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# Neuro-immune lessons from an annelid: the medicinal leech

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**Abstract**:

An important question that remains unanswered is how the vertebrate neuroimmune system can be both friend and foe to the damaged nervous tissue. Some of the difficulty in obtaining responses in mammals probably lies in the conflation in the central nervous system (CNS), of the innate and adaptive immune responses, which makes the vertebrate neuroimmune response quite complex and difficult to dissect. An alternative strategy for understanding the relation between neural immunity and neural repair is to study an animal devoid of adaptive immunity and whose CNS is well described and regeneration competent. The medicinal leech offers such opportunity. If the nerve cord of this annelid is crushed or partially cut, axons grow across the lesion and conduction of signals through the damaged region is restored within a few days, even when the nerve cord is removed from the animal and maintained in culture. When the mammalian spinal cord is injured, regeneration of normal connections is more or less successful and implies multiple events that still remain difficult to resolve. Interestingly, the regenerative process of the leech lesioned nerve cord is even more successful under septic than under sterile conditions suggesting that a controlled initiation of an infectious response may be a critical event for the regeneration of normal CNS functions in the leech. Here are reviewed and discussed data explaining how the leech nerve cord *sensu stricto* (*i.e.* excluding microglia and infiltrated blood cells) recognizes and responds to microbes and mechanical damages.

Keywords: Annelid, CNS, antimicrobial peptides, neuro-immunity, sensing receptors, regeneration

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## 1. INTRODUCTION

Because of its multiple vital functions, it is critical that the CNS be successfully defended against pathogens. For a long time, this organ was considered to be immunologically inert and isolated from the peripheral immune system. However, it is now well established that immune surveillance and inflammatory responses do occur within this compartment (Wrona et al, 2006). Indeed, in response to either cerebral injury or systemic bacterial infection, the CNS launches a well-organized immunological reaction that encompasses both neural components and peripheral immune system cells. Within the mammalian CNS, resident glial cells, including astrocytes and microglia, have been shown to initiate a characteristic innate immune response by producing and releasing antimicrobial peptides (AMPs), cytokines and chemokines (Becher et al., 2000). These circulating molecules promote the destruction of the invading bacteria, the permeabilization of the blood brain barrier (BBB), and the recruitment of peripheral leukocytes to the CNS and the activation of their effector functions, including further production of cytokines as well as phagocytosis by peripheral macrophages. The specific outcome of this neuroinflammatory response, which has both beneficial and detrimental aspects, depends on the context of the insult and on the duration of the inflammation. On the positive side, increased immune activity rapidly initiates the killing of bacteria and the removal of apoptotic cells and cellular debris, while also playing an important role in neuroprotection and repair by inducing the production of neurotrophic factors. In fact, several recent observations suggest that induction of regeneration of normal CNS function may depend critically upon the co-initiation of an immune response (Gendelman et al., 2002, 2003; Nguyen et al., 2002; Pavlov and Tracey, 2015; Russo and McGavern, 2015). On the negative side, excessive and/or chronic glial reactivity, in conjunction with the presence of adaptive immune cells within the CNS, can damage the CNS by inducing neuronal death and by blocking axonal myelination. An important question that remains unanswered is how the vertebrate neuroimmune system can be both friend and foe to the damaged nervous tissue. Some of the difficulty in obtaining an answer in mammals probably lies in the conflation in the CNS of the innate and adaptive immune responses, which makes the vertebrate neuroimmune response quite complex and difficult to dissect. An alternative strategy for understanding the relation between neural immunity and neural repair is to study an animal

devoid of adaptive immunity and whose CNS is well described and regeneration competent. The medicinal leech offers such opportunity.

In this review, we discuss the unique characteristics of the medicinal leech as model for studying the immune response of the CNS.

## 1. THE MEDICINAL LEECH AS A MODEL TO STUDY THE LINK BETWEEN IMMUNITY AND REGENERATION OF THE CNS

Several features make the CNS of the medicinal leech *Hirudo verbana* (often wrongly referred and sold in Europe as *Hirudo medicinalis*) particularly attractive as a model system for the exploration of interactions between bacteria, the nervous and immune systems. These features include simplicity, a fixed number of neurons, and consistency from animal to animal, which allow the recognition, characterization and repeated study of identified neurons, at all developmental stages and following specific perturbations, such as mechanical or septic trauma. The leech CNS is comprised of a fixed number (Nicholls and Van Essen, 1974) of mid-body segmental ganglia, plus larger "head" and tail "brains", linked to each other by longitudinal nerves known as connectives. Most segmental ganglia have a complement of ~400 neurons and 8 giant glial cells, along with a large population of microglia. Almost all of the ~400 neurons have homologs in every ganglion; a majority has been characterized morphologically as well as physiologically, and for many their synaptic connectivity, neurotransmitters and roles in behaviour have been determined (Muller et al., 1979; Muller and Carbonetto, 1979). Moreover, by contrast with mammals, studies in the leech can be focused exclusively on the neural immune response of the CNS itself. Indeed, the leech nerve cord normally lies within a ventral blood sinus, but it is encapsulated by a tough fibrous sheath that may, like the mammalian BBB, limit the exchange of macromolecules and cells with the blood. However, the intact CNS can be easily removed from the animal and cultured in the absence of peripheral immune system components and blood cells that might infiltrate the CNS after injury (Fig. 1a).

The most important feature is the capacity of the medicinal leech CNS to regenerate and restore normal function in response to injury. If the nerve cord of this annelid is crushed or partially cut, axons grow across the lesion and conduction of signals through the damaged region is restored within a few days, even when the nerve cord is removed from the animal and maintained in

culture. When the mammalian spinal cord is injured, regeneration of normal connections is more or less successful and implies multiple events that still remain difficult to resolve. In the leech, the process of regeneration begins with a rapid activation of microglial cells leading to their accumulation at the lesion site (Muller and Carbonetto, 1979). Microglial cells are resident macrophages in mammals (Hanisch and Kettenmann, 2007; Parry et al., 1997), arthropods (Smith et al., 1987) and leeches (von Bernhardi and Muller, 1995) which respond rapidly to brain injury by moving to the lesion and accumulating there. Whether they subsequently divide as in mammals and arthropods, or not as in leeches, recruited microglia phagocyte cellular debris (Neumann et al., 2009). Although blood cells are still in a good shape after a one week culture, microglial cells die rapidly (in less than 24 hours). By developing a procedure to deplete the nerve cord in microglial cells (Schikorski et al., 2008), we evidenced that an optimal regeneration require microglial cells for initiation and blood cells to facilitate and accelerate the process (Boidin-Wichlacz et al., 2012).

Interestingly, the regenerative process of the lesioned nerve cord is even more successful under septic than under sterile conditions suggesting that initiation of a controlled infectious response may be a critical event for the regeneration of normal CNS functions in the leech (Fig. 1b).

