Chapter 5

Neuroprotection and Immunity in the Medicinal Leech *Hirudo medicinalis*: What About Microglia?

Jacopo Vizioli, Francesco Drago, Christophe Lefebvre
University of Lille – Science and Technology, Villeneuve D'Ascq, France

THE MEDICINAL LEECH CENTRAL NERVOUS SYSTEM

Hirudo medicinalis, and all other leeches, belong to the phylum Annelida and are members of the Lophotrochozoa, which also includes mollusks, brachiopods, flatworms, and nemerteans. The medicinal leech, *H. medicinalis*, was studied initially in the field of neuroscience because of the particular structure of its central nervous system (CNS).[1] The leech CNS is a tubular nerve cord composed of 1 head ganglion, 21 body ganglia, and 7 merged tail ganglia. All these ganglia are connected by a beam of fibers termed the connectives consisting of two large lateral bundles of nerve fibers and the medial Faivre's nerve. The neuronal cell bodies are located within the ganglia and extend their own axons into the connectives. In addition to the neurons, a few giant glial cells surround the axons in the connectives and six-packet glial cells sheathe the neuronal cell bodies in each ganglion. Another type of small resident immune cell, the microglia, is found in each connective and ganglion. The leech nerve cells were studied notably for their electrophysiological properties. Because the nerve cord accessibility allows for the chemical destruction of single cells, specific neurons were discriminated functionally and mapped in each ganglion, leading to the description of three groups of sensory neurons—named touch (T), pressure (P), and nociceptive (N) cells—and one type of motor neuron (M).[2] In vivo electrophysiological measures of single-altered neurons were correlated to locomotive behavior. The leech CNS undergoes synapse regeneration as a natural and functional mechanism leading to the restoration of locomotion. In this context, some authors showed that individual neurons develop new synaptic connections with a high degree of specificity following a lesion.[3,4]

Lessons in Immunity: From Single-Cell Organisms to Mammals
http://dx.doi.org/10.1016/B978-0-12-803252-7.00005-9
Copyright © 2016 Elsevier Inc. All rights reserved.

This ability to regenerate was also verified by valuable in vitro experiments from leech ganglia isolated and maintained in culture.[5–7] The nerve repair processes were progressively investigated by taking into account the importance of glial cell types. The individual destruction of giant glial cells showed no alteration in the mechanism of synaptic reconnection suggesting that these cells are not essential in this process.[8,9] In contrast, the involvement of the microglia is very important, as described below.

MICROGLIA AS BRAIN IMMUNE CELLS

Microglial cells are brain-resident macrophages involved in neurogenesis, neuronal growth, and immune-related functions. These CNS-resident immune cells were first named "microglia" by del Rio-Hortega who studied them in the medicinal leech using the silver carbonate method.[10] In mammals, they are described generally as the first effectors in the case of inflammation, trauma, or other neuronal pathologies,[11] but microglia should be mostly considered as multitasking cells involved in a large panel of functions under physiological and pathological states (eg, phagocytosis, vessel patterning, synaptic refinement, or immunosurveillance).[12] The origin of microglia is linked to yolk-derived macrophages that, through blood circulation, migrate and colonize the brain.[13] Subsequently, the resident microglia help neurogenesis and axonal growth and release neurotrophic factors necessary for brain development. In the adult brain, two populations of macrophage/microglia exist, commonly described as resident and infiltrating cells. Resident microglia constitute a pool of macrophages tightly associated with neurons and act as sentinel cells in homeostasis and brain protection. In the case of nerve tissue injury, microglia are activated: the resident cells develop a ramified shape, transform into a retracted form devoid of filopodia, and start migrating toward the affected area, becoming "reactive microglia." When disease or injury occurs (trauma, infectious, or autoimmune diseases), blood-derived monocytes and bone marrow-derived cells infiltrate the brain, pass through the blood–brain barrier, and migrate to the affected area. The resident and infiltrating macrophages/microglia are similar morphologically and are not easily distinguishable, but the expression of specific surface molecules permits differentiation between them.[14,15] The end result is a large panel of microglia/macrophages that exhibit both neuroprotective and neurotoxic effects. The discrimination of functional features between these two cell types—usually classified in specific "microglial phenotype"—is essential for elucidating neuroimmune responses involving activated cells. This is why alternative models, such as the medicinal leech, which present negligible infiltration of blood immune cells, are advantageous in developing new insights into the CNS-resident immune cell response. Microglia-like cells have been reported occasionally in some invertebrate species. Indeed, the presence of a subset of glial cells sharing structural and functional similarities with vertebrate microglia was identified in mollusks and insects, but their characterization remains poorly

explored.[16] These cells are now studied principally in vertebrates such as the mouse and zebra fish.[17] The second one, in particular, is a promising model for structural and functional studies on microglia because of the possibility to couple genetic approaches with neuroimaging techniques.[18] The leech remains the only invertebrate model for the study of microglia functions due to the structure of its CNS.[17] The comparison between leech and mouse/zebra fish models would help the understanding of evolutionary-conserved processes regulating microglia functions in brain immunity.

