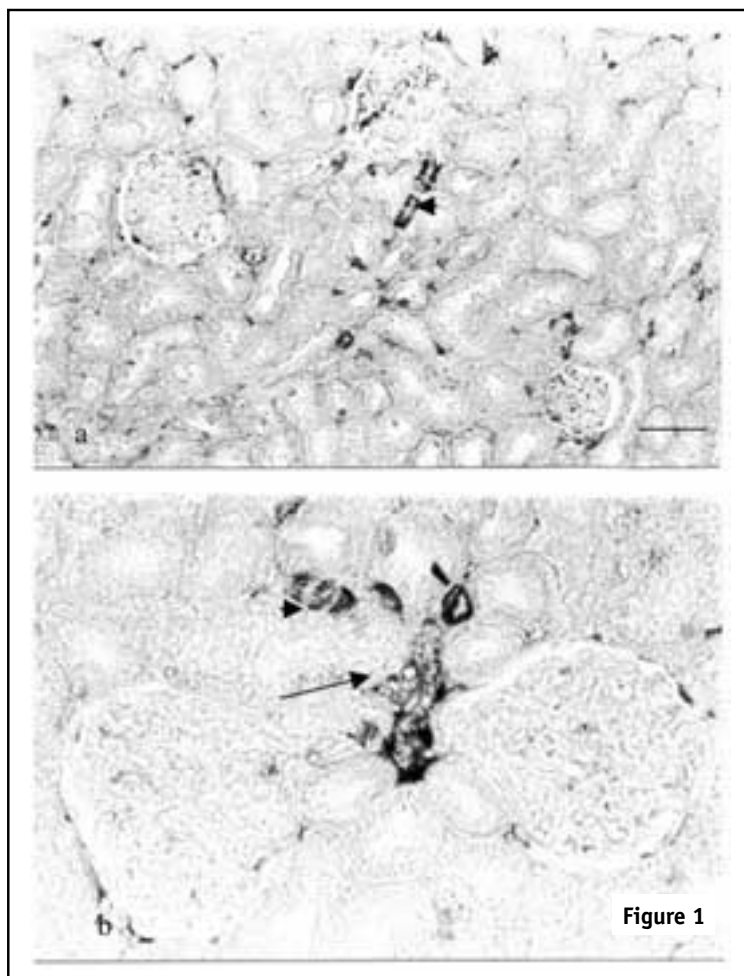


Fig. 1. Rat renal cortex. Immunostaining is localized to the vascular pole of the glomerulus. It is also observed in other vascular segments.

