Microglia of Medicinal Leech (*Hirudo medicinalis*) Express a Specific Activation Marker Homologous to Vertebrate Ionized Calcium-Binding Adapter Molecule 1 (Iba1/alias Aif-1)

Francesco Drago,[1] Pierre-Eric Sautière,[1] Françoise Le Marrec-Croq,[1] Alice Accorsi,[2] Christelle Van Camp,[1] Michel Salzet,[1] Christophe Lefebvre,[1] Jacopo Vizioli[1]

[1] Université Lille 1, Laboratoire de Spectrométrie de Masse Biologique Fondamentale et Appliquée, EA4550, Villeneuve d'Ascq 59655, France

[2] Department of Life Sciences, University of Modena and Reggio Emilia, Modena 41125, Italy

Received 29 November 2013; accepted 7 April 2014

ABSTRACT: **The Ionized calcium-Binding Adapter molecule 1 (Iba1), also known as Allograft Inflammatory Factor 1 (AIF-1), is a 17 kDa cytokine-inducible protein, produced by activated macrophages during chronic transplant rejection and inflammatory reactions in Vertebrates. In mammalian central nervous system (CNS), Iba1 is a sensitive marker associated with activated macrophages/microglia and is upregulated following neuronal death or brain lesions. The medicinal leech *Hirudo medicinalis* is able to regenerate its CNS after injury, leading to a complete functional repair. Similar to Vertebrates, leech neuroinflammatory processes are linked to microglia activation and recruitment at the lesion site. We identified a gene, named Hm*iba1*, coding a 17.8 kDa protein showing high similarity with Vertebrate AIF-1. The present work constitutes the first report on an Iba1 protein in the nervous system of an invertebrate. Immunochemistry and gene expression analyses showed that *Hm*Iba1, like its mammalian counterpart, is modulated in leech CNS by mechanical injury or chemical stimuli (ATP). We presently demonstrate that most of leech microglial cells migrating and accumulating at the lesion site specifically expressed the activation marker *Hm*Iba1. While the functional role of Iba1, whatever species, is still unclear in reactive microglia, this molecule appeared as a good selective marker of activated cells in leech and presents an interesting tool to investigate the functions of these cells during nerve repair events.** © 2014 Wiley Periodicals, Inc. Develop Neurobiol 74: 987–1001, 2014

Keywords: **microglia activation; Iba1; allograft inflammatory factor 1; medicinal leech; nerve repair**

INTRODUCTION

The Ionized calcium-Binding Adapter molecule 1 (Iba1; Ito et al., 1998) is a 17 kDa cytokine-responsive macrophage molecule almost identical to three other proteins called Allograft Inflammatory Factor-1 (AIF-1; Utans et al., 1995), Daintain (Chen et al., 1997), and Microglial Response Factor-1 (MRF-1; Kato et al., 2000). These molecules are highly conserved Ca^{2+}-binding EF-hand proteins that were firstly identified in rat and human heart grafts subject to chronic rejection (Utans et al., 1995,

Correspondence to: J. Vizioli (Jacopo.Vizioli@univ-lille1.fr).

Contract grant sponsor: Agence Nationale de la Recherche (projet MIMIC); contract grant number: ANR-11-JSV4-005.

Contract grant sponsor: Université Lille 1.

Contract grant sponsor: Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche (MENESR), France.

Conflict of Interest: The authors declare that they have no competing interests.

Published online 10 April 2014 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/dneu.22179

1996). In Mammals, the action of proinflammatory factors like interferon-gamma (IFN-γ), induces the expression of AIF-1 in macrophages and neutrophils in a large number of pathologies such as inflammatory allograft rejections, autoimmune diseases, brain injuries, vasculopathies and cancer (Zhao et al., 2013). Although these proteins share a highly conserved amino acid sequence, their functional identity remains to be elucidated (Deininger et al., 2002). Recent reports showed that proteins of the AIF-1 superfamily, well distributed in Vertebrates, are also present in a few other groups of animals, including Sponges (Kruse et al., 1999), Mollusks (De Zoysa et al., 2010; Zhang et al., 2010, 2013; Li et al., 2012) and Echinoderms (Ovando et al., 2012). The comparison of such sequences shows a good similarity of protein primary structures, suggesting a significant conservation of functional properties of AIF-1 factors among Metazoa. Accumulated evidence indicated that AIF-1 exerts its functions in many organs and could be correlated with several kinds of inflammation-related diseases regulating macrophage activation. So far no receptors for AIF-1/Iba1 have been described either in Vertebrates or in Invertebrate species.

AIF-1 and its alternative forms MRF-1 and Iba1 are cell markers also expressed in monocytic lineage of the nervous systems of Vertebrates and are associated with several neuropathologies. In rat experimentally transplanted glioblastoma and gliosarcoma, AIF-1 expression was detected in activated macrophages/microglial cells either in compact or in infiltrative tumor (Deininger et al., 2000). In Mammals, the expression of *iba1*/*aif-1* is linked to microglia activation and is induced after spinal cord injury (Schwab et al., 2001) or brain trauma (Beschorner et al., 2000). MRF-1, constitutively expressed in resting microglia, is upregulated in cultured rat cells exposed to ATP treatment (Kaya et al., 2002; Tanaka and Koike, 2002) and in response to neuronal apoptosis or necrosis (Tanaka et al., 2000). Iba1-positive macrophages/microglial cells were also observed in rat models of autoimmune diseases (e.g., encephalomyelitis, neuritis, and uveitis) (Schluesener et al., 1998). In the human brain, this protein is a sensitive marker for microglial activation correlated to the intensity of tissue damage both in inflammation and cerebral hypoxia (Postler et al., 2000). In neurobiology, this factor is mostly known as Iba1 and, together with CD11b (alias Ox42) and coronin-1a (Ahmed et al., 2007), constitutes an effective tool to identify reactive microglia in different pathologies or *in vitro* investigations. In particular, Iba1 is a diagnostic marker used for routine immunohistochemistry on either microglial cell lines or nerve pathologies like spinal cord injury (Dijkstra et al., 2000) and brain tumors (Tran et al., 1998). Iba1 crosslinks actin filaments and is located at membrane ruffles and phagocytic cups of activated macrophage/microglia (Ohsawa et al., 2000). However, its functions remain poorly investigated; the generation of Aif-1-null transgenic mice would permit a better understanding of the role of this gene on several inflammatory diseases (Casimiro et al., 2013).

The medicinal leech (*Hirudo medicinalis*) is a well-known model for the capacity of its central nervous system (CNS) to regenerate and restore normal function in response to injury. The leech CNS consists of a ventral nerve cord formed by segmental ganglia linked to each other by connective fibers. This structure contains a few, giant glial cells and thousands of resident microglial cells that are spread all along the nerve cord (Coggeshall and Fawcett, 1964; Jansen and Nicholls, 1972; Le Marrec-Croq et al., 2013). Unlike in Vertebrates, leech CNS does not possess any astrocytes or oligodendrocytes. Nerve repair events involve the action of resident microglia and begin with a rapid activation of microglial cells leading to a morphological modification and to their accumulation at the lesion site (Morgese et al., 1983; Le Marrec-Croq et al., 2013). ATP and NO are involved in microglia migration towards the site of injury (Duan et al., 2009; Arafah et al., 2013); the inhibition of these diffusible molecules reduces microglial accumulation, consequently delaying the sprouting of damaged axons (Ngu et al., 2007). Recent reports showed that microglia recruitment also depends on the action of cytokines (Schikorski et al., 2009; Croq et al., 2010) and complement-like factors (Tahtouh et al., 2009, 2012). Even if these reports supplied novel information for the comprehension of cell chemotaxis, the activation process remains unclear and no specific markers have been so far characterized in reactive microglia. This work contributes to dissect the mechanisms of activation and migration of microglia as well as to functionally characterize these cells during CNS repair in *H. medicinalis*. In this context, we focused our attention on the research of specific microglial markers permitting the identification of activated cells all along the nerve cord during healing processes. We identified in a *Hirudo* CNS Expressed Sequence Tag (EST) library a gene homologous to mammalian *aif−1*. This is, to our knowledge, the first report of such a factor in Invertebrate CNS. We studied the regulation of this gene, named Hm*iba1* for *Hirudo medicinalis* Ionized calcium-Binding Adapter molecule 1 (alias Hm*aif-1*), and the distribution of the