In *H. medicinalis*, resident microglia are the "immune triggers" for axonal regeneration since a very low number of blood cells infiltrates the CNS upon injury.[19] Within 24 h following damage to the connectives, resident microglial cells migrate to the lesion. When microglial accumulation is inhibited, a significant reduction in axonal sprouting of damaged neurons occurs, which illustrates the importance of microglia in the natural repair of injured axons.[20] As previously mentioned, the giant glial cells are not involved in the initiation of nerve repair.[8,9] The neuroimmune response in leeches is supported by microglia as well as by neurons. Taking into account the nerve repair process, we may suggest a particular neuroprotective phenotype for leech microglia.

IMMUNE RESPONSE AGAINST PATHOGENS

Differential display proteomic analyses showed that the leech CNS is an immunocompetent organ able to respond to bacterial challenges by modulating the profile of several proteins, including cytoskeletal and metabolic proteins, foldases, kinases, and neurohemerythrin.[21] The modulation of these molecules might reflect a cytoskeletal reorganization linked to cell migration, vesicular trafficking, and/or phagocytosis. Some of the regulated proteins, such as neurohemerythrin and gliarin, are expressed specifically in glial and microglial cells suggesting that, as in the vertebrates, these cells are involved in leech CNS immune responses.[22]

The immune competency of the leech nervous system and its role in producing antimicrobial peptides (AMPs) upon bacterial challenges have been demonstrated.[23] Two AMPs called neuromacin and *Hm*lumbricin (*Hm* for *Hirudo medicinalis*) were purified and identified from the nerve cord of immune-challenged leeches. Neuromacin is active only against gram-positive bacteria. *Hm*lumbricin, because of the similarity to its earthworm counterpart, is predicted to be active against fungi, gram-positive bacteria, and gram-negative bacteria. Both AMPs were observed in the injured area of connectives a few hours after axotomy while only neuromacin was detected in microglia surrounding neuron bodies. *Hm*lumbricin and neuromacin genes were not modulated by the axotomy itself, but they were rapidly upregulated upon an immune challenge. Both genes were induced by gram-positive bacteria stimulation, and the *Hm*lumbricin expression was also upregulated by CNS exposure to zymosan. These results indicate that the leech CNS is able to mount a pathogen-specific response that discriminates among different microbial components.[21]

Interestingly, the addition of native *Hm*lumbricin and neuromacin promoted the wound healing of an injured nervous system, and this effect is inhibited by the addition of specific antibodies. While it is doubtful that these AMPs contribute to axonal outgrowth directly, their presence in the injured area might improve the cleansing events associated with a lesion and thus enhance the nerve repair process in *H. medicinalis*.

Analysis of the leech genome revealed the presence of key molecules involved in pathogen recognition, which is necessary to establish a specific neuroimmune response toward microbial components.[24–26] Two members of the MyD88 family (*Hm*MyD88 and *Hm*SARM), adaptors of Toll-like receptors (TLRs), were characterized.[27] Lipopolysaccharide (LPS) may induce a redistribution of *Hm*MyD88 at the surface of neurons demonstrating for the first time the response of neurons to LPS exposure through a MyD88-dependent signaling pathway. Thus leech neurons and microglia express pattern recognition receptors regulating the differential production of AMPs, such as neuromacin and *Hm*lumbricin, as well as the release of the cytokine *Hm* endothelial monocyte-activating polypeptide II (*Hm*EMAPII).[23,24,28] These results indicate that a neuroimmune response is specifically triggered in leeches upon a septic challenge or CNS injury.[25,26,29]

The antimicrobial response triggered by pathogens is also associated with a cell-mediated immune response. In mammals, microglial cells have an important phagocytic role in the CNS, removing damaged neurons, cell debris, and apoptotic cells.[30] Similarly, in leeches, microglia gathered at the lesion to clear the cellular debris associated to tissue damage.[31] In vitro, leech microglia can phagocytize fluorescein isothiocyanate-labeled bacteria (*Aeromonas hydrophila*).[29] Bacteria were detectable inside the cells within 10 min of incubation and the phagocytosis process continued for at least 6 h. These data suggest a rapid clearing mechanism by resident microglia that in a short time phagocytizes the debris issued from the lesion and cleans the area.

MICROGLIA RECRUITMENT

The previous data show that leech microglial cells have properties similar to their mammalian counterparts through their mobility, phagocytic activity, and morphological changes during the activation processes.

