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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 evolutionarily distant models such as molluscs, crustaceans, insects and mammals. Fundamental players in this crosstalk are professional phagocytes: macrophages in vertebrates and immunocytes in invertebrates. Although they have different developmental origins, macrophages and immunocytes possess comparable functions and differentiate under the control of evolutionarily 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 haemocytes into neural cells has been documented recently. In light of developmental, molecular and functional evidence, we propose that the immune–neuroendocrine role of circulating phagocytes pre-dates the split of protostomian and deuterostomian superphyla and has been conserved during the evolution of the main groups of metazoans.

Key words: 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 & 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 defence 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 scarce knowledge on haematopoiesis 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 & Hartenstein, 2013; Accorsi, Ottaviani & Malagoli, 2014). For the purposes of this review, we adopt a general functional label for the circulating phagocytes of the different species considered, and refer to 'immunocytes', i.e. circulating cells exploiting innate immune functions similarly to vertebrate phagocytes. The term immunocyte was coined for mammalian cells (Berman, 1963; Carr & Blalock, 1988; Smith & Blalock,

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1988), but can be extended to invertebrates due to functional similarities among the phagocytes of metazoans (Ottaviani & Franceschi, 1997; Malagoli, Casarini & Ottaviani, 2006; Ottaviani, Malagoli & Franceschi, 2007; Ottaviani *et al.*, 2008; Ottaviani, 2011).

While Metchinkoff's experiments on phagocytosis led to the first description of the cellular component of the immune system, Paul Ehlrich (1897) investigated the humoral component that we now know to include not only specific antibodies, but also molecules such as lysozymes, antimicrobial peptides and interferons. The cellular and the humoral immune components cooperate to provide the foundation of immune function in vertebrates and invertebrates (Lemaitre & Hoffmann, 2007). However, besides 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 the immune response and neuro-hormones, evidencing the ancient origins of their interrelationship. We will particularly refer to mammals as vertebrate models and to molluscs, insects and crustaceans as invertebrate models.

II. NEURO-IMMUNOMODULATION OF THE IMMUNE RESPONSE IN VERTEBRATES

POMC is a large precursor protein that contains various active peptides, formed by cleavage of POMC into structurally and functionally distinct fragments (Mains, Eipper & Ling, 1977; Roberts & Herbert, 1977). In mammals, POMC precursors are glycosylated with a molecular weight that varies from 31 to 36 kDa in relation to the level of glycosylation. The sequence of POMC mRNA and the structure of the POMC gene was first determined in humans by Chang, Cochet & Cohen (1980). The POMC gene structure, which is highly conserved, consists of three exons and two large introns and it is present as a single copy per haploid genome; the POMC peptide consists 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 (aa 1–31), β-LPH (61–91)], δ-endorphin (61–87), α-endorphin (61–76) and β-melanocyte-stimulating hormone (β–MSH, 41–58). Adrenocorticotropin hormone (ACTH) (1–39) forms the POMC central region, and contains both α-MSH (1–31) and the corticotropin-like intermediate lobe peptide (CLIP) (18–39). The N-terminal region includes the γ-MSH fragment (Hadley & Haskell-Luevano, 1999). In all vertebrates, transcription of the POMC gene and the proteolytic maturation of its precursor are mainly under the control of hypothalamic corticotropin-releasing hormone (CRH). The evolution of POMC genes was reviewed recently in vertebrates (Dores & Baron, 2011) and invertebrates (Malagoli, Accorsi & Ottaviani, 2011). As discussed in greater detail in Section IV, information on POMC peptides and their relative sequences has been derived from studies on the flatworm *Schistosoma mansoni* (Duvaux-Miret *et al.*, 1990, 1992*a*,*b*), the leech *Theromyzon tessulatum* (Salzet *et al.*, 1997; Salzet, Capron & Stefano, 2000*a*) and the mussels *Mytilus galloprovincialis* and *Mytilus edulis* (Ottaviani, Capriglione & Franceschi, 1995; Stefano, Salzet-Raveillon & Salzet, 1999). The analysis of POMC peptides in *M. edulis* indicates poor conservation of the overall sequence, but higher levels of similarity in correspondence of the peptides contained within POMC. These include a γ-MSH-like peptide (aa 68–79) and an ACTH-like-peptide (106–145). The ACTH sequence included an α-MSH at positions 106–119 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 (Pages *et al.*, 1983; Callaerts, Huybrechts & de Loof, 1988) and opioid binding sites (Santoro, Hall & Zukin, 1990) were observed in the fruit fly *Drosophila melanogaster*, but genes encoding for POMC-derived molecules do not appear to be present in this well-studied invertebrate model.

Among the fragments of POMC, ACTH has a key role in immunomodulation. 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 are classified into two groups – glucocorticoids and mineralcorticoids – that often have distinct physiological and pathological functions, but such distinctions are less clear in non-mammalian species (Denver, 2009). Corticosteroids influence the development and maturation of the brain, the immune system, and many other organ systems. Furthermore, they also regulate complex functions, such as energy storage and metabolism control, stimulate gluconeogenesis and feeding behaviour and influence the consolidation of learning and memory (Denver, 2009).

After the first demonstrations that in mammals POMC mRNA can be present outside the central nervous system (CNS) (Lolait *et al.*, 1986; Westly *et al.*, 1986), attempts were made to characterize transcript length and peptide functions. POMC-derived peptides are numerous and their action on the immune response is frequently controversial, perhaps because of the variability in types of experimental stimuli applied (Smith, 2003).

