The circulating phagocytes: the ancient and conserved interface between immune and neuroendocrine function

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ABSTRACT

Immune and neuroendocrine functions display significant overlap in highly divergent and evolutionary distant models, such as molluscs, crustaceans, insects and mammals. Fundamental players in this crosstalk are professional phagocytes, labelled as macrophages in vertebrates and as immunocytes in invertebrates. Although they have different developmental origins, macrophages and immunocytes possess comparable functions and differentiate under the control of evolutionary conserved transcription factors. Macrophages and immunocytes share their pools of receptors, signalling molecules and pathways with neural cells and the neuro-

endocrine system. In crustaceans, adult transdifferentiation of circulating hemocytes into neural cells has been recently documented. In light of developmental, molecular and functional evidence, we propose that the immune-neuroendocrine role of circulating phagocytes predates the split of protostomian and deuterostomian *superphyla* and has been conserved during the evolution of the main groups of metazoans.

Keywords: immunocytes, macrophage, invertebrate, vertebrate, immune and neuroendocrine function, evolution

CONTENTS

I. INTRODUCTION

The immune system of complex organisms is based on the equilibrated and continuous crosstalk between cellular and molecular components (Lemaitre and Hoffmann, 2007; Murphy, 2011). At the outset of immunology, the Russian scientist Elie Metchnikoff (1883, 1884) observed that some invertebrate cells, which Metchnikoff called phagocytes, were able to defend the organisms by encapsulating and engulfing non-self material. Through his experiments, Metchnikoff established, for the first time, that phagocytes represent the first line of defense against infections and that they can be detected in all organisms, from invertebrates to vertebrates.

The classification of invertebrate circulating phagocytes has been hampered by the scarce knowledge on hematopoiesis in the majority of invertebrate models and the great variety in developmental origins and cellular morphologies that comparative immunologists have described so far (Ottaviani, 2005, 2006; Grigorian and Hartenstein, 2013; Accorsi *et al.,* 2014). For the purpose of this review, we will adopt a general functional label for the circulating phagocytes of the different species considered, and will refer to “immunocytes”, *i.e.*, circulating cells exploiting innate immune functions similarly to the vertebrate phagocytes. The term immunocyte has been coined for mammalian cells (Berman, 1963; Smith and Blalock, 1988; Carr and Blalock, 1988), but could be extended to invertebrates as well due to the functional similarities between phagocytes

of metazoans (Ottaviani and Franceschi, 1997; Malagoli *et al.*, 2006; Ottaviani *et al*., 2007, 2008; Ottaviani, 2011).

While Metchinkoff performed his fundamental experiments on phagocytosis leading to the first description of the cellular component of the immune system, Paul Ehlrich (1897), made fundamental discoveries on the humoral component that we now know to include not only specific antibodies, but also innate molecules such as lisozymes, antimicrobial peptides and interferons. The cellular and the humoral immune components, then, cooperate and constitute the foundation of immune function in vertebrates and invertebrates (Lemaitre and Hoffmann, 2007). However, beside immune-related soluble factors, several other molecules have been found to influence the activity of mammalian immunocytes. Among these, great interest was raised by proopiomelanocortin (POMC)-, proenkephalin- and prodynorphin-derived peptides, whose discovery, in mononuclear leukocytes, set the basis for the field of neuroimmunology and research on neuroimmune modulation (Kowalski, 1997; Blalock, 1999; Smith, 2003). The aim of this review is to explore, in a comparative perspective, the interconnections between immune response and neuro-hormones, evidencing the ancient origins of their interrelationship. We will especially refer to mammals as vertebrate models and to molluscs, insects and crustaceans as invertebrate models.

