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Short communication

Enhanced antibacterial activity in *Hydra* polyps lacking nerve cells

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Abstract

The nervous system evolved within cnidarians. When assessing antibacterial activity in the freshwater polyp *Hydra*, we observed a strong correlation between the number of neurons present and the antibacterial activity. Tissue lacking neurons had a drastically enhanced antibacterial activity against Gram-positive (*Bacillus subtilis*) and Gram-negative (*E. coli*) bacteria compared to control tissue. The results indicate direct and strong neural influences on immunity in the phylogenetically oldest animals having a nervous system.

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1. Introduction

Antibacterial activity is the first line of defence against pathogens and crucial part of all innate immune systems. Antibacterial peptides are found in great diversity throughout the animal and plant kingdoms and have been suggested to play a fundamental role in the successful evolution of complex multicellular organisms [1]. Recently, there is evidence in invertebrates and vertebrates that the immune system interacts with the nervous system [2–4]. Molecules previously thought to be restricted to either neural or immune systems are found playing overlapping roles. For example, the neuropeptides substance P, calcitonin G related peptide (CGRP), and vaso-intestinal polypeptide (VIP) are upregulated following exposure to infectious pathogens

or allergens and can directly influence immunological diseases [5]. CGRP also provokes immunosuppressive effects following exposure to ultraviolet irradiation [6]. Similarly, tachykinins and neurokinin A are released following injury or inflammation. Adrenocorticotropin hormone (ACTH) is involved in immune (chemotaxis and phagocytosis) and neuroendocrine (stress response) functions in invertebrates and vertebrates [3]. Neuropeptides also directly participate in immune reactions since precursors for neuropeptides such as proenkephalin and chromogranin B can be processed into peptides (enkelytin/peptide B) with antibacterial properties [4]. Despite these findings, there are still large gaps in our understanding of the neuroimmuno interaction—mostly due to the lack of appropriate in vivo experimental model systems to evaluate the contribution of neurons in immune reactions. As shown below, the freshwater polyp *Hydra*, a Cnidarian and member of the most basal animal phylum having a nervous system, represents such a model.

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Hydra polyps consist of only two epithelia, the ectoderm and the endoderm surrounding a gastric cavity [7]. Each of the epithelial layers is made up of a cell lineage, while the remaining cells are part of the interstitial cell lineage which reside among the epithelial cells of both layers. Multipotent interstitial stem cells give rise to neurons, cnidocytes, secretory cells and gametes [8]. The nervous system in *Hydra* is simple, consisting of a nerve net of sensory neurons and ganglion cells [9,10]. *Hydra* neurons differentiate continuously from multipotent stem cells [8] and are constantly intercalated into the nerve net at a rate appropriate to the rate of epithelial cell division [11].

There is as yet little knowledge as to the mechanism of how *Hydra* or any other cnidarian polyp defend themselves against infectious microorganisms. At the cellular level, the epithelium can be considered as the protective skin and an integral part of the *Hydra* innate immune defense against microbial infection. Ectodermal epithelial cells play an active role as phagocytes in elimination of 'excess', nonself, and apoptotic cells [12,13]. Thus, although *Hydra* has no special immunocytes, epithelial cells in addition to their role in producing signal peptides used for axial patterning [14] and neuron differentiation [15] appear to be capable of immune responses. At the molecular level, nothing is known about the components of the *Hydra* innate immune system. In contrast, molecules participating in functioning and regulation of the *Hydra* nervous system are well described. Numerous neuropeptides have been isolated from *Hydra* and other cnidarians. Their structure has been elucidated [9] and the corresponding preprohormones have been cloned [9,16]. A systematic approach to identify morphogenetically active peptides (*The Hydra Peptide Project*) has revealed that *Hydra* not only contains neuropeptides but also peptides derived from epithelial cells [17]. Thus, in contrast to higher metazoa, the majority of signaling molecules in Cnidaria appear to be peptides.

When investigating the antibacterial activity in *Hydra*, we unexpectedly observed a strong correlation between the number of neurons present and the level of the antibacterial response. Using a mutant strain containing temperature-sensitive inter-

stitial stem cells we observed strong antibacterial activity in epithelial tissue lacking any neurons suggesting that in *Hydra* neurons influence the innate immune response. Thus, since it was probably within cnidarians, or a related ancestor group, that nervous systems first evolved [9,18], the cross-talk between nervous and immune system appears to be an evolutionary ancient invention.

