Characterization and immune function of two intracellular sensors, HmTLR1 and HmNLR, in the injured CNS of an invertebrate

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ARTICLE INFO

Article history:
Received 30 August 2010
Received in revised form 24 September 2010
Accepted 25 September 2010
Available online 29 October 2010

Keywords:
Toll-like receptor
Nod-like receptor
Neuroimmunity
Invertebrate
Medicinal leech
Hirudo medicinalis

ABSTRACT

Unlike mammals, the CNS of the medicinal leech can regenerate damaged neurites, thus restoring neural functions. Our group recently demonstrated that the injured leech nerve cord is able to mount an immune response, which promotes the regenerative processes. This defense mechanism is microorganism-specific, suggesting that the leech CNS is able to discriminate among microbial components. We report here the characterization of two receptors potentially implicated in this detection: HmTLR1 and HmNLR. Interestingly, HmTLR1 presents an endosomal distribution in neurons and appears as a chimera combining the mammalian intraendosomal domain of TLR3 and the cytoplasmic section of TLR13, while HmNLR is cytosolic and has the highest homology to NLRC3 receptors. Both receptors show patterns of induction upon stimulation that suggest their involvement in the leech neuroimmune response. This work constitutes the first demonstration in an invertebrate of (i) an intracellular TLR and (ii) a cytosolic PRR related to the NLR family.

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1. Introduction

Innate immunity corresponds to the first line of defense common to all metazoa. Phylogenetically ancient, it is mediated by molecules named PRRs (Pattern Recognition Receptors) encoded within the host genome and able to recognize PAMPs (Pathogen-Associated Molecular Patterns) specific to diverse microorganisms (Dolan et al., 2007). In their fight against pathogens, animals align two main battalions of PRRs: (i) the Toll Like Receptors (TLR), a family of transmembrane proteins containing a Leucine-Rich Repeat (LRR) domain and a cytoplasmic Toll/IL1 receptor (TIR) domain and (ii) cytoplasmic receptors such as the Nod-like Receptors (NLRs) characterized by a central oligomerization domain (NACHT) and an associated LRR array. TLRs and NLRs belong to the Leucine-Rich Repeat superfamily, in which the structural motifs created by the LRR repeats are considered as highly versatile and evolvable protein–ligand interaction motifs (Dolan et al., 2007).

In mammals, about thirteen TLR paralogues have been identified, among them 10 in human and 12 in mice that together are numbered from 1 to 13 (Leulier and Lemaître, 2008). According to the lengths of their LRR domains, phylogenetic analyses and amino acid homologies, vertebrate TLRs have been classified into six groups whose members usually recognize a common particular class of ligand. In effect, the TLR2 subfamily (TLR1, 2, 6) recognizes lipopeptide, the TLR3 subfamily (single member) is specific for double stranded RNA (dsRNA), the TLR4 subfamily (single member) for lipopolysaccharide (LPS), the TLR5 subfamily (single member) for flagellin, and the TLR9 subfamily (TLR7–9) for heme and nucleic acids (Jin and Lee, 2008). By contrast with the other TLRs, members of the TLR11–13 subfamily remain poorly described. To date, only one paper suggests that TLR11 recognizes uropathogenic bacteria (Zhang et al., 2004). The expression of TLRs differs with cell types, as does their cellular localization. In vertebrates, some have been found to be expressed extracellularly (TLR1, 2, 4, 5, 6 and 11) and others intracellularly (TLR3, 7, 8 and 9) in myeloid cells and also in non myeloid cells (McGettrick and O’Neill, 2010). To the best of our knowledge, there is no published evidence of intracellular toll or TLRs in invertebrates.

Generally referred to as intracellular sensors, very few NLRs have a specific ligand identified (reviewed in Benko et al., 2008). NOD2 appears as a general sensor of bacterial elements, recognizing muramyl dipeptide (MDP) which is the minimal motif common to...
all peptidoglycans. NOD1 is more specific, detecting a degradation product of the wall of Gram-negative bacteria (GM-TriDAP). In addition, NLRP1 is activated by anthrax lethal toxin, and NLRC4 (IPAF or CARD12) seems to be the intracellular counterpart of TLR5, detecting flagellin. Beyond pathogenic microorganisms recognition, some NLR family members have been shown to respond to internal elicitors. For example, NLRP3 (=Nalp3) seems to be activated by MDP as well as cellular potassium efflux. The NLRP3 inflammasome also forms in response to stress and danger signals such as uroic acid crystals released by necrotic cells, UVB irradiation or xenogenous compounds (aluminium, asbestos, silica). Apart from these better-known sensors, the other members of the NLR family, including more than 20 representatives in mammals, are still orphan receptors. Moreover, to date, NLRs have only been characterized in vertebrates – even if many putative NLRs have been predicted from the genomes of the purple sea urchin (Rast et al., 2006) and amphioxus (Huang et al., 2008) – and in particular, no NLR homologue has been found in the well-characterized genomes of the ecdysozoans Drosophila and Caenorhabditis elegans.