II. NEURO-IMMUNOMODULATION OF THE IMMUNE RESPONSE IN VERTEBRATES ~~MAMMALS~~

POMC is a large precursor protein that contains various active peptides, formed by cleavage of POMC into structurally and functionally different fragments (Mains *et al.,* 1977; Roberts and Herbert, 1977). In mammals, POMC precursors are glycosylated with a molecular weight that varies from 31 to 36 kDa which changes in relation to the level of glycosylation. The sequence of POMC mRNA and the structure of the POMC gene has been first determined in humans by Cohen *et al.* in the 1980 (Chang *et al.,* 1980). The POMC gene structure, which is highly conserved, consists of three exons and two large introns and is present as a single copy per haploid genome; the POMC peptide consist of 241 amino acids (aa) in humans, 239 aa in cows and 209 aa in rats and mice (Eberle, 1988). The POMC C-terminal fragment, 91 aa long constitutes β-lipotropin (β-LPH). The cleavage of β-LPH produces β-endorphins (β-endorphin 1-31, β-LPH 61-91), δ-endorphin (61-87), α-endorphin (61-76) and β-melanocyte-stimulating hormone (β–MSH, 41-58). The adrenocorticotropin hormone (ACTH) (1-39) forms the POMC central region, and contains both α-MSH (the first 1-31 aa) and the corticotropin-like intermediate lobe peptide (CLIP) (18-39). The N-terminal region includes the γ-MSH fragment (Hadley and Haskell-Luevano, 1999). In all vertebrates, the transcription of the POMC gene and the proteolytic maturation of its precursor are mainly under control of the hypothalamic corticotropin-releasing hormone (CRH).

The evolution of POMC genes has been recently reviewed in vertebrates (Dores and Baron, 2011) and invertebrates (Malagoli *et al*., 2011). As indicated in greater detail in Section IV, information on POMC peptides and the relative sequences has been provided in the flatworm *Schistosoma mansoni* (Duvaux-Miret *et al.*, 1990, 1992a,b), in the leech *Theromyzon tessulatum* (Salzet *et al*., 1997, 2000a) and in the mussels *Mytilus galloprovincialis* and *Mytilus edulis* (Ottaviani *et al*., 1995; Stefano *et al.,* 1999). The analysis of POMC peptides in *M. edulis* indicate a poor conservation of the overall sequence, but a higher levels of similarity in correspondence of the peptides contained within POMC. These included a γ-MSH-like peptide (68-79) and an ACTH-like-peptide (106-145). The ACTH sequence included at positions 106-119 a α-MSH; and, in positions 120-143, a CLIP-like peptide. A β-LPH-like peptide (154-192), β-MSH-like peptide (163-170) and a met-enkephalin-like peptide (177-181) were also present (Stefano *et al*., 1999). Enkephalin-like molecules were retrieved during the '80s (Pages et al., 1983; Callaerts et al., 1988) and opioid binding sites were observed in the fruit fly (Santoro et al., 1990), but genes encoding for POMC-derived molecules does not appear to be present in the well-studied invertebrate model *Drosohpila melanogaster*.

Among the fragments of POMC, a key role in immunomodulation is played by ACTH. After CRH stimulation, corticotropin is secreted into the systemic circulation and binds to receptors expressed in adrenocortical cells to increase the biosynthesis of corticosteroids, in particular

glucocorticoids. In vertebrates, corticosteroids and their receptors have been classified into two distinct groups: glucocorticoids and mineralcorticoids that often play distinct physiological and pathological functions, but such distinctions are less clear in non-mammalian species (Denver, 2009). Corticosteroids influence development and maturation of the brain, the immune system, as well as many other organ systems. Furthermore, they also regulate complex functions, such as, energy storage and metabolism control, stimulate gluconeogenesis and feeding behavior and they influence the consolidation of learning and memory (Denver, 2009).

After the first demonstrations that in mammals POMC mRNA is present also outside the central nervous system (Lolait *et al.,* 1986; Westly *et al*., 1986), several experiments have been performed in order to characterize transcript length and peptide functions. POMC-derived peptides are numerous and their action on the immune response is frequently controversial, probably because of the variability in type of the experimental stimulus applied (Smith, 2003).