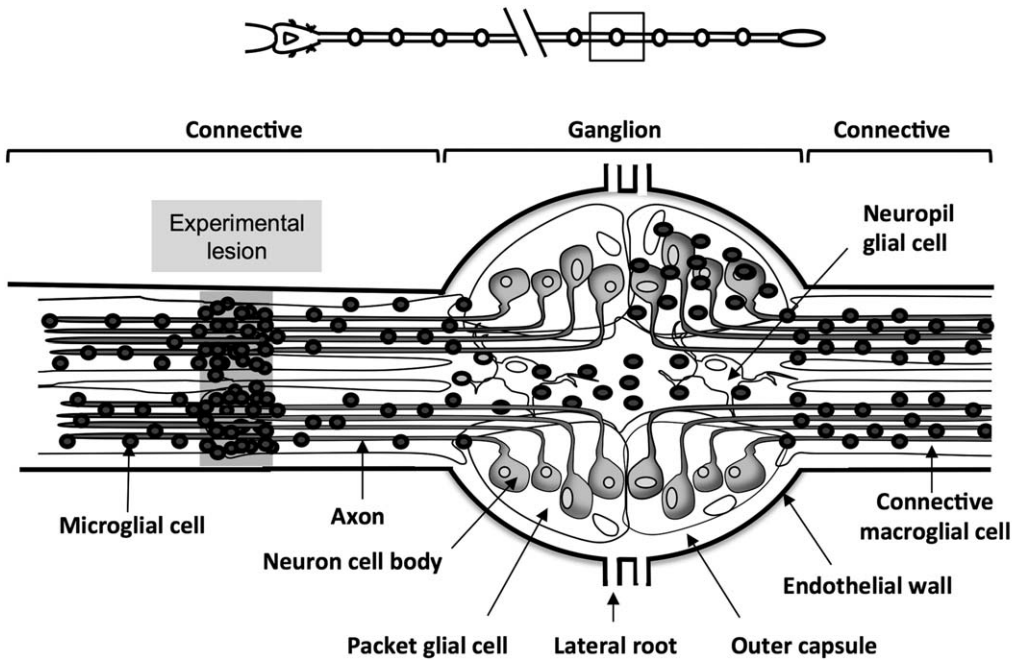

Figure 1 Leech CNS structure. Upper diagram shows the organization of leech CNS: segmental ganglia are represented with connective fibers as well as fused head (left) and tail (right) ganglia. Lower diagram details the structure of a ganglion and the adjacent connectives. Microglial cells surrounding neuron bodies are only represented in one packet of cells. On the left side is represented the accumulation of microglia in connectives following an experimental injury.

correlated proteins in microglia in leech CNS upon injury or ATP stimulation. This study established that Hm*iba1* is an inducible gene expressed in the nerve cord of the leech and that this protein is selectively present in some activated microglial cells.

MATERIALS AND METHODS

Leech CNS Structure and Preparation

The leech CNS (Fig. 1) consists of a ventral nerve cord containing 21 segmental ganglia and few merged head and tail ganglia. Such CNS is enveloped by a collagen sheath (outer capsule) and is located into a ventral blood sinus. Ganglia are linked to each other by hundreds of axons that form longitudinal nerves known as connectives (Coggeshall and Fawcett, 1964; Jansen and Nicholls, 1972). Smaller lateral nerves (lateral roots) connect ganglia with body sensory and effectory organs. Each ganglion contains about 400 neuron bodies arranged into six groups, each one enveloped by a single glial cell (packet glial cell). Two big glial cells are present the neuropil region (neuropil glial cells). Each connective fiber contains a single giant cell (connective macroglial cell) surrounding the axons. In leech CNS, a large population of microglial cells is spread all along the nerve cord and plays a crucial neurotrophic role. In the case of axotomy or inflammatory events, these resident microglia migrate towards the site of lesion and their activity, so far largely unknown, is essential for nerve regeneration and functional recovery of the system (Jansen and Nicholls, 1972; Le Marrec-Croq et al., 2013). Unlike in Vertebrates, the leech CNS does not possess any astrocytes or oligodendrocytes. All protocols regarding the use of leeches were carried out in strict accordance with the French legislation and European Treaty, and were in compliance with the Helsinki Declaration. Adult leeches (*Hirudo medicinalis*) were purchased from Ricarimpex (Eysines, France) and maintained in artificial pond water for one week before use. Leech CNS were removed under sterile conditions from animals anaesthetized in 10% cold ethanol and rinsed in Ringer solution. CNS used in the experiments were lesioned by forceps crushing at the connective level and then incubated at different times at 18°C in complete L-15 medium (85% Leibovitz's L-15 Medium, Gibco, Invitrogen, Carlsbad, CA) complemented with 2 m*M* L-glutamine, 10 UI/mL penicillin, 10 μg/mL streptomycin, 10 m*M* Hepes, 0.6% glucose, and 10% fetal calf serum).

Hm*iba1* cDNA Cloning

A gene homologous to Vertebrate *aif-1*, so named Hm*aif-1* for *Hirudo medicinalis aif-1*, was identified in the EST database of *H. medicinalis* CNS (Hirudinea Genomics Consortium). The cDNA encoding the *Hm*AIF-1 (GenBank accession number KF437461) was amplified by PCR from

total leech CNS cDNA as template. Amplification was performed using specific forward (5′CCCCGGATCCATGAG TTTGGACCTCAAAGAC3′) and reverse (5′CCCCCT CGAGTCACTTCGTCCCACTGTTG3′) primers containing restriction sites for further cloning in expression vectors. PCR amplifications were carried out on a Thermal Cycler (Mastercycler Gradient, Eppendorf, Hambourg, Germany) with 150 ng of cDNA in a solution containing 2 U of Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA), 10 m*M* of each PCR primer, 10 m*M* dNTPs, 1.5 m*M* $MgCl_2$, and 1× of PCR buffer in a final volume of 50 μL. The reaction cycles were performed as follows: 94°C for 1 min, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, and 40 s at 72°C. A single PCR product was obtained and cloned into the pGEM T-easy vector (Promega, Madison, WI) following the manufacturer's protocol.

Anti-*Hm*Iba1 Antibody Production

Specific polyclonal anti-*Hm*Iba1 antibodies were raised in rabbit (New Zealand White, SPF) against a synthetic peptide corresponding to the predicted N-terminal region (Met_1-Lys_{16}) of the *Hm*Iba1 protein (Proteogenix, France). Ovalbumin was used as carrier molecule. Rabbit preimmune serum was collected before immunization and used for control experiments. Antibody validation was done by ELISA (Proteogenix, France) and by Western blotting.

Western Blotting

Three isolated nerve cords were injured, rinsed (T0h) or cultured (T6h) in L-15 complete medium and pooled for protein extraction as previously described (Vergote et al., 2006). The same protocol was performed in ATP stimulation assays, starting with an incubation step (1 h at 18°C) to expose the nerve cords to different concentrations of ATP (0.01, 0.05, 0.1, 0.5, and 1 m*M*). Samples were then rinsed, cultured for additional 5 h in L-15 complete medium and treated for protein extraction as above. For each experimental condition, SDS-PAGE were conducted with 11.5% polyacrylamide gel (Tastet et al., 2003). CNS protein extract (10 μg) was homogenized (v/v) in Laemmli sample buffer and loaded on the gel. Migration was performed in TGS buffer pH 8.3 (25 m*M* Tris, 192 m*M* glycine, and 0.1% SDS). The gel was ran at 70 V for 15 min and at 140 V for 45 min. The separated proteins were transferred to Amersham™ Hybond™-ECL nitrocellulose membranes (GE Healthcare, France). The membrane was incubated for 30 min at RT in blocking solution (Tween 0.05% w/v, milk powder 5% w/v in PBS 0.1 *M*, pH 7.2) and then overnight at 4°C in rabbit polyclonal anti-*Hm*Iba1 (1:20,000 in blocking solution containing 1% ovalbumin). In control experiments, anti-*Hm*Iba1 antibodies were substituted with rabbit preimmune serum (1:20,000). After rinsing with PBS-Tween, the membrane was incubated for 1 h at RT in secondary goat antirabbit polyclonal antibodies conjugated with horseradish peroxidase (Jackson Immunoresearch, West Grove, PA; dilution 1:20,000 in PBS-Tween). Finally, after another rinsing with PBS, immunoreactive bands were revealed using the ECL Kit SuperSignal West Dura ChemoLuminescent Substrate (Thermo Scientific, Rockford, IL). Chemiluminescence analyses were performed by ImageQuant LAS-4000 mini system (Fujifilm, Tokyo, Japan) and quantified by Multigauge software.

Whole Mount Immunohistochemistry

Single leech CNS were dissected out, experimentally injured by forceps crushing of connective fibers, and finally cultured *in vitro* in complete L-15 medium at 18°C for the following times: T0h, T6h, T24h, T3d, T7d, and T10d. In ATP-stimulation experiments, isolated nerve cords were crushed, incubated for 1 h in 0.1 or 1 m*M* ATP, then rinsed and incubated for 5 h or 23 h more in culture medium. Samples were fixed for 1 h at RT in 4% PFA, rinsed several time in PBS and permeabilized for 24 h at 4°C in 0.1 *M* glycine, 1% Triton X-100 in PBS. Nonspecific background staining was blocked with saturation buffer (1% Triton X-100, 3% normal donkey serum, 1% BSA, and 1% ovalbumin in PBS-glycine 0.1 *M*) for 5 h at 4°C. Samples were then incubated overnight at 4°C with primary anti-*Hm*Iba1 antibodies (1:5000) and mouse anti-gliarin monoclonal antibodies (mAb 9A8; Xu et al., 1999; 1:500) diluted in saturation buffer. Mouse monoclonal anti-gliarin antibody was kindly gifted by Dr J. Johansen (Iowa State University, IA). After rinsing with permeabilization solution, samples were incubated for 1 h at 37°C in secondary donkey antirabbit and antimouse antibodies (1:2000) conjugated with Alexa 488 or Alexa 555 (Invitrogen), respectively. Cell nuclei were counterstained with Hoechst 33342 (Invitrogen; 1:30,000) for 20 min at 4°C and samples were finally mounted on the slide in Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). Control experiments were performed following the same immunostaining protocol without the primary antibody or replacing the anti-*Hm*Iba1 antibodies with rabbit pre-immune serum. Sample observation was performed with a Zeiss LSM 700 Laser Scanning Confocal Microscope.