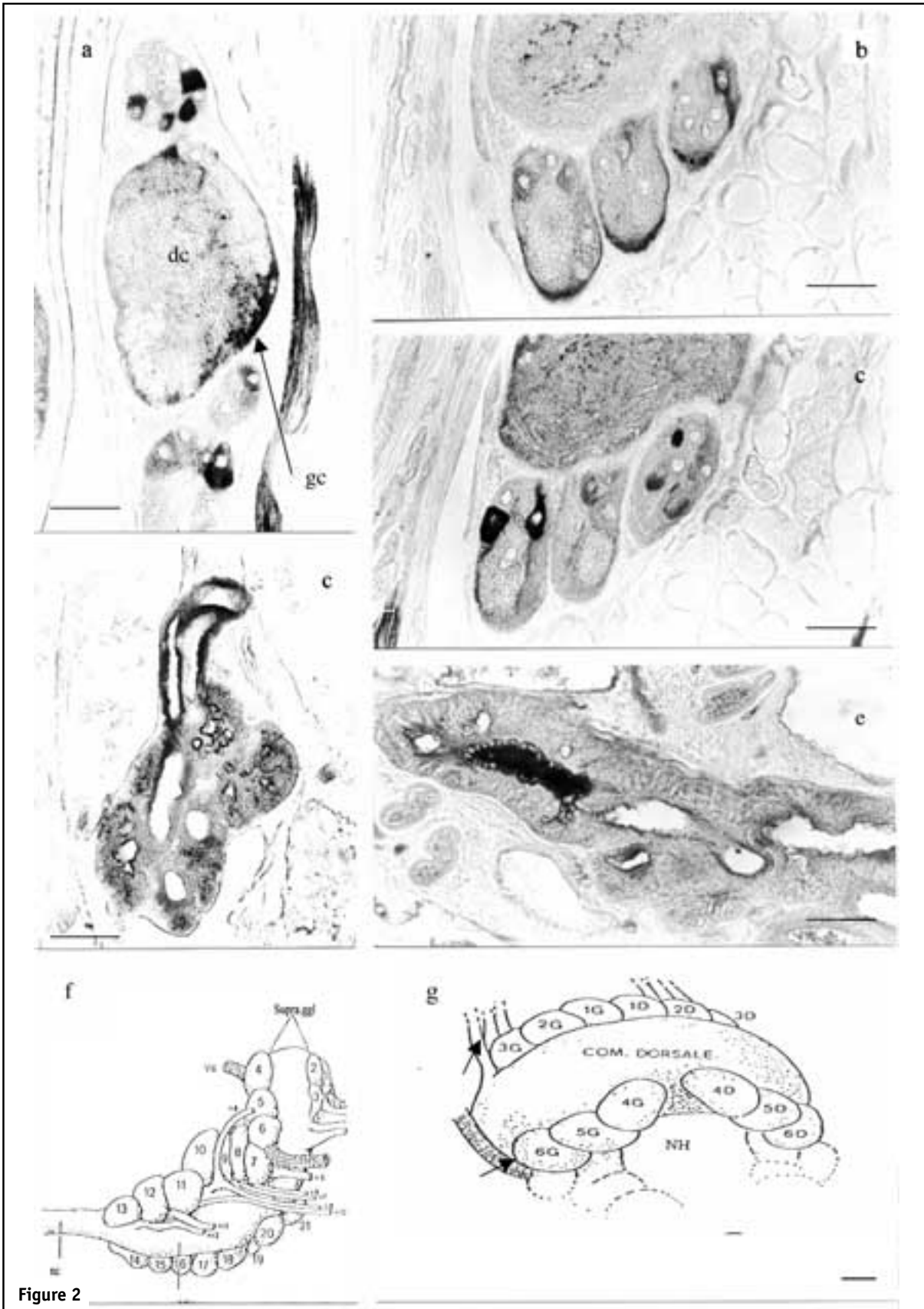


Figure 2

- a: Immunostaining with anti-leech renin in leech neurons, glial cells (gc, arrow) located in the dorsal commissure (dc).
 b, c: Adjacent sections of the leech supraesophageal ganglion treated with anti-renin (b) or with anti-ACE (c). Some neurons and glial cells are stained with the two antisera.
 d: The nephridial canal and numerous canaliculi (ca) showing renin immunostaining.

- e: Immunostaining was detected in the lumen of the nephridial canal.
 f: Scheme of *T. tessulatum* brain (supraesophageal ganglion (supra.ggl) and subesophageal ganglion (sub.ggl.)) nc: nerve cord, vs: blood vessel. Each compartment is numbered (1–21).
 g: Scheme of *T. tessulatum* supraesophageal ganglion. The anterior compartments are numbered 1 to 3 and the dorso-ventral ones from 4 to 6. The dorsal commissure is indicated as Com Dorsal. NH indicates the neurohemal area.