POMC is the common precursor for both the melanocortin-related peptides (ACTH/α-MSH, β-MSH, and γ-MSH) and the opioid β-endorphin, whereas the other members of the opioid/orphanin gene family, *i.e.*, proenkephalin, prodynorphin and proorphanin, are mainly precursors of opioid peptides (Dores and Baron, 2011). The involvement of opiod peptides in the immune response and inflammation of mammals has been

repeatedly reported (Sacerdote *et al.*, 2003; Smith, 2003). However, recent *in vivo* experiments and literature surveys indicate that mammalian immunocytes do not display the necessary receptors to justify the action of opioids on the immune system (Al-Hashimi *et al*., 2013). At today, the opioid receptors are classified as follows: μ or mu (MOP), δ or delta (DOP), κ or kappa (KOP) and orphanin FQ receptor (NOP). In mammals, proenkephalin is the common precursor for DOP agonists, prodynorphin is the common precursor for the KOP agonists and proorphanin is the precursor of NOP ligands (Dores and Baron, 2011). While NOP mRNA has been detected in mammalian immunocytes, this was not the case for MOP, DOP and KOP, which are also the targets for synthetic opioids (Al-Hashimi *et al*., 2013). On these basis, none of the several hypotheses that might justify the action of opioids on immune system, is at present conclusive (McCarthy *et al*., 2001; Al-Hashimi *et al*., 2013).

Even though the fine molecular details of immune-neuroendocrine interactions remain to be elucidated (Malagoli *et al*., 2011; Al-Hashimi *et al*., 2013), what is most important, for the purpose of this review, is the additional cultural step offered by the demonstration of the existence of the biochemical background necessary for bidirectional cross-talk between the immune and neuroendocrine system. Thanks to the presence of numerous mediators, the immune system of mammals has been proposed to work as a sensory organ, able to elicit a neural and physiological response towards non cognitive stimuli, such as pathogens. While in turn, the

neuroendocrine system may react to cognitive stimuli by producing molecules able to modify the activity of leukocytes (Blalock, 1999). The tight collaboration between the immune and neuroendocrine system has been revealed in mammals during the 80's, but subsequently several experiments have been successively performed that clarified the evolution and diversification of that connection (Stefano and Salzet, 1999; Salzet, 2000; Salzet *et al*., 2000b).

III. DIFFERENT ORIGINS OF INVERTEBRATE IMMUNOCYTES

One of the most difficult tasks for comparative immunologists is the distinction between homologous and similar (or convergent) functions. The label of immunocyte here proposed is useful for the description of functions, but inadequate to highlight the diversity existing among invertebrate hematopoiesis and hematopoietic sites. Hematopoiesis has been widely studied in gastropod molluscs, insects and crustaceans. While research on hematopoiesis in molluscs is short of detailed information, the knowledge about insects and crustaceans is significantly broader, with two fundamental models being *Drosophila melanogaster* and *Pacifastacus leniusculus*, respectively (Lin and Söderhäll, 2011; Grigorian and Hartenstein, 2013).

In gastropods, hematopoiesis may directly involve the circulating hemocytes (Sminia, 1972; Ottaviani, 1983). Other hematopoietic districts in gastropods are the epithelial and connective tissues of the walls of the

blood sinus, the region of heart-kidney, the mantle (Kinoti, 1971), as well as the ventricular cavity and lung (Sminia 1981). Moreover, the presence of a recognizable amebocyte producing organ has been described in *Biomphalaria glabrata* (Jeong *et al*., 1983) and in *Planorbarius corneus* (Ottaviani, 1988). Recently, in *Pomacea canaliculata* mitotic figures were found within the pericardial cavity (Accorsi *et al*., 2014). Despite the numerous observations suggesting or demonstrating hematopoietic activity in molluscs, information about hemocyte maturation and differentiation is still lacking.