*Hirudo*, therefore, is an excellent model system for exploring fundamental questions about the interaction of the nervous and innate immune systems, including (a) what is the nature of the innate immune response mounted by the nervous system? (b) How does the nerve cord *sensu stricto* (*i.e.* excluding microglia and infiltrated blood cells) respond to microbes, mechanical damages and other stresses?

## 2. NEURONAL MICROBIAL SENSING OF THE LEECH

Invertebrates, being devoid of adaptive immunity dependent on RAG (*i.e.* Recombination-Activating Genes), are interesting model systems for exploring the molecular basis of innate immunity. For example, the initial evidence for the pivotal role of the Toll receptor family in immunity was discovered in *Drosophila*, and only later in mammals (Imler and Zheng, 2004). Another example is the discovery of the first antimicrobial peptides (AMPs) by Hans Boman in the moth *Hyalophora cecropia* (Steiner et al., 1981). AMPs are now considered as important effectors of the innate immune systems of both invertebrates and vertebrates. Most reports on immune effectors in invertebrates have tended to focus on their involvement in the systemic anti-

infectious response. More and more studies described the presence of immune molecules in the nervous systems of insects and nematodes, both members of the ecdysozoan group. Indeed, several Toll-like receptors (TLR) and some molecules of the TLR signalling pathway have been detected in glial and neuronal cells of *Drosophila*, and appear to have a role in neural development in the larvae (Wharton and Crews, 1993). In *Caenorhabditis elegans*, an ortholog of the *Drosophila* toll gene was shown to be expressed in pharyngeal neurons, where it participates in defensive behaviour by discouraging the worm from ingesting pathogenic bacteria (Pujol et al., 2001). Increasingly, a role for the nervous system in recognizing microbes has been showed not only in the induction of host immunity but also in broader effects on host physiology associated with the microbiota, the metabolism, the behavior, and the pathophysiology of diseases (Kawli et al., 2010).

The innate immune response is an evolutionarily ancient defense strategy against pathogenic agents that has been documented widely in living organisms, including invertebrates and vertebrates. Its major functions include: (1) recruiting immune system cells to infection sites though the production of chemokines and cytokines; (2) activating the complement cascade in order to identify pathogens, activate cells to promote pathogen clearance and stimulate the adaptive immune response; (3) interacting specifically with pathogens through membrane or cytosolic receptors in immune circulating cells in order to remove pathogens from organs and tissues.

To search for the major players for these functions, we screened the *Hirudo* transcriptome database for invertebrate homologs of vertebrate genes belonging to these different categories and then investigate their immune and/or neuro-regenerative functions by combining cellular, molecular, biochemical and morpho-functional approaches (Macagno et al., 2010). Among the various factors identified, we will lay particular emphasis on: (1) Pattern Recognition Receptors (PRR) and their associated signalling pathways; (2) Immune effectors such as AMPs and cytokines produced by neurons.

### 2.1. Pattern Recognition Receptors (PRR) expressed in the leech CNS

The innate immune system uses different PRR that sense Microbe-Associated Molecular Patterns (MAMPs). These include TLRs, Retinoic-acid-inducible gene-1 (RIG-1)-Like Receptors

(RLRs), and the Nod-Like Receptors (NLRs), all of which contain Leucine Rich Repeat domains (LRRs). PRRs contain two key functional domains *i.e.* the LRR domain that interacts directly or indirectly with microbial signature shared by major classes of microbes, whereas the second is more like protein-protein interaction domains activating the downstream signalling event, leading to the transcription of immunity effector genes such as anti-microbial substances, cytokines. Such danger signaling receptors seem to be well conserved in leeches and are all expressed in the CNS of the leech (Table 1). Their presence was predictable according to the demonstrated ability of this organ to mount an antimicrobial response specific of the microbial agent it is exposed to (Schikorski et al., 2008, 2009; Tasiemski and Salzet, 2010).

***Toll-Like Receptors (TLRs)***: TLRs are critical components of the innate immune responses of both vertebrates and invertebrates (Leulier et al., 2008; Imler and Hoffmann, 2000a, b, 2001; Imler et al., 2000; Imler and Zheng, 2004; Tauszig et al., 2000). TLRs are type I transmembrane receptors with an ectodomain containing several LRR motifs flanked by conserved cystein-rich motifs, a feature they share with several other types of receptors, including GPIba, the neurotrophin receptor Trk, and CD14 (Imler and Hoffmann, 2000b). Within the cytoplasm, TLRs have a ~150 amino-acid domain with strong homology to a corresponding region in the Receptor for Interleukin 1 (IL-1R), though the ectodomain of IL-1R has of three immunoglobulin-like motifs instead of LRRs.

There are 10-13 known mammalian TLRs, depending on species, and many of the components of the cytoplasmic pathway from TLRs to the nucleus have been identified. For example, TLR4 is an essential component of the lipopolysaccharide (LPS) receptor complex, together with the membrane associated, GPI-linked, CD14. TLRs also mediate NF-kB activation in response to a broad repertoire of microbial molecules, including the responses to (A) nucleic acids, mediated by TLR3 (double-stranded (ds) RNA), TLR7 (single-stranded RNA enriched in U residues) and TLR9 (unmethylated CpG motifs); (B) bacterial lipopeptides and/or fungal PAMPs, mediated by the TLR2/TLR6 and TLR2/TLR1 heterodimers; and (C) bacterial proteins, e.g., TLR5 is required for recognition of flagellin, whereas TLR11 mediates activation by profilin of the protozoan parasite *Toxoplasma gondii* or protein components from uropathogenic strains of *Escherichia coli* (Imler and Hoffmann, 2000a, 2001).

Among invertebrates, the innate immune response has been first and most extensively investigated in ecdysozoan species: *Drosophila melanogaster* (De Gregorio et al., 2002;

Ferrandon et al., 1998) and *C. elegans* (Pujol et al., 2001). A large number of TLRs are now identified in various metazoan phyla: Cnidaria, Annelida, Mollusca, Arthropoda, Echinodermata and Chordata (Hemmrich et al., 2007; Rauta et al., 2014). The existence of TLRs in annelids has already been deduced from *in silico* analysis of the genomes of *Capitella* and *Helobdella* (Davidson et al., 2008). As for the medicinal leech, the repertoire of *Capitella* consists primarily of the vertebrate-like rather than a protostome like domain organisation. Interestingly, the blastp homology of *Hm*TLR1 with the vertebrate TLR13 and TLR3 is consistent with this observation. In addition to the sequence homology, *Hm*TLR1 is expressed by both microglia and neurons and seems to exert functions comparable to those described for the mammalian TLR3 in the brain (see above). Indeed, in vertebrates, microglia has been reported to express mRNAs for TLRs 1 to 9 whereas neurons and oligodendrocytes (Prehaud et al., 2005) express only transcripts encoding TLR3 (Bsibsi et al., 2002).