The expression of PRRs has been demonstrated in many cells of the peripheral immune system in both invertebrate and vertebrate animals. In addition to mounting an innate immune response, PRR-induced signaling also transmits its evaluation of the pathogens to the adaptive immune system. In the mammalian central nervous system (CNS), neurons and all major glial cells including microglia, astrocytes and oligodendrocytes have been shown to express many TLRs, whereas reports of brain NLR expression are scarce (Nod2 in astrocyte and microglia, Sterka et al., 2006; NLRP1, 5 and 10 in cerebellar granule neurons id (Frederick Lo et al., 2008)). These PRRs allow the brain resident cells to perceive the presence of a large variety of pathogens and in response, to elicit synergistic immune responses which can be either beneficial by eliminating pathogens and by promoting axonal growth, or deleterious by inflicting irreparable damages to the brain (Nguyen et al., 2002). These contradictory effects may be explained by the complexity and the size of the vertebrate CNS, which lead to most of the studies being performed on cultures of purified brain cells. As suggested by Aravalli et al. (2007), such in vitro studies can generate misleading information due to the artificial nature of the experiments and/or the lack of purity of the brain cell types. An alternative is to use PRR knockout animals or invertebrate models, which offer simpler systems that allow both in vivo and/or ex vivo analyses on an entire CNS. Invertebrates are excellent models for exploring the molecular basis of innate immunity. For example, the pivotal role of the TLRs was discovered first in the fruit fly Drosophila melanogaster and later in mammals (Imler and Zheng, 2004). Most reports on immune responses in invertebrates have tended to focus on the peripheral anti-infectious response, while very few studies are related to the immune response developed by the CNS. Several TLRs and some members of the TLR signalling pathway have been detected in glial and neuronal cells of Drosophila and appear to play functions in the neural development of the larva (Wharton and Crews, 1993). In another ecdysozoan model, the nematode C. elegans, a toll gene was shown to be expressed in pharyngeal neurons where it participates in defensive behaviour by discouraging the worm from ingesting pathogenic bacteria (Pradel et al., 2007).

Unlike vertebrates, the medicinal leech, a lophotrochozoa model system, is able to regenerate its CNS after a lesion, restoring locomotion and neural functions within a few weeks (Wang et al., 2005). Our group previously showed that the regenerative process of the injured CNS of the medicinal leech is enhanced when it is also facing a microbial challenge; and that the leech nerve cord is able to establish a specific neuroimmune response by discriminating microbial components (Schikorski et al., 2008, 2009). In the present study, we sought to characterize two PRRs at a molecular level and to determine whether these sensors are implicated in the neural immunity of the leech CNS. We report here for the first time in an invertebrate (i) a TLR with an intracellular localization and (ii) a cytosolic PRR related to the vertebrate NLR family.

2. Materials and methods

2.1. Microorganisms

The Gram-positive and Gram-negative bacteria, respectively Micrococcus nishinomiyaensis and Aeromonas hydrophila, were isolated from the natural environment of Hirudo medicinalis as previously described (Schikorski et al., 2008). These bacterial colonies, which live in freshwater, were selected from agar plates under aerobic conditions at room temperature using a random isolation grid.

2.2. Animals and treatments

Adult H. medicinalis were purchased from Ricarimpex (Bordeaux, France) and maintained in autoclaved pond water changed daily, for 1 week before starting any experimental procedure.

2.2.1. Microbial challenges ex vivo

Ten dissected nerve cords were collected per condition. Connectives between ganglia were injured in a standard manner using a pair of sterilized fine iridectomy scissors. Axotomized nerve cords were separately incubated in L-15 medium containing different microbial components: 3 × 10^7 CFU/ml of heat killed Gram positive (M. nishinomiyaensis) or negative (A. hydrophila) bacteria, 100 ng/ml of E. coli LPS (0111:B4 strain, Invivogen), 100 µg/ml of zymosan (Invivogen), 10 µg/ml of Muramyl dipeptide (MDP, Invivogen), 2 µg/ml of lipoteichoic acid (LTA) (Invivogen), 100 µg/ml of mnanose (Invivogen), 10 µg/ml of Poly(I:C) (Invivogen), 10 µg/ml of Poly(I:C) combined with the transfection reagent LyoVec (Invivogen) for different time (T0, t = 6 h) at room temperature. The supernatant of cells cultured with Visceral stomatitis virus (VSV) for several days was used to stimulate the axotomized nerve cord ex vivo. Incubations without microbial components were performed in the same conditions as controls (H2O).

2.2.2. Regeneration process

Collected nerve cords were axotomized between each ganglion and cultured up to 8 days under sterile conditions. Ganglia were collected for RNA preparation at point 0 (T0), 6 h, 1 day, 3 days and 8 days. Total RNA from 6 nerve cords were used in the RT-qPCR reactions.

2.3. cDNA cloning

2.3.1. EST bank screening

Partial sequences encoding HmTLR1 and HmNLR have been retrieved from the leech H. medicinalis nervous system EST database (genoscope: http://www.cns.fr/externe/English/Projets/Projet PE/PE.html).

The complete nucleotidic sequences were obtained by Rapid Amplification of 5′ cDNA End (5′ RACE). Total RNAs from leech nervous system were extracted using Trizol (Life Technologies). RNAs (3 µg) were transcribed into single-stranded cDNA using specific antisense oligonucleotides deduced from the HmTLR1 or HmNLR cDNA EST sequence. (Superscript II, Invitrogen, protocol of the manufacturer). After first strand cDNA synthesis and addition of a poly-d(T) tail at its 3′ end using a terminal transferase (GIBCO, protocol of the manufacturer), one fourth of the reaction was amplified by PCR using an oligo-dG anchor primer and internal antisense primers also deduced from the 3′ sequence obtained from the EST. PCR were performed for 40 cycles using one unit of Taq polymerase
(GoTaq, Promega) in 1.5 mM of MgCl₂. The cycling parameters were: 94 °C for 2 min to activate the enzyme and then 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min.