Following an experimental lesion, a massive accumulation of microglial cells can be observed at the injury site (Fig. 5.1A). Microglia start moving within a few minutes postaxotomy. Their accumulation can be observed at the lesion site within 2 h and continues up to 24 h. As indicated earlier, this mechanism is essential for synapse reconstitution, axonal sprouting, and functional recovery in leeches.[18] This mobility postinjury was functionally investigated in order to specify the molecular mechanisms mediating their time-course accumulation. Several chemotactic signals coming from neurons or glial cells were identified.

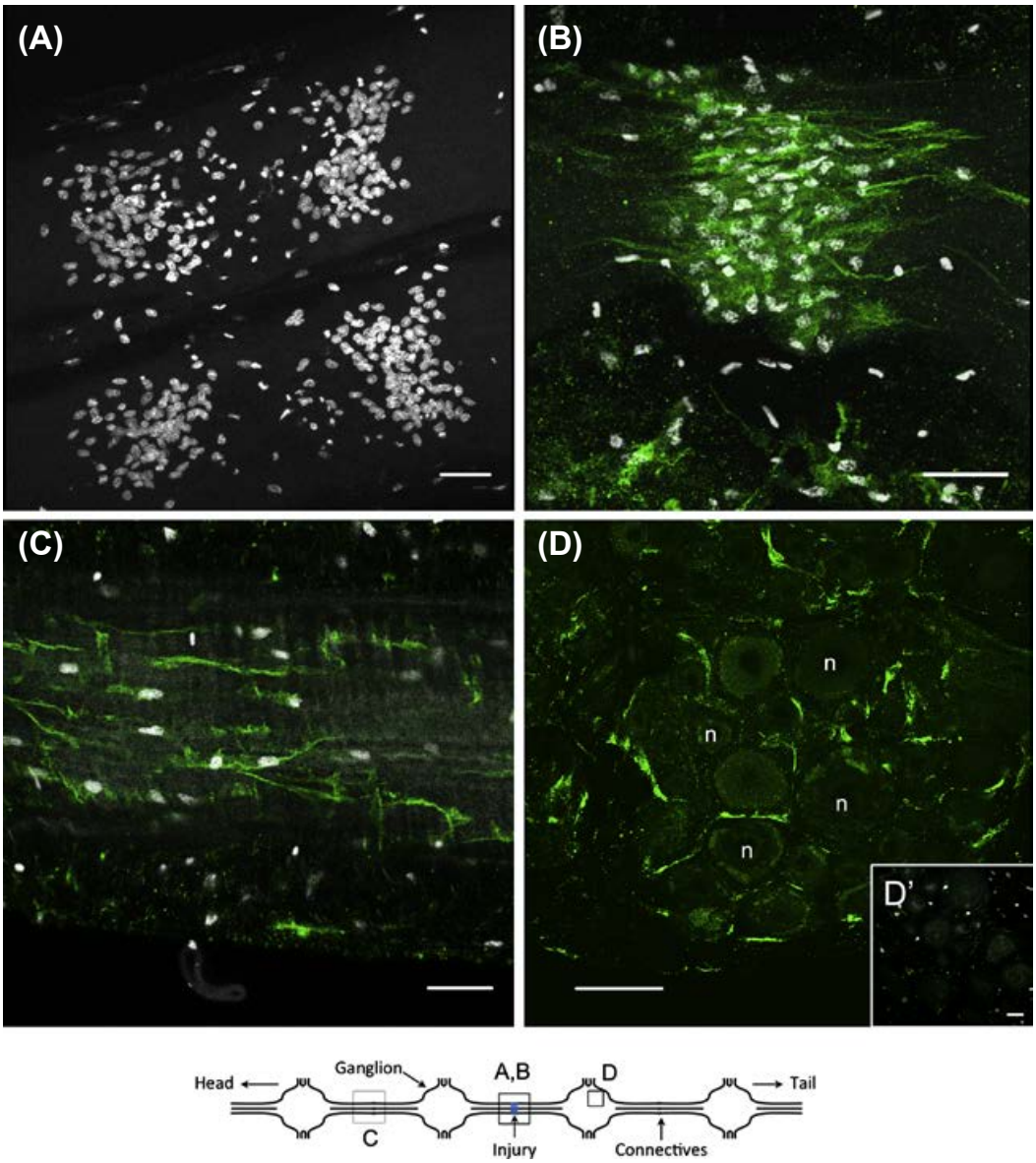

FIGURE 5.1 Confocal microscopy imaging showing the activated microglia from *Hirudo medicinalis* nerve cords cultured at different time points. (A) Nuclear staining of connective microglial cells accumulated at the lesion site in the leech CNS (24 h postaxotomy). (B–D) Immunohistochemistry on whole-mounted leech CNS carried out with anti-*Hm*Iba1 antibodies. (B) Accumulation of *Hm*Iba1-positive cells at the lesion site (6 h postaxotomy). (C) Immunostaining of resident microglia in a nonlesioned connective segment (24-h-cultured nerve cord). (D) *Hm*Iba1-positive microglial cells surrounding neuron bodies (n) of a ganglion (3-day-cultured nerve cord). (D′) *Hm*Iba1 immunostaining of a freshly dissected ganglion (T0h). Cell nuclei in A, B, C, and D′ were counterstained with Hoechst 33342. Scale bars correspond to 20 µm. In the bottom, a schematic representation of leech CNS organization indicating (boxes) the connective sites and the ganglion area illustrated in figures (A)–(D).