Our analysis of the *Hirudo* EST libraries led to the identification of 4 other TLRs that are all expressed in the nervous system but whose functions remain undescribed (Table 1). One of them, namely *Hm*TLR3 was recently further analyzed and appeared to be up-regulated in the bacterially challenged nerve cord (unpublished data). Interestingly, the LRR domain of *Hm*TLR3 presents homologies with the vertebrates TLR8, known to be implicated both in the antiviral response by recognizing single strand ARN and in the neuronal development (Ma et al., 2007). The great homology of *Hm*TLRs with vertebrate TLRs supports our interest to use the leech model to understand the immune mechanisms developed by the CNS in mammals. PAMP recognition by leech TLRs has yet to be elucidated to fully demonstrate the conservation between the two systems. Indeed, it still has to be determined whether this is a direct recognition as observed in mammals or an indirect recognition as described for the fruit fly (Leulier et al., 2008).

***Nod-Like Receptors***. The NLRs, consisting of more than 200 related family members, are present in the cytosol and recognize intracellular MAMPs together with RLRs, TLRs, and C-type lectin families (Motta et al., 2015). In addition to their response to intracellular pathogens, NLRs have been shown to play important roles in distinct biological processes ranging from regulation of antigen presentation, sensing metabolic changes in the cell, modulation of inflammation, embryo development, cell death, and differentiation of the adaptive immune response (Lupfer

and Kanneganti, 2013). While no NLR homologue has been found in the genomes of the ecdysozoans *Drosophila* and *Caenorhabditis elegans,* a sequence related to NLR was found in the CNS of the medicinal leech (Cuvillier-Hot et al., 2011). *Hm*NLR shares best homologies with NLRC3 receptors, considered as evolutionarily basal to the NLR family in vertebrates. NLRC3 protein is an important cytosolic PRR that negatively regulates innate immune response in mammals. In zebrafish, recent data demonstrate that NLRC3-like by preventing inappropriate macrophage activation, contribute to normal microglial cell development (Microglia derived from primitive macrophages that migrate into the brain during embryogenesis) (Shiau et al., 2013). Such role cannot be supported in the leech CNS since *Hm*NLR has a brain tissue expression restricted to neurons. Confocal microscopy data more precisely evidences an accumulation of *Hm*NLR in the submembranous compartment. This cytosolic distribution reminds the localization of activated Nod2, whose membrane recruitment in human cells appears as necessary for NF-kB activation post microbial challenge (Barnich et al., 2005).

The N-terminal tail of *Hm*NLR (recognition domain) displays no conserved domain, nor does it match with any known molecule in blast analysis. However, its orthologs detected in the genome of *Capitella*, an annelid Polychaeta, do present a CARD domain upstream of the LRR domain. Considering that the clitellates - among which the Hirudinae – probably derived from a polychaete-like ancestor (Sperlin et al., 2009), it is possible that in the course of the evolution of leeches the ligand-binding domain was conserved but not the upstream effector domains, suggesting innovative transduction pathways. Interestingly, in the amphioxus genome also, some sequences similar to vertebrate NLR without a NACHT domain were described (Huang et al., 2008), highlighting a NLR repertoire in non-vertebrates more complex than that of vertebrates. The characterization of a NLR homologue in annelids reinforces the hypothesis of an ancient origin for this family of cytosolic sentinels.

***Viral sensing receptors.*** Multiple observations support the presence of viral sensing receptors in the nerve cord of the leech. Several immune transcripts have been showed to be over-expressed in the nerve cord after viral activation though polyI:C agents (Schikorski et al., 2008) (Fig. 2). Silencing experiments performed to shut down *Hm*TLR1 expression also resulted in increased expression of the AMP *Hm*-lumbricin reflecting a siRNA sensing system in the leech nerve cord. Among the PRRs that detect the intracellular presence of MAMPs, a virus sensing receptor RIG-

1 like DExD/H box RNA helicases (RLR) was detected in *Hirudo* EST (V. Cuvillier et al, unpublished data). The presence of a great number of RNA helicases among the leech transcripts, coupled with the presence of the RIG-1 orthologs, suggests the capacity for mounting an efficient anti-viral immune response in *Hirudo*. Transcripts with strong homology to a number of antiviral response factors, including Dicer, Drosha and Argonaute, were found in the *Hirudo* database (Table 2) and we suspect the AMP lumbricin to possess anti-viral activities because of its strong expression after viral challenges and its cytosolic localization. In leech, highly conserved molecules related to serpins, eglin c, or leech-derived tryptase inhibitor (LDTI), which in other systems are known to be active against viruses like HIV or Hepatitis C Virus NS3 protease (Auerswald et al., 1994; Martin et al., 1998), will need to be taken into account in dissecting out the leech antiviral response.

***Functions of PRRs in the response to neuronal injury and/or microbial infection of the leech CNS:*** Under sterile conditions, genes encoding *Hm*TLR1 or *Hm*NLR appeared to be differentially modulated during the regenerative process (Fig.2). Although the gene encoding *Hm*TLR1 was observed to be downregulated along with the CNS repair, the level of *Hm*NLR transcripts appeared to be up regulated a few days (3 days) post axotomy, suggesting a role of the last one in the mid-term events engaged during neural regeneration (Cuvillier-Hot et al., 2011). The regenerative process is known to be effective 7 days post injury of the leech CNS). Interestingly, neuronal injury in mammals leads to the induction of NLRP1 and NLRP5, which are believed to regulate caspase activation and apoptosis in injured neurons (Frederick Lo et al., 2008). This dual role of NLR family members – sensing pathogens as well as damages – fits especially well to the neural context where immunity and tissue repair appear more and more as intimately connected (Eming et al., 2009). Various hypotheses could explain the decrease of *Hm*TLR1 transcripts: (i) this receptor participates in limiting axonal growth as reported for TLR3, (ii) *Hm*TLR1 is not engaged in the regeneration of the injured CNS at all and/or (iii) *Hm*TLR1 is required for the regenerative process but because of the long lifespan of this protein a neosynthesis is not needed.

The variation of the gene expression was also quantified in nerve cords experimentally infected by various microbial derivatives (Fig. 2) (Cuvillier-Hot et al., 2011). Interestingly, a comparable pattern of expression was obtained for both genes suggesting a communication between our two