All PCR products were cloned using pGemT-easy vector (according to the protocol provided by the manufacturer) and transformed into competent Escherichia coli JM 109 cells (Promega). Plasmids DNA were sequenced with a FM13/RM13 sequencing kit (Applied biosystem) according to the manufacturer’s instructions.

2.4. Sequence and phylogenetic analyses

Translated sequences of HmTLR1 and HmNLR were used to search for conserved domains using the Simple Molecular Architecture Research Tool (SMARTTM Schultz et al., 1998) web server. The sequences that best match with our sequences in Blastp analyses were retrieved from GenBank and used for the construction of phylogenetic trees using PhyML 3.0 (www.phylogeny.fr, Dereeper et al., 2008).

2.5. Gene expression analysis

2.5.1. Gene expression in purified cells

The ganglia from 6 isolated nerve cords were carefully decapsulated by removing the collagen layer that envelopes the nerve cord with microscissors. Neurons (>10 μm) and microglial cells (5 μm) were mechanically dissociated and resuspended in 200 μl of complete L-15 medium. The cells were then filtered through a 7 μm nylon mesh, as described in Tahtouh et al. (2009). According to their small size, purified microglial cells were collected with the eluate, while purified neurons were retained on the mesh. The latter were collected by gently scraping the inner face of the mesh in clean culture medium. RNA from purified cells were then extracted (Qiazol, Qiagen) and used for CDNA synthesis (Superscript II, Invitrogen) and PCR amplification (GoTaq, Promega).

2.5.2. Real time PCR quantification

20 axotomized nerve cords cultured at different time in presence or absence of microbial components were used per condition. RNA extraction, cDNA synthesis, and real time PCR procedures were realized as already described (Schikorski et al., 2008). The primers used for HmTLR1 (forward primer: 5'-CGAGTAATTA-GAGCCGCAA-3'; reverse primer: 5'-GTCCGTTAAAAGTCCTGC-3') and HmNLR (forward primer: 5'-ACACAAAGCATTACAAAC-3'; reverse primer: 5'-TCTATGCCAGAAGGACTCTC-3') were designed with the Primer3 Input software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The 18S was used as the reference gene (18S forward: 5'-TCGCGTATTTCGTAGGTC-3', 18S reverse: 5'-AGAAATGCGCTCACCAC-3'). Real Time reactions were conducted on a CFX96 qPCR system (BioRad) using a hot start, then 40 cycles at 94 °C, 15 s; 56 °C, 30 s; 72 °C, 30 s, and a final extension step at 72 °C for 3 min. Analysis of relative gene expression data was performed using the ΔΔCt method. For each couple of primers, a plot of the log CDNA dilution versus ΔCt was generated to validate the qPCR experiments. The slopes of regression lines for primers of HmTLR1 and HmNLR versus 18S are 0.0664 and 0.0797, respectively, suggesting equivalent efficiencies of amplification compared to the reference gene.

2.6. In situ immunodetection

2.6.1. Antisera

HmTLR1 protein was detected using the mouse antiserum as previously characterized in Schikorski et al. (2009). The HmNLR antiserum was produced by Agrobio. The chemically synthesized immunogenic sequence (FGESRQVRKHDHASQ) was coupled to BSA and used for the immunization procedure of two chickens.

2.6.2. Immunocytochemistry

Neurons and microglial cells mechanically dissociated from nerve cords were incubated for 6 h with bacteria (M. nishinomiyaensis and A. hydrophila, 3 x 10⁷ CFU/ml each) and then fixed centrifuged on slides. They were treated for immunocytochemical procedure as follow. Cells were first incubated with mouse anti-HmTLR1 (1:400) and chicken anti-HmNLR antibodies (1:400) and then with Texas-red (red) conjugated goat anti-mouse secondary antibody (1:100 – Jackson) and FITC (green) conjugated rabbit anti-chicken secondary antibody (1:100 – Jackson) for 1 h at 37 °C. An antibody recognizing the Early Endosomal Antigen 1 (EEA1, Santa Cruz) was also used as a marker of early endosomes in co-labeling procedures with anti-HmTLR1. As control, the immunolabeling procedure was carried out with the preimmune sera. Slices were then mounted in glycercol (Dako) and examined using a confocal microscope (Zeiss LSM 510).

2.6.3. Whole mount immunohistochemistry

Nerve cords were fixed just after removal (T0), after 6 h of culture in sterile conditions (6 h H2O) or after 6 h of culture in presence of bacteria (M. nishinomiyaensis and A. hydrophila, 3 x 10⁷ CFU/ml each) or MDP (10 μg/ml). For some ganglia, the surrounding thick capsule was carefully opened and removed post-fixation to better see neurons and to give a better access to antibodies. Samples were then incubated with anti-HmNLR (1:800) and anti-HmTLR1 antibodies (1:800) overnight at 4 °C. Primary antibodies were removed and samples were incubated with Texas-Red (red) conjugated goat anti-mouse secondary antibody (1:100 – Jackson) and FITC (green) conjugated goat anti-chicken secondary antibody (1:100 – Jackson) for 1 h at 37 °C. Finally, nerve cords were incubated with Hoechst (Invitrogen) for 20 min for nuclear staining. Controls and observations were performed as described above.