2. Materials and methods

2.1. Animals and culture conditions

We used *Hydra oligactis* and three strains of *Hydra magnipapillata*. *H. magnipapillata* strain 105 is wildtype; strain sf-1 is a mutant strain containing a temperature-sensitive interstitial cell lineage [19]. Strain A10 is a chimeric strain consisting of the ectodermal and endodermal epithelial cell lineages from strain 105 and the interstitial cell lineage from sf-1. Unless otherwise stated, animals were cultured using standard methods in a constant temperature room maintained at 18 ± 0.5 °C, with 12 h light and 12 h dark period. At the permissive temperature, antibacterial activity is similar in wild type *H. magnipapillata* strain 105 and mutant strain sf-1.

2.2. Interstitial cell elimination and culture of nerve free *Hydra*

To eliminate the interstitial cells, sf-1 animals raised at 18 °C (permissive temperature) were cultured at 25 °C (nonpermissive temperature) for 16 days. Control polyps cultured at 18 °C have a nerve cell density of 0.14 nv/epi [20]. Different cell types of the interstitial cell lineage are lost from the tissue at different rates [19–21]. Interstitial cells are eliminated from the treated tissue within the first 12–16 h of treatment. Nematoblasts decrease within 48 h of treatment. Elimination of nerve cells occurs at a slower rate and requires 8–10 days for an overall loss [20]. Due to the loss of nerve cells and nematocytes, animals become unable to eat by themselves. These nonfeeding epithelial animals were cultured in standard medium, fed by hand, and cleaned daily using methods described [8].

2.3. Protein extraction

For each experiment 25 polyps were used from control cultures and 60 polyps from the nerve-free culture. Polyps were macerated in 8% citric acid, equal amount of ethanol was added and extracts were kept at -20°C until use. After thawing, cell debris was removed by centrifugation and supernatants were corrected and neutralized (pH 7.0). Samples were placed in Speed Vac to evaporate the liquid. Precipitates were resuspended in $5\ \mu\text{l}$ acetic acid prior to be used for the measurement of antimicrobial activities.

2.4. Microorganisms and antibacterial assay

Antimicrobial activity was determined by measuring growth inhibition of *E.coli* strain XL blue and *Bacillus subtilis* strain DSM 347. We followed the conditions described by Lehrer et al. [22] with minor modifications. Briefly, a volume containing $1-4 \times 10^6$ bacterial CFU was added to 10 ml warm 10 mM sodium phosphate buffer, pH 7.4 (NAPB) that contained 3 mg of powdered TSB medium, 1% w/v agarose and 0.02% v/v Tween 20. The bacteria containing agar were poured into $100 \times 1000 \times 15\ \text{mm}^3$ petri dish to form a uniform layer. A template and a 3 mm diameter gel punch were used to make 16 evenly spaced wells. After adding $5\ \mu\text{l}$ of extract per well, the plates were incubated for 3 h at 37°C , and then overlaid with 10 ml of sterile overlay agar (6% w/v solution of TSB and 1% w/v agarose). After incubation for 18–24 h at 37°C , the diameter of the clear zone surrounding the wells was measured. The diameter of clearing was expressed in units ($0.1\ \text{mm} = 1\ \text{U}$) and was calculated after subtracting the diameter of the central well ($3\ \text{mm} = 30\ \text{U}$). Results were confirmed by repeating the assay three times. Acetic acid controls without *Hydra* extract showed no bacterial clearing.

3. Results and discussion

H. magnipapillata strain sf-1 animals cultured at the permissive temperature (18°C) contain high levels of nerve cells (about 0.14–0.16 nv/epi).

Animals cultured at the nonpermissive temperature lose their neurons within 8–10 days of temperature treatment [20,21]. Fig. 1 shows a typical epithelial *H. magnipapillata* strain sf-1 polyp (Fig. 1(B)) as well as a mutant polyp kept at the permissive temperature (Fig. 1(A)). We used epithelial *Hydra* polyps lacking any neurons as a model system to investigate the influence of neurons on antimicrobial activity. For assessing antimicrobial activity, a radial diffusion assay was performed. *H. magnipapillata* strain sf-1 polyps were cultured at the permissive or nonpermissive temperature for up to 12 days. Thereafter protein extract was prepared and aliquots of $5\ \mu\text{l}$ added to gels containing *E. coli* XL blue. After incubation at 37°C for 16 h, zones of bacterial clearing became readily apparent (Fig. 1). Tissue temperature-treated for 4 days showed similar antimicrobial activity than control tissue indicating that presence or absence of interstitial cells and nematoblasts has no influence on the immune response. Temperature treatment for longer periods of time (8–12 days), however, reduces the numbers of neurons and results in drastically enhanced zones of bacterial clearing, about three times that of control tissue (Fig. 2). The data indicate that in the absence of neurons *Hydra* epithelial tissue has enhanced activity against *E. coli* XL blue. To demonstrate that the antibacterial activity is localized within *Hydra* cells, we dissociated sf-1 polyps into single cells and washed the cells several times by centrifugation. All antibacterial activity was restricted to the cell pellet. To confirm that the antimicrobial activity was indeed contributed by the absence of neurons and not by temperature treatment per se, we carried out three control experiments. First, in *H. magnipapillata* strain 105 polyps we could not detect any enhanced or attenuated activity in tissue kept at 25°C for 15 days. Second, when using chimeric strain A10 which consists of the epithelial cell lineages from strain 105 and the interstitial cell lineage from sf-1, similar results could be observed than in strain sf-1. Third, in another species, *H. oligactis*, antibacterial activity was similar in animals kept at 18 and 25°C indicating that *Hydra* epithelial cells in the presence of nerve cells do not respond to temperature increase by increased antibacterial activity. We, therefore, conclude that the enhanced antimicrobial activity in epithelial sf-1 tissue is due to the absence of neurons.