In *Drosophila*, hematopoiesis takes place at two different stages during ontogenesis: a first population of hemocytes arises from the head mesoderm during early embryogenesis, followed by a second population that derives from the mesodermal lymph glands during the larval stage (Traver and Zon, 2002; Makhijani and Brückner, 2012). To this regard, Holz *et al.* (2003) suggested the use of the terms embryonic hemocytes and lymph gland hemocytes to distinguish the origin of the hemocytes circulating in adult fruit flies. Both embryonic and lymph gland hemocytes may acquire some characteristics of a macrophage. Their determination/differentiation as hemocytes depends on *serpent* and they can differentiate into podocytes, crystal cells and plasmatocytes (Lanot *et al*., 2001; Holz *et al*., 2003). Even if embryonic and lymph gland hemocytes are both present in *Drosophila* larvae, the two hemocyte

populations originate from two different mesodermal regions and are determined at different developmental stages (Holz *et al*., 2003).

P. leniusculus is a long-living crustacean presenting a well-recognizable hematopoietic tissue that produces new hemocytes all through the animal's lifetime. The difference with *Drosophila* is apparent because *P. leniusculus* does not have larval hemocytes that persist into the adult but instead it is characterized by a rather regular and continuous hematopoiesis (Lin and Söderhäll, 2011). The hematopoietic tissue of *P. leniusculus* contains five types of cells (Type 1-5 cells). Type 1 may be the precursor stem cells for the two possible cell lineages, one originating hyalinocytes (directly from Type 1 precursors) and a second originating semigranular and granular hemocytes (through the Type 2-5 developmental stages) (Lin and Söderhäll, 2011). In the tiger shrimp *Penaeus monodon* it has been proposed the existence of two lineages, one developing semigranular cells from hyaline cell and the other developing granular cells from semigranular precursors (van de Braak, 2000). In Decapods, hyalinocytes and semi-granular hemocytes are the cells more closely resembling the molluscan immunocytes, because they perform professional phagocytosis (Söderhäll *et al*., 1986). The transcriptional regulation of hemocyte maturation has not been elucidated in detail in crustaceans as it has been in *Drosophila*, but the involvement of GATA transcription factors and Runx protein has been demonstrated. The continuous and proportioned proliferation of hemocyte precursors is

regulated by the cytokines Astakine 1 and Astakine 2. The former promotes the proliferation of hemocyte precursors (Söderhäll *et al.,* 2005) and stimulates the maturation along the lineage of semigranular cells. Astakine 2 is involved in hemocyte maturation but it also exerts neuroendocrine functions (Watthanasurorot *et al*., 2013).

Despite the differences in the anatomy of hematopoietic disctricts and the morphology and functions of circulating immunocytes, molecular analyses have revealed genetic and functional similarities during the maturation of insect, crustacean and mammalian immunocytes. The family of GATA transcription factors, their co-factors (U-shaped/FOG), the AML1 domain family transcription factors (Lozenge/Runx1) (Hoffmann *et al*., 1999; Fossett and Schulz, 2001), as well as Notch signaling (Duvic *et al*., 2002), are essential for immune cell determination and differentiation into specific cytotypes.

This brief overview on the hematopoiesis in key invertebrate models shows that the term immunocyte (or the broader term hemocyte) may include cells sharing only little similarity in terms of development and maturation. The homologies among invertebrate hemocytes and between invertebrate and vertebrate immunocytes are far from being established. This notwithstanding, the interactions between immune and neural functions have been observed in several invertebrate models, including molluscs, insects and crustaceans.

IV. NEUROENDOCRINE ASPECTS OF INVERTEBRATE IMMUNOCYTE FUNCTIONS: PRESENCE AND EFFECTS OF POMC-DERIVED FRAGMENTS

In the late ‘80s, the experiments performed by Blalock and Smith in mammals (Weigent and Blalock, 1987; Smith and Blalock, 1988), prompted the research of pioneer comparative neuro-immunologists, which were working mainly with molluscs. The evidence of POMC-derived peptides in molluscan immunocytes was demonstrated using different techniques, such as immunocytochemistry, radioimmunoassay (RIA) and flow cytometry (Ottaviani and Franceschi, 1997). The freshwater snail *P. corneus*, one of the core models for the immune-neuroendocrine studies, displayed two types of immunocytes circulating in the hemolymph. One immunocytes was endowed with phagocytic activity, it was able to adhere to glass, to produce agglutinins and to bind Con A. The second type of immunocytes did not display phagocytic capacity, it did not adhere to microscope glass slides, but it could form rosettes with sheep red blood cells. The proliferation of not adhering hemocytes was stimulated by PHA and they did bind immunoglobulins specifically raised against markers of vertebrate T lymphocytes (Ottaviani, 1983, 1992). The phagocytic hemocyte positively bound anti-human ACTH and anti-human β-endorphin antibodies, while not adhering immunocytes did not contain ACTH- and β-endorphin-like material. POMC-like peptides were also observed in the phagocytic cells of *P. corneus* digestive gland, not to be confused with