3. Results

3.1. Characterization of two sensing receptors in the leech

Analysis of the cDNA library of the leech CNS coupled to 5’ RACE-PCR allowed us to obtain the complete sequence (Genbank # HM196380) of a molecule presenting the signature of a TLR: LRR domains followed by a transmembrane domain and a TIR domain (Fig. 1). This protein was called HmTLR1 as being the first TLR characterized in H. medicinalis. SMART™ sequence analysis of the Nterminal part of HmTLR1 revealed the presence of one LRRNT followed by six LRRs (Fig. 1A,B). Based on the organization of the extracellular LRR array, two types of TLRs have been described in the literature (Leulier and Lemaitre, 2008): (i) the single cysteine cluster TLRs with an array of LRRs capped by a cysteine-rich domain located at the Cterminal end of LRRs (LRRCT) and (ii) the multiple cysteine cluster TLRs which contain in addition to the LRRCT domain capping the LRR array, LRRNT and LRRCT domains, located within the array in a tandem orientation. HmTLR1 is unique in exhibiting an array of LRRs capped by one LRRNT only. By contrast to most TLRs described in invertebrates and vertebrates species, no LRRCT domains were identified from the analysis of the ectodomain of HmTLR1. Analysis with the signal peptide software of the amino acid sequence deduced from the cDNA shows that the preHmTLR1 exhibits a signal peptide as classically described for members of the TLR family.

As a first step, Blastp analysis was carried out with the entire amino acid sequence of HmTLR1 and the best hits were retrieved to build a phylogenic tree (Fig. 1C). Data reveal great homologies mainly with TLR13 characterized in vertebrate species such as the mouse Mus musculus and the opossum Monodelphis domestica and
to a lesser extent with TLRs of animals living in freshwater such as the zebrafish *Danio rerio*, the goldfish *Carassius auratus*, the salmon *Salmo salar* or the rainbow trout *Oncorhynchus mykiss*. No homology with molecules identified in other lophotrochozoa was noticed. As a second step, BLASTp analyses of the LRR and the TIR domains of *HmTLR1* were performed separately, in order to get information on the possible functions and the signalling pathways associated with this receptor. LRRs correspond to protein–ligand interaction motifs found in numerous proteins with diverse functions including innate immunity and nervous system development (Dolan et al., 2007). As shown in the Fig. 2, the LRR domain of *HmTLR1* significantly matches ($e$-values < $e^{-12}$) with the sequences of LRRs implicated...
in (i) pathogen recognition, such as the LRR domain of the TLR3 and those of some Variable Lymphocyte Receptors (VLRs) and (ii) tissue remodelling and/or axonal guidance in vertebrates, including the LRRs of vasorin, decorin and netrin known to participate in tissue remodelling and/or axonal guidance in vertebrates. Thus, both regenerative and immune functions could be attributable to this receptor. This is in line with the recent data reporting that TLR3, in addition to its well described role as a dsRNA sensor into the intracellular domain of TLR3, in addition to its well described role as a dsRNA sensor into the intracellular domain of TLR3 (Fig. 4A). BLASTp analyses indicate that the predicted sequence (117 kDa – Fig. 4A). BLASTp analyses indicate that the predicted sequence (117 kDa – Fig. 4A).

3.2. Neural expression and co-localization of HmTLR1 and HmNLR

HmTLR1 and HmNLR transcripts were both found in purified neurons, whereas only HmTLR1 seems to be expressed in microglial cells in basal conditions (Fig. 4B). These results are confirmed by the immunodetection of the proteins in neurons and microglial cells for HmTLR1 and in neurons only for HmNLR (Fig. 4C, D).

Cellular localization of HmTLR1 and HmNLR were further investigated in neurons by immunocytochemistry and confocal microscopy analyses. In the absence of infection, HmTLR1 was detected within vesicles throughout the cytoplasm of neurons (Fig. 4D). Immunocytochemistry analysis using an Ab directed against EAA1, a marker of early endosomes, revealed that a fraction of HmTLR1 is associated with endosomes (Fig. 4E). Thus in neurons, HmTLR1 is localized within the endosomal compartment, similar to the endosomal distribution of TLR3 in mouse neuronal cells (Jackson et al., 2006). By contrast, the subcellular localization of HmNLR protein appears to be cytosolic as would be predicted by the absence of a signal peptide (Fig. 4D). Double labeling of isolated neurons shows a clear co-localization of HmTLR1 and HmNLR (Fig. 4F), with a marked accumulation of both proteins at the site of axon injury (arrow). This common pattern of expression opens up the possibility of cross-talk and/or interactions between these two receptors.

3.3. Modulation of the PRR gene expression during the process of repair and upon microbial challenge of the leech CNS

In order to explore possible functions of the characterized PRRs in the leech CNS, variations of the transcript levels were quantified firstly during the regeneration process (Fig. 5) and secondly upon microbial challenge (Fig. 6).