Gene Expression Analysis

Leech CNS were dissected as described above and left for 1h in 1m*M* ATP in PBS at 18°C, rinsed and incubated for additional 5 h or 23 h in complete L-15 medium. The same procedure was done for control samples incubated 1 h in PBS or directly left in complete medium. All the experiments were performed on pools of nerve cords issued from three adult leeches cultured in the absence of peripheral immune system components and blood cells. For total RNA extraction, nerve cords were immersed in 500 μL of Qiazol (Qiagen, Hilden, Germany) into 2 mL tubes Precellys®24 (Bertin Technologies, France) pre-filled with 1.4 mm ceramic beads and homogenized by shaking (2 times for 45 s at 6500 rpm) in a Precellys®24 homogenizer (Bertin Technologies, France). RNA extraction was performed

according to manufacturer's protocol and suspended in RNase-free water. The extracted total RNA was treated with RQ1-DNase1 (Promega) to prevent contamination by genomic DNA. cDNA was generated from 2 μg of total RNA using random primers and Superscript II Reverse Transcriptase kit (Invitrogen) in a final volume of 20 μL. cDNA were treated with RNaseH (Promega) to optimize the amplification reaction product. Real-time quantitative PCR (qPCR) were performed with the Quantifast SG SYBR green PCR kit (Qiagen, Hilden, Germany) by combining 2 μL of cDNA template (diluted 1:4), 2 μL of primer mix (10 μ*M*) and 21 μL of Master Mix in a final volume of 25 μL. Specific primers were designed for the qPCR analyses for Hm*iba1* gene (5′-ACAGGAACGATCTGCTACA C-3′, 5′-TCACTTTCGATTCCTTTTCC-3′) and for a leech 18*s* ribosomal protein gene, used as housekeeping reference (5′-GGAGGAGCGCGTTTATTAAG-3′, 5′-GGGCACA CACTTGAAACATC-3′). The qPCR reactions were conducted on CFX 96 Real-Time System (Biorad, Richmond, CA) with the following conditions: 5 min at 95°C (1 cycle), 10 s at 95°C, and 30 s at 56°C (39 cycles) followed by a final melting curve to control the amplified specificity. Experiments were done on triplicate samples and repeated at least on two different sets of cDNA template. Analysis of relative gene expression of Hm*iba1* was calculated using the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001). For each primer pair, the log of cDNA dilutions was plotted against the ΔCt values to validate the quantitative PCR assays (data not shown). Statistical analyses were performed by ANOVA Tukey's test using the GraphPad© Prism® 6.0 software.

RESULTS

Molecular Characterization of Hm*iba1*

A cDNA sequence coding a gene homologous to mammal *iba1*, alias *aif-1*, was identified in the EST library from the leech CNS and the *H. medicinalis* genome (Macagno et al., 2010). Based on the available sequence, specific primers were designed and used for RT-PCR amplification. cDNA was then cloned and sequenced. The gene, named Hm*iba1* (GenBank accession number KF437461), has a predicted full-length cDNA of 794 bp including 259 bp 5′ corresponding to the untranslated region (UTR) and 126 bp as 3′ UTR. The open reading frame (ORF) encodes a 155 amino acid protein devoid of putative signal peptide and with a calculated molecular mass of 17.87 kDa. The Hm*iba1* genomic sequence revealed the presence of seven exons and six introns. The ORFs were 30, 60, 67, 42, 100, 63, and 103 bp in length from ORF1 to ORF7, respectively (data not shown). Analysis of the cDNA sequence predicted the presence of a central pair of Ca^{2+} binding EF-hand motifs (51–75 and 84–111 regions), typically present in the proteins belonging to the EF-hand family (Deininger et al., 2002; Fig. 2). *Hm*Iba1 shows some variability in the Ca^{2+} binding sites generally present in this family of proteins [Fig. 3(A)], lacking part of the eight conserved residues necessary to bind calcium in Vertebrates (Schulze et al., 2008). The *Hm*Iba1 putative amino acid sequence presents a 52% identity with the human protein and an average identity of 50.25 and 55% with other AIF-1 proteins of Vertebrates and invertebrates, respectively. Phylogenic analysis [Fig. 3(B)] indicates the presence of two groups of AIF-1 proteins in Metazoans, corresponding to invertebrate and vertebrate species. *Hm*Iba1 shows a closer relationship with molluscan than with other metazoan AIF-1 molecules.

*Hm*Iba1 Immunolocalization

Polyclonal antibodies were raised against the N-terminal region of the putative *Hm*Iba1 protein. Total CNS protein extracts from freshly dissected (T0h) and cultured (T6h) nerve cords were used for western blotting analyses. The result showed the presence of an immunoreactive band of ∼18 kDa (Fig. 4) in both samples. This value corresponds to the predicted molecular weight of the *Hm*Iba1 protein (17.87 kDa). In control experiments done with rabbit preimmune serum, no specific immunoreactive bands were observed. Protein quantification was established by comparing the absorbance of the signal per surface unit (AU/mm^2). An increased amount of protein of around 65.6% was observed in T6h cultured samples (60.39 ± 3.55 AU/mm^2) when compared to naïve (T0h) nerve cords (39.61 ± 3.55 AU/mm^2).

The specific anti-*Hm*Iba1 serum was also used for immunohistochemistry to assess the presence and localization of the protein on whole mounted CNS cultured at different times with or without ATP stimulation. To identify the glial cells in leech CNS, coimmunostaining were performed using polyclonal anti-*Hm*Iba1 and monoclonal anti-gliarin antibodies. Gliarin is a molecule belonging to intermediate filament protein superfamily, first described as a general marker of glial and microglial cells of leech CNS (Luthi et al., 1994; Xu et al., 1999). This anti-gliarin monoclonal antibody was recently used to observe and characterize the morphology of resting and migrating microglial cells in leech CNS (Jansen and Nicholls, 1972; Le Marrec-Croq et al., 2013). Time course experiments were performed on freshly dissected (T0h) or injured nerve cords cultured at various time points (from 6 h up to 10 days; Fig. 5). Microglial cells are resident cells evenly distributed

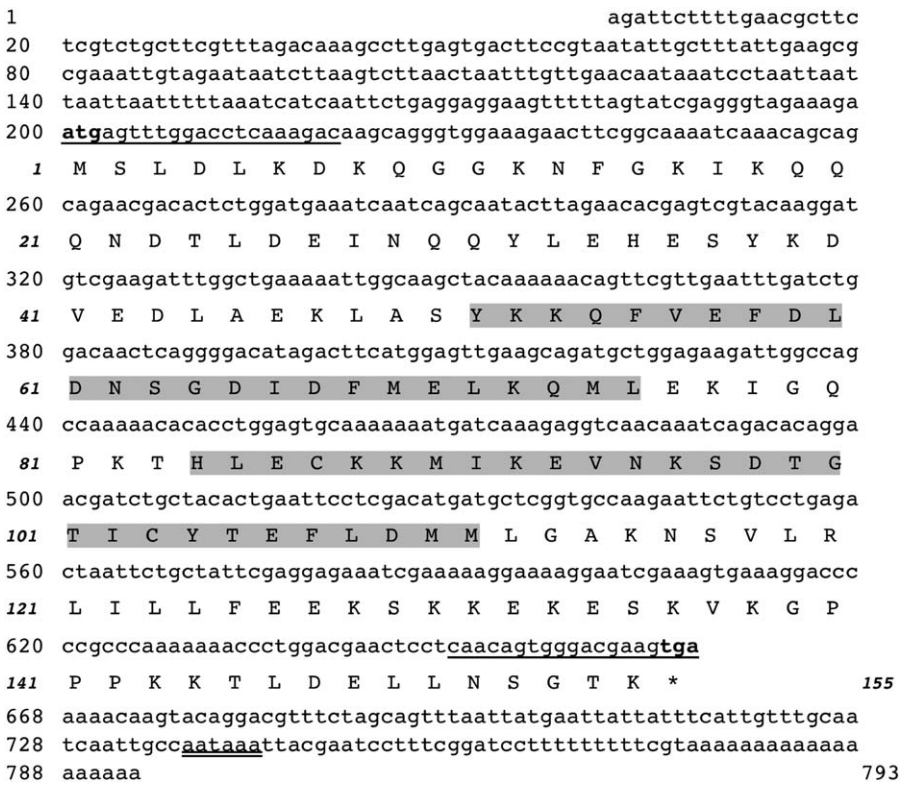

Figure 2 Complete cDNA and putative primary sequence of leech Hm*iba1*. Forward and reverse primer sequences used for amplification are underlined. Start and stop codons are in bold. The RNA polyadenylation signal sequence is double-underlined. The regions highlighted in gray represent the two predicted Ca^{2+} binding EF-hand motifs.

in segmental ganglia and in connective tissues. Hoechst dye counterstaining was used to identify the position of cell nuclei in CNS structures. This approach, coupled to confocal microscopy observation, permitted to associate single cell nuclei to cytoplasmic immunostaining located on different focal plans. In all the samples, the size and the distribution of *Hm*Iba1 immunopositive structures corresponded only to microglia and not to axon or giant glial cells. This element confirmed *Hm*Iba1 as a specific microglial marker. The description of results obtained on whole mount immunohistochemistry experiments is done separately for connective fibers and segmental ganglia. After the injury of CNS, microglial cells are activated and rapidly recruited at the lesion site. For this reason, we mainly focused the study of *Hm*Iba1 immunolocalization on connective regions. In naïve samples (T0h), a very little number of gliarin and *Hm*Iba1 positive cells were observed in the injured as well as in unlesioned regions of connectives [Fig. 5(A)]. In the following time points, a significant increase in the number of positive cells was detected at the lesion site postaxotomy. Cells concentrated at the injured area appeared spindle-shaped and showed a high nucleus/cytoplasm ratio. Six hours post lesion, most of accumulated microglia were *Hm*Iba1-positive, while a little number of gliarin-expressing cells was observed [Fig. 5(B)]. Several *Hm*Iba1$^+$ cells were visible at the periphery of the lesion, still moving towards the injury site. This situation was still present 24 h post-lesion [Fig. 5(C)]. The two markers colocalize in relatively few cells located at the site of injury or spread inside the connectives [inset of Fig. 5(C)]. In these cells, gliarin was mostly located in the perinuclear area while *Hm*Iba1 generally filled the periphery of the cytoplasm.