POMC is the common precursor for both 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 & Baron, 2011). The involvement of opioid peptides in the immune response and

inflammation of mammals has been reported repeatedly (Sacerdote, Limiroli & Gaspani, 2003; Smith, 2003). However, recent *in vivo* experiments and literature surveys indicate that mammalian immunocytes do not display the required receptors to allow action of opioids on the immune system (Al-Hashimi *et al.*, 2013). At present, opioid receptors are classified as: μ (MOP), δ (DOP), κ (KOP) and orphanin FQ (NOP). In mammals, proenkephalin is the common precursor for DOP agonists, prodynorphin for KOP agonists and proorphanin for NOP ligands (Dores & Baron, 2011). While NOP mRNA has been detected in mammalian immunocytes, this is not the case for MOP, DOP and KOP, which are also targets for synthetic opioids (Al-Hashimi *et al.*, 2013). At present, none of the several hypotheses that propose an action of opioids on the immune system is supported conclusively (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), it is important to recognize the presence of the biochemical background necessary for bidirectional crosstalk between the immune and the 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 neural and physiological responses towards non-cognitive stimuli, such as pathogens, while the neuroendocrine system may react to cognitive stimuli by producing molecules able to modify the activity of leukocytes (Blalock, 1999). A tight collaboration between the immune and neuroendocrine system was revealed in mammals during the 1980s, and subsequent studies clarified the evolution and diversification of that connection (Stefano & Salzet, 1999; Salzet, 2000; Salzet, Vieau & Day, 2000*b*).

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 'immunocyte' proposed here is useful for the description of function, but does not highlight the diversity existing within invertebrate haematopoiesis and haematopoietic sites. Haematopoiesis has been studied in gastropod molluscs, insects and crustaceans. While research in molluscs is more limited, knowledge from insects and crustaceans is significantly broader, with two fundamental models being *Drosophila melanogaster* and *Pacifastacus leniusculus*, respectively (Lin & Söderhäll, 2011; Grigorian & Hartenstein, 2013).

In gastropods, haematopoiesis may directly involve circulating haemocytes (Sminia, 1972; Ottaviani, 1983). Other haematopoietic districts in gastropods include the epithelial and connective tissues of the walls of the blood sinus, the heart–kidney region, the mantle (Kinoti, 1971), and the ventricular cavity and lung (Sminia, 1981). An amoebocyte-producing organ has been described in *Biomphalaria glabrata* (Jeong, Lie & Heyeman, 1983) and in *Planorbarius corneus* (Ottaviani, 1988). Recently, in *Pomacea canaliculata* mitotic figures were found within the pericardial cavity (Accorsi *et al.*, 2014). Despite numerous observations suggesting or demonstrating haematopoietic activity in molluscs, information about haemocyte maturation and differentiation is still lacking.

In *D. melanogaster*, haematopoiesis takes place at two different stages during ontogenesis: a first population of haemocytes 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 & Zon, 2002; Makhijani & Brückner, 2012). Holz *et al.* (2003) suggested the use of the terms embryonic haemocytes and lymph gland haemocytes to distinguish the origin of haemocytes circulating in adults. Both embryonic and lymph gland haemocytes may acquire some characteristics of a macrophage. Their determination/differentiation as haemocytes 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 haemocytes are both present in *D. melanogaster* larvae, the two 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 with recognizable haematopoietic tissue that produces new haemocytes throughout the animal's lifetime. Compared with *D. melanogaster*, *P. leniusculus* does not have larval haemocytes that persist into the adult but instead possesses regular and continuous haematopoiesis (Lin & Söderhäll, 2011). The haematopoietic tissue of *P. leniusculus* contains five types of cells. Type 1 may be precursor stem cells for the two possible cell lineages, one originating hyalinocytes (directly from Type 1 precursors) and a second originating semigranular and granular haemocytes (*via* Type 2–5 developmental stages) (Lin & Söderhäll, 2011). In the tiger shrimp *Penaeus monodon* the existence of two lineages has been proposed, one developing semigranular cells from hyaline cells and the other developing granular cells from semigranular precursors (van de Braak *et al.*, 2000). In decapods, hyalinocytes and semi-granular haemocytes are the cells most resembling molluscan immunocytes, because they perform professional phagocytosis (Söderhäll, Smith & Johansson, 1986). The transcriptional regulation of haemocyte maturation has not been elucidated in detail in crustaceans, but the involvement of GATA transcription factors and Runt-related transcription factor (Runx) protein has been demonstrated. The continuous proliferation of haemocyte precursors is regulated by the cytokines astakine 1 and 2. The former promotes the proliferation of haemocyte precursors (Söderhäll *et al.*, 2005) and stimulates their maturation along the lineage of semigranular cells. Astakine 2 is involved in haemocyte maturation but also exerts neuroendocrine functions (Watthanasurorot *et al.*, 2013).

Despite differences in the anatomy of haematopoietic districts 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/Folded gastrulation (FOG), the myeloid leukemia 1 protein (AML1) domain family transcription factors (Lozenge/Runx1) (Hoffmann *et al.*, 1999; Fossett & Schulz, 2001), and Notch signalling (Duvic *et al.*, 2002), are essential for immune cell determination and differentiation into specific cytotypes.

This brief overview on haematopoiesis in key invertebrate models shows that the term immunocyte (or the broader term haemocyte) may include cells sharing little similarity in terms of development and maturation. Homologies among invertebrate haemocytes and between invertebrate and vertebrate immunocytes are far from being established. Notwithstanding this, 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

Early experiments in mammals (Weigent & Blalock, 1987; Smith & Blalock, 1988), prompted the research of pioneer comparative neuro-immunologists working mainly with molluscs. Evidence for POMC-derived peptides in molluscan immunocytes was derived from different techniques such as immunocytochemistry, radioimmunoassay and flow cytometry (Ottaviani & Franceschi, 1997). The freshwater snail *P. corneus*, one of the core models for immune–neuroendocrine studies, possesses two types of immunocytes circulating in the haemolymph. One was endowed with phagocytic activity, was able to adhere to glass, produced agglutinins and bound concanavalin (Con A). The second type did not display phagocytic capacity, did not adhere to glass slides, but could form rosettes with sheep red blood cells. The proliferation of non-adhering haemocytes was stimulated by phytohaemagglutinin (PHA) and they did bind immunoglobulins specifically raised against markers of vertebrate T lymphocytes (Ottaviani, 1983, 1992). The phagocytic haemocyte positively bound anti-human ACTH and anti-human β-endorphin antibodies, while non-adhering immunocytes did not contain ACTH- and β-endorphin-like material. POMC-like peptides were also observed in phagocytic cells of the *P. corneus* digestive gland, not in circulating haemocytes (Ottaviani *et al.*, 1990). ACTH-like molecules were also detected in circulating phagocytes of several other molluscan species including the freshwater snail *Viviparus ater* (Ottaviani *et al.*, 1995) and the mussel *Mytilus galloprovincialis* (Franchini, Fontanili & Ottaviani, 1994), as well as in the haemolymph of the blue mussel *M. edulis* (Smith *et al.*, 1991). Similar observations were made in the insect *Leucophaea maderae* (Smith *et al.*, 1991) and the earthworm *Eisenia foetida* (Cooper, Franchini & Ottaviani, 1995). Although circulating cells in different models should not be considered automatically as homologues, remarkably the immunocytochemical experiments always evidenced POMC-like products in cells endowed with phagocytic activity.