circulating hemocytes (Ottaviani *et al*., 1990). ACTH-like molecules were also detected in the circulating phagocytes of several molluscan species, such as the freshwater snail *Viviparus ater* (Ottaviani *et al.,* 1995), the mussel *Mytilus galloprovincialis* (Franchini *et al.,* 1994), as well as in the hemolymph of the blue mussel *M. edulis* (Smith *et al.*, 1991). Similar observations were performed in the insect *Leucophaea maderae* (Smith *et al.,* 1991) and in the earthworm *Eisenia foetida* (Cooper *et al.,* 1995). Though circulating cells in different models should not be automatically considered as homologue, remarkably the immunocytochemical experiments evidenced POMC-like products always in cells endowed with phagocytic activity.

ACTH receptor-like mRNA was detected in molluscan immunocytes and human peripheral blood mononucleated cell (PBMC) by *in situ* hybridization using a digoxigenin-labelled bovine complementary DNA probe (Ottaviani *et al.,* 1998). Previous studies in human PBMC had demonstrated the presence of high- and low-affinity ACTH receptors (Smith *et al.,* 1987).

Immunocytochemical experiments also showed that ACTH-like molecules are present in macrophages of all the vertebrate classes, while they were only observed in the lymphocytes of tetrapods, *i.e.,* amphibians, reptiles, birds and mammals (Ottaviani *et al.,* 1992).

Morphological evidence was rapidly flanked by functional experiments, showing that human POMC-derived peptides may promote the activation

of molluscan immunocytes. Similarly to human cytokines, human POMC-derived products may promote a functional response in molluscan immunocytes. Indeed, human ACTH (1-24) affected cell motility, chemotaxis and phagocytosis of molluscan immunocytes via adenylate cyclase/cAMP/protein kinase A and protein kinase C pathways, as observed for human macrophages (Ottaviani *et al*., 1997, 2000; Kletsas *et al.,* 1998; Sassi *et al.,* 1998).

As it has been observed for humans (Smith, 2003), the full-length ACTH and derived fragments may affect the cell migration in invertebrate models either positively or negatively, in relation to the ACTH fragment utilized and the species considered (Ottaviani and Franceschi, 1997). Likewise, the bacterial phagocytic tests performed in presence of human ACTH gave controversial results, that did not correlate with those obtained in the chemotaxis experiments, in consequence of the doses and the models adopted (Ottaviani *et al*., 1994).

The body of evidence collected during the ‘90s in molluscs, seemed to have an obvious conclusion in the identification of the genes encoding for opioid peptides (POMC, proenkephalin, and prodynorphin) in molluscs and other invertebrate models (Dores and Baron, 2011). Surprisingly, this was not the case and the presence of POMC- and other opioid-derived peptides outside vertebrates is still open to debate (Dores and Baron, 2011). The search for orthologous genes encoding for opioid peptides in all the invertebrate genomes and transcriptomes currently available in