ATP is considered a general microglial activator exerting a "go" signal for migration. Its extracellular release by nerve cells is regulated through innexin/pannexin channels.[32] Its recognition by microglia might require purinergic receptors since a specific antagonist was observed to attenuate the migration.[33]

Nitric oxide (NO) is responsible for recruiting leech microglia within the first minutes following a lesion.[34,35] In addition, NO activates the accumulation of microglia at low concentrations far from the lesion site and stops their migration at high concentrations at the injury site.[34] These data suggest that NO contributes to microglial cell movement by regulating their directionality, and NO has been identified as the first diffusible molecule for the microglial movement toward the lesion.

Furthermore, the balance of NO and ATP-release, contributing to the microglia accumulation at the injury site, might be regulated by endocannabinoids, namely, N-arachidonylethanolamide and 2-arachidonoyl glycerol.[36] Arachidonic acid is also involved in this regulatory process. Indeed, injury releases arachidonic acid that blocks the ATP-release through the inhibition of innexin/pannexin channels. Consequently, arachidonic acid acts as an endogenous regulator spreading out a stop signal to microglia at lesions.[37] In addition, the involvement of ATP and NO correlates the microglial recruitment with the initiation of axonal sprouting.[20]

Other immune effectors homologous to vertebrate molecules (called *Hm*EMAPII, *Hm*IL-16, and *Hm*C1q) playing an important role in the microglia recruitment were identified in the leech CNS.

EMAPII is a marker of microglial cell reactivity[38] and is highly produced by activated microglia in neurodegenerative pathologies.[39] The first chemotactic function of EMAPII on microglia has been described in the leech CNS.[28] *Hm*EMAPII production in leeches is associated with TLR-dependent pathways.[24,28]

Interleukin-16 (IL-16) was initially identified in mammals as a lymphocyte chemoattractant factor,[40,41] and it is produced by lymphocytes and microglia as a proinflammatory cytokine.[42] Human IL-16 attracts $CD4^+$ lymphocytes under pathological conditions.[43] In the brain, it would regulate inflammatory processes after axonal damage.[44,45] Its leech homolog, *Hm*IL-16, is produced in neurons following a lesion, is released at the axonal ends, and serves to chemoattract microglia.[46] Studies using neutralizing antibodies on leech microglia accumulation suggest the involvement of receptors homologous to CXCR3 and CD4 that are used for mammalian EMAPII and IL-16, respectively.[28,46]

C1q is considered as an inflammatory mediator in mammals and seems to be a key molecule in neurodegenerative pathologies.[47,48] As shown in the leech CNS for *Hm*C1q,[49] its mammalian form is known to drive microglial activation after neuronal and/or microglial production.[50] Two different receptors, called *Hm*C1qBP and *Hm*CalR (calreticulin), were characterized in *H. medicinalis* and would be expressed by distinct microglial subpopulations.[51,52] In mammals, these C1q-interacting receptors have not been demonstrated in nerve cells but only in peripheral dendritic cells.[53]

In *H. medicinalis*, ex vivo experiments can be performed on cultured segments of isolated nerve cords, and microglial cells are still able to accumulate following the experimental lesion of the tissue. This ex vivo approach using nuclear dyes allows us to observe the recruitment of microglia under different

conditions. In vitro chemotactic assays were developed as well using primary cultures of leech microglia. Both approaches showed that leech microglia can be partially chemoattracted by the human forms of EMAPII, IL-16, and C1q.[28,46,49]

As explained earlier, cells gather at an experimental lesion under specific chemoattractant signals, suggesting the existence of several microglia subpopulations that differ in migration chronology and activation patterns. This differential reactivity indicates that specific microglial cells may have different functions in the context of axonal repair in early (minutes/hours) as well as late (days) events.

As suggested in mammals, resident microglia must be considered as a mosaic of different reactive subsets responsive to several neurochemical signals.[54] Since a high conservation of recognition mechanisms for chemotactic signals was observed between leeches and mammals, the *H. medicinalis* microglia represent an interesting model to decipher the molecular signaling in microglia recruitment.