receptors upon microbial challenge of the injured leech nerve cords. Gram-positive bacteria and Muramyl DiPeptide (MDP) appear as the best inducers of *Hm*TLR1 and *Hm*NLR genes. With these conditions, our sensing receptors colocalized at the injured sites which, because of the solid fibrous capsule surrounding the leech CNS, corresponds to the exclusive ways of entrance/contact of/with microorganisms. These data also evoke a communication between *Hm*TLR1 and *Hm*NLR in our model (Cuvillier-Hot et al., 2011). Chauhan and colleagues showed that NOD2 synergizes TLR-induced inflammatory cytokine production in murine microglia and astrocytes, illustrating the interplay that may exist between co-expressed TLR and NLR receptors (Chauhan et al., 2009). Further investigations should be envisaged to study the potential interplay between leech *Hm*TLR1 and *Hm*NLR in the CNS, both under homeostatic conditions and following injury and / or infection. The role of *Hm*-TLR1 was elucidated (Schikorski et al., 2009). Indeed, silencing of the *HmTlr1* gene in the leech CNS demonstrated that upon microbial challenge, this receptor is involved in the induction of the gene encoding the chemokine *Hm*p43/EMAPII, an immune effector known to recruit phagocytic cells at the lesion site. These data are reminiscent of some observations of rat microglial cells, which have been reported to produce EMAPII after systemic injections of TLR agonists, such as polyinosine-polycytidylic acid (a TLR3 ligand) and R848 (a TLR 7/8 ligand) (Zhang and Schwarz, 2002). The regulation of EMAPII by a TLR in both leech and mammals reinforces the great conservation between these two models. Moreover, the existence of an immunity mediated by a TLR in the leech CNS have showed for the first time an immune function of a TLR in a non-ecdysozoan model (i.e., in an invertebrate model that is different from *C. elegans* and *D. melanogaster*) (Leulier and Lemaitre, 2008). We hypothesize that a co-evolutive process of the sensor receptors might have took place between the parasite (*i.e.* the medicinal leech) and its hosts (*i.e.* vertebrates including amphibians, fresh water fishes and mammals) presumably resulting from their close contact with the same microorganisms.

## 2.2 PRR signalling pathways in the leech nervous system

In many species, including invertebrates and vertebrates, PRR activation triggers an intracellular signalling pathway, followed by the regulation of defense genes. A survey of the medicinal leech transcriptome and genome databases allowed revealing the presence in the leech of the main

signaling molecules involved in the canonical vertebrate TLR pathways (Fig. 3 and table 1) (Macagno et al., 2010). This stands in sharp contrast to other invertebrates, such as insects and nematodes, for which the PRR pathways thus far appear to be less conserved, with many components missing. Whether all the identified leech putative homologs indeed play similar functional roles remains to be shown by further analysis, but their presence in the transcriptome database adds once again support to the hypothesis that annelid genetic programs are more closely related to those of vertebrates than are those of arthropods (Macagno et al., 2010) (Gagniere et al., 2010).

At the level of the nervous system, data clearly showed that the leech nerve cord is able to establish a specific neuroimmune response by discriminating microbial components after microbial challenge and/or post injury. As detailed before, leech neural cells express various PRRs, and in response produce immune specific effectors to fight encountered microbes and/or promoting nerve repair (see below). Neurons and microglia express sensing receptors like *Hm*-TLR1, which is associated with chemokine production (i.e. EMAPII) in response to septic challenge or lesion. To gain insights into the TLR signalling pathways involved in this neuroimmune response, members of the Myeloid Differentiation factor 88 (MyD88) family were investigated (Rodet et al., 2015). In mammals, it includes 5 adaptor proteins containing a TIR domain, MyD88, MyD88-adapter-like (Mal), TIR-domain-containing adaptor protein-inducing IFN beta (TRIF), TRIF-Related Adaptor Molecule (TRAM) and Sterile alpha and Armadillo-Motif-containing protein (SARM). All TLRs, except TLR3, recruit MyD88 to mediate innate immune signalling.

In the leech nerve cord, two members of the MyD88 family: *Hm*-MyD88 and *Hm*-SARM have been recently evidenced to be tightly regulated not only upon immune challenge but also during CNS repair, suggesting their involvement in both processes (Rodet et al., 2015). Interestingly, a stimulation of leech neurons with lipopolysaccharide (LPS) triggered a redistribution of *Hm*-MyD88 and *Hm*-TLR1 at the cell surface. To the best of our knowledge, these data showed for the first time that differentiated neurons of the CNS could respond to LPS through a MyD88-dependent signalling pathway, while in mammals, studies describing the direct effect of LPS on neurons and the outcomes of such treatment remain scarce and controversial.

## 3. IMMUNE EFFECTORS PRODUCED BY NEURONS

Once activated, sensing receptors of the leech CNS drive the production and release of numerous molecular effectors like AMPs and cytokines. These factors promote the regenerative process directly through their neurotrophic properties or indirectly thought the recruitment of immune cells (microglia and blood cells) that accumulate and release neurotrophic factors at the lesion site.

### 3.1. Neuronal antimicrobial substances with neurotrophic properties

The leech nervous system produces infection-inducible AMPs (Schikorski et al., 2008). *Hm*-lumbricin and neuromacin have been shown to be produced by microglial cells and by neurons themselves in response to CNS injury. Microbial components differentially induce the transcription, by microglial cells, of both antimicrobial peptide genes, the products of which accumulate rapidly at sites in the CNS undergoing regeneration following axotomy. A preparation of leech CNS depleted of microglial cells, allowed demonstrating the production of AMPs by neurons themselves.

Neither neuromacin-like nor lumbricin-like molecules have been found in the genomes of ecdysozoan invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster*, underlining the importance of enlarging the number of invertebrate models dedicated to study innate immunity.

Surprisingly, in addition to manifesting antibacterial properties, neuromacin and *Hm*-lumbricin exert impressive regenerative effects on the leech CNS. In vertebrates, one study provides evidence for the positive effects of an antimicrobial peptide on the restoration of the functions of a lesioned peripheric nerve. Indeed, the addition of neutrophil defensin NP-1 on the lesioned sciatic nerve in rats leads to increase the rate of growth of regenerative nerve fibers by 30% (Nozdrachev et al., 2006). These data observed in the leech were the first evidencing the participation of AMPs produced by the nervous system itself in the regeneration process of the CNS. However, multiple examples of neuropeptides, such as the alpha-Melanocyte Stimulating Hormone in human and proenkephalin A-derived peptides in both mammals and/or leeches, have also been described to possess antimicrobial properties *in vitro* (Tasiemski and Salzet, 2010).

Thus, as in humans, antimicrobial peptides are involved in the innate immune system of the leech CNS.

Neuromacin is a relative of theromacin, a cysteine-rich AMP first identified from the body fluid of the leech *Theromyzon tessulatum* (Tasiemski et al., 2004) and later in *Hirudo* (*Hm*-theromacin) (Tasiemski and Salzet, 2010). Theromacin is synthesized by the large fat cells and the blood cells while neuromacin is produced by the epithelial cells of the gut, the neurons and the microglial cells of the leech CNS (Boidin-Wichlacz et al., 2012; Tasiemski et al., 2015; Tasiemski et al., 2004). Theromacin possesses a longer C-terminal domain than neuromacin. That results in two different conformations, resulting in different biological activities for the two peptides (Jung et al., 2012). Further investigations revealed that neuromacin, like theromacin, belongs to the macin family a new family of AMP within the superfamily of scorpion toxin-like proteins.