ACTH receptor-like mRNA was detected in molluscan immunocytes and human peripheral blood mononucleated cells (PBMCs) by *in situ* hybridization using a digoxigenin-labelled bovine complementary DNA probe (Ottaviani, Franchini & Hanukoglu, 1998). Previous studies in human PBMCs 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 vertebrate classes, while they were only observed in lymphocytes of tetrapods (amphibians, reptiles, birds and mammals) (Ottaviani *et al.*, 1992).

Functional experiments have shown that human POMC-derived peptides may promote the activation of molluscan immunocytes. Indeed, human ACTH (aa 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, Franchini & Franceschi, 1997; Kletsas *et al.*, 1998; Sassi, Kletsas & Ottaviani, 1998; Ottaviani *et al.*, 2000).

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

The body of evidence collected from molluscs seemed to indicate the presence of genes encoding for opioid peptides (POMC, proenkephalin, and prodynorphin) in molluscs and other invertebrate models (Dores & Baron, 2011). Surprisingly, the presence of POMC- and other opioid-derived peptides outside vertebrates is still open to debate (Dores & Baron, 2011). A search in Genbank and in species-specific genome/transcriptome databases we made in June 2015 for orthologous genes encoding for opioid peptides in all invertebrate genomes and transcriptomes currently available did not reveal any orthologues. As noted recently by Malagoli *et al.* (2011), POMC-related coding genes have only been retrieved in the parasitic flatworm *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 demonstrated indirectly by *in situ* hybridization in several 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 led some authors to hypothesize a possible transfer of genetic material from host to parasite (Phares & Cox, 1987; Malagoli *et al.*, 2011). Further studies identified POMC-derived peptides in the blue mussel *M. edulis* (Stefano *et al.*, 1999); this remains one of the few studies providing molecular evidence in support of the existence of POMC protein and POMC-derived peptides in invertebrates. 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 present relatively low similarity in terms of sequence, making the identification of the encoding genes 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), synthetic opioids also influence invertebrate immunocyte functions, such as cellular adherence and migration (Stefano, Cadet & Scharrer, 1989*a*; Stefano *et al.*, 1989*b*). These effects were observed on insect, molluscan and human immunocytes (Stefano *et al.*, 1989*a*, 1993). Image-analysis experiments demonstrated that immunocytes from the mussel *M. edulis*, the cockroach *L. maderae* and human blood respond to low opioid concentrations by adhering and clumping. Moreover, the administration of exogenous opioids to the mussel may elicit direct movement of immunocytes in which a deoxycorticosterone (DOC) receptor for endogenous enkephalins may be involved (Stefano *et al.*, 1989*a*).

In accordance with the functional evidence, the opioid-containing propeptides proenkephalin and prodynorphin have also been identified in invertebrate neural ganglia and circulating immunocytes (Leung & Stefano, 1984; Salzet & Stefano, 1997*a*,*b*; Stefano & Salzet, 1999; Salzet, 2000; Salzet *et al.*, 2000*b*; Tasiemski *et al.*, 2000; Salzet & Tasiemski, 2001). Proenkephalin material has been observed in immunocytes of the leech *T. tessulatum*. *T. tessulatum* proenkephalin displays approximately 25% similarity with its amphibian counterpart, but is significantly smaller (15 kDa *versus* 30 kDa) (Salzet & Stefano, 1997*a*). In functional terms, the leech proenkephalin plays a double role of opioid and antimicrobial peptide precursor, because it acts as a precursor for both met-enkephalin and antimicrobial peptide B (Salzet *et al.*, 2000*b*; Tasiemski *et al.*, 2000). In *T. tessulatum*, immune stimulation with lipopolysaccharide (LPS) promotes a significant increase of levels of met-enkephalin and of antimicrobial peptide B, peaking 15 min after LPS challenge (Tasiemski *et al.*, 2000). The timing of the response and the diverse effects of the molecules liberated from proenkephalin implied the presence of a unified neuro-immune protective response mediated by leech immunocytes independently from the origin of the stressor (i.e. neural or immune stimulus) (Tasiemski *et al.*, 2000). In the blue mussel *M. edulis*, immunocytes contain proenkephalin material more closely related to mammalian proenkephalin, in terms of size and similarity, than to leech proenkephalin (Salzet & Stefano, 1997*a*). In both leech and mussel immunocytes DOP receptors have been identified (Stefano *et al.*, 1992; Liu, Casares & Stefano, 1996; Salzet & Stefano, 1997*a*) giving further support to the idea of a complex neuro-immune crosstalk in the regulation of invertebrate immunocytes.