A similar immunostaining of cells gathered at the damaged area was observed up to 10 days later, with the accumulation peaking 3 days post lesion [Fig. 5(C–F)]. In samples cultured for 10 days, the amount of microglia accumulated at the injury site diminished and cells get a rounded shape [Fig. 5(F)]. Gliarin was mostly expressed in the first days after injury and was poorly observed in the following time points. In negative controls, CNS were treated with rabbit preimmune serum [Fig. 5(G)] and no positive signals were observed at any time point.

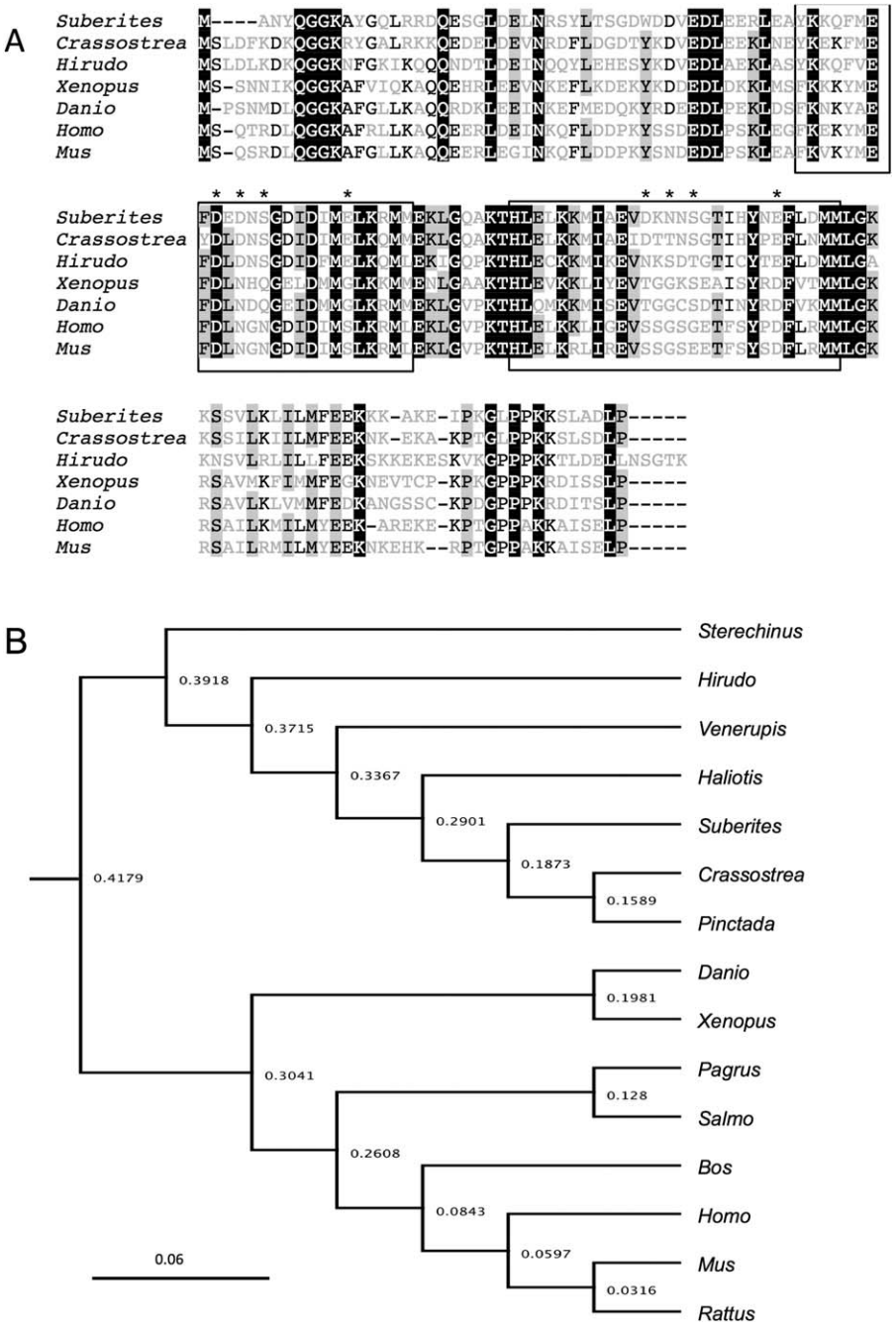

Figure 3 A: Multiple alignment of AIF-1 indicating identity between sequences from different selected species. Highlighted residues correspond to high identity rate between AIF-1 proteins (black = 100% and gray = 80%). Lower rate values are indicated with black (60%) and gray (<50%) letters. The boxed sequences represent the EF-hand motifs and asterisks indicate Ca^{2+} binding residues. B: Phylogenetic tree based on the primary structure of AIF-1 from the following species: *Hirudo medicinalis* (KF437461) *Xenopus laevis* (NP_001086148), *Pagrus major* (BAA36938), *Salmo salar* (ACI69971), *Crassostrea gigas* (EKC34896), *Sterechinus neumayeri* (ACO40483), *Pinctada martensi* (JF929906), *Haliotis discus hannai* (ABH10674), *Danio rerio* (NP_942571), *Suberites domuncula* (CAC38780), *Venerupis philippinarum* (ACU83234), *Mus musculus* (BAA28216), *Homo sapiens* (AAD18087), *Bos taurus* (AAK30155), and *Sus scrofa* (P81076). Node values indicate percent bootstrap confidence derived from 1000 replicates. The bar shows the genetic distance. Phylogenetic analysis was carried out by Geneious® Basic v6.1 software (Drummond et al., 2012).

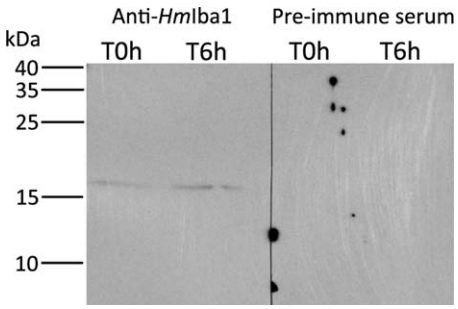

Figure 4 Western blot analysis performed using specific rabbit polyclonal anti-*Hm*Iba1 antibodies on protein extract (CNS) prepared from freshly dissected (T0h) and cultured (T6h) nerve cords. A specific immunoreactive band was detected at about 18 kDa, according to the molecular weight ladder (kDa). No specific signals were observed on the negative control experiments performed using primary rabbit preimmune serum.

In the ganglia of naïve nerve cords (T0h) very few microglial cells were *Hm*Iba1 positive [Fig. 5(H)]. A higher amount of *Hm*Iba1 immunopositive cells was observed in the ganglia of cultured samples. This positivity peaked at T3d [Fig. 5(I)] and gradually decreased to the basal level in the following days (data not shown). The *Hm*Iba1$^+$ cells, mostly located in the inter-neuronal spaces, had an elongated shape and occasionally displayed also immunoreactivity for gliarin. Interestingly, such immunostaining pattern was observed for segmental ganglia flanking the lesion. In all samples, gliarin was mainly present in largely ramified cells forming a network around neuron bodies.

Recent studies reported a direct link between ATP stimulation and microglial cell reactivity in leech (Duan et al., 2009), but the effects described were mostly associated with cell movement and migration. No evidence exists so far between ATP treatment and the expression of specific activation markers in such cells. In order to determine the effect of ATP on microglial cell activation and estimate the possible variation in *Hm*Iba1 production and cell distribution, we performed some assays stimulating nerve cords with ATP. Immunohistochemistry with anti-gliarin and anti-*Hm*Iba1 was performed on leech CNS dissected and injured, stimulated with 0.1 m*M* ATP or 1 m*M* ATP and incubated for additional times in culture medium (Fig. 6). The same treatment was used for control samples, not exposed to ATP. Colocalization for gliarin and *Hm*Iba1 was detected in some microglial cells spread in untreated connective fibers [Fig. 6(A,B)]. In ATP-treated nerve cords, microglial cells normally accumulated at the crushed areas and the immunostaining results did not differ from that reported for the untreated samples of Figure 5 (not shown). At T6h post 0.1 m*M* ATP-treatment [Fig. 6(C)] we did not detect significant variations of *Hm*Iba1 in connective microglia while an increased number of cells resulted *Hm*Iba1$^+$ at T24h [Fig. 6(D)]. A stronger enhancement of *Hm*Iba1 was detected in the microglia of 1 m*M* ATP-treated nerve cords [Fig. 6(E)]. Lined between axons and spread along the nerve cord, these *Hm*Iba1$^+$ cells looked stretched, with a flattened nucleus and in contact with each other through their elongated cytoplasmic ends [inset of Fig. 6(E)]. In untreated ganglia [Fig. 6(G,H)], at T6h and T24h, the distribution of *Hm*Iba1 and gliarin was comparable to that described above for cultured CNS (Fig. 5). As observed in connectives, an increase of interneuronal *Hm*Iba1-positive cells was observed in segmental ganglia after 0.1 m*M* ATP stimulation at T6h and at T24h compared with untreated samples [Fig. 6(I,J)]. Interestingly, a considerable enhancement of *Hm*Iba1 immunostaining was observed for ganglia exposed to 1 m*M* ATP, while such treatment had a poor effect on gliarin distribution all along the nerve cord [Fig. 6(K)]. Negative control samples, treated with rabbit preimmune serum, did not show immunostaining reaction [Fig. 6(F,L)].