The antimicrobial character of the macins is clearly demonstrated by their ability to permeabilize membranes of *B. megaterium* within a few minutes, suggesting the bacterial membrane as target, and its disruption as the mode of action (Jung et al., 2012). However, the macins showed significant differences in their mechanistic behavior, pore-forming activity, activity not inhibited by the presence of salt, were solely observed for neuromacin. This resistance to salt allows neuromacin to exert its antimicrobial properties not only in the SNC but also in the gut lumen where the osmolyte concentrations explode after each leech blood meal. We hypothesized that neuromacin was probably selected instead of theromacin to cope with the variation in salt concentrations in the gut environment. In this organ, neuromacin provides a protection against invasive pathogenic bacteria and contribute to the unusual simplicity of the gut microflora of the leech (Tasiemski et al., 2015). As this salt resistance was pH-dependent, it appears to be mediated through the de-/protonation of the histidine residues that are missing in theromacin. Moreover, neuromacin leads to aggregation of liposomes as well as Gram-negative bacteria whereas theromacin does not. In fact, on the contrary of the surface properties of neuromacin, the bipolar character of theromacin is insufficient for aggregation according to the *barnacle model i.e.* dual electrostatic as well as hydrophobic peptide-membrane interaction applied in parallel to two individual bacterial cells. Therefore, each bacterial cell can stick to several others, leading to the formation of huge cell aggregates (Jung et al., 2012).

Besides their antimicrobial activity, neuromacin and theromacin also exert a nerve-repair activity (Jung et al., 2012). In *Hirudo*, the production sites of the leech macins are in accordance with their regenerative effects on injured nerve cords. Indeed, theromacin that is produced by circulating blood cells is released into the plasma that surrounds the nervous system whereas neuromacin is directly produced by nerve cells and accumulates at the wounded site of the central nervous system (Boidin-Wichlacz et al., 2012) (Schikorski et al., 2008). The leech plasma enriched in AMPs, among them theromacin but also destabilase (a lysozyme like molecule), stimulated the regeneration of the central nervous system suggesting a nerve-repair activity of these antibiotic molecules. The neuronal repair process in leech does not include the *de novo* regeneration of entire neurons (Duan et al., 2005). It is described so far as migration of microglia to the site of lesion and an axon outgrowth which probably is cytoskeleton driven. Interestingly, both neuromacin and theromacin not only induce nerve repair, i.e., the axonal regrowth in leech, but they also increase the number of viable murine neuroblastoma cells. This observation might extend the role of leech macins in nerve repair as they are able to modulate cytoskeletal functions in leech and enhance the proliferation of neuroblastoma cells. Therefore, the nerve-repair process in leech might also include the proliferation of neurons *de novo*. The mechanism of proliferation induction remains to be determined. The fast uptake and initial inhomogeneous distribution of theromacin might be a hint that endocytosis is involved.

The development of antimicrobial pharmaceuticals based on AMPs represents a promising alternative approach in human anti-biotherapy. Beside their potential as templates for the development of alternative antibiotics the AMPs' various "secondary" activities might be beneficial for the development of pharmaceuticals suitable in other medical fields. In particular, the nerve-repair activity of the leech macins or the proliferation effect exerted by theromacin, neuromacin but also hydramacin are of particular interest (Jung et al., 2012). Further investigations of the nerve-repair activity might eventually lead to findings that support the development of pharmaceuticals effective against neural diseases, e.g., paraplegia, which is to date purely speculative. Neuromacin was patented in this direction.

### 3.2. Neuronal cytokines with chemoattractant properties

Among the effectors already discovered in leeches, *Hm*TLR1 is linked to the cytokine related to EMAP II in the context on the brain immune response after crush (Schikorski et al., 2009) (Table 3). *Hm*EMAP II is the first cytokine-related molecule characterized in nervous system invertebrates. The cytokine EMAPII has been suggested to be a marker of microglial cell reactivity in the mammalian CNS (Schluesener et al., 1999; Schluesener et al., 1997; Tas and Murray, 1996). Activated microglia of injured brain tissue ensuing from inflammation or neurodegeneration have been shown to produce high levels of EMAPII (Mueller et al., 2003). This cytokine was initially isolated from cultures of methylcholanthrene A-induced fibrosarcoma cells and constitutes the mature product of the C-terminal region of a 43 kDa precursor referred as the multi-synthetase complex p43, a component of the aminoacyl-tRNA synthetase complex involved in protein synthesis in mammals (Shalak et al., 2001). Numerous studies have described the pleiotropic biological activities of EMAPII and its precursor (van Horssen et al., 2006). At the peripheral level, *in vitro* studies have demonstrated that EMAPII: (i) participates in the recruitment of polymorphonuclear leukocytes and mononuclear phagocytes, (ii) promotes endothelial apoptosis, (iii) enhances the expression of some other cytokines, and (iv) stimulates dermal proliferation, wound repair, and graft neovascularization (Mueller et al., 2003). Interestingly, even if EMAPII is now considered as a modulator of inflammatory reactions within the peripheral innate immune response, its exact biological function in the neural immune response of the CNS has yet to be elucidated.

The detection of *Hm*p43/EMAPII in the microglial cells accumulated at the injured site of the leech CNS underlines some similarities of the inflammatory response of the nerve cord in our model with that of the human brain (Mueller et al., 2003). In the leech, however, neuronal cells also contribute to the production of *Hm*EMAPII, as demonstrated in the preparation of leech CNS depleted of microglial cells. By favoring the recruitment of microglial cells to the axotomized site, EMAPII indirectly contributes to neural repair and to the antimicrobial response of the leech CNS. Indeed, recruited microglial cells have been described to participate in the phagocytosis of damaged tissues and in the regeneration process by producing laminin, an extracellular matrix molecule known to promote neurite outgrowth and antimicrobial peptides, which exert neurotrophic activities (Schikorski et al., 2008; von Bernhardi and Muller et al. , 1995).

Besides EMAP, other cytokines are produced by the leech nervous system *e.g.* the one related to human interleukin-16 (IL-16). *Hm*-IL-16 protein present in the neurons, is rapidly transported and stored along the axonal processes to promote the recruitment of microglial cells to the injured axons. The ability of *Hm*-IL-16 to recruit microglial cells to sites of CNS injury suggests a role for *Hm*-IL-16 in the crosstalk between neurons and microglia in the leech CNS repair. Interestingly, in addition to its chemo attractive property, *Hm*IL-16 is able to promote human CD4+ T cells migration thus showing functional analogies of *Hirudo* IL-16 (*Hm*IL-16) with human IL-16 (Croq et al., 2009).

Other molecules (granulin, SOCS, TNF alpha…..) already known for their cytokinic activity in the nervous system of mammals have been detected in the leech CNS. Further investigations are needed to determine their neuroimmune functions in *Hirudo* (Table 3).