NEUROINFLAMMATORY MARKERS

Similarly to vertebrates, the microglia of *H. medicinalis* are activated upon bacterial challenges or CNS injury. The processes associated with a CNS lesion involve the reactivity of several hundreds of microglial cells. In the first hours following the injury, the activation process is linked to (1) a morphological modification of cells that undergo a change from a stellate to a spindled shape, (2) a migration along connective fibers toward the injured area, and (3) their accumulation in the axotomy zone (Fig. 5.1A). For 20 years, gliarin was the only general glial marker described in the leech CNS.[55,56] This molecule, belonging to the intermediate filament protein superfamily, highlighted the different morphologies of microglial cells according to their location and the lesion status of the nerve cord.[29] A novel microglial marker, *Hm*Iba1, was characterized in *Hirudo*.[57] This protein is significantly similar to the vertebrate ionized calcium-binding adaptor molecule 1 (Iba1), also known as Aif-1 (allograft inflammatory factor 1). Iba1 is a largely used microglial marker in mammalian models because of its specific expression in activated cells. The expression of Iba1 in mammals is linked to different pathologies such as cancer, autoimmune diseases, or brain injuries, but its function remains poorly understood.[58] Similarly to its vertebrate counterpart, *Hm*Iba1 is specifically expressed in leech microglia upon an experimental lesion (Fig. 5.1). *Hm*Iba1 is the first marker for activated microglia described to date in invertebrates, and its modulation upon inflammatory conditions looks similar to that which occurs in rodent models.[57] Interestingly, this protein is only present in some of the hundreds of cells gathered at the lesion site a few hours postaxotomy (Fig. 5.1B). Other microglial cells accumulate at the lesion despite their lack of *Hm*Iba1 signals. This result supports the hypothesis of different microglial populations migrating to the challenged area and displaying different functions and/or activation states.

Indeed, *Hm*Iba1 immunostaining is not specifically associated to migrating cells but only indicates their activation state. Fig. 5.1C shows some *Hm*Iba1-positive cells in a nonlesioned connective 24 h after lesion of the neighboring connective. A similar result was observed for resident microglia in ganglia (Fig. 5.1D) that indicates that microglia that are not directly involved in migration and accumulation events are nonetheless in an activated state. In naïve conditions, these cells in both connectives (data not shown) and ganglia (Fig. 5.1D′) show a weak expression level of *Hm*Iba1.

Similarly to vertebrate Iba1, the leech gene is induced by ATP.[57] Because of the similarities of leech and mammalian microglia described earlier, novel activation markers associated to neuroinflammatory events in the leech have been identified. Preliminary studies from leech CNS suggest the presence of some additional proteins (CD11b, CD45, progranulin), generally expressed in vertebrate macrophages and microglia upon inflammatory conditions.[26,59] These results suggest a conservation of basic mechanisms controlling cell activation and inflammation during postaxotomy events in leech microglia compared to mammalian models.

TOWARD NERVE REPAIR: MICROGLIA/NEURONS CROSSTALK INTO THE SPOTLIGHT

Upon an experimental lesion, leech microglia promote wound repair and axonal sprouting. The molecular crosstalk between microglia and neurons in this neuroprotection is largely unknown. In the CNS, intercellular communication is mediated by (1) synapses, (2) the secretion of soluble molecules, or (3) the release of extracellular vesicles (EVs) containing various effectors. The third mechanism seems to be important in the leech: a massive presence of EV-related structures interacting with the neuron surface was observed during the coculture of leech microglia and neurons.[60] When neurons were cultured alone, many fewer EVs were detected, suggesting that EVs mainly derive from microglial cells.

Under physiological conditions and various disease states, cells secrete two types of EVs. The first type, the exosomes (50–100 nm in diameter), are generated inside multivesicular bodies (MVBs) and are released upon MVB fusion with the plasma membrane. The second type of EVs, the ectosomes, also called shed vesicles/particles (100 nm–1 μm in diameter), bud from the plasma membrane. The protein composition of EVs varies depending on the cellular origin and plays a role in the phenotypic response or programmed cell death events. Moreover, the EVs contain (1) mRNAs that can be translated to proteins by cells receiving the vesicles and (2) miRNAs that regulate posttranscriptional processes in target cells. The EVs, released from many cell types, are being studied as potential markers of physiological and pathological conditions. Their relevance in neurogenesis, as well as in pathogenesis of several CNS disorders, has only begun to be explored, according to their production by neurons and glial cells.[61,62] The EVs are involved in neurite outgrowth after axonal damage

as well as during axonal regeneration.[63] Moreover, the ability of EVs to cross the blood–brain barrier and their potential to be manipulated genetically have created a great interest in EVs as natural vectors for the delivery of therapeutic agents (RNAs and/or proteins).[64,65] Proteomic studies are currently being undertaken to investigate the content of leech-activated microglia EVs associated to general (ATP) or specific (C1q, EMAPII, and IL-16) stimulation.[66] The characterization of EV contents (proteins and RNAs) and their correlation with neurite outgrowth constitute an interesting approach to bringing new insights into the microglial role leading to neuroprotection and highlight their functions in the leech as well as in vertebrate models.