Less information is available on prodynorphins in invertebrate models, first discovered in the CNS of the leech *T. tessulatum* (Salzet & Stefano, 1997*b*). Similarly to leech proenkephalin (Salzet & Stefano, 1997*a*), leech prodynorphin is significantly smaller than its human counterpart (14 kDa *versus* 28 kDa). The leech prodynorphin is a precursor for four different peptides: Leu-enkephalin, α-Neo-endorphin, dynorphin A and dynorphin B (Salzet & Stefano, 1997*b*). Prodynorphin-related peptides have subsequently been found in mussel haemolymph and immunocytes (Stefano, Salzet-Raveillon & Salzet, 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 haemocytes of insects (Ford *et al.*, 1996) and molluscs (Stefano *et al.*, 1989*a*), implying the presence of numerous receptors for opioids also in invertebrates (Stefano *et al.*, 1998; Stefano & 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 $\mu 3$ and $\mu 4$, are involved in the regulation of lateral ciliary activity in the visceral ganglia (Cadet, 2004). In this model, morphine or the synthetic opioid peptide [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) significantly enhance ciliary beating in a naloxone-sensitive manner, confirming the specificity of the receptors (Cadet, 2004). Interestingly, the expression of μ opiate receptors varies seasonally 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-Ala^2, D-Met^5]-enkephalinamide (DAMA) on immunocyte motility and conformational changes (Stefano *et al.*, 1989*a*). Genes encoding for opioid ligands have not been identified in *D. melanogaster* to date, 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 identification of two distinct opioid binding sites in *D. melanogaster* head membranes (Santoro *et al.*, 1990). A recent bioinformatic analysis of the available sequences of neuropeptides and their receptors in metazoans failed to find evidence for opioid receptors in protostomians (Jékely, 2013). However, this may be related to the small number of available sequences for protostomian opioid receptors, since the same analysis stated homology between annelid, molluscan and vertebrate opioids (Jékely, 2013).

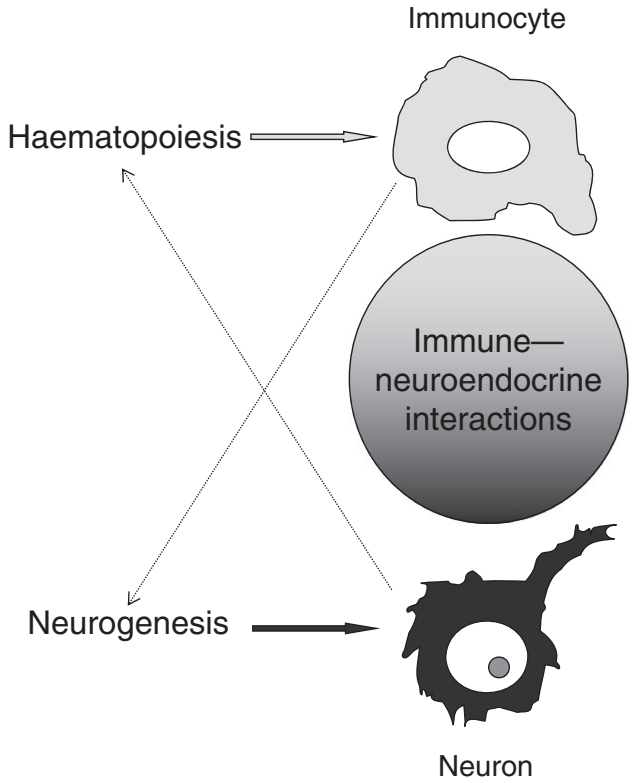

Fig. 1. Integrated view of the immune–neuroendocrine system. The immune–neuroendocrine system bases its functioning on different cells that share similar mediators and receptors, i.e. immunocytes (top) and neurons (bottom). Circulating haemocytes present specific immune functions, but they may also react 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 haemocyte maturation before their release into the 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 interrelation between immune and neuroendocrine functions is an ancient and conserved feature of metazoans.

VI. NEUROENDOCRINE ASPECTS OF INVERTEBRATE IMMUNOCYTE DEVELOPMENT

Even in the absence of abundant molecular evidence, immune and neuroendocrine crosstalk seems an ancient feature that has been conserved in metazoans (Fig. 1). Besides observations collected in molluscs (Malagoli, Accorsi & Ottaviani, 2011; Ottaviani *et al.*, 2013), *P. leniusculus* astakines, a prokineticin protein family, intervene in haematopoiesis and in the circadian control of the melanization enzyme, pro-phenoloxidase. A tight connection between neural factors and haematopoiesis (Fig. 1) has been observed also in *D. melanogaster*. Besides lymph gland activity, larval haematopoiesis also relies on the colonization of haematopoietic pockets by embryonic haemocytes. During larval development, haemocytes proliferate and are released into the circulation. If the haemocytes are removed by manipulation from their haematopoietic pockets, they spontaneously return to them, attracted by a trophic microenvironment generated by the peripheral nervous system (Makhijani *et al.*, 2011). Mutants with deficiencies in the peripheral nervous system also have a reduced haemocyte population, whereas the ectopic expression of a proneural gene promotes the ectopic accumulation of haemocytes. The tight anatomical interconnection in *D. melanogaster* between neural cells and proliferating and maturing immunocytes suggests that the nervous system exerts an important control on the maturation of immune functions (Makhijani & Brückner, 2012). Bidirectional interplay between neural and immune cells (Fig. 1) also has been described 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 precursor cell lineage is spatially separated into 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 24 h period, serotonin significantly enhanced the total number of cells in the first-generation niche, but this did not correspond to an increase in mitosis. 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 haematopoietic districts are the most likely candidates for ensuring a continuous supply of new cells to the neurogenic niche (Makhijani & Brückner, 2012). In mammals, the possibility of transdifferentiation of bone marrow cells into neurons has been explored *in vitro* (Sanchez-Ramos, 2002) and after their graft into the brain, haematopoietic cells seem to acquire some neural features (Gottschling *et al.*, 2007). Although there may be other explanations (Coyne *et al.*, 2006), haematopoietic/neural transdifferentiation represented an hypothesis extensively explored by researchers (Makhijani & Brückner, 2012). In the crayfish *P. clarkii*, adult transdifferentiation of circulating haemocytes into neurons has been demonstrated (Benton *et al.*, 2014). 5-ethynyl-20-deoxyuridine (EdU)-labelled haemocytes from a donor crayfish were transferred into a recipient crayfish where they colonized the neurogenic niche and migrated to brain clusters 9 and 10, where they started to synthesize the appropriate neurotransmitters. When the same experiments were performed with EdU-labelled cells of the hepatopancreas, neither colonization of the neurogenic niche nor transdifferentiation into neurons occurred, demonstrating that competence to become neuronal precursors is specific to haemocytes (Benton *et al.*, 2014). These results deeply undermine our vision of the relationship between the CNS and the immune system in mammals, with important patho-physiological implications.