Genbank and/or in species-specific genome/transcriptome databases did not reveal any orthologues. Consequently, as recently reviewed by Malagoli *et al.* (2011), at present POMC-related coding genes have been retrieved in the parasitic flat worm *Schistosoma mansoni* (Duvaux-Miret *et al*., 1990) and the annelid *T. tessulatum* (Salzet *et al*., 1997). The presence of mRNA encoding for POMC-derived peptides has been indirectly demonstrated by *in situ* hybridization in different taxa (Franchini *et al*., 1994; Ottaviani *et al*., 1995). The significant similarity between the POMC-derived peptides of the parasite *S. mansoni* and those of humans allowed the hypothesis of a possible transfer of genetic material from host to parasite (Phares and Cox, 1987; Malagoli *et al.,* 2011). Further studies evidenced POMC-derived peptides in the blue mussel *M. edulis* (Stefano *et al.,* 1999), but this remains one of the few studies providing molecular evidence in support of the existence of POMC protein and POMC-derived peptides in invertebrates. This notwithstanding and considering the difficulties encountered in isolating full length POMC mRNA in mammals (Blalock, 1999), it may be that some domains of POMC peptides are widely conserved among metazoans in terms of structure but they present a relatively low similarity in terms of sequences, making the identification of the encoding genes all the more difficult.

V. NEUROENDOCRINE ASPECTS OF INVERTEBRATE IMMUNOCYTE FUNCTIONS: PRESENCE AND EFFECTS OF OTHER OPIOIDS

Similarly to the effects described for human ACTH on molluscan immunocytes (Ottaviani *et al*., 1997; Kletsas *et al.,* 1998; Sassi *et al.,* 1998), also synthetic opioids influence invertebrate immunocyte functions, such as cellular adherence and migration (Stefano *et al*., 1989a, b). The effects were observed on insect, molluscan and human immunocytes (Stefano *et al*., 1989a; Stefano *et al*., 1993). Image analysis experiments demonstrated that immunocytes from the mussel *M. edulis,* from the cockroach *L. maderae* and from human blood respond to low opioid concentrations by adhering and clumping. Moreover, the administration of exogenous opioids to the mussel may elicit a direct movement of immunocytes in which the possible existence of a DOC receptor for endogenous enkephalins could be predicted (Stefano *et al*., 1989a).

In accordance with the functional evidence, the opioid-containing propeptides proenkephalin and prodynorphin have also been retrieved in invertebrate neural ganglia and circulating immunocytes (Leung and Stefano, 1984; Salzet and Stefano, 1997a, b; Stefano and Salzet, 1999; Salzet, 2000; Salzet *et al*., 2000b; Tasiemski *et al*., 2000; Salzet and Tasiemski, 2001;). Proenkephalin material has been observed in the immunocytes of *T. tessulatum*. *T. tessulatum* proenkephalin display approximately the 25% of similarity with its amphibian counterpart, but it is significantly smaller (15 kDa vs 30 kDa) (Salzet and Stefano, 1997a). In functional terms, the leech proenkephalin plays the double role of opioid

and antimicrobial peptide precursor, because it encodes for both Met-enkephalin and for the antimicrobial peptide B (Tasiemski *et al*., 2000; Salzet *et al*., 2000b). In *T. tessulatum*, the immune stimulation with LPS promotes the significant increase of the levels of Met-enkephalin and of the antimicrobial peptide B, peaking at 15 min after LPS challenge. The timing of the response and the diverse effects of the molecules liberated from proenkephalin allowed to hypothesize the presence of a unified neuro-immune protective response mediated by leech immunocytes in order to efficiently contrast immediate threats to the organism, independently from the origin of the stressor (*i.e.*, neural or immune stimulus) (Tasiemski *et al*, 2000). Also the blue mussel *M. edulis* immunocytes contain proenkephalin material which results more closely related to mammalian proenkephalin, in terms of size and similarity, than the leech proenkephalin (Salzet and Stefano, 1997a). Both in leech and mussel immunocytes the DOP receptors have been identified (Stefano *et al*., 1992; Liu *et al*., 1996; Salzet and Stefano, 1997a) giving further support to the idea of a complex neuro-immune cross talk in the regulation of invertebrate immunocytes.