CONCLUSIONS

The medicinal leech represents an intriguing model to study different phenomena in neurobiology from immune response to nerve repair. Its CNS constitutes a relatively simple system easily accessible in vivo and very useful for ex vivo studies. It has multiple roles, including as an immune competent organ able to mount an effective humoral and cell-mediated immune response against pathogens. The *H. medicinalis* CNS is particularly interesting because of its ability to repair itself naturally following injury. In this context, microglial cells play a pivotal role, being at the interface between immunity and axonal repair. These resident cells exhibit multiple functions. Their study will bring new insights into the comprehension of neuroimmune and neuroinflammatory mechanisms controlling nerve repair in several animal models.

REFERENCES

1. Coggeshall RE, Fawcett DW. The fine structure of the central nervous system of the leech, *Hirudo Medicinalis*. *J Neurophysiol* 1964;**27**:229–89.
2. Nicholls JG, Baylor DA. Specific modalities and receptive fields of sensory neurons in CNS of the leech. *J Neurophysiol* 1968;**31**:740–56.
3. Baylor DA, Nicholls JG. Patterns of regeneration between individual nerve cells in the central nervous system of the leech. *Nature* 1971;**232**:268–70.
4. Jansen JK, Nicholls JG. Regeneration and changes in synaptic connections between individual nerve cells in the central nervous system of the leech. *Proc Natl Acad Sci USA* 1972;**69**: 636–9.
5. Wallace BG, Adal MN, Nicholls JG. Regeneration of synaptic connections by sensory neurons in leech ganglia maintained in culture. *Proc R Soc Lond B Biol Sci* 1977;**199**:567–85.
6. Muller KJ, Scott SA. Correct axonal regeneration after target cell removal in the central nervous system of the leech. *Science* 1979;**206**:87–9.
7. Muller KJ, Scott SA. Removal of the synaptic target permits terminal sprouting of a mature intact axon. *Nature* 1980;**283**:89–90.
8. Elliot EJ, Muller KJ. Synapses between neurons regenerate accurately after destruction of ensheathing glial cells in the leech. *Science* 1982;**215**:1260–2.
9. Elliott EJ, Muller KJ. Sprouting and regeneration of sensory axons after destruction of ensheathing glial cells in the leech central nervous system. *J Neurosci* 1983;**3**:1994–2006.

10. Del Rio-Hortega P. Cytology and cellular pathology of the nervous system. In: Penfield W, editor. *Microglia*. New York (NY): P.B. Hoebaer; 1932. p. 483–534.
11. Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. *Physiol Rev* 2011;**91**:461–553.
12. Casano AM, Peri F. Microglia: multitasking specialists of the brain. *Dev Cell* 2015;**32**:469–77.
13. Swinnen N, Smolders S, Avila A, et al. Complex invasion pattern of the cerebral cortex bymicroglial cells during development of the mouse embryo. *Glia* 2013;**61**:150–63.
14. Prinz M, Priller J, Sisodia SS, Ransohoff RM. Heterogeneity of CNS myeloid cells and their roles in neurodegeneration. *Nat Neurosci* 2011;**14**:1227–35.
15. Butovsky O, Jedrychowski MP, Moore CS, et al. Identification of a unique TGF-beta-dependent molecular and functional signature in microglia. *Nat Neurosci* 2014;**17**:131–43.
16. Sonetti D, Ottaviani E, Bianchi F, et al. Microglia in invertebrate ganglia. *Proc Natl Acad Sci USA* 1994;**91**:9180–4.
17. Sieger D, Peri F. Animal models for studying microglia: the first, the popular, and the new. *Glia* 2013;**61**:3–9.
18. Eyo UB, Dailey ME. Microglia: key elements in neural development, plasticity, and pathology. *J Neuroimmune Pharmacol* 2013;**8**:494–509.
19. Boidin-Wichlacz C, Vergote D, Slomianny C, Jouy N, Salzet M, Tasiemski A. Morphological and functional characterization of leech circulating blood cells: role in immunity and neural repair. *Cell Mol Life Sci* 2012;**69**:1717–31.
20. Ngu EM, Sahley CL, Muller KJ. Reduced axon sprouting after treatment that diminishes microglia accumulation at lesions in the leech CNS. *J Comp Neurol* 2007;**503**:101–9.
21. Vergote D, Macagno ER, Salzet M, Sautiere PE. Proteome modifications of the medicinal leech nervous system under bacterial challenge. *Proteomics* 2006;**6**:4817–25.
22. Vergote D, Sautiere PE, Vandenbulcke F, et al. Up-regulation of neurohemerythrin expression in the central nervous system of the medicinal leech, *Hirudo medicinalis*, following septic injury. *J Biol Chem* 2004;**279**:43828–37.
23. Schikorski D, Cuvillier-Hot V, Leippe M, et al. Microbial challenge promotes the regenerative process of the injured central nervous system of the medicinal leech by inducing the synthesis of antimicrobial peptides in neurons and microglia. *J Immunol* 2008;**181**:1083–95.
24. Cuvillier-Hot V, Boidin-Wichlacz C, Slomianny C, Salzet M, Tasiemski A. Characterization and immune function of two intracellular sensors, *Hm*TLR1 and *Hm*NLR, in the injured CNS of an invertebrate. *Dev Comp Immunol* 2011;**35**:214–26.
25. Tasiemski A, Salzet M. Leech immunity: from brain to peripheral responses. *Adv Exp Med Biol* 2010;**708**:80–104.
26. Macagno ER, Gaasterland T, Edsall L, et al. Construction of a medicinal leech transcriptome database and its application to the identification of leech homologs of neural and innate immune genes. *BMC Genomics* 2010;**11**:407.
27. Rodet F, Tasiemski A, Boidin-Wichlacz C, et al. *Hm*-MyD88 and *Hm*-SARM: two key regulators of the neuroimmune system and neural repair in the medicinal leech. *Sci Rep* 2015;**5**:9624.
28. Schikorski D, Cuvillier-Hot V, Boidin-Wichlacz C, Slomianny C, Salzet M, Tasiemski A. 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* 2009;**183**:7119–28.
29. Le Marrec-Croq F, Drago F, Vizioli J, Sautiere PE, Lefebvre C. The leech nervous system: a valuable model to study the microglia involvement in regenerative processes. *Clin Dev Immunol* 2013;**2013**:274019.
30. Fu R, Shen Q, Xu P, Luo JJ, Tang Y. Phagocytosis of microglia in the central nervous system diseases. *Mol Neurobiol* 2014;**49**:1422–34.