For many years, the migration and presence of macrophages in the CNS of mammals has been considered as a signal representing damage. This has been shown to be incorrect by the abundance of data demonstrating an active and protective role of these cells with respect to the proper functioning of the CNS (Redmond & 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, immunocytes display common features like professional phagocytosis of foreign particles and continuous crosstalk with neural components.

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

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

(5) Recent findings connecting haematopoiesis 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 crosstalk.

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IX. REFERENCES

Accorsi, A., Ottaviani, E. & Malagoli, D. (2014). Effects of repeated hemolymph withdrawals on the haemocyte populations and hematopoiesis in *Pomacea canaliculata*. *Fish and Shellfish Immunology* **38**, 56–64.

Al-Hashimi, M., Scott, S. W. M., Thompson, J. P. & Lambert, D. G. (2013). Opioids and immune modulation: more questions than answers. *British Journal of Anaesthesia* **111**, 80–88.

Benton, J. L., Kery, R., Li, J., Noonin, C., Söderhäll, I. & Beltz, B. S. (2014). Cells from the immune system generate adult-born neurons in crayfish. *Developmental Cell* **30**, 322–333.

Benton, J. L., Zhang, Y., Kirkhart, C. R., Sandeman, D. C. & Beltz, B. S. (2011). Primary neuronal precursors in adult crayfish brain: replenishment from a non-neuronal source. *BMC Neuroscience* **12**, 53.

Berman, L. (1963). The immunologically competent cell ("immunocyte") system-an attempt at a delineation of cellular relationships. *Blood* **21**, 246–249.

Blalock, J. E. (1999). Proopiomelanocortin and the immune-neuroendocrine connection. *Annals of the New York Academy of Sciences* **885**, 161–172.

van de Braak, C. B., Taverne, N., Botterblom, M. H., van der Knaap, W. P. & Rombout, J. H. (2000). Characterisation of different morphological features of black tiger shrimp (*Penaeus monodon*) haemocytes using monoclonal antibodies. *Fish and Shellfish Immunology* **10**, 515–530.

Cadet, P. (2004). Nitric oxide modulates the physiological control of ciliary activity in the marine mussel *Mytilus edulis* via morphine: novel mu opiate receptor splice variants. *Neuroendocrinology Letters* **25**, 184–190.

Callaerts, P., Huybrechts, R. & De Loof, A. (1988). Distribution of a methionine enkephalin-like substance in gonads of *Drosophila melanogaster*. *International Journal of Invertebrate Reproduction and Development* **13**, 147–156.

Carr, D. J. & Blalock, J. E. (1988). "Classical" neuroendocrine peptide hormones produced by cells of the immune system. *Brain, Behavior, and Immunity* **2**, 328–334.

Chang, A. C. Y., Cochet, M. & Cohen, S. N. (1980). Structural organization of human genomic DNA encoding the pro-opiomelanocortin peptide. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 4890–4894.

Cooper, E. L., Franchini, A. & Ottaviani, E. (1995). Earthworm coelomocytes possess immunoreactive cytokines and POMC-derived peptides. *Animal Biology* **4**, 25–29.

Coyne, T. M., Marcus, A. J., Woodbury, D. & Black, I. B. (2006). Marrow stromal cells transplanted to the adult brain are rejected by an inflammatory response and transfer donor labels to host neurons and glia. *Stem Cells* **24**, 2483–2492.

Denver, R. J. (2009). Structural and functional evolution of vertebrate neuroendocrine stress systems. *Annals of the New York Academy of Sciences* **1163**, 1–16.

Dores, R. M. & Baron, A. J. (2011). Evolution of POMC: origin, phylogeny, posttranslational processing, and the melanocortins. *Annals of the New York Academy of Sciences* **1220**, 34–48.

Drago, F., Sautière, P. E., Le Marrec-Croq, F., Accorsi, A., Van Camp, C., Salzet, M., Lefebvre, C. & Vizioli, J. (2014). Microglia of medicinal leech (*Hirudo medicinalis*) express a specific activation marker homologous to vertebrate ionized calcium-binding adapter molecule 1 (Iba1/alias aif-1). *Developmental Neurobiology* **74**, 987–1001.

Duvaux-Miret, O., Dissous, C., Gautron, J. P., Pattou, E., Kordon, C. & Capron, A. (1990). The helminth *Schistosoma mansoni* expresses a peptide similar to human beta-endorphin and possesses a proopiomelanocortin-related gene. *New Biology* **2**, 93–99.

Duvaux-Miret, O., Stefano, G. B., Smith, E. M., Dissous, C. & Capron, A. (1992*a*). Immunosuppression in the definitive and intermediate hosts of the human parasite *Schistosoma mansoni* by release of immunoactive neuropeptides. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 778–781.

Duvaux-Miret, O., Stefano, G. B., Smith, E. M., Mallozzi, L. A. & Capron, A. (1992*b*). Proopiomelanocortin-derived peptides as tools of immune evasion for the human trematode *Schistosoma mansoni*. *Acta Biologica Hungarica* **43**, 281–286.

Duvic, B., Hoffmann, J. A., Meister, M. & Rovet, J. (2002). Notch signaling controls lineage specification during *Drosophila* larval hematopoiesis. *Current Biology* **12**, 1923–1927.

Eberle, A. N. (1988). *The Melanotropins: Chemistry, Physiology and Mechanisms of Action*. Karger, Basel.

Ehlrich, P. (1897). Die Werbemessung des Diphtherieheilserums und deren theoretische Grundlagen. *Klinische Jahrbuch* **6**, 299–326.

Ford, R., Jackson, D. M., Tetrault, L., Torres, J. C., Assanah, P., Harper, J., Leung, M. K. & Stefano, G. B. (1996). A behavioral role for enkephalins in regulating locomotor activity in the insect *Leucophaea maderae*: evidence for high affinity kappa-like opioid binding sites. *Comparative Biochemistry and Physiology* **85C**, 61–66.