## 4. CONCLUSION AND FUTURE DIRECTIONS

As in mammals, the leech nervous system uses a common panel of proteins to initiate antiinfectious responses and regrowth programs. The relative simplicity of the leech CNS in combination with its having complex mechanisms to react to infection suggests that the study of the neural immunity in *H. medicinalis* will contribute to a better understanding of the implication of immune molecules in the neural repair of the CNS in mammals. Interestingly a significant and antigen-specific increase of the level of AMPs was recently detected in the nerve cords dissected from leeches exposed to different alive bacteria added to their water environment (Fig. 4). This differential gene expression of the leech AMPs observed in the CNS might result from a peripheral signal induced by the bacteria in contact with the leech skin as observed for *C. elegans* but also from bacteria that enter the gut and interact with the leech immunity and gut microflora (Kawli et al., 2010). Further investigations in this direction are planned to clearly establish whether there is a link between epidermal and neural immunity or/and between the gut microbial community and the neural immunity of the leech. The fact that the medicinal leech constitutes a model system of gut symbiosis reinforces the interest to explore this field (Bomar and Graf, 2012; Nelson and Graf, 2012; Nyholm and Graf, 2012; Ott et al., 2014). An effect of the microbiome that is not restricted to the digestive tract might be assumed taking into account the growing evidence of the multiple roles that exert gut symbiotic bacteria on their host

physiology and adaptation (Maranduba et al., 2015; Ray et al., 2015). Moreover it would be also interesting to determine whether *Hirudo* uses its nervous system to respond to diverse microbial cues, and engages it in a protective behavioral avoidance response to environmental pathogens and/or a selective behavior of the environmental bacteria composing its gut microbiota. Indeed, this behavior also constitutes an immune response, with sensors and recognition systems that drives a protective response following a learning experience.

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690
691

692 **Figure legends**

693

694 **Figure 1:** (a) Schematic cross section of *H. medicinalis*' body. The CNS is enclosed within the
695 ventral blood sinus. (b) Defined amount of bacteria (mix of heat-killed M. nishino: *Micrococcus*
696 *nishinomiyaensis* and A. hydrophila: *Aeromonas hydrophila* at $3x10^7$ CFU/ml) promotes the
697 regeneration process relative to sterile conditions (Schikorski, 2008). The Gram-positive and
698 Gram-negative bacteria M. nishino and A. hydrophila, respectively, were isolated from the
699 natural environment of *Hirudo*. Sectioning of one side of the paired connective nerve linking
700 adjacent segmental ganglia was performed on excised nerve cords maintained in culture. To
701 monitor the progress of nerve repair, micrographs of the damaged nerve cords were taken every
702 24 h, in the presence or absence of bacteria. Under sterile conditions, as documented in the left
703 column, restoration of the connective nerve across the cut begins at ~4 days post-axotomy (panel
704 J 4) and is finished 4 days later. In comparison, nerve repair is evident sooner in the presence of
705 a controlled number of bacteria, reconnection starting after 2 days or 3 days (right column).

706

707 **Figure 2**: Modulation of the gene expression of leech PRRs and immune effectors in nerve cords
708 incubated for 6 hours with various microbial components (2 µg/ml of LTA: lipoteichoïc acid; 10
709 µg/ml of MDP: muramyl dipeptide; 100 ng/ml of LPS: lipopolysaccharides), heat killed bacteria
710 at a concentration favoring the regenerative process (see Fig. 1b); VSV: Vesicular stomatitis
711 virus or viral mimetic (10 µg/ml of polyI:C). Incubations without microbial components or
712 bacteria were performed in the same conditions as controls. For the details see: Cuvillier-Hot et
713 al., 2011; Schikorski et al.,2008; Schikorski et al., 2009.

714

715 **Figure 3:** TLR pathways in the leech CNS. Analysis of the *Hirudo* transcriptome database
716 reveals the presence of putative homologs of nearly all factors reported to play critical roles in
717 human TLR pathways. Framed E values give the homologies of the leech proteins with their
718 counterparts identified in *Ce* : *Caenorhabditis elegans*, *Dm* : *Drosophila melanogaster* and *Hs* :
719 *Homo sapiens.* Some of them (E values 0) has already been entirely characterized.

720

721 **Figure 4:** The level of AMP expression is enhanced in the CNS of leeches that have swum for 6
722 hours in water heavily enriched in a mix of alive bacteria (*A. nishinomyaensis* and *A. hydrophila*

723 $10^7$ CFU per ml) in comparison with leeches that have swum in water not enriched in bacteria.
724 Adult leeches were maintained at room temperature in sterile artificial pond water for one week
725 before starting the experiment (*i.e.* addition or not of bacteria to the water tank). Dissection of
726 the nerve cords, RT-qPCR and statistical analysis were performed according to the methods
727 described in Schikorski et al., 2008.

**Table 1:** Repertoire of PRRs and elements of the PRR signalling pathways expressed in the leech CNS.

| | **E-values** |
|---|---|
| 4 Toll-Like Receptors (TLRs) | 2e-17 to 2e-19 |
| *Hm* Nod Like Receptor (NLR) | 0 |
| *Hm* RIG-1 like Receptors (RLRs) | 0 |
| Immunoglobulin superfamily receptors | 6.4e-16 to 1.5e-118 |
| *Hm*-Myeloid differentiation factor 88 (MyD88) | 0 |
| Evolutionarily conserved signalling intermediate in toll pathways (ECSIT) | 8.1e-20 |
| *Hm*-Sterile alpha and TIR motif-containing protein (SARM) | 0 |
| Toll-interacting protein (TOLLIP) | 1.1e-32 |
| Interleukin-1 receptor-associated kinase 4 (IRAK-4) | 3.7e-34 |
| Tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF-3) | 1.3e-52 |
| TNF receptor associated factor 6 (TRAF-6) | 1.7e-21 |
| P38 mitogen activated protein kinase (MAPK) | 6.3e-95 |
| NF-kappa-B p105 subunit (Contains: NFkB p50 subunit) | 4.5e-29 |
| IKK-like protein | 4.4e-45 |
| Interferon Regulatory Factor (IRF) | 1.3e-16 to 2.2e-17 |
| similar to interferon gamma-inducible protein 30 | 8e-32 |
| Fas Associated *via* Death Domain (FADD) | 2.3e-18 |
| Caspases 3/7 | 3.8e-53 to 2.7e-59 |