As a first set of experiments, we observed the level of expression of HmTLR1 and HmNLR in conditions of neural regeneration: nerve cords maintained in culture for up to eight days under sterile conditions were lesioned at T0 by cutting completely through half of a connective nerve that links two adjacent ganglia. The time intervals were chosen in reference to the observations reported by Müller, who demonstrated that synaptic connections and normal functions of axotomized leech neurons were restored eight days after injury (Müller and Carbonetto, 1979). Cultures were stopped at different time post axotomy. Over 8 days, a decrease of the HmTLR1 messenger level was observed suggesting that either this sensing receptor is not required or it exerts a suppressive effect on the regenerative process as reported for TLR3 (Fig. 5A). By contrast, HmNLR gene is induced in the few days that follow axotomy but then returns to basal expression at 8 days, suggesting a role in the mid-term events occurring post injury (Fig. 5B).

Gene expression patterns of HmTLR1 and HmNLR during an induced immune response were also investigated by real time RT-PCR. The experiments were conducted with leech isolated nerve
Fig. 3. Sequence analysis of HmNLR. (A) Amino acid sequence deduced from the cDNA encoding HmNLR. (B) Schematic organization of the protein obtained from the SMART web server. (C) Phylogenetic analysis of the HmNLR gene. The sequences that best match with HmNLR in BLASTp were retrieved from GenBank and a phylogenetic tree was built using the phylogeny.fr web server. Numbers correspond to percentages of bootstrap values over 100 replicates. Gene accession numbers: 170649657 (NLRP12, C. moloch); 194674897 (NLRP12, B. taurus); 226462209 (LRR-containing protein, M. pusilla); 222137288 (NLRC3, I. punctatus); 73959310 (NLRC3, C. familiaris); 194219286 (NLRC3, E. caballus); 194678537 (NLRC3, B. taurus).

cords incubated for 6 h in the presence of various components that derive from or mimic the presence of different microorganisms e.g. bacteria, fungi or viruses. As illustrated in Fig. 6, HmTLR1 and HmNLR transcript levels were rapidly enhanced by some specific microbial challenges, confirming as suggested in previous studies from our group that the leech nerve cord is able to discriminate microbial components present in its environment. As noticed above, no induction of expression was measured 6 h following dissection under sterile conditions, suggesting that the inductions observed thereafter were not due to the axotomy itself, but rather to the incubation of the nerve cords with microbial substances. The data presented in Fig. 6 clearly indicate that a 6 h treatment of the nerve cord with Gram-positive bacteria living in the environment of the leech induces a significant increase of both
Fig. 4. HmTLR1 and HmNLR are expressed in the central nervous system of the leech. (A) The anti-HmNLR Ab reveals a single band at the expected size by western blot analysis of a leech extract. (B) Detection of HmTLR1 and HmNLR transcripts by PCR amplification in leech purified neurons and microglial cells. (C) No immunolabeling was observed when incubating isolated neurons with both HmTLR1 (Texas Red) and HmNLR (FITC) preimmune sera. (D) HmTLR1 protein is detected in dissociated neurons and microglial cells by immunocytochemistry (Texas Red), while HmNLR (FITC) are clearly co-expressed in neurons, and seem to accumulate at the injured sites that correspond here to the region where the axon had been pulled out by the dissociation procedure (arrow).

HmTLR1 and HmNLR mRNA levels, the first more pronounced than the second. Note that the killed bacteria used for the challenge were added to the culture medium at a concentration identified by our group as exerting regenerative effects on the axotomized leech CNS (Schikorski et al., 2008). To a lesser extent, both genes are also induced in nerve cords incubated with LPS, a Gram-negative wall derivative; LTA has only a slight effect on HmTLR1 expression. Interestingly in both cases, zymosan, a component of yeast membrane, strongly reduces gene expression, suggesting that fungi may have an immunosuppressive effect on leech CNS. This may be in line with our observations that an involuntary contamination of the culture medium by filamentous fungi has deleterious effects on the regeneration process of the leech nerve cord. Finally, VSV, which is a negative-sense RNA virus known to have a neuronal tropism in mammals, has no effect on these genes, raising the question of the cellular compatibility of this vertebrate pathogen with the leech cells. However, both genes appear to be up regulated by the extracellular virus mimetic poly(I:C) and/or the cytosolic poly(I:C) + LyoVec suggesting a possible implication of these receptors in an antiviral response of the leech CNS. Interestingly, the HmNLR gene induction by poly(I:C) is observable only in the presence of a transfection reagent (LyoVec) whereas the HmTLR1 gene induction does not require that poly(I:C) enters the cytosolic compartment. This argues in favor of a regulation of the HmNLR gene by a cytoplasmic sensor, while HmTLR1 expression seems influenced by intracellular and extracellular sensors activated by the presence of viral components.

MDP appears clearly as the best inducer of both genes. This microbial component being produced in a larger extent by Gram positive than by Gram negative bacteria, this result is consistent with the induction preferentially observed in the presence of Micrococcus rather than Aeromonas bacteria. This observation suggests the presence of a sensor sensitive to MDP in the leech nerve cord, echoing the vertebrates Nod1 and Nod2. This immune receptor would regulate the expression of both sensors, would it be HmNLR itself or an unknown receptor.