31. Morgese VJ, Elliott EJ, Muller KJ. Microglial movement to sites of nerve lesion in the leech CNS. *Brain Res* 1983;**272**:166–70.
32. Samuels SE, Lipitz JB, Dahl G, Muller KJ. Neuroglial ATP release through innexin channels controls microglial cell movement to a nerve injury. *J Gen Physiol* 2010;**136**:425–42.
33. Duan Y, Sahley CL, Muller KJ. ATP and NO dually control migration of microglia to nerve lesions. *Dev Neurobiol* 2009;**69**:60–72.
34. Chen A, Kumar SM, Sahley CL, Muller KJ. Nitric oxide influences injury-induced microglial migration and accumulation in the leech CNS. *J Neurosci* 2000;**20**:1036–43.
35. Kumar SM, Porterfield DM, Muller KJ, Smith PJ, Sahley CL. Nerve injury induces a rapid efflux of nitric oxide (NO) detected with a novel NO microsensor. *J Neurosci* 2001;**21**:215–20.
36. Arafah K, Croix D, Vizioli J, Desmons A, Fournier I, Salzet M. Involvement of nitric oxide through endocannabinoids release in microglia activation during the course of CNS regeneration in the medicinal leech. *Glia* 2013;**61**:636–49.
37. Samuels SE, Lipitz JB, Wang J, Dahl G, Muller KJ. Arachidonic acid closes innexin/pannexin channels and thereby inhibits microglia cell movement to a nerve injury. *Dev Neurobiol* 2013;**73**:621–31.
38. Schluesener HJ, Seid K, Meyermann R. Effects of autoantigen and dexamethasone treatment on expression of endothelial-monocyte activating polypeptide II and allograft-inflammatory factor-1 by activated macrophages and microglial cells in lesions of experimental autoimmune encephalomyelitis, neuritis and uveitis. *Acta Neuropathol* 1999;**97**:119–26.
39. Mueller CA, Schluesener HJ, Conrad S, Meyermann R, Schwab JM. Spinal cord injury induces lesional expression of the proinflammatory and antiangiogenic cytokine EMAP II. *J Neurotrauma* 2003;**20**:1007–15.
40. Center DM, Cruikshank W. Modulation of lymphocyte migration by human lymphokines. I. Identification and characterization of chemoattractant activity for lymphocytes from mitogen-stimulated mononuclear cells. *J Immunol* 1982;**128**:2563–8.
41. Cruikshank W, Center DM. Modulation of lymphocyte migration by human lymphokines. II. Purification of a lymphotactic factor (LCF). *J Immunol* 1982;**128**:2569–74.
42. Center DM, Kornfeld H, Ryan TC, Cruikshank WW. Interleukin 16: implications for CD4 functions and HIV-1 progression. *Immunol Today* 2000;**21**:273–80.
43. Schluesener HJ, Seid K, Kretzschmar J, Meyermann R. Leukocyte chemotactic factor, a natural ligand to CD4, is expressed by lymphocytes and microglial cells of the MS plaque. *J Neurosci Res* 1996;**44**:606–11.
44. Mittelbronn M, Dietz K, Schluesener HJ, Meyermann R. Local distribution of microglia in the normal adult human central nervous system differs by up to one order of magnitude. *Acta Neuropathol* 2001;**101**:249–55.
45. Skundric DS, Cai J, Cruikshank WW, Gveric D. Production of IL-16 correlates with $CD4^+$ Th1 inflammation and phosphorylation of axonal cytoskeleton in multiple sclerosis lesions. *J Neuroinflammation* 2006;**3**:13.
46. Croq F, Vizioli J, Tuzova M, et al. A homologous form of human interleukin 16 is implicated in microglia recruitment following nervous system injury in leech *Hirudo medicinalis*. *Glia* 2010;**58**:1649–62.
47. Bergamaschini L, Donarini C, Gobbo G, Parnetti L, Gallai V. Activation of complement and contact system in Alzheimer's disease. *Mech Ageing Dev* 2001;**122**:1971–83.
48. Kishore U, Gaboriaud C, Waters P, et al. C1q and tumor necrosis factor superfamily: modularity and versatility. *Trends Immunol* 2004;**25**:551–61.
49. Tahtouh M, Croq F, Vizioli J, et al. Evidence for a novel chemotactic C1q domain-containing factor in the leech nerve cord. *Mol Immunol* 2009;**46**:523–31.