Hm*iba1* Gene Expression

ATP plays a significant role in microglia activation stimulating cell migration in mouse (Miller and Stella, 2009); the same effect was also observed in leech (Duan et al., 2009). In addition, 1 m*M* ATP greatly upregulates the expression of MRF-1 (alias AIF-1/Iba1) protein in cultured rat microglia (Tanaka and Koike, 2002). Based on these data, we investigated by real-time quantitative PCR the effect of Hm*iba1* in leech CNS upon 1 m*M* ATP stimulation. Freshly dissected nerve cords were experimentally crushed and bathed with ATP or vehicle PBS. Control samples were maintained in culture medium. The CNS were then cultured for additional 5 h or 23 h and used for total RNA extraction. Quantitative PCR results (Fig. 7) showed that Hm*iba1* gene is expressed in cultured leech CNS. The rate of expression at T0h (freshly dissected nerve cords) was arbitrarily fixed to 1. In the different experiments, the amount of transcript decreased of about 37–40% after a few hours (T6h) of culture *ex vivo* and remained stable at least up to 24 h later in control and in PBS-treated nerve cords. The Hm*iba1* gene resulted upregulated 6 h after stimulation with ATP, with a 6-fold increase of RNA amount relative to T0h

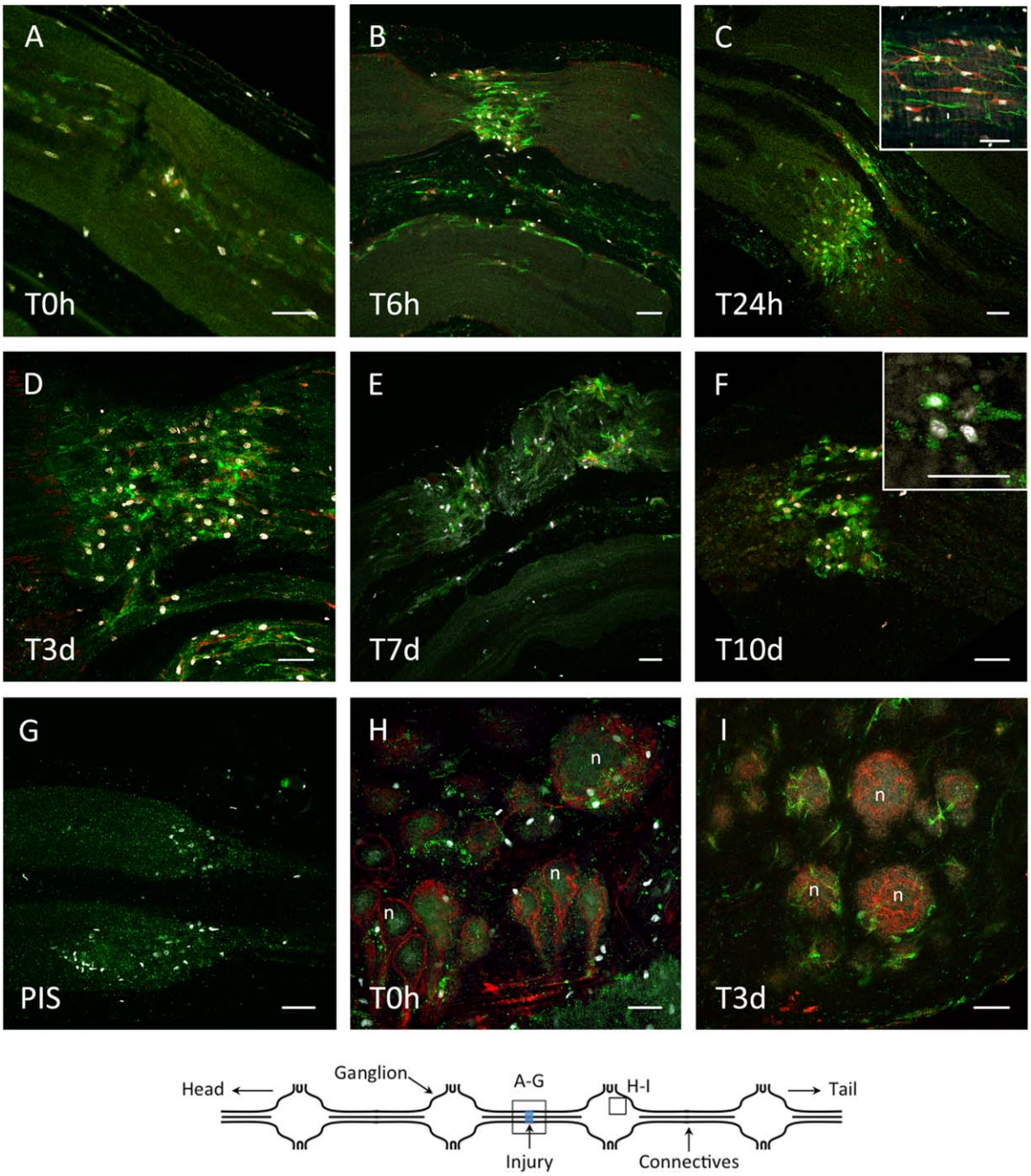

Figure 5 Immunohistochemistry on whole mounted leech CNS. Samples were injured and incubated at different time points (T0 hour, 1, 3, 7, and 10 days). Double immunostaining was carried out with anti-*Hm*Iba1 (green) and antigliarin (red) antibodies that specifically labeled microglia. Cell nuclei were counterstained with Hoechst 33342 (white). A–F: Immunochemistry on lesioned connectives performed at different time points showing the accumulation of immunopositive microglia at the site of axotomy. The inset of Figure C shows a detail of *Hm*Iba1 and gliarin colocalization inside some elongated microglial cells. The inset of Figure F displays the rounded morphology of residual *Hm*Iba1 positive cells accumulated at the lesion site. G: Control experiment on injured connective done with primary rabbit pre-immune serum (PIS); similar controls were performed for all the experimental conditions (not shown). H–I: Anti-*Hm*Iba1 and anti-gliarin immunostaining of a naïve (T0h) and a cultured (T3d) segmental ganglion. Some neuron bodies in the ganglia are labeled (*n*). In the bottom, a schematic representation of leech CNS indicating (boxes) the connectives lesion site and ganglion area illustrated in Figures A–G and H–I, respectively. Picture brightness was slightly and homogeneously modified to improve the visibility of cell nuclei or unstained tissue background. Scale bars correspond to 20 μm.