The similarity of the induction profiles lead us to hypothesize that a co-regulation process of HmTLR1 and HmNLR might take place in the leech CNS. To check whether a molecular cross-talk could take place between the two receptors in vivo, their cellular localization was investigated in injured nerve cords incubated...
can observe in Fig. 9 that the MDP inhibition of microglia recruitment of vertebrate macrophages (Nagao et al., 1979, 1982). We intriguingly, this result is remindful of the blocking effect of MDP on the modulation does not abolish the accumulation of the receptors at the site under a MDP treatment (Fig. 8A–C) (Morgese et al., 1983). Interesting-cohesion that favors the entry of poly(I:C) into the cytosolic compart-ment. Graphics represent the best results of two independent experiments that were calculated versus the control treatment (H2O), based on the experimental measures performed in triplicates (*p<0.05).

with killed bacteria. Whole mount immunohistochemical labeling (Fig. 7) shows (i) the co-expression and the up-regulation of HmTLR1 and HmNLR in challenged nerve cords and (ii) their accumulation at the lesion site. It is worth noting that we often observed an asymmetric response at the lesion site, one end showing more intense labeling than the other, as can be observed on Fig. 7G and H. Moreover, the accumulation of HmTLR1-positive vesicles at the labelled cut-end (see arrow in Fig. 7h) suggests a role for antero-grade transport in the accumulation of the two receptors at the site of axotomy.

The same experiments were also conducted in the presence of MDP, on one hand because of the great capacity of this bacte-rial component to induce the gene expression of the HmTLR1 and HmNLR genes (see previous section) and on the other because of its inhibitory effect on the microglia recruitment to the lesion site (Fig. 8). Indeed, it appeared that the usual accumulation of microglial cells at lesion sites in the CNS (Fig. 8A–C), failed to occur under a MDP treatment (Fig. 8A–C) (Morgese et al., 1983). Interest-ingly, this result is remindful of the blocking effect of MDP on the migration of vertebrate macrophages (Nagao et al., 1979, 1982). We can observe in Fig. 9 that the MDP inhibition of microglia recruitment does not abolish the accumulation of the receptors at the site of axotomy (Fig. 9B). In agreement with the results of quantitative RT-PCR (Fig. 6), an enhancement of the immunohistochemical signal is visible, especially for HmNLR, when the CNS is incubated with this microbial component (Fig. 9A, cf. 6 h MDP versus 6 h).

Confocal microscopy analyses confirm the presence of both immunoreceptors in the cell bodies of neurons and in the axons of the injured connectives, suggesting a neuronal somata production that is presumably followed by the axonal transport of the receptors to the lesion site.

Thus, in the presence of whole (killed bacteria) or partial (MDP) bacterial cell wall, HmTLR1 and HmNLR are up-regulated in neu-rons and accumulate at the lesion site. Taken together, these results clearly suggest a neuroimmune function of the two sensors in the leech CNS.

4. Discussion

Many LRR molecules have been described in invertebrates. Some of them, generally those having extracellular (or intravesicular) LRRs (e.g. Ao et al., 2008; Povelones et al., 2009; Powell et al., 2009), have clear immune functions. Thus far however, neither endosomal TLR nor NLR homologues have been characterized and functionally studied in an invertebrate, despite the fact that many TLRs and NACHT containing sequences have been retrieved from in silico analyses of whole genomes (Rast et al., 2006; Huang et al., 2008; Hibino et al., 2006; Davidson et al., 2008). In this respect, we describe here for the first time in a protostomian model two intracellular sensors involved in the neuroimmune response of the medicinal leech.