Less information is available on prodynorphins in invertebrate models, that were first discovered in the CNS of the leech *T. tessulatum* (Salzet and Stefano, 1997b). Similarly to the leech proenkephalin (Salzet and Stefano, 1997a), also the leech prodynorphin is significantly smaller than the human counterpart (14 kDa vs 28 kDa). Notwithstanding, the leech

prodynorphin is a precursor for four different peptides, namely Leu-enkephalin, α-Neo-endorphin, dynorphin A and dynorphin B (Salzet and Stefano, 1997b). Prodynorphin-related peptides have subsequently been retrieved also in mussel hemolymph and immunocytes (Stefano *et al*., 1998). In this case the peptide is longer than that sequenced in the leech, as mussel prodynorphin still contains Leu-enkephalin, α-Neo-endorphin, dynorphin A and dynorphin B plus an orphanin FQ-like peptide (Stefano *et al*., 1998).

Several opioid substances can stimulate hemocyte of insects (Ford *et al*., 1996) and molluscs (Stefano *et al*., 1989a), thus suggesting the presence of numerous receptors for opioids also in invertebrates (Stefano *et al*., 1998; Stefano and Kream, 2008). μ opiate receptors have been extensively studied in neurons of *M. edulis* and it has been suggested that two alternatively spliced μ opiate receptors, similar to human μ3 and μ4, are involved in the regulation of lateral ciliary activity in the visceral ganglia of the blue mussel (Cadet, 2004). In this model, morphine or the synthetic opioid peptide DAMGO significantly enhance ciliary beating in a naloxone sensitive manner, confirming the specificity of the evidenced receptors (Cadet, 2004). Of interest, the expression of μ opiate receptors follows seasonal variations in mussel pedal ganglia (Mantione *et al*., 2010). Morpho-functional studies indicated the existence of a γ receptor in mussel immunocytes, mediating the effects of the γ-selective ligand [D-Ala2,D-Met5]enkephalinamide (DAMA) on immunocyte motility and

conformational changes (Stefano *et al*., 1989a). Genes encoding for opioid ligands have not been identified in *D. melanogaster* so far, while this is not the case for opioid receptors. Binding analyses performed with radioactive forms of etorphine, a universal opioid ligand, dihydromorphine, a MOP-selective ligand and ethylketocyclazocine, a KOP-selective ligand, allowed to identify two distinct opioid binding sites in *D. melanogaster* head membranes (Santoro et al., 1990). As it has been mentioned before for the ligands (Dores and Baron, 2011), a recent bioinformatic analysis of the available sequences of neuropeptides and their receptors in metazoans failed to evidence opioid receptors in protostomians (Jékely, 2013). However, this outcome may be related to the small number of available sequences for protostomian opioid receptors, since the same analysis stated the homology between annelid, molluscan and vertebrate opioids (Jékely, 2013).

VI. NEUROENDOCRINE ASPECTS OF INVERTEBRATE IMMUNOCYTE DEVELOPMENT

Even in the absence of abundant molecular evidence, the immune and neuroendocrine crosstalk between based on a common pool of mediators seems an ancient feature that has been conserved in metazoans. Beside the observations on CRH-ACTH-biogenic amines collected in molluscs, we have mentioned above the *P. leniusculus* astakines, a prokineticin protein families which intervene in hematopoiesis and in the circadian

control of the melanization enzyme, pro-phenoloxidase. A tight connection between neural factors and hematopoiesis has been observed also in *Drosophila*. Beside lymph gland activity, the larval hematopoiesis also relies on the colonization of hematopoietic pockets by embryonic hemocytes. As long as the larval development proceeds, the hemocytes proliferate and are released in circulation. If the hemocytes are removed by manipulation from their hematopoietic pockets, they spontaneously return to them, attracted by a trophic microenvironment generated by the peripheral nervous system (Makhijani *et al*., 2011). Mutants presenting deficiencies in the peripheral nervous system also have a reduction in hemocyte population, whereas the ectopic expression of a proneural gene promotes the ectopic accumulation of the hemocytes. The tight anatomical interconnection in *Drosophila* between neural cells and proliferating and maturing immunocytes suggests that the nervous system of *D. melanogaster* exerts an important control on the maturation of immune functions (Makhijani and Brückner, 2012). The bidirectional interplay between neural and immune cells has been described also during adult neurogenesis in the brain of the crayfish *Procambarus clarkii* along the central olfactory pathway. In *P. clarkii* it is relatively easy to follow adult neurogenesis because the lineage of precursor cells are spatially separated in different and recognizable niches. Unexpectedly, the first generation pool of neuronal precursors is not self-renewing but its replenishment depends on other cells of the neurogenic niche. Through *in*