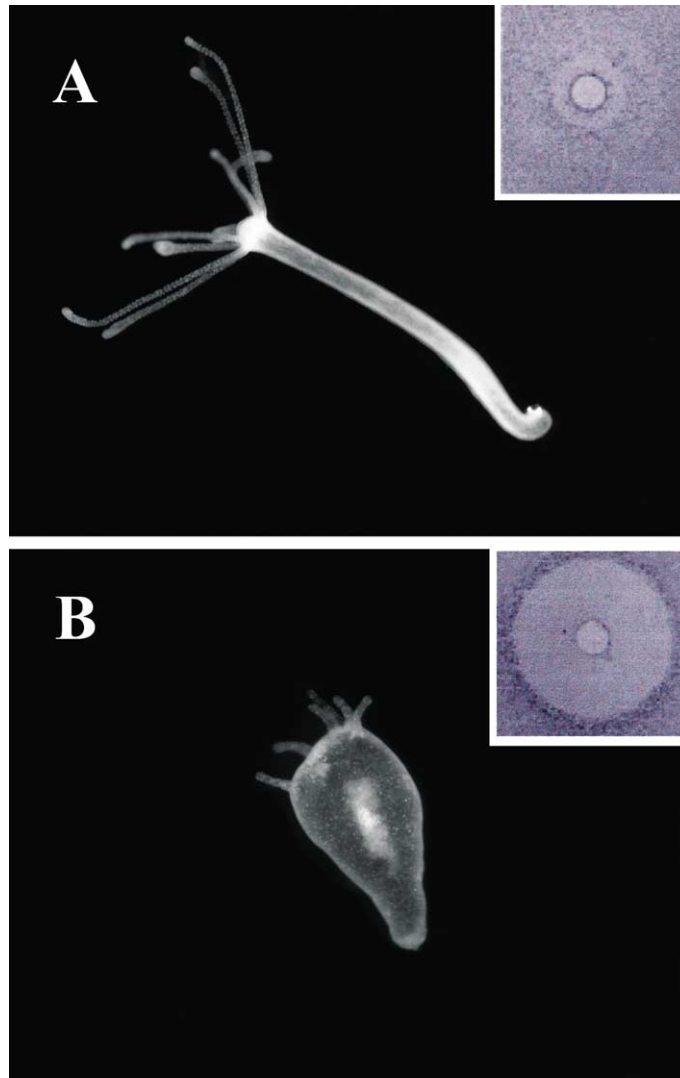


Fig. 1. *H. magnipapillata* strain sf-1 polyps cultured at permissive (A; $\times 16$) and nonpermissive (B; $\times 40$) temperature for 12 days. Note the typical morphology of the epithelial polyp. Insets: corresponding radial diffusion assays against *E. coli* indicating increased antibacterial activity in polyps lacking neurons.

Using the same experimental procedure we next examined whether nerve-free *Hydra* tissue also has enhanced antimicrobial activity against Gram-positive bacteria. *H. magnipapillata* strain sf-1 polyps were cultured at the nonpermissive temperature for up to 16 days. Thereafter protein extract from 30 polyps was prepared and aliquots of 5 μ l added to gels containing *B. subtilis* strain DSM 34. As shown in Fig. 3, after incubation at 37 °C for 16 h, zones of

bacterial clearing became readily apparent indicating the ability of *Hydra* tissue to suppress *Bacillus* growth. Within the first 8 days at elevated temperature tissue growth inhibition of *B. subtilis* was similar in temperature treated and control tissue. Only in polyps which were temperature-treated for 12–16 days and, therefore, lacking any neurons, enhanced zones of bacterial clearing, about 1.6 times that of the activity in control tissue, could be observed (Fig. 3). Although