The comparison of Hirudo TLR sequence with databanks and the phylogenetic data presented here reveal a close proximity of the leech receptor with that of vertebrates living in fresh water. The medicinal leech being an ectoparasite of the latter, this may suggest that the chimeric structure characteristic of HmTLR1 (see Section 3) might find its origin in a coevolution of the medicinal leeches with freshwater vertebrates (including amphibians, fishes and mammals), which are the typical hosts of this hematophageous model. We hypothesize that a co-evolutive process of the sensors might have taken place between the parasite and its hosts, presumably resulting from their close contact with the same microorganisms. 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 chiefly consists of TLRs that display the vertebrate-like rather than the protostome-like domain organisation. Interestingly, the BLASTp homology of HmTLR1 with the vertebrate TLR13 and TLR3 is consistent with this observation. In addition to the sequence homology, HmTLR1 seems to exert functions comparable to those described for the mammalian TLR3 in the brain. Indeed, in a previous paper our group has demonstrated the implication of HmTLR1 in the gene induction of the chemoattractant cytokine EMAPII. Silencing of the HmTLR1 gene in the CNS demonstrated that upon microbial challenge, this receptor is involved in the induction of the gene encoding Hmp43/EMAPII (Schikorski et al., 2009). Those data were reminiscent of some observations of rat microglial cells, which have been reported to produce EMAPII after systemic injections of TLR agonists, such as poly(I:C) (a TLR3 ligand) and R848 (a TLR 7/8 ligand) (Zhang et al., 2005). The regulation of EMAPII by a TLR in both leeches and vertebrates highlights the great con-servation between these two animal groups. Moreover this study, which underlined the existence of an immunity mediated by a TLR in the leech CNS, was the very first demonstration of an immune function of a TLR in both leeches and vertebrates highlighting the great con-servation between these two animal groups. Moreover this study, which underlined the existence of an immunity mediated by a TLR in the leech CNS, was the very first demonstration of an immune function of a TLR in a non-ecdysozoan system (i.e., in a protostome model that is different from C. elegans and D. melanogaster) (Leulier and Lemaître, 2008). The strong homology of HmTLR1 with TLR13 whose ligand and function are still not described in the literature, supports our interest in using the leech as a model for understanding the immune mechanisms developed by the CNS in general.
Fig. 7. *Hm*TLR1 and *Hm*NLR immunolabeling in the leech CNS challenged with bacteria. *Hm*TLR1 (Texas red) and *Hm*NLR (FITC) were detected by whole mount immunohistochemistry performed on isolated leech nerve cords just after dissection (*T* = 0 h), after 6 h of culture in sterile conditions (*T* = 6 h without bacteria) and after 6 h of culture in the presence of a mix of killed Gram(+) and Gram(−) bacteria (*T* = 6 h with bacteria). One interganglionic connective was cut before treatment, creating a lesion where microglial cells accumulate after an incubation of 6 h as revealed by nuclear staining (F and I versus C). *Hm*TLR1 and *Hm*NLR expression appear as upregulated in ganglia exposed to bacteria (G and H) compared to basal conditions (A and B) or to culture in sterile medium (D and E). Moreover, both receptors clearly accumulate at the lesion site specifically in presence of bacteria (g and h versus a and b and d and e, magnifications of the lesion sites). Merged zooms on the lesion site confirm a co-localization of *Hm*TLR1 and *Hm*NLR in case of immune challenge (g + h versus a + b and d + e). Note that *Hm*TLR1 appears as expressed in vesicles directed at the axonal cut ends (h, arrow). Controls performed with preimmune sera showed no labeling (data not shown).
In addition to HmTLR1, another sensing receptor, namely HmNLR, has been characterized from the leech nervous system. As the other members of its family, HmNLR meets the structural requirements to be functionally involved in ligand recognition and binding. It is of special relevance that the best BLASTp match for HmNLR is a NLRC3 homologue (formerly called NOD3 or CLR16.2). Indeed, this specific member is considered as basal to the NLR family in vertebrates (Hughes, 2006). So far, no specific ligand has been identified for NLRC3 but it has been assigned a negative regulatory function. Similarly NLRP12 (Monarch-1, PYPAF7), which is also related to HmNLR, has no known ligand but has a clear immunosuppressive role. More specifically, NLRP12 appears to block IRAK-1 (IL-1R-associated kinase-1) activation and to destabilize NIK (NF-kB inducing kinase), thus inhibiting the NF-kB activation pathway and inflammatory gene expression (Williams et al., 2005; Lich and Ting, 2007). Accordingly, NLRP12 gene is down-regulated by TLR agonists (Lord et al., 2009) and the absence of a functional NLRP12 protein has been linked to hereditary periodic fever (Jeru et al., 2008). The N-terminal part of HmNLR displays no conserved domain, nor does it match with any known molecule in BLASTp analysis. However, its paralogues 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 are the Hirudinae – probably derived from a polychaete-like ancestor, it is possible that in the course of evolution, the ligand-binding domain was conserved but not the upstream effector domains, suggesting innovative transduction pathways. Interestingly, some sequences similar to vertebrate NLR without a NACHT domain were also described in the amphioxus genome, highlighting a NLR repertoire in non-vertebrates more complex than that of vertebrates (Huang et al., 2008). The characterization of a NLR homologue in annelids reinforces the hypothesis of an ancient origin for this family of cytosolic sentinels. The conservation of the ligand-binding domain underlines the strength of the selective pressures that is exerted on the immune mechanisms dedicated to the detection of potential pathogens.

By contrast with members of the TLR family, involvement of NLR sensors in neuroimmunity is poorly documented in vertebrates. In
Fig. 9. HmTLR1 and HmNLR immunolabeling in the leech CNS challenged with MDP. HmTLR1 (Texas red) and HmNLR (FITC) were detected by whole mount immunohistochemistry analysis performed on isolated leech nerve cords, fixed immediately after dissection (T0) or after 6 h of culture in presence (6 h MDP) or not (6 h) of MDP. Controls were performed with pre-immune sera (preimmune). For preimmune treatment and T0 condition, the bright field was superposed to the confocal image in order to distinguish the shadow of the structures. (A) Both receptors are expressed into the cellular bodies of neurons. The signal becomes more intense after MDP treatment. (B) HmTLR1 and HmNLR accumulate at the axotomized site of the nerve cord incubated with MDP.
mammals, NLRs have been mainly studied in immune cells and mucous tissues (e.g. Wilmanski et al., 2008). In mammals, NLR3 is mainly described in lymphocytes and is linked with inhibition of T-cell activity (Conti et al., 2005). In fishes however, it is present in various organs including brain tissue (Sha et al., 2009) with the exception of lymphocytes (Laing et al., 2008). This suggests rather broad immune functions for NLR3, at least in basal vertebrates. Concerning neural functions of NLR receptors, very few data are available. Nod2 have been reported as an important component of the immune response machinery in microglia and astrocytes (Chauhan et al., 2009). In neurons, only NLRP1 and NLRP5 have been deeply investigated in the past few years, for their involvement in the control of inflammation and apoptosis in mammalian neurons (de Rivera Vaccari et al., 2009; Frederick Lo et al., 2008). This obvious lack of knowledge highlights the interest of leech model system to investigate the role of NLR-like molecules in neuroimmunity.