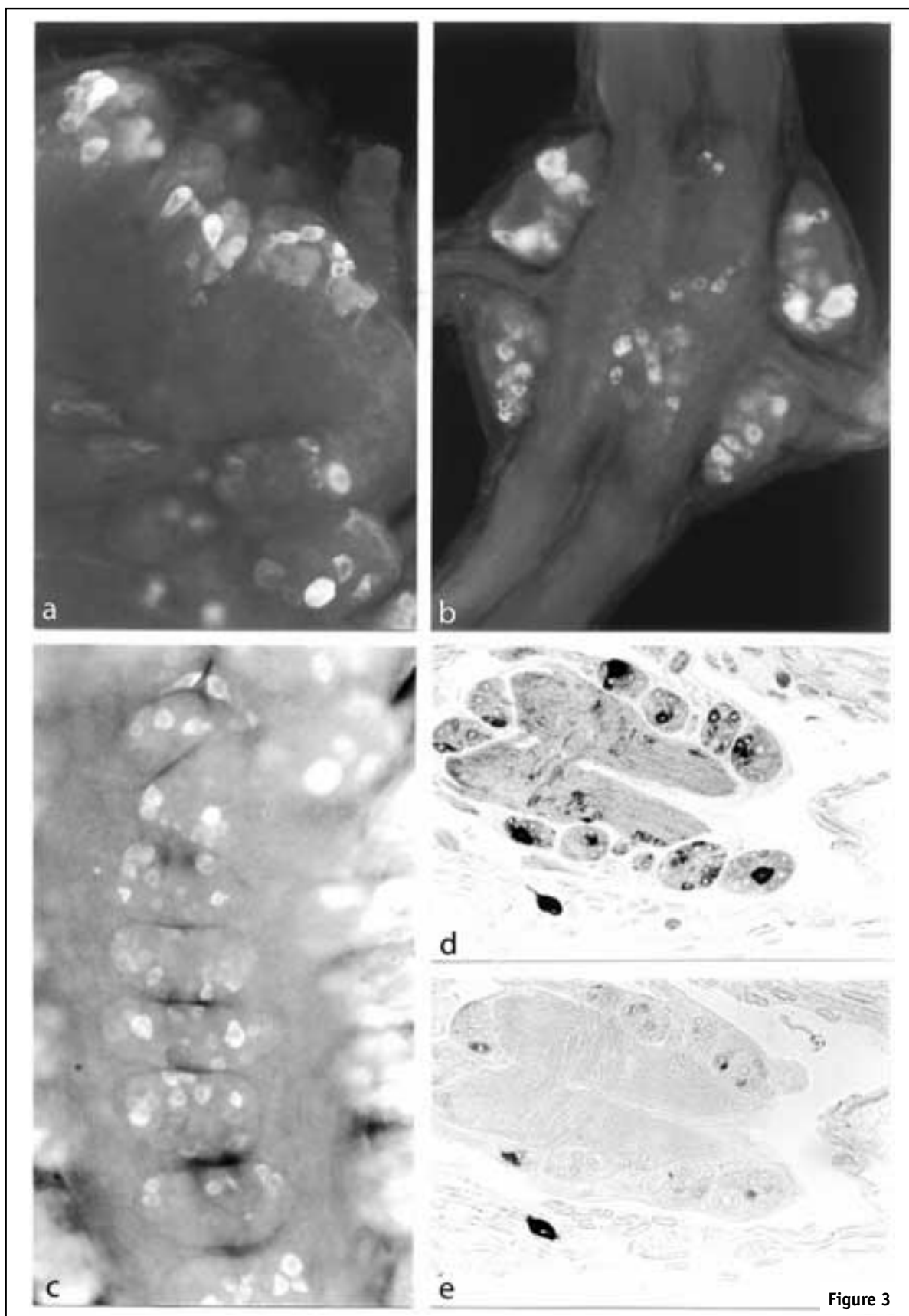


Figure 3

a, b, c: Immunohistochemical fluorescent micrographs of whole mount preparations of *T. tessulatum* central nervous system labeled with anti-renin. (a) Dorsal view of the brain showing numerous immunoreactive neurons at the level of the anterior compartment of the supraesophageal ganglion, the segmental ganglion of the nerve cord (b) and at the level of the lateral compartment of the subesophageal ganglion (c). (b) Ventral view of the nerve cord ganglion. Numerous immunoreactive neurons are present in the lateral and

median compartments (c), ventral view of subesophageal ganglion at level of median compartments containing numerous immunoreactive cells.

d, e: Adjacent sections of the leech subesophageal ganglion treated either with anti-renin (d) or with anti-renin pre-adsorbed with homologous antigen (e). Pre-adsorption of the antiserum with renin fragment abolished the immunostaining present at level of neurons and fibers and only a few neurons are still stained.

Discussion

Evidences are now given of the presence of aspartyl protease enzymes in blood-feeding parasites, including schistosomes, hookworms, and malaria parasites [30, 31]. For example, clone encoding the aspartic proteinase (PFAPD) from *Plasmodium falciparum* strain HB3 was obtained during the course of a project designed to sequence and identify the protein coding regions of the parasite's genome. The protein encoded by the clone contains a sequence identical to the N-terminal sequence determined for an aspartic proteinase isolated from the digestive vacuole of *P. falciparum* and demonstrated to participate in the hemoglobin digestive pathway [30]. The translated polypeptide sequence encompasses a number of features characteristic of aspartic proteinases, having > 30% identity and > 50% similarity overall to human cathepsin D, cathepsin E and renin. A model of the three-dimensional structure of PFAPD was constructed using rule-based procedures. This confirms that the primary sequence may be folded as a single chain into a three dimensional structure closely resembling those of other known aspartic proteinases. In blood-sucker leeches, we also found an aspartyl-protease enzyme related to renin [15]. Evidences of hemoglobin cleavage products were also found e.g. hemorphin-like substances [32], we can so also speculate that this enzyme is implicated in hemoglobin degradation. However, the immunocytochemical data, we here obtained favors, its involvement in osmoregulation like in the mosquito *Aedes aegypti* [21].

Moreover, as the first reported for leech renin, using pepstatin A affinity columns and enzymatic essays, we found an enzyme that catalyzes the breakdown of TDP with a molecular mass of 32 kDa. Its N-terminal sequence demonstrates that this enzyme belongs to the aspartyl protease family. A pro-renin like enzyme was also discovered during the purification with a molecular mass of 55 kDa. These data were consistent with those found in the mosquito and in other invertebrates [21]. Several aspartyl proteases are known to be secreted as pro-enzymes and undergo self-activation upon exposure to acidic pH. Upon activation, an NH₂-terminal pro-peptide of up to 50 amino acids long is released. Sequence alignment showed homology between the NH₂-terminal sequence of the mosquito enzyme [21] and the pro-peptide sequences, particularly bovine chymosin. This suggests that the active enzyme may be produced as a zymogen with a short pro-peptide. We now suspect the same applies for the leech renin-like enzyme. Moreover, as previously mentioned, these two enzymes are localized in the renal system: nephridia in leeches, Malpighian vessels in mosquitoes, and kidneys in vertebrates.

Thus, this report demonstrates that leeches have both the endogenous ligand for angiotensin, the angiotensin-like peptide [14], and the necessary biosynthesis systems in the brain and in excretory tract (nephridia), as in vertebrates. Zerbst-Boroffka *et al.* [26, 27] and Salzet [10, 13] showed that, beginning 15 minutes after taking a feed of blood, diuresis occurs over eight hours and leech angiotensin levels increase greatly. By contrast, ACE endogenous inhibitor [28], and leech osmoregulatory factors [16, 17] are lower. Moreover, leech angiotensin is known to stimulate chloride secretion in leech stomach tegument [29] and skin [29], reflecting an involvement in the control of osmotic balance by the renin-angiotensin system in leeches.

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