Fossett, N. & Schulz, R. A. (2001). Functional conservation of hematopoietic factors in *Drosophila* and vertebrates. *Differentiation* **69**, 83–90.

Franchini, A., Fontanili, P. & Ottaviani, E. (1994). Expression of pro-opiomelanocortin (POMC)-mRNA in phagocytic hemocytes of *Mytilus galloprovincialis*. In *Contributions to Aanimal Biology* (eds R. Argano, C. Cirotto, E. Grassi Milano and L. Mastrolia), pp. 233–236. Halocynthia Association, Palermo.

Gottschling, S., Eckstein, V., Saffrich, R., Jonas, A., Uhrig, M., Krause, U., Seckinger, A., Miesala, K., Horsch, K., Straub, B. K. & Ho, A. D. (2007). Primitive and committed human hematopoietic progenitor cells interact with primary murine neural cells and are induced to undergo self-renewing cell divisions. *Experimental Hematology* **35**, 1858–1871.

Grigorian, M. & Hartenstein, V. (2013). Hematopoiesis and hematopoietic organs in arthropods. *Development Genes and Evolution* **223**, 103–115.

Hadley, M. E. & Haskell-Luevano, C. (1999). The proopiomelanocortin system. *Annals of the New York Academy of Sciences* **20**, 1–21.

Hoffmann, J. A., Kafatos, F. C., Janeway, C. A. & Ezekowitz, R. A. (1999). Phylogenetic perspectives in innate immunity. *Science* **284**, 1313–1318.

Holz, A., Bossinger, B., Strasser, T., Janning, W. & Klapper, R. (2003). The two origins of hemocytes in *Drosophila*. *Development* **130**, 4955–4962.

Jékely, G. (2013). Global view of the evolution and diversity of metazoan neuropeptide signaling. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 8702–8707.

Jeong, K. H., Lie, K. J. & Heyeman, D. (1983). The ultrastructure of the amebocyte-producing organ in *Biomphalaria glabrata*. *Developmental & Comparative Immunology* **7**, 217–228.