In the leech CNS, we report here that HmNLR has a brain tissue expression restricted to neurons, by contrast to HmTLR1, which is expressed by both microglia and neurons as described for the mammalian TLR3. Indeed, in vertebrates, microglia has been reported to express mRNAs for TLRs 1–9, whereas neurons and oligodendrocytes (Prehaud et al., 2005) express only transcripts encoding TLR3 (Bsibsi et al., 2002). Confocal microscopy data clearly locate HmNLR in the cytoplasm: precise observation even suggests an accumulation of HmNLR in the submembranous compartment of neurons (Fig. 4D). This sub-cellular localization has already been observed for activated Nod2, whose membrane recruitment appears as necessary for NF-kB activation in the presence of MDP in human cells (Barnich et al., 2005), and which is able to associate with proteins of the cortical cytoskeleton probably by interacting with erbin (Kufer et al., 2006).

Interestingly, HmTLR1 also exhibits an intracellular localization. Indeed co-immunocytochemistry analysis with a marker of early endosome (EEA1) shows an accumulation of some HmTLR1 in the endosomal compartment of leech neurons in basal conditions. This result was predictable because of the BLASTp homology of HmTLR1 with TLR3, but not expected, since so far no intracellular distribution of a TLR had been reported in a protostomian model, including in the genetic models D. melanogaster and C. elegans (Leulier and Lemaître, 2008). In mammals, endosomal localization of TLR3 appears to be crucial for its activation in neurons (Vercammen et al., 2008), and high levels of TLR3 are found in neurons in neurodegenerative diseases or viral infections of the brain (Laфон et al., 2006). Unusually, endosomal TLR3 does not signal through MyD88 but signals instead via the recruitment of TRIF, a TIR domain-containing adaptor inducing interferon (Yamamoto et al., 2003). Further investigations will be conducted in order to determine whether the signaling pathway associated with HmTLR1 implicates either MyD88 or TRIF in our model.

The significant levels of expression of HmTLR1 and HmNLR in the leech CNS suggest important functions in response to neuronal injury and/or infection. Quantitative PCR analyses were performed in order to get information on the role of these receptors in the leech CNS of our model. Under sterile conditions, genes encoding HmTLR1 or HmNLR appeared to be differentially modulated during the regenerative process. The latter is known to be effective 7 days post injury in the leech CNS. The gene encoding HmTLR1 was observed to be downregulated along with the CNS repair. Various hypotheses could explain this result: (i) HmTLR1 participates in limiting axonal growth as reported for TLR3, (ii) HmTLR1 is not engaged in the regeneration of the injured CNS at all and/or (iii) HmTLR1 is required for the regenerative process but because of the long lifespan of this protein de novo synthesis is not needed. On the contrary, the level of HmNLR transcripts appeared to be up regulated in leech nerve cords a few days (3 days) post axotomy, suggesting a role in the mid-term events occurring during neural regeneration. This observation resembles the induction of NLRP1 and NLRP5 observed after neuronal injury in mammals; they are believed to regulate caspase activation and apoptosis in injured neurons (Frederick Lo et al., 2008). Similarly, some evidences suggest that inflammasomes may be responsible for some of the brain damages characteristic of Alzheimer’s disease, may be through the disturbance of K+ efflux (Salminen et al., 2008). This dual role of NLR family members in the signaling of pathogen as well as damages, fits especially well in the neural context where immunity and tissue repair appear more and more intimately connected (Eming et al., 2009).

The variation of the gene expression was also quantified in nerve cords experimentally infected by various microbial derivatives. Interestingly, a comparable pattern of expression was obtained for both genes suggesting a co-regulation of the two receptors upon microbial challenge of the injured leech nerve cords. Gram-positive bacteria and MDP appear as the best inducers of HmTLR1 and HmNLR genes. With these conditions, whole mount immunohistochemistry analyses point out a co-accumulation of our sensing receptors at the injured sites, which because of the solid fibrous capsule surrounding the leech CNS, correspond to the exclusive entry point for microorganisms. This data also evokes a connection between HmTLR1 and HmNLR in our model. Direct interaction seems unlikely considering their respective structural but cross-linkages in their respective signaling networks are possible. Chauhan and colleagues recently 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 work is warranted to study the links between leech HmTLR1 and HmNLR in the CNS, both under homeostatic conditions and following injury and/or infection.

Altogether the presented data introduce two novel sensing receptors expressed in the CNS of an invertebrate. The similarities of HmTLR1 and HmNLR with vertebrate receptors in terms of sequence, activity and intracellular distribution, pave the way to investigate a co-evolutionary process which may have occurred between the leech and its host due to an exposure to common microorganisms. Moreover, this work, which constitutes the first evidence of a participation of TLR and NLR in the neuroimmune response of an invertebrate, reinforces the interest in using the leech to study the conserved immune mechanisms involved in neural defense and regeneration.

Acknowledgements

This work was supported by the Centre National de la Recherche Scientifique (CNRS), the Ministère de l’Enseignement Supérieur et de la Recherche and the Genoscope. The authors are grateful to Pr Didier Hober and Dr PE Lobert for having managed the VSV manipulations, and to Pr Eduardo Macagno for English editing.

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