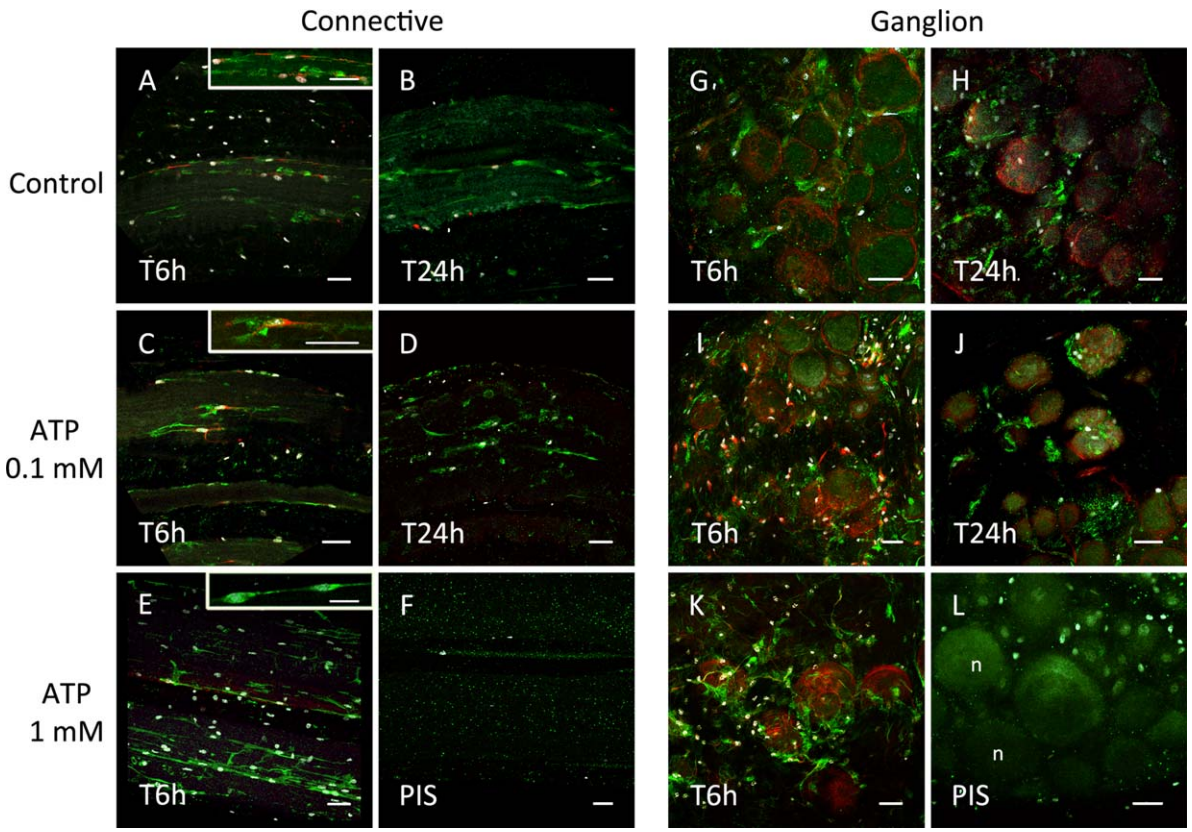

Figure 6 Immunohistochemistry on whole mounted CNS upon ATP stimulation. Injured nerve cords were incubated for 1 h in ATP before a recovering step (5 h or 23 h) in culture medium, fixation, and immunostaining. Control samples were simply incubated in culture medium after injury. Double immunostaining was performed with anti-*Hm*Iba1 (green) and antigliarin (red) antibodies as above. Cell nuclei were counterstained with Hoechst 33,342 (white). Figures A-E show the immunostaining on connectives unstimulated (A, B) and treated with ATP 0.1 m*M* (C, D) or 1 m*M* (E). Insets of Figures A, C, and E show a detail of immunopositive cells at T6h. G–K: display the effects on segmental ganglia of the same treatments described above. Experimental negative controls were performed on 24 h-cultured nerve cords using rabbit preimmune serum (PIS) (F, L). Some of the neuron cell bodies were labeled (*n*) in (L). Picture brightness was slightly and homogeneously modified to make better visible cells nuclei or unstained tissue background. Scale bars correspond to 20 μm.

expression. This overexpression is still present at T24h with a 2.5-fold increase.

DISCUSSION

In adult mammals, brain resident ramified/resting microglia become activated by modifying their morphology to amoeboid shape in response to infection, injury or inflammation of the nervous system. Reactive microglia proliferate, phagocytize degenerating elements and release either cytotoxic agents or cytokines (Imura et al., 2013). Interestingly, similar events also occur in leech CNS, where activated microglia are able to change morphology modifying their shape (Bernhardi and Nicholls, 1999; Kettenmann et al., 2011; Le Marrec-Croq et al., 2013). Activation firstly involves a retraction of filopodia, transforming microglia into globular-shaped cells, followed by the acquisition of an elongated morphology during the migration. These cells, as reported in the present article, get a spindle shape [see inset Fig. 6(E)] and migrate along axon fibers to reach the lesion point. Several days following the injury, cells accumulated at the lesion return to a rounded morphology [see inset Fig. 5(F)] (Le Marrec-Croq et al., 2013). An opposite modification in morphology, from rounded to ramified shape, was described for cultured microglial cells exposed to conditioned

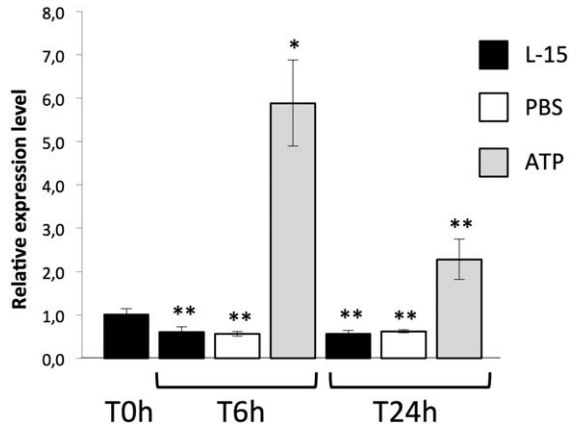

Figure 7 Real time qPCR quantification of the expression level of Hm*iba1* in leech CNS upon ATP stimulation. Experiments were done comparing injured nerve cords cultured in L15 complete medium (black) with those incubated 1 h in PBS (white) or 1 m*M* ATP (gray), before a recovering period (5 h or 23 h) in culture medium. The relative amounts of transcripts were calculated by the $\Delta\Delta C_t$ method. A leech 18S ribosomal gene was used as internal reference. Statistical analyses were performed on data standardized against T0h control samples arbitrarily set to 1. Significance ($*p < 0.05$, $**p < 0.01$ vs. Control) was calculated by ANOVA Tukey's test (bar represents standard error).

medium issued from injured nerve cords (Bernhardi and Nicholls, 1999). These data highlight the plasticity of leech microglia permitting the shifting from resting to activated state upon different physiological stimuli. In addition leech microglia phagocytize and express cytokines (Le Marrec-Croq et al., 2013), showing an intriguing functional convergence with mammalian cells. Nevertheless, major differences distinguish these two systems: leech CNS lacks glial cells like astrocytes and oligodendrocytes. In addition, infiltrating macrophages are almost absent in this organism, because very few blood cells penetrate the injured nervous system (Boidin-Wichlacz et al., 2012). Therefore, the inflammatory control is exclusively exerted by the resident microglia that are the main sentinels monitoring CNS immune response. Moreover, neurectomy permits the export and culture *ex vivo* of the leech nerve cord, avoiding penetrating cells from blood circulation. These elements make the *Hirudo* CNS a good model to dissect out the role of resident microglia in inflammatory and healing events. In this context, we focused our researches on mechanisms inducing microglial cell activation and migration. These processes are quite complex and partially influenced by the action of diffusible factors, like ATP and NO (Duan et al., 2009). Microglia chemotaxis was shown to depend on the axonal release of cytokines (*Hm*EMAPII and *Hm*IL-16; Schikorski et al., 2009; Croq et al., 2010) or complement-like factors (*Hm*C1q; Tahtouh et al., 2009, 2012; Le Marrec-Croq et al., 2014). This recruitment may be linked to the differential expression of specific receptors at the surface of microglial cell subsets (Le Marrec-Croq et al., 2013). Because of the similarities of leech and mammalian microglia described above, we looked for possible activation markers in *H. medicinalis* during neuroinflammatory events. In Mammals, Iba1 is a marker selectively expressed in reactive microglia. This molecule is generally known as Allograft Inflammatory Factor 1 (AIF-1) in non-nervous tissues and constitutes a well-established macrophage marker. We identified a gene homologous to human *iba1* in the EST library of leech CNS. This gene, named Hm*iba1* (alias Hm*aif-1*), codes a protein presenting two putative Ca^{2+}-binding EF-hand motifs, typically present in the AIF-1 family members. The residues responsible for Ca^{2+}-binding properties are not fully conserved in *Hm*Iba1. Amino acid substitutions in these motifs have already been pointed out in other vertebrate (Deininger et al., 2002) and invertebrate AIF-1 (Ovando et al., 2012), but the functional role of such modifications remains largely unknown. Most of the proteins belonging to the AIF-1 family have been reported and studied in Mammals, even if homologs were identified in non-mammalian Vertebrates, like amphibians, birds and several boney fishes. Their characterization remains extremely limited in invertebrates. AIF-1 was initially described in marine sponges (Porifera) where this molecule is involved, as in Mammals, in immune cell activation. This was the first documentation for a cytokine-mediated alloimmune response in invertebrates (Kruse et al., 1999). A molecule homologous to AIF-1 was recently characterized in the Antarctic sea urchin *Sterechinus neumayeri* (Echinoderms). This molecule was shown to be induced in coelomocytes exposed to bacterial challenge (Ovando et al., 2012). Invertebrate AIF-1–like molecules were mainly described in Mollusks, notably in Gastropods (De Zoysa et al., 2010) and Bivalves (Zhang et al., 2010, 2013; Li et al., 2012). In these animals, the respective AIF-1 genes are expressed in a variety of unstimulated tissues, suggesting a role not necessarily linked to allogenic or immune inflammation and are upregulated in different organs upon bacterial challenge. Phylogenetic analysis showed that leech *Hm*Iba1 is more closely related to molluscan than to other animal AIF-1. This observation is consistent with the evolutionary proximity of Annelids and Mollusks, grouped in the Lophotrochozoan taxon

(Adoutte et al., 2000). AIF-1 is characterized by a high conservation of the amino acid sequence throughout the evolution of Vertebrates and invertebrates. It suggests that proteins belonging to this family may have similar functions. Further studies will be performed on peripheral tissues of *Hirudo* to establish if *Hm*Iba1 is expressed in macrophage-like cells and if, like in other animals, it is involved in inflammatory response to injury or immune challenge. The works so far published on Invertebrates highlighted the role of AIF-1 in cell-mediated immune response but it is still unclear if any invertebrate AIF-1 acts as an immune-modulating factor in the nervous system, as observed in Vertebrates. In fact, this work is the first report of an AIF-1 molecule in the nervous system of an Invertebrate species.