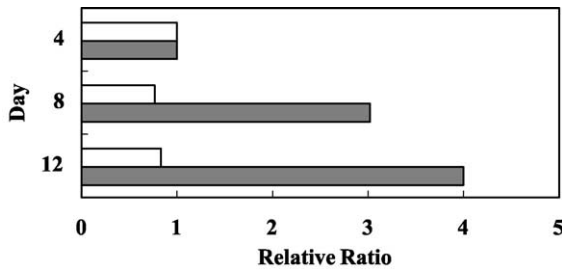


Fig. 2. Antimicrobial activities against *E. coli* in normal and nerve-free *H. magnipapillata* strain sf-1. Radial diffusion assay with *Hydra* extracts prepared 4, 8, and 12 days after temperature shift. More than 30 polyps were used for each group. The relative ratio of the zone (18 °C, 4 days) was calculated as one. White bar, normal sf-1 polyps at permissive temperature. Grey bar, polyps cultured at 25 °C for 4, 8 and 12 days.

the difference between control and nerve-free tissue was smaller than in experiments using *E. coli* XL blue, the results clearly show a stronger response against Gram-positive bacteria when neurons are absent. The reason for the reduced activity against Gram-positive bacteria is not known.

The *Hydra* body consists of a head at one end of a hollow gastric column, and a foot at the opposite end (Fig. 1(A)) which enables the animals to adhere to the substrate. Bacterial biofilms are prevalent on most of the surfaces used by *Hydra* as substrate in nature or under laboratory condition. To test whether there is a local response to substrate-associated microbes, we

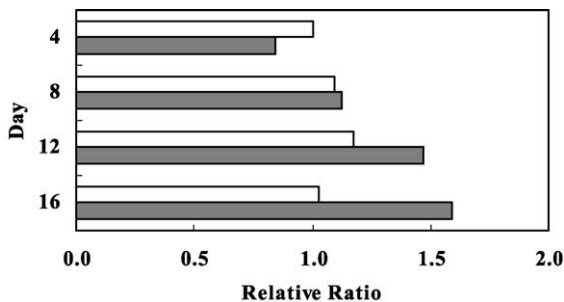


Fig. 3. Antimicrobial activities against *B. subtilis* in normal and nerve-free *H. magnipapillata* strain sf-1. Radial diffusion assay with *Hydra* extracts prepared 4, 8, 12, and 16 days after temperature shift. More than 30 polyps were used for each group. The relative ratio of the zone (18 °C, 4 days) was calculated as one. White bar, normal sf-1 polyps at permissive temperature. Grey bar, polyps cultured at 25 °C up to 16 days.

extracted protein from head, gastric region and foot of *H. magnipapillata* sf-1 cultured at the permissive temperature. When testing inhibition of growth of *E. coli*, tissue from the head region had four times lower activity than tissue from gastric region or foot (Table 1). There were no significant local differences in the activity against *B. subtilis* (Table 1). In sum, all epithelia along the body column appear to be capable of antimicrobial response with a slightly enhanced activity against Gram-negative bacteria in tissue from the lower body region.

The results reported here revealed two novel facts concerning innate immunity in *Hydra*. First, the findings indicate for the first time that *Hydra* tissue contains antimicrobial activity against Gram-negative and Gram-positive bacteria. This protection, most likely, is at least in part due to the synthesis of potent antimicrobial peptides. Second and most surprisingly, antibacterial activity in *Hydra* is positively correlated with the absence of nerve cells. In other animal groups communication and reciprocal regulation between the nervous and immune systems have been proposed to be essential for the stability of the organism [2,4,23–25]. Our observation indicates that this neuro-immune interaction can be traced back in evolution from vertebrates to *Hydra*, a member of an animal phylum in which the nervous system first evolved. So far, many studies have focused either on the evolution of the immune system or on the evolution of the nervous system [26]. The observations presented here indicate that both systems evolved in close relation to each other together. At this stage, we cannot determine what role the *Hydra* nervous system plays in the regulation of the antimicrobial response. The data, however,

Table 1

Antibacterial activities along different body parts in *H. magnipapillata* sf-1 cultured at the permissive temperature. Shown is area of bacterial clearance with relative ratio: head = 1, $n = 200$ polyps

	<i>E. coli</i>	<i>B. subtilis</i>
Head	1.00	1.00
Upper body	4.16	0.97
Lower body	3.75	1.04
Foot	2.89	0.92

raise the possibility that *Hydra* neurons may actively take part in regulation of the antibacterial response and negatively control production of antibacterial peptides. Alternatively, the putative neuroimmune ‘cross-talk’ in *Hydra* could be a competition-based interaction between neuropeptide and antibacterial peptide production. Interestingly, a regulatory interaction between *Hydra* epithelial cells and nerve cells in regard to synthesis of active peptides has been described earlier for the neuropeptide ‘head activator’ [27]. Whatever the precise mechanism, studying the neuroimmune interactions in *Hydra* will provide insight in a phylogenetically old, intriguing system that has developed to cope effectively with infections of various types.

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