50. Farber K, Cheung G, Mitchell D, et al. C1q, the recognition subcomponent of the classical pathway of complement, drives microglial activation. *J Neurosci Res* 2009;**87**:644–52.
51. Tahtouh M, Garcon-Bocquet A, Croq F, et al. Interaction of *Hm*C1q with leech microglial cells: involvement of C1qBP-related molecule in the induction of cell chemotaxis. *J Neuroinflammation* 2012;**9**:37.
52. Le Marrec-Croq F, Bocquet-Garcon A, Vizioli J, et al. Calreticulin contributes to C1q-dependent recruitment of microglia in the leech *Hirudo medicinalis* following a CNS injury. *Med Sci Monit* 2014;**20**:644–53.
53. Vegh Z, Kew RR, Gruber BL, Ghebrehiwet B. Chemotaxis of human monocyte-derived dendritic cells to complement component C1q is mediated by the receptors gC1qR and cC1qR. *Mol Immunol* 2006;**43**:1402–7.
54. Pannell M, Szulzewsky F, Matyash V, Wolf SA, Kettenmann H. The subpopulation of microglia sensitive to neurotransmitters/neurohormones is modulated by stimulation with LPS, interferon-gamma, and IL-4. *Glia* 2014;**62**:667–79.
55. Xu Y, Bolton B, Zipser B, Jellies J, Johansen KM, Johansen J. Gliarin and macrolin, two novel intermediate filament proteins specifically expressed in sets and subsets of glial cells in leech central nervous system. *J Neurobiol* 1999;**40**:244–53.
56. Luthi TE, Brodbeck DL, Jeno P. Identification of a 70 kD protein with sequence homology to squid neurofilament protein in glial cells of the leech CNS. *J Neurobiol* 1994;**25**:70–82.
57. Drago F, Sautiere PE, Le Marrec-Croq F, et al. Microglia of medicinal leech (*Hirudo medicinalis*) express a specific activation marker homologous to vertebrate ionized calcium-binding adapter molecule 1 (Iba1/alias aif-1). *Dev Neurobiol* 2014;**74**:987–1001.
58. Zhao YY, Yan DJ, Chen ZW. Role of AIF-1 in the regulation of inflammatory activation and diverse disease processes. *Cell Immunol* 2013;**284**:75–83.
59. Vizioli J, Accorsi A, Croq F, et al. Neuroinflammation and microglia activation studies: a novel strategy using an invertebrate model, the medicinal leech. *Glia* 2011;**59–S1**:S153.
60. Le Marrec-Croq F, Van Camp C, Drago F, et al. Extracellular vesicles (EVs) from leech microglia: a tool for understanding the dialog with damaged neurons. *Glia* 2015;**63–S1**:E212–3.
61. Bahrini I, Song JH, Diez D, Hanayama R. Neuronal exosomes facilitate synaptic pruning by up-regulating complement factors in microglia. *Sci Rep* 2015;**5**:7989.
62. Gupta A, Pulliam L. Exosomes as mediators of neuroinflammation. *J Neuroinflammation* 2014;**11**:68.
63. Lopez-Verrilli MA, Picou F, Court FA. Schwann cell-derived exosomes enhance axonal regeneration in the peripheral nervous system. *Glia* 2013;**61**:1795–806.
64. El Andaloussi S, Mager I, Breakefield XO, Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov* 2013;**12**:347–57.
65. Hu G, Drescher KM, Chen XM. Exosomal miRNAs: Biological properties and therapeutic potential. *Front Genet* 2012;**3**:56.
66. Drago F, Arab T, Van Camp C, et al. Differentially activated microglia release Extracellular Vesicles (EVs) showing specific contents and functions in a model of nerve repair. *Glia* 2015;**63–S1**:E321–2.