Immunohistochemistry analysis on whole-mounted nerve cords confirmed the hypothesis of *Hm*Iba1 cell-specific expression in leech microglia, since neither other glial cells nor neurons resulted positive for this marker. The constitutive amount of the *Hm*Iba1 protein in naïve CNS was negligible; nevertheless, its level rapidly increases in tissues submitted to experimental injury. A few hours after lesion the protein was mostly present in microglia gathered at the injury site as well as in those located in segmental ganglia. It indicates that axotomy induces a general alert in CNS, inducing the activation of several microglial cells that start producing *Hm*Iba1. Since the accumulated microglial cells are not *Hm*Iba1 positive in their totality, this molecule, even if specific, does not represent a marker for general microglia activation in leech. Double-immunostaining experiments were performed with anti-gliarin and anti-*Hm*Iba1 antibodies, to establish if these two markers colocalize in the microglia. Gliarin is an intermediate filament protein previously described as specifically expressed in leech glial cells (Xu et al., 1999) and upregulated upon CNS bacterial challenge (Vergote et al., 2006). Our results corroborated the presence of gliarin in microglia and showed that it is present in some cells spread into connectives or enveloping neuron bodies in segmental ganglia. The colocalization of the two markers was occasionally observed in connectives. Gliarin is poorly present in naïve CNS; its production slightly increases in samples submitted to axotomy and decreases a few days later. This transient expression may reflect an activation state linked to early cytoskeleton reorganization in microglia of injured tissues. These data indicate that this protein is not a good marker for long-term microglial activation process in connectives. Although not specific, gliarin constitutes the only microglial marker so far described in leech and its colocalization with *Hm*Iba1 here evidenced, directly validates this latter as microglial specific factor. Further studies on gliarin will permit to highlight the kinetic and the distribution of this molecule in the different cell types following nerve cord injury.

An interesting element emerging from the data presently reported is that anti-*Hm*Iba1 and anti-gliarin antibodies respectively stain microglial cell subsets that constitute a limited portion of the thousands of cells present in the nerve cord. In addition, even when accumulated at the lesion site, the activated cells are not all positive for these markers. These elements suggest the presence of relative activation states of microglial cells able to become rapidly reactive upon alert signals and reinforce the hypothesis of the presence of functional heterogeneous microglia sub-populations in leech nerve cord, as previously suggested (Tahtouh et al., 2012; Le Marrec-Croq et al., 2013, 2014). Most of these cells might have different functions, reactivity, activation kinetics, or specific recruitment and exert their direct or indirect activity in the repair process without any expression of *Hm*Iba1 or gliarin.

To establish if the presence of *Hm*Iba1 in leech CNS is modulated by ATP stimulation, we treated the cultured nerve cords with different nucleotide concentrations. Immunohistochemistry experiments showed that ATP globally increases the amount of *Hm*Iba1$^+$ cells throughout the nerve cord, while it appeared less operative on gliarin expression. *Hm*Iba1 production resulted weakly induced by 0.1 m*M* ATP stimulation but appeared strongly enhanced by 1 m*M* ATP treatment. In connectives, in particular, the *Hm*Iba1-positive cells became elongated and stretched between axons, resembling microglia migrating along fibers. This observation is consistent with a previous report demonstrating that ATP stimulates in a dose-dependent way the movement and migration of microglia towards the lesion site, with a peak of activity at 0.1 m*M* ATP (Duan et al., 2009). Although such concentration efficiently activates microglia movement, our data show that it poorly affects the production of *Hm*Iba1, the latter favored by a higher amount of nucleotide. Interestingly, a similar effect was described in cultured rat microglia where 1 h of treatment with 1 m*M* ATP greatly enhanced the production and release of MRF1 (alias AIF-1/Iba1; Tanaka and Koike, 2002). These data indicate that ATP plays a crucial role in *Hm*Iba1 regulation and constitutes an additional element reinforcing the functional similarity between mammalian and leech microglia. To estimate the effect of ATP treatment on Hm*iba1* gene expression in leech CNS, we performed q-PCR analyses on templates produced

from nerve cords exposed to ATP treatment vs. control samples incubated in PBS or left in culture medium.

Results revealed that Hm*iba1* gene is constitutively expressed in leech CNS. This basal transcription rate of mRNA might make microglial cells reactive and ready to produce the protein in the hours following the lesion. The experimental results suggest that the real constitutive amount of Hm*iba1* corresponds to that detected at T6h and T24h in control and PBS-treated samples (Fig. 7). The slightly higher amount of transcript observed at T0h might rather be linked to the stress induced in leech by surgery procedure (anesthesia and dissection steps) involving an important rate of injuries and axotomy in CNS. Such initial stress associated to CNS dissection and sample treatment has already been mentioned for microglia issued from rat brain organotypic cultures preparation (Hailer et al., 2005). These events may provoke the release of several alert signals in leech CNS (endogenous ATP, NO, cytokines, ...) inducing a rapid and transient transcription of the Hm*iba1* gene that returns to the basal expression level in cultured nerve cords a few hours later. This physiological process may explain the results obtained for the controls.

A significant increase of RNA level was observed in ATP-treated nerve cords. Such induction of Hm*iba1* transcript remains visible at least up to 24 h after stimulation, indicating a stable expression of the gene probably guaranteeing the production of the molecule in the days subsequent to the activation. These data, however, are in contrast with a previous report indicating that in rat microglia the treatment with 1 m*M* ATP induces a gradual and significant downregulation of *mrf-1* transcripts in the hours following the addition of the nucleotide (Kaya et al., 2002). Such selective suppression would not affect the production and release of the MRF-1 protein that the same authors assert to be strongly enhanced by ATP treatment (Tanaka and Koike, 2002). Recent studies in leech CNS originally showed that ATP was released from giant glial cells (neuroglia) in the connective via innexin channels to activate microglia, presumably through P2Y receptors (Samuels et al., 2010). Further studies will permit to elucidate if, in leech, the ATP-reactive cells might represent a subset of microglia involved in functions specially linked to Ca^{2+} metabolism and sensitive to ATP stimulation by the possible presence of purinergic receptors. Future investigations are necessary to ascertain if leech stimulated microglia are also able to secrete ATP by exocytosis, as recently demonstrated in mammalian cells (Imura et al., 2013). The role of *Hm*Iba1 in leech CNS is still undefined, so functional assays will be performed using the recombinant protein to search its interactant(s) in CNS protein extract. In Vertebrates, AIF-1 (Iba1) is known to interact with L-fimbrin, forming complexes in membrane ruffles and phagocytic cups (Ohsawa et al., 2004). A gene homologous to L-fimbrin was identified in the EST library from leech CNS. The study of relationships of *Hm*Iba1 with L-fimbrin as well as with other possible interactants will help to understand the role of this factor during microglia activation, migration or phagocytosis events. Further investigations are necessary to identify novel selective microglial markers in leech, to understand their regulation patterns and to study the cross talking events occurring between microglia and neurons. These elements could also introduce new insights about the activation processes and the inflammatory mechanisms in mammalian microglia.

The authors thank Elodie Richard (BioImaging Center of Lille, BICeL-Campus Lille 1) for access to confocal microscopy facilities and technical advice.

REFERENCES

Adoutte A, Balavoine G, Lartillot N, Lespinet O, Prud'homme B, de Rosa R. 2000. The new animal phylogeny: Reliability and implications. Proc Natl Acad Sci USA 97:4453–4456.

Ahmed Z, Shaw G, Sharma VP, Yang C, McGowan E, Dickson DW. 2007. Actin-binding proteins coronin-1a and IBA-1 are effective microglial markers for immunohistochemistry. J Histochem Cytochem 55:687–700.

Arafah K, Croix D, Vizioli J, Desmons A, Fournier I, Salzet M. 2013. Involvement of nitric oxide through endocannabinoids release in microglia activation during the course of CNS regeneration in the medicinal leech. Glia 61:636–649.

Bernhardi RV, Nicholls JG. 1999. Transformation of leech microglial cell morphology and properties following coculture with injured central nervous system tissue. J Exp Biol 202 (Pt 6):723–728.

Beschorner R, Engel S, Mittelbronn M, Adjodah D, Dietz K, Schluesener HJ, Meyermann R. 2000. Differential regulation of the monocytic calcium-binding peptides macrophage-inhibiting factor related protein-8 (MRP8/S100A8) and allograft inflammatory factor-1 (AIF-1) following human traumatic brain injury. Acta Neuropathol 100:627–634.

Boidin-Wichlacz C, Vergote D, Slomianny C, Jouy N, Salzet M, Tasiemski A. 2012. Morphological and functional characterization of leech circulating blood cells: role in immunity and neural repair. Cell Mol Life Sci 69: 1717–1731.

Casimiro I, Chinnasamy P, Sibinga NE. 2013. Genetic inactivation of the allograft inflammatory factor-1 locus. Genesis 51:734–740.

Chen ZW, Ahren B, Ostenson CG, Cintra A, Bergman T, Moller C, Fuxe K, et al. 1997. Identification, isolation, and characterization of daintain (allograft inflammatory factor 1), a macrophage polypeptide with effects on insulin secretion and abundantly present in the pancreas of prediabetic BB rats. Proc Natl Acad Sci USA 94:13879–13884.

Coggeshall RE, Fawcett DW. 1964. The fine structure of the central nervous system of the Leech, Hirudo Medicinalis. J Neurophysiol 27:229–289.

Croq F, Vizioli J, Tuzova M, Tahtouh M, Sautiere PE, Van Camp C, Salzet M, et al. 2010. A homologous form of human interleukin 16 is implicated in microglia recruitment following nervous system injury in leech Hirudo medicinalis. Glia 58:1649–1662.

De Zoysa M, Nikapitiya C, Kim Y, Oh C, Kang DH, Whang I, Kim SJ, et al. 2010. Allograft inflammatory factor-1 in disk abalone (Haliotis discus discus): molecular cloning, transcriptional regulation against immune challenge and tissue injury. Fish Shellfish Immunol 29: 319–326.

Deininger MH, Meyermann R, Schluesener HJ. 2002. The allograft inflammatory factor-1 family of proteins. FEBS Lett 514:115–121.

Deininger MH, Seid K, Engel S, Meyermann R, Schluesener HJ. 2000. Allograft inflammatory factor-1 defines a distinct subset of infiltrating macrophages/microglial cells in rat and human gliomas. Acta Neuropathol 100:673–680.