vivo and *in vitro* experiments, Benton *et al.* (2011) demonstrated that over a 24h period, serotonin significantly enhanced the total number of the cells into the first generation niche, but this did not correspond to an increase in mitoses. This observation suggested that an external and non-neuronal source of cells must be present. Further *in vitro* experiments led to the hypothesis that stem cells released by hematopoietic districts are the most likely candidates for ensuring a continuous supply of new cells to the neurogenic niche (Makhijani and Brückner, 2012). In mammals, the possibility of a transdifferentiation of bone marrow cells into neurons has been explored *in vitro* (Sanchez-Ramos, 2002) and after their graft into the brain, hematopoietic cells seemed to have acquired some neural features (Gottschling *et al*., 2007). Though other events may justify these observations (Coyne *et al*., 2006), the hematopietic/neural transdifferentiation represented an hypothesis extensively explored by researchers (Makhijani and Brückner, 2012). In the crayfish *P. clarkii* the adult transdifferentiation of circulating hemocytes into neurons has been demonstrated (Benton *et al*., 2014). The 5-ethynyl-20-deoxyuridine (EdU)-labeled hemocytes from a donor crayfish were transferred into a recipient crayfish. Once into the recipient crayfish, the labelled hemocytes colonized the neurogenic niche, and then migrated to brain clusters 9 and 10, where they started to synthetize the appropriate neurotransmitters. When the same experiments were performed with EdU-labeled cells of the hepatopancreas, neither the colonization of the neurogenic niche, nor the

transdifferentiation into neurons occurred, demonstrating that the competence of becoming neuronal precursors is specific of hemocytes (Benton *et al*., 2014). These important results deeply undermine our vision of the relationship between the central nervous system (CNS) and the immune system in mammals, with important patho-physiological implications. For many years, the migration and the presence of macrophages in the CNS of mammals has been considered as a signal representing damage. This dogma has been shown to be unfounded by the abundance of data demonstrating an active and protective role of these cells with respect to the proper functioning of the CNS (Redmond and Chan, 2012; Schwartz *et al*., 2013; Drago *et al*., 2014).

VII. CONCLUSIONS

(1) Vertebrate and protostomian immunocytes play a central role in immune and neuroendocrine responses.

(2) Despite their diverse origin and various morphologies in both vertebrates and protostomian invertebrates, the immunocytes display common features like professional phagocytosis of foreign particles and a constant cross talk with neural components.

(3) The immunocyte differentiation may begin in different tissues or organs, but it relies on the action of a pool of highly conserved transcription factors (Fig. 1).

(4) The intersection of immunocytes with the neuroendocrine component has been repeatedly observed.

(5) The recent findings connecting hematopoiesis with neural activities, and neurogenesis with immune cell precursors, provide a strong developmental basis for the ancient origin and the widespread conservation of immune-neuroendocrine cross-talk.

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Figure Legend

Fig. 1. Integrated view of the immune-neuroendocrine system. Immune-neuroendocrine system bases its functioning on different cells that share similar mediators and receptors, *i.e.,* immunocytes (top) and neurons

(bottom). Circulating hemocytes present specific immune functions, but they may also react to signals released by neural cells and may intervene in neurogenesis. Similarly, neurons and other neural components present specific activities but they can be influenced by soluble factors produced by immune components. Neural components may also play an important role in driving hemocyte maturation before its release into circulation. At least partially, this scheme can be applied to phylogenically distant models such as molluscs (Ottaviani *et al.,* 2008), arthropods (Makhijani *et al.,* 2011; Benton *et al.,* 2014) and vertebrates (Smith, 2003) suggesting that the interrelation of the immune-neuroendocrine functions is an ancient and conserved feature of metazoans.