Kinoti, G. K. (1971). Observations on the infection of bulinid snails with *Schistosoma mattheei*. II. The mechanism of resistance to infection. *Parasitology* **62**, 161–170.
Kletsas, D., Sass, D., Franchini, A. & Ottaviani, E. (1998). PDGF and TGF-beta induce cell shape changes in invertebrate immunocytes via specific cell surface receptors. *European Journal of Cell Biology* **75**, 362–366.
Kowalski, J. (1997). Effect of enkephalins and endorphins on cytotoxic activity of natural killer cells and macrophages/monocytes in mice. *European Journal of Pharmacology* **326**, 251–255.
Lanot, R., Zachary, D., Holder, F. & Meister, M. (2001). Postembryonic hematopoiesis in *Drosophila*. *Developmental Biology* **230**, 243–257.
Lemaitre, B. & Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. *Annual Review of Immunology* **25**, 697–743.
Leung, M. K. & Stefano, G. B. (1984). Isolation and identification of enkephalin in pedal ganglia of *Mytilus edulis* (mollusca). *Proceedings of the National Academy of Sciences of the United States of America* **81**, 955–958.
Lin, X. & Söderhäll, I. (2011). Crustacean hematopoiesis and the astakine cytokines. *Blood* **117**, 6417–6424.
Liu, Y., Casares, F. & Stefano, G. B. (1996). $\delta 2$ opioid receptor mediates immunocyte activation. *Chinese Journal of Neuroimmunology and Neurology* **3**, 69–72.
Lolait, S. J., Clements, J. A., Markwich, A. J., Cheng, C., Mcnally, M., Smith, A. I. & Funder, J. W. (1986). Pro-opiomelanocortin messenger ribonucleic acid and posttranslational processing of beta endorphin in spleen macrophages. *The Journal of Clinical Investigation* **77**, 1776–1779.
Mains, R. E., Eipper, B. A. & Ling, N. (1977). Common precursor to corticotropins and endorphins. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 3014–3018.
Makhijani, K., Alexander, B., Tanaka, T., Rulifson, E. & Brückner, K. (2011). The peripheral nervous system supports blood cell homing and survival in the *Drosophila* larva. *Development* **138**, 5379–5391.
Makhijani, K. & Brückner, K. (2012). Of blood cells and the nervous system: hematopoiesis in the *Drosophila* larva. *Fly (Austin)* **6**, 254–260.
Malagoli, D., Accorsi, A. & Ottaviani, E. (2011). The evolution of pro-opiomelanocortin: looking for the invertebrate fingerprints. *Peptides* **32**, 2137–2140.
Malagoli, D., Casarini, L. & Ottaviani, E. (2006). Algal toxin yessotoxin signalling pathways involve immunocyte mussel calcium channels. *Cell Biology International* **30**, 721–726.
Mantione, K. J., Cadet, P., Casares, F., Zhu, W. & Stefano, G. B. (2010). Seasonal variations in mu opiate receptor signaling in the nervous system of the blue mussel, *Mytilus edulis*: temperature controls physiological processes. *Invertebrate Survival Journal* **7**, 141–145.
McCarthy, L., Wetzel, M., Sliker, J. K., Eisenstein, T. K. & Rogers, T. J. (2001). Opioids, opioid receptors, and the immune response. *Drug and Alcohol Dependence* **62**, 111–123.
Metchnikoff, E. (1883). Untersuchungen uber die intracellular Verdauung bei Wirbellosen Thieren. *Arbeit Zoologischen Instituten, Universitat Wien* **5**, 141–168.
Metchnikoff, E. (1884). Über eine sprosspilzkrankheit der daphnien; Beithag zur über den kamp der phagocyten gengen krankheitserreger. *Virchows Archiv für Pathologische Anatomie und Physiologie und für Klinische Medizin* **96**, 177–195.
Murphy, K. (2011). *Janaway's Immunobiology*, Eighth Edition. Garland Science, New York.
Ottaviani, E. (1983). The blood cells of the freshwater snail *Planorbis corneus* (Gastropoda, Pulmonata). *Developmental & Comparative Immunology* **7**, 209–216.
Ottaviani, E. (1988). Histological and immunocytochemical studies on the origin of haemocytes in the freshwater snail *Planorbarius corneus* (L.) (Gastropoda, Pulmonata). *Zeitschrift für Mikroskopisch-Anatomische Forschung* **102**, 649–654.
Ottaviani, E. (1992). Immunorecognition in the gastropod molluscs with particular reference to the freshwater snail *Planorbarius corneus* (L.) (Gastropoda, Pulmonata). *Bollettino di Zoologia* **59**, 129–139.
Ottaviani, E. (2005). Insect immunorecognition. *Invertebrate Survival Journal* **2**, 142–151.
Ottaviani, E. (2006). Molluscan immunorecognition. *Invertebrate Survival Journal* **3**, 50–63.
Ottaviani, E. (2011). Immunocyte: the invertebrate counterpart of the vertebrate macrophage. *Invertebrate Survival Journal* **8**, 1–4.
Ottaviani, E., Accorsi, A., Rigillo, G., Malagoli, D., Blom, J. M., Tascedda, F. (2013). Epigenetic modification in neurons of the mollusc *Pomacea canaliculata* after immune challenge. *Brain Research* **1537**, 18–26.
Ottaviani, E., Capriglione, T. & Franceschi, C. (1995). Invertebrate and vertebrate immune cells express pro-opiomelanocortin (POMC) mRNA. *Brain, Behavior, and Immunity* **9**, 1–8.
Ottaviani, E. & Franceschi, F. (1997). The invertebrate phagocytic immunocyte: clues to a common evolution of immune and neuroendocrine systems. *Immunology Today* **18**, 169–174.
Ottaviani, E., Franchini, A., Cossarizza, A. & Franceschi, C. (1992). ACTH-like molecules in lymphocytes. A study in different vertebrate classes. *Neuropeptides* **23**, 215–219.
Ottaviani, E., Franchini, A. & Fontanili, P. (1994). The effect of corticotropin-releasing factor and pro-opiomelanocortin-derived peptides on the phagocytosis of molluscan hemocytes. *Experientia* **50**, 837–839.
Ottaviani, E., Franchini, A. & Franceschi, C. (1997). Pro-opiomelanocortin-derived peptides, cytokines, and nitric oxide in immune responses and stress: an evolutionary approach. *International Review of Cytology* **170**, 79–141.
Ottaviani, E., Franchini, A. & Hanukoglu, I. (1998). *In situ* localization of ACTH receptor-like mRNA in molluscan and human immunocytes. *Cellular and Molecular Life Sciences* **54**, 139–142.
Ottaviani, E., Franchini, A., Malagoli, D. & Genedani, S. (2000). Immunomodulation by recombinant human interleukin-8 and its signal transduction pathways in invertebrate hemocytes. *Cellular and Molecular Life Sciences* **57**, 506–513.
Ottaviani, E., Malagoli, D., Capri, M. & Franceschi, C. (2008). Ecoimmunology: is there any room for the neuroendocrine system? *BioEssays* **30**, 868–874.
Ottaviani, E., Malagoli, D. & Franceschi, C. (2007). Common evolutionary origin of the immune and neuroendocrine systems: from morphological and functional evidences to in silico approaches. *Trends in Immunology* **28**, 497–502.
Ottaviani, E., Petraglia, F., Montagnani, G., Cossarizza, A., Monti, D. & Franceschi, C. (1990). Presence of ACTH and β-endorphin immunoreactive molecoles in the freshwater snail *Planorbarius corneus* (L.) (Gastropoda, Pulmonata) and their possible role in phagocytosis. *Regulatory Peptides* **27**, 1–9.
Pages, M., Jimenez, F., Ferrus, A., Peralta, E., Ramirez, G. & Gelpi, E. (1983). Enkephalin-like immunoreactivity in *Drosophila melanogaster*. *Neuropeptides* **14**, 87–98.
Phares, C. K. & Cox, G. S. (1987). Molecular hybridization and immunological data support the hypothesis that the tapeworm, *Spirometra mansonoides*, has acquired a human growth hormone gene. In *Molecular Paradigms for Eradicating Helminthic Parasites* (ed. A. J. Macinnis), pp. 391–405. Alan R. Liss Company, New York.
Redmond, S. A. & Chan, J. R. (2012). Neuroscience. Revitalizing remyelination--the answer is circulating. *Science* **336**, 161–162.
Roberts, J. L. & Herbert, E. (1977). Characterization of a common precursor to corticotropin and β-lipotropin: cell free synthesis of the precursor and identification of corticotropin peptides in the molecule. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5300–5304.
Sacerdote, P., Limiroli, E. & Gaspani, L. (2003). Experimental evidence for immunomodulatory effects of opioids. *Advances in Experimental Medicine and Biology* **521**, 106–116.
Salzet, M. (2000). Invertebrate molecular neuroimmune processes. *Brain Research and Brain Research Review* **34**, 69–79.
Salzet, M., Capron, A. & Stefano, G. B. (2000*a*). Molecular crosstalk in host-parasite relationships: schistosome- and leech-host interactions. *Parasitology Today* **16**, 536–540.
Salzet, M., Vieau, D. & Day, R. (2000*b*). Crosstalk between nervous and immune systems through the animal kingdom: focus on opioids. *Trends in Neurosciences* **23**, 550–555.
Salzet, M., Salzet-Raveillon, B., Cocquerelle, C., Verger-Bocquet, M., Pryor, S. C., Rialas, C. M., Laurent, V. & Stefano, G. B. (1997). Leech immunocytes contain proopiomelanocortin: nitric oxide mediates hemolymph proopiomelanocortin processing. *The Journal of Immunology* **159**, 5400–5411.
Salzet, M. & Stefano, G. B. (1997*a*). Invertebrate proenkephalin: delta opioid binding sites in leech ganglia and immunocytes. *Brain Research* **768**, 224–232.
Salzet, M. & Stefano, G. B. (1997*b*). Prodynorphin in invertebrates. *Molecular Brain Research* **52**, 46–52.
Salzet, M. & Tasiemski, A. (2001). Involvement of pro-enkephalin-derived peptides in immunity. *Developmental and Comparative Immunology* **25**, 177–185.
Sanchez-Ramos, J. R. (2002). Neural cells derived from adult bone marrow and umbilical cord blood. *Journal of Neuroscience Research* **69**, 880–893.
Santoro, C., Hall, L. M. & Zukin, R. S. (1990). Characterization of two classes of opioid binding sites in *Drosophila melanogaster* head membranes. *Journal of Neurochemistry* **54**, 164–170.
Sassi, D., Kletsas, D. & Ottaviani, E. (1998). Interactions of signaling pathways in ACTH (1-24)-induced cell shape changes in invertebrate immunocytes. *Peptides* **19**, 1105–1110.
Schwartz, M., Kipnis, J., Rivest, S. & Prat, A. (2013). How do immune cells support and shape the brain in health, disease, and aging? *The Journal of Neuroscience* **33**, 17587–17596.
Sminia, T. (1972). Structure and function of blood and connective tissue cells of the fresh water pulmonate *Lymnaea stagnalis* studied by electron microscopy and enzyme histochemistry. *Cell and Tissue Research* **130**, 497–526.
Sminia, T. (1981). Gastropods. In *Invertebrate Blood Cells* (Volume **1**, eds N. A. Ratcliffe and A. F. Rowley), pp. 191–232. Academic Press, London.
Smith, E. M. (2003). Opioid peptides in immune cells. *Advances in Experimental Medicine and Biology* **521**, 51–68.
Smith, E. M. & Blalock, J. E. (1988). A molecular basis for interactions between the immune and neuroendocrine systems. *International Journal of Neuroscience* **38**, 455–464.