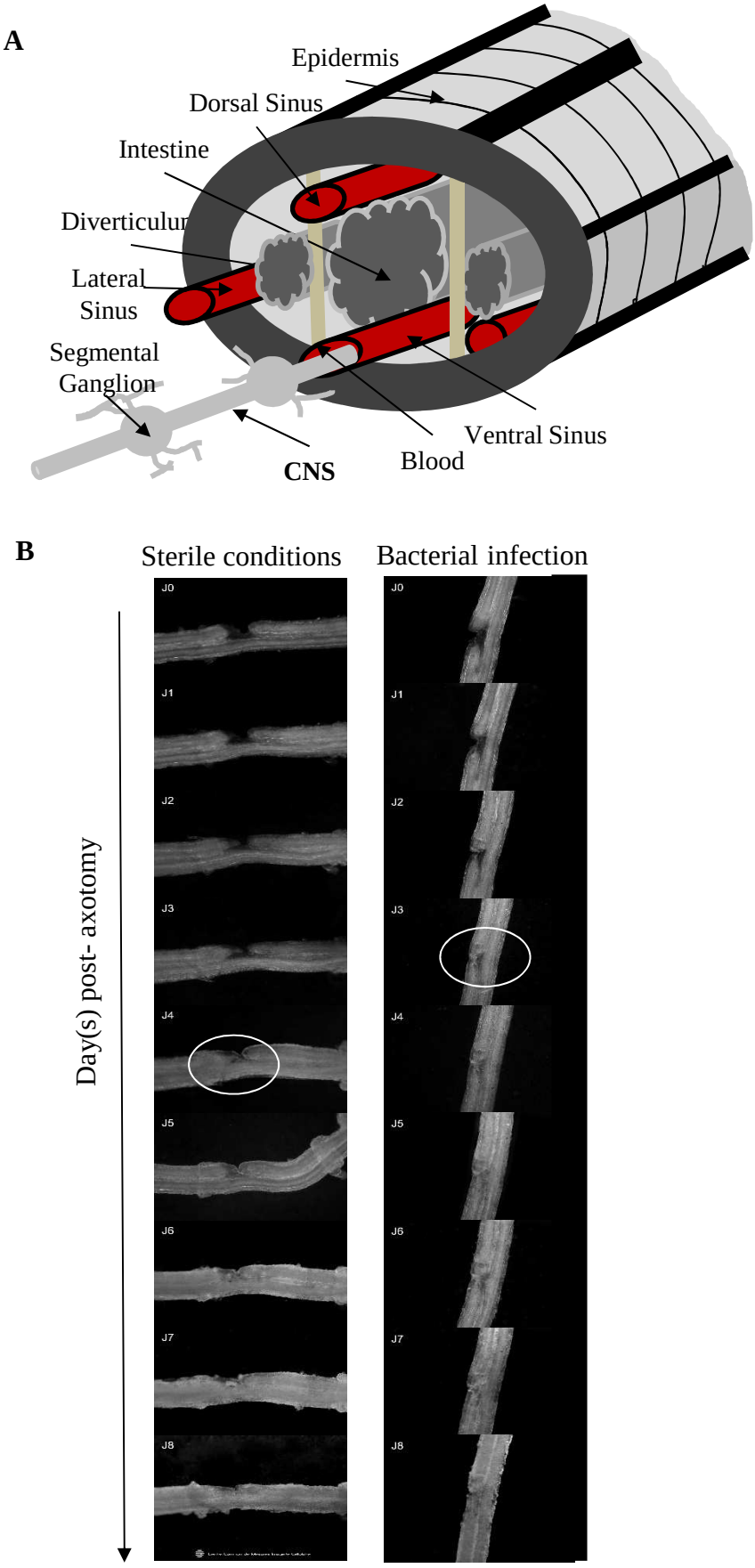
**Table 2:** Antimicrobial response elements produced by the leech CNS.

| | **E-values** |
|---|---|
| *Hm* Lumbricin (AMP) | 0 |
| *Hm* Theromyzin (AMP) | 0 |
| *Hm* Neuromacin (AMP) | 0 |
| Destabilase 1 (lysozyme-like) | 0 |
| Eglin-C | 2.3e-36 |
| LPS Binding Protein/ Bactericidal Permeability Increasing protein (LBP/BPI) | 6.2e-40 |
| Dicer | 7.3 e-91 |
| Drosha | 6.2e-76 |
| Argonaute-like protein | 1.9e-98 |

**Table 3:** ~~**(a)**~~ Cytokine related molecules synthesized by the leech CNS (~~**(b)** Level of expression in the CNS incubated with a controlled number of bacteria promoting the regenerative process~~.

| | **E values** |
|---|---|
| *Hm* p43/Endothelial Monocyte-Activating Polypeptide 2 (EMAP2) | 0 |
| TNF alpha | 6.6e-23 |
| Granulins | 1.8e-34 |
| SOCS | 6.9e 27 |
| *Hm* Interleukin-16 | 0 |

Figure 1


A
Epidermis
Dorsal Sinus
Intestine
Diverticulum
Lateral Sinus
Segmental Ganglion
CNS
Blood
Ventral Sinus
B
Sterile conditions
Bacterial infection
Day(s) post- axotomy
J0
J1
J2
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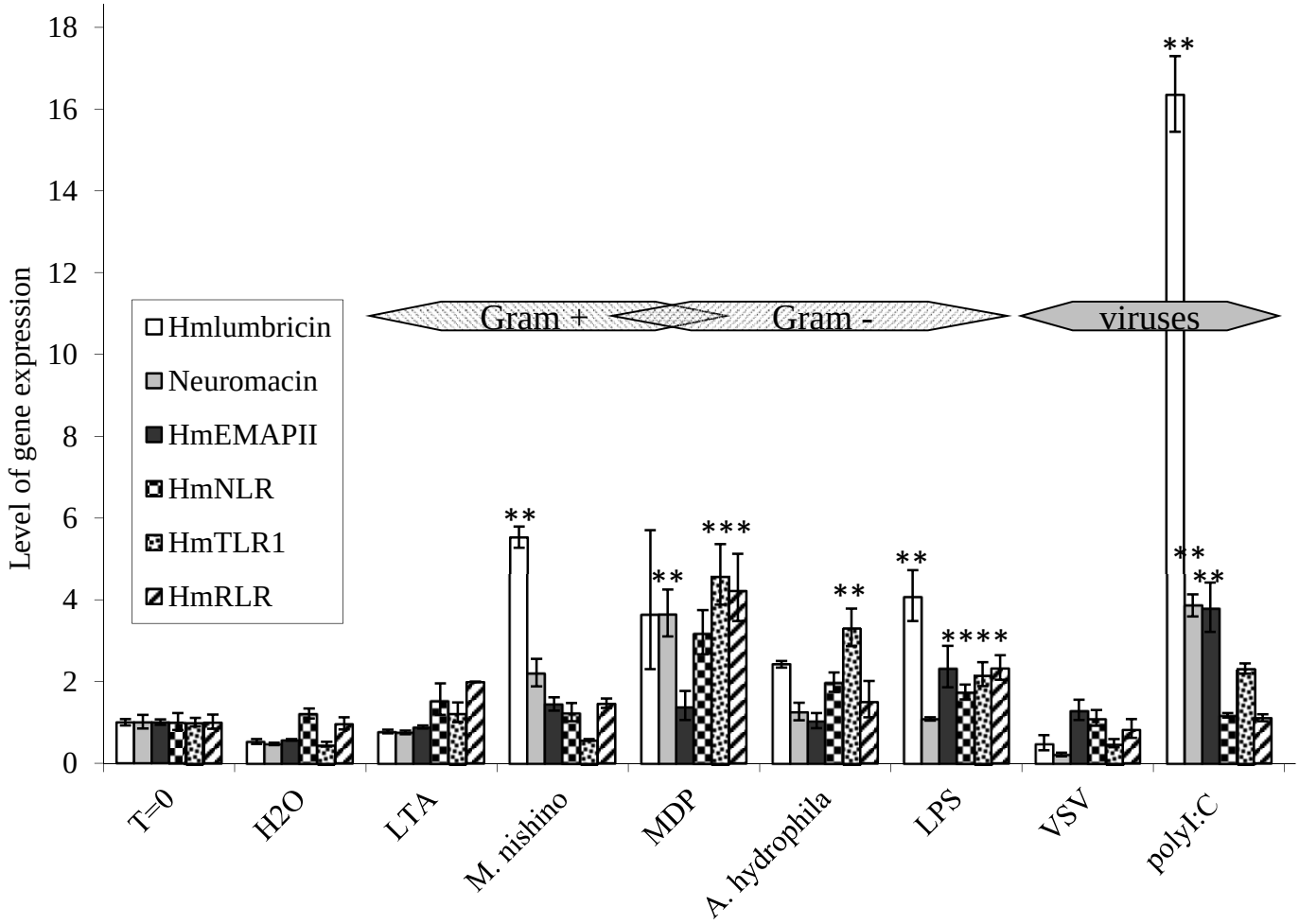
J6
J7
J8