Dijkstra S, Geisert EJ, Gispen WH, Bar PR, Joosten EA. 2000. Up-regulation of CD81 (target of the antiproliferative antibody; TAPA) by reactive microglia and astrocytes after spinal cord injury in the rat. J Comp Neurol 428:266–277.

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, et al. 2012. Geneious v6.1. Available at: http://www.geneious.com.

Duan Y, Sahley CL, Muller KJ. 2009. ATP and NO dually control migration of microglia to nerve lesions. Dev Neurobiol 69:60–72.

Hailer NP, Vogt C, Korf HW, Dehghani F. 2005. Interleukin-1beta exacerbates and interleukin-1 receptor antagonist attenuates neuronal injury and microglial activation after excitotoxic damage in organotypic hippocampal slice cultures. Eur J Neurosci 21:2347–2360.

Imura Y, Morizawa Y, Komatsu R, Shibata K, Shinozaki Y, Kasai H, Moriishi K, Moriyama Y, Koizumi S. 2013. Microglia release ATP by exocytosis. Glia 61:1320–1330.

Ito D, Imai Y, Ohsawa K, Nakajima K, Fukuuchi Y, Kohsaka S. 1998. Microglia-specific localisation of a novel calcium binding protein, Iba1. Brain Res Mol Brain Res 57:1–9.

Jansen JK, Nicholls JG. 1972. Regeneration and changes in synaptic connections between individual nerve cells in the central nervous system of the leech. Proc Natl Acad Sci USA 69:636–639.

Kato H, Tanaka S, Oikawa T, Koike T, Takahashi A, Itoyama Y. 2000. Expression of microglial response factor-1 in microglia and macrophages following cerebral ischemia in the rat. Brain Res 882:206–211.

Kaya N, Tanaka S, Koike T. 2002. ATP selectively suppresses the synthesis of the inflammatory protein microglial response factor (MRF)−1 through Ca(2+) influx via P2X(7) receptors in cultured microglia. Brain Res 952:86–97.

Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. 2011. Physiology of microglia. Physiol Rev 91:461–553.

Kruse M, Steffen R, Batel R, Muller IM, Muller WE. 1999. Differential expression of allograft inflammatory factor 1 and of glutathione peroxidase during auto- and allograft response in marine sponges. J Cell Sci 112 (Pt 23):4305–4313.

Le Marrec-Croq F, Drago F, Vizioli J, Sautiere PE, Lefebvre C. 2013. The leech nervous system: A valuable model to study the microglia involvement in regenerative processes. Clin Dev Immunol 2013:274019.

Le Marrec-Croq F, Garcon-Bocquet A, Vizioli J, VanCamp C, Drago F, Franck J, Wisztorski M, et al. 2014. Calreticulin contributes to C1q-dependent recruitment of microglia in the leech Hirudo medicinalis following a CNS injury. Medical Science Monitor 20:644–653.

Li J, Chen J, Zhang Y, Yu Z. 2012. Expression of allograft inflammatory factor-1 (AIF-1) in response to bacterial challenge and tissue injury in the pearl oyster, Pinctada martensii. Fish Shellfish Immunol 34:365–371.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408.

Luthi TE, Brodbeck DL, Jeno P. 1994. Identification of a 70 kD protein with sequence homology to squid neurofilament protein in glial cells of the leech CNS. J Neurobiol 25:70–82.

Macagno ER, Gaasterland T, Edsall L, Bafna V, Soares MB, Scheetz T, Casavant T, et al. 2010. Construction of a medicinal leech transcriptome database and its application to the identification of leech homologs of neural and innate immune genes. BMC Genomics 11:407.

Miller AM, Stella N. 2009. Microglial cell migration stimulated by ATP and C5a involve distinct molecular mechanisms: Quantification of migration by a novel near-infrared method. Glia 57:875–883.

Morgese VJ, Elliott EJ, Muller KJ. 1983. Microglial movement to sites of nerve lesion in the leech CNS. Brain Res 272:166–170.

Ngu EM, Sahley CL, Muller KJ. 2007. Reduced axon sprouting after treatment that diminishes microglia accumulation at lesions in the leech CNS. J Comp Neurol 503:101–109.

Ohsawa K, Imai Y, Kanazawa H, Sasaki Y, Kohsaka S. 2000. Involvement of Iba1 in membrane ruffling and phagocytosis of macrophages/microglia. J Cell Sci 113 (Pt 17):3073–3084.

Ohsawa K, Imai Y, Sasaki Y, Kohsaka S. 2004. Microglia/macrophage-specific protein Iba1 binds to fimbrin and

enhances its actin-bundling activity. J Neurochem 88: 844–856.

Ovando F, Gimpel C, Cardenas C, Machado Cunha Da Silva JR, De Lorgeril J, Gonzalez M. 2012. Cloning and expression analysis of allograft inflammatory factor type 1 in coelomocytes of antarctic sea urchin (Sterechinus neumayeri). J Shellfish Res 31:875–883.

Postler E, Rimner A, Beschorner R, Schluesener HJ, Meyermann R. 2000. Allograft-inflammatory-factor-1 is upregulated in microglial cells in human cerebral infarctions. J Neuroimmunol 104:85–91.

Samuels SE, Lipitz JB, Dahl G, Muller KJ. 2010. Neuroglial ATP release through innexin channels controls microglial cell movement to a nerve injury. J Gen Physiol 136:425–442.

Schikorski D, Cuvillier-Hot V, Boidin-Wichlacz C, Slomianny C, Salzet M, Tasiemski A. 2009. Deciphering the immune function and regulation by a TLR of the cytokine EMAPII in the lesioned central nervous system using a leech model. J Immunol 183:7119–7128.

Schluesener HJ, Seid K, Kretzschmar J, Meyermann R. 1998. Allograft-inflammatory factor-1 in rat experimental autoimmune encephalomyelitis, neuritis, and uveitis: Expression by activated macrophages and microglial cells. Glia 24:244–251.

Schulze JO, Quedenau C, Roske Y, Adam T, Schuler H, Behlke J, Turnbull AP, et al. 2008. Structural and functional characterization of human Iba proteins. FEBS J 275:4627–4640.

Schwab JM, Frei E, Klusman I, Schnell L, Schwab ME, Schluesener HJ. 2001. AIF-1 expression defines a proliferating and alert microglial/macrophage phenotype following spinal cord injury in rats. J Neuroimmunol 119:214–222.

Tahtouh M, Croq F, Vizioli J, Sautiere PE, Van Camp C, Salzet M, Daha MR, et al. 2009. Evidence for a novel chemotactic C1q domain-containing factor in the leech nerve cord. Mol Immunol 46:523–531.

Tahtouh M, Garcon-Bocquet A, Croq F, Vizioli J, Sautiere PE, Van Camp C, Salzet M, et al. 2012. Interaction of HmC1q with leech microglial cells: involvement of C1qBP-related molecule in the induction of cell chemotaxis. J Neuroinflammation 9:37.

Tanaka S, Kato H, Koike T. 2000. Microglial Response Factor (MRF)-1: Constitutive expression in ramified microglia and upregulation upon neuronal death induced by ischemia or glutamate exposure. Zool Sci 17:571–578.

Tanaka S, Koike T. 2002. Selective inflammatory stimulations enhance release of microglial response factor (MRF)-1 from cultured microglia. Glia 40:360–371.

Tastet C, Lescuyer P, Diemer H, Luche S, van Dorsselaer A, Rabilloud T. 2003. A versatile electrophoresis system for the analysis of high- and low-molecular-weight proteins. Electrophoresis 24:1787–1794.

Tran CT, Wolz P, Egensperger R, Kosel S, Imai Y, Bise K, Kohsaka S, et al. 1998. Differential expression of MHC class II molecules by microglia and neoplastic astroglia: relevance for the escape of astrocytoma cells from immune surveillance. Neuropathol Appl Neurobiol 24: 293–301.

Utans U, Arceci RJ, Yamashita Y, Russell ME. 1995. Cloning and characterization of allograft inflammatory factor-1: a novel macrophage factor identified in rat cardiac allografts with chronic rejection. J Clin Invest 95:2954–2962.

Utans U, Quist WC, McManus BM, Wilson JE, Arceci RJ, Wallace AF, Russell ME. 1996. Allograft inflammatory factory-1. A cytokine-responsive macrophage molecule expressed in transplanted human hearts. Transplantation 61:1387–1392.

Vergote D, Macagno ER, Salzet M, Sautiere PE. 2006. Proteome modifications of the medicinal leech nervous system under bacterial challenge. Proteomics 6:4817–4825.

Xu Y, Bolton B, Zipser B, Jellies J, Johansen KM, Johansen J. 1999. Gliarin and macrolin, two novel intermediate filament proteins specifically expressed in sets and subsets of glial cells in leech central nervous system. J Neurobiol 40:244–253.

Zhang L, Zhao J, Li C, Su X, Chen A, Li T, Qin S. 2010. Cloning and characterization of allograft inflammatory factor-1 (AIF-1) from Manila clam Venerupis philippinarum. Fish Shellfish Immunol 30:148–153.

Zhang Y, Li J, Yu F, He X, Yu Z. 2013. Allograft inflammatory factor-1 stimulates hemocyte immune activation by enhancing phagocytosis and expression of inflammatory cytokines in Crassostrea gigas. Fish Shellfish Immunol 34:1071–1077.

Zhao YY, Yan DJ, Chen ZW. 2013. Role of AIF-1 in the regulation of inflammatory activation and diverse disease processes. Cell Immunol 284:75–83.