Smith, E. M., Brosnan, P., Meyer, W. J. & Blalock, J. E. (1987). An ACTH receptor on human mononuclear leukocytes. Relation to adrenal ACTH-receptor activity. *The New England Journal of Medicine* **317**, 1266–1269.

Smith, E. M., Hughes, T. K., Scharrer, B., Leung, M. K. & Stefano, G. B. (1991). The production and action of ACTH-related peptides in invertebrate hemocytes. *Advances in Neuroimmunology* **1**, 7–16.

Söderhäll, I., Kim, Y. A., Jiravanichpaisal, P., Lee, S. Y. & Söderhäll, K. (2005). An ancient role for a prokineticin domain in invertebrate hematopoiesis. *The Journal of Immunology* **174**, 6153–6160.

Söderhäll, K., Smith, V. J. & Johansson, M. (1986). Exocytosis and phagocytosis by isolated haemocyte populations of crustaceans: evidence for cell co-operation in the cellular defence reactions. *Cell and Tissue Research* **245**, 43–49.

Stefano, G. B., Cadet, P. & Scharrer, B. (1989*a*). Stimulatory effects of opioid neuropeptides on locomotory activity and conformational changes in invertebrate and human immunocytes: evidence for a subtype of delta receptor. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 6307–6311.

Stefano, G. B., Leung, M. K., Zhao, X. H. & Scharrer, B. (1989*b*). Evidence for the involvement of opioid neuropeptides in the adherence and migration of immunocompetent invertebrate hemocytes. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 626–830.

Stefano, G. B., Digenis, A., Spector, S., Leung, M. K., Bilfinger, T. V., Makman, M. H., Scharrer, B. & Abumrad, N. N. (1993). Opiate-like substances in an invertebrate, an opiate receptor on invertebrate and human immunocytes, and a role in immunosuppression. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 11099–11103.

Stefano, G. B. & Kream, R. (2008). Endogenous opiates, opioids, and immune function: evolutionary brokerage of defensive behaviors. *Seminars in Cancer Biology* **18**, 190–198.

Stefano, G. B., Melchiorri, P., Negri, L., Hughes, T. K. & Scharrer, B. (1992). D-Ala2.-Deltorphin I binding and pharmacological evidence for a special subtype of delta opioid receptor on human and invertebrate immune cells. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 9316–9320.

Stefano, G. B. & Salzet, M. (1999). Invertebrate opioid precursors: evolutionary conservation and the significance of enzymatic processing. *International Reviews of Cytology* **187**, 261–286.

Stefano, G. B., Salzet-Raveillon, B. & Salzet, M. (1998). *Mytilus edulis* hemolymph contain prodynorphin. *Immunology Letters* **63**, 33–39.

Stefano, G. B., Salzet-Raveillon, B. & Salzet, M. (1999). *Mytilus edulis* hemolymph contains pro-opiomelanocortin: LPS and morphine stimulate differential processing. *Brain Research. Molecular Brain Research* **63**, 340–350.

Tasiemski, A., Verger-Bocquet, M., Cadet, M., Goumon, Y., Metz-Boutigue, M. H., Aunis, D., Stefano, G. B. & Salzet, M. (2000). Pronkephalin A-derived peptides in invertebrate innate immune processes. *Brain Research. Molecular Brain Research* **76**, 237–252.

Traver, D. & Zon, L. I. (2002). Walking the walk: migration and other common themes in blood and vascular development. *Cell* **108**, 731–734.

Watthanasurorot, A., Saelee, N., Phongdara, A., Roytrakut, S., Jiravanichpaisal, P., Söderhäll, K. & Söderhäll, I. (2013). Astakine 2--the dark knight linking melatonin to circadian regulation in crustaceans. *PLoS Genetics* **9**, e1003361.

Weigent, D. A. & Blalock, J. E. (1987). Interactions between the neuroendocrine and immune systems: common hormones and receptors. *Immunological Reviews* **100**, 79–108.

Westly, H. J., Kleiss, A. J., Kelley, K. W., Wong, P. K. & Yuen, P. H. (1986). Newcastle disease virus-infected splenocytes express the proopiomelanocortin gene. *The Journal of Experimental Medicine* **163**, 1589–1594.

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