Figure 2


Level of gene expression
18
16
14
12
10
8
6
4
2
0
Hmlumbricin
Neuromacin
HmEMAPII
HmNLR
HmTLR1
HmRLR
Gram +
Gram -
viruses
**
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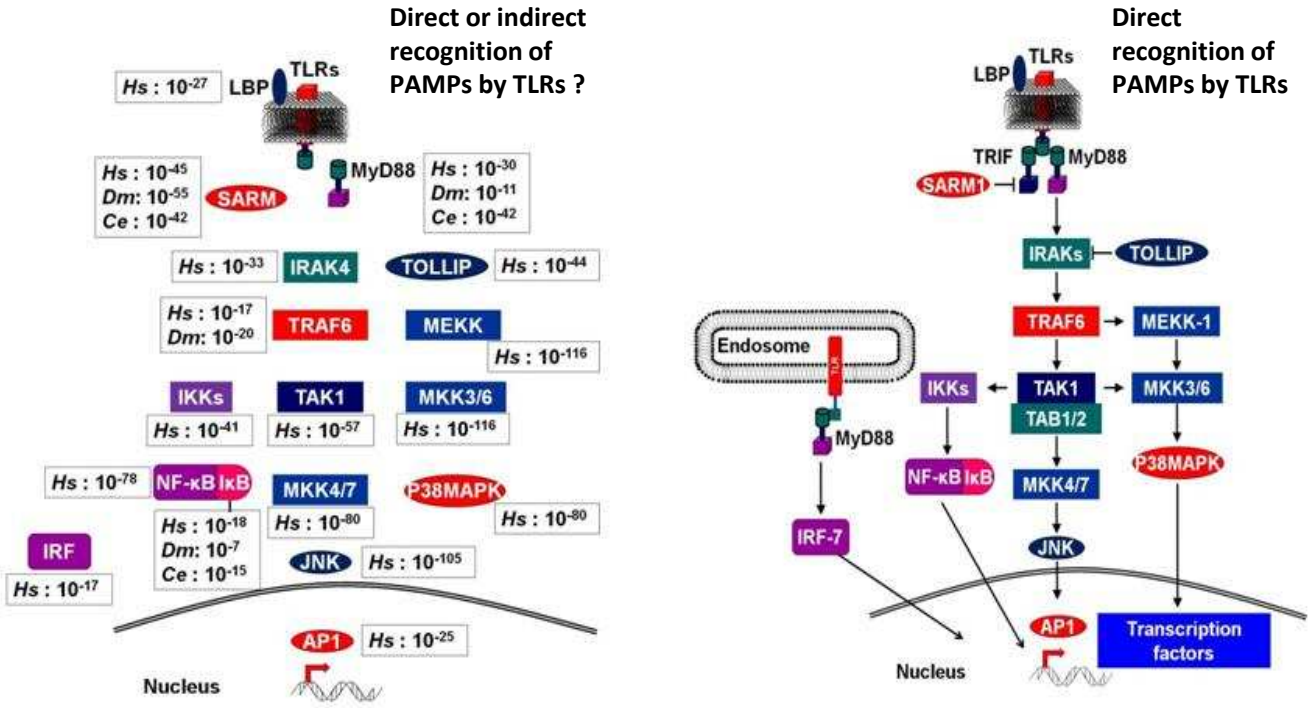
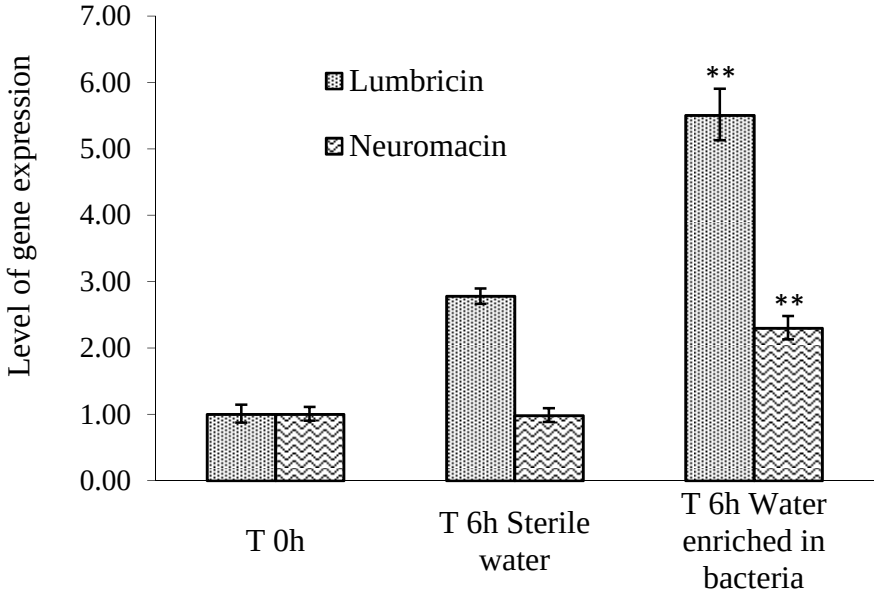
**
T=0
H2O
LTA
M. nishino
MDP
A. hydrophila
LPS
VSV
polyI:C

Figure 3




**LEECH** **HUMAN**

Figure 4


7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Level of gene expression
Lumbricin
Neuromacin
**
**
T 0h
T 6h Sterile water
T 6h Water enriched in bacteria

The medicinal leech is an excellent model system for exploring fundamental questions about the interaction of the nervous and innate immune systems

As in mammals, the leech CNS uses a common panel of proteins to initiate antiinfectious responses and regenerative programs

Leech neurons produce antimicrobial peptides having neurotrophic activities

Leech neurons express microbial sensing receptors