

# Annelid Neuroimmune System

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**Abstract** : Neuropeptides have been found in nervous central or immune systems of Annelids. Since these signaling molecules are found free in the hemolymph, they are considered as hormones. Hormonal processes along with enzymatic processing similar to that found in vertebrates occur in annelids. Furthermore, amino acid sequence determination of annelids precursor gene products reveals the presence of the respective peptides that exhibit high sequence identity to their mammalian counterparts. Nevertheless, specific neuropeptides to annelids or invertebrates have also been in these animals. These peptides are flanked by potential proteolytic signal sites for the various known enzymes confirming that annelids neuropeptide precursors are processed in a similar manner to that described in mammals *i.e.* implicating prohormone convertase enzymes.



## 1. INTRODUCTION

Neuropeptides are widespread and play a major role in the animal kingdom, including in protozoa. The similar amino acid sequences of some of the neuropeptides, *i.e.*, oxytocin/vasopressin, angiotensins, in vertebrates and in invertebrates demonstrate that they are stable and old in evolutionary terms. These signaling peptides seem to have appeared before that of a primitive nervous system, suggesting that they were first implicated in intercellular communication [1]. Therefore, they may have evolved from a small number of signaling proteins encoded by ancestral genes that have been conserved and amplified over time [1].

## 2. WHAT CONSTITUTES A NEUROENDOCRINE SYSTEM?

In vertebrates, this system is based on chemical signaling between neural and endocrine structures. Final outcomes may be realized via the chemical messengers traveling from circulatory conduits to their specific target sites. This process may, in part, rely on neurosecretion of the signaling molecules [2]. The complexity of this system can be visualized quickly when one considers its operating regulations that result from both classical neurotransmitters, cytokines, growth factors and hormones neuroendocrine, autocrine and paracrine communications. Apart from a neuroendocrine system also exists a neuroimmune communication, which consists to a reciprocal signaling between neuroendocrine and immune cells which use the same molecules to coordinate their activity, *i.e.*, adrenocorticotropin ACTH [3]. We have recently

demonstrated that neurovascular regulation [4] of neurosecretion occurs in rat median eminence fragments via a nitric oxide mediated process [5]. Thus, our concept of a neuroendocrine system is constantly growing given its complexity but may be simply summarized as the result of bi-directional communications between neural and endocrine structures over a distance greater than that occurring by synaptic communication.

## 3. DO ANNELIDS HAVE A NEUROENDOCRINE SYSTEM?

Annelids neural tissues do not contain anatomical correlates of hypothalamus or pituitary. However, they possess localized ganglionic regions rich in mammalian-like neuroendocrine signaling molecules *e.g.* angiotensins [6-8], oxytocin/vasopressin peptide family [7], opioids [9-12] (tables 1 and 2). Furthermore, these molecules appear free in the animals' hemolymph, demonstrating distant signaling [13-15] via several target tissues [15], including immune cells [16-20]. Thus, the fact that a classical closed conduit system to carry signaling molecules does not exist in Annelids should not detract from an endocrine presence because the baseline functioning of the system depends on distance between the origin of a signal molecule and its target tissue/receptor.

## 4. ARE NEUROENDOCRINE SIGNALING MOLECULES IN ANNELIDS AND VERTEBRATES SIMILAR?

This question represents an important aspect regarding the existence of an Annelids neuroendocrine system. Among the 30 neuropeptides isolated, so far, in annelids (Tables 1 and 2), the most part already sequenced are related to the ones previously isolated in vertebrates. They can be divided into 4 groups of signaling molecules *i.e.* the angiotensins,

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**Table 1. Characterized Annelid Neuropeptides from Leeches**

23	Sequence	Name
<i>Theromyzon tessulatum</i> <i>Erpobdella octoculata</i> <i>Hirudo medicinalis</i> <i>Hirudo nipponia</i> <i>Whitmania pigra</i>	SYVMEHFRWDKFGFRKIKRRPIKVVYNGAEDESAEAFPLE	ACTH-like
	DRVYIHPFHLLXWG	Angiotensin I
	DRVYIHPF	Angiotensin II
	RVYIHPF	Angiotensin III
	IPEPYVWD	LORF (leech osmoregulator factor)
	FMRF-amide	FMRF-amide
	FM(O)RF-amide	FMRF-amide sulfoxide
	FLRF-amide	FLRF-amide
	GDPFLRFamide	GDPFLRF-amide
	PLG	MIF-1
	YGGFL	Leucine-enkephalin
	YGGFM	Méthionine-enkephalin
	YGGFLRKYPK	$\alpha$ neoendorphin
	YVMGHFRWDKamide	MSH-like peptide
	GSGVSNGGTEMIQLSHIRERQRYWAQDNLRRRFLKamide	Leech Egg Laying Hormone
	DRVYIHPF-amide	Angiotensin II-amide
	CFIRNCPKG-amide	Lysine-conopressin
	FMRF-amide; FM(O)RF-amide	FMRF-amide
	GDPFLRF-amide	GDPFLRF-amide
	FLRF-amide	FLRF-amide
	IPEPYVWD; IPEPYVWD-amide	LORF
	FMRF-amide, FM(O)RF-amide	FMRF-amide
	FLRF-amide	FLRF-amide
AMGMLRM-amide	Myomoduline-like peptide	
WRLRSDETVRGTRAKCEGEWAHACLCLGGNamide	Leech excitatory peptide	

the oxytocin/vasopressin, the myotropic peptides and the opioid families. With the exception to the myotropic peptides, which are more specific to the annelids, the other molecules families have also been identified in vertebrates in which they play crucial roles as neurohormones. These data further strengthen the existence of a neuroendocrine system in the annelids.

#### 4.1. Angiotensin-Like Peptides

Biochemical identification of a "central" angiotensin II (AII)-like peptide in the leech *Erpobdella octoculata* was demonstrated and found to be amidated [6]. This constituted the first characterization of an angiotensin-like peptide in an invertebrate, demonstrating its conservation during the course of evolution. An identification of the proteins immunoreactive to anti-AII was found both in brain extracts and in *in vitro* translated brain RNA products [7]. The pro-AII precursor detected in the brain extracts possesses a *ca* 19 kDa molecular mass and is also a "multiple hormone precursor" as it is also recognized by two other antisera : a polyclonal  $\gamma$ -MSH and a specific monoclonal antibody directed against leech neurons, Tt159 [8]. Furthermore, we found in leeches a *ca.* 11-kDa peptide with a sequence of DRVYIHPFHLLXWG, which exhibits a 78.5% sequence identity to the N-terminus of the angiotensinogen and a 100% sequence identity to AI (Table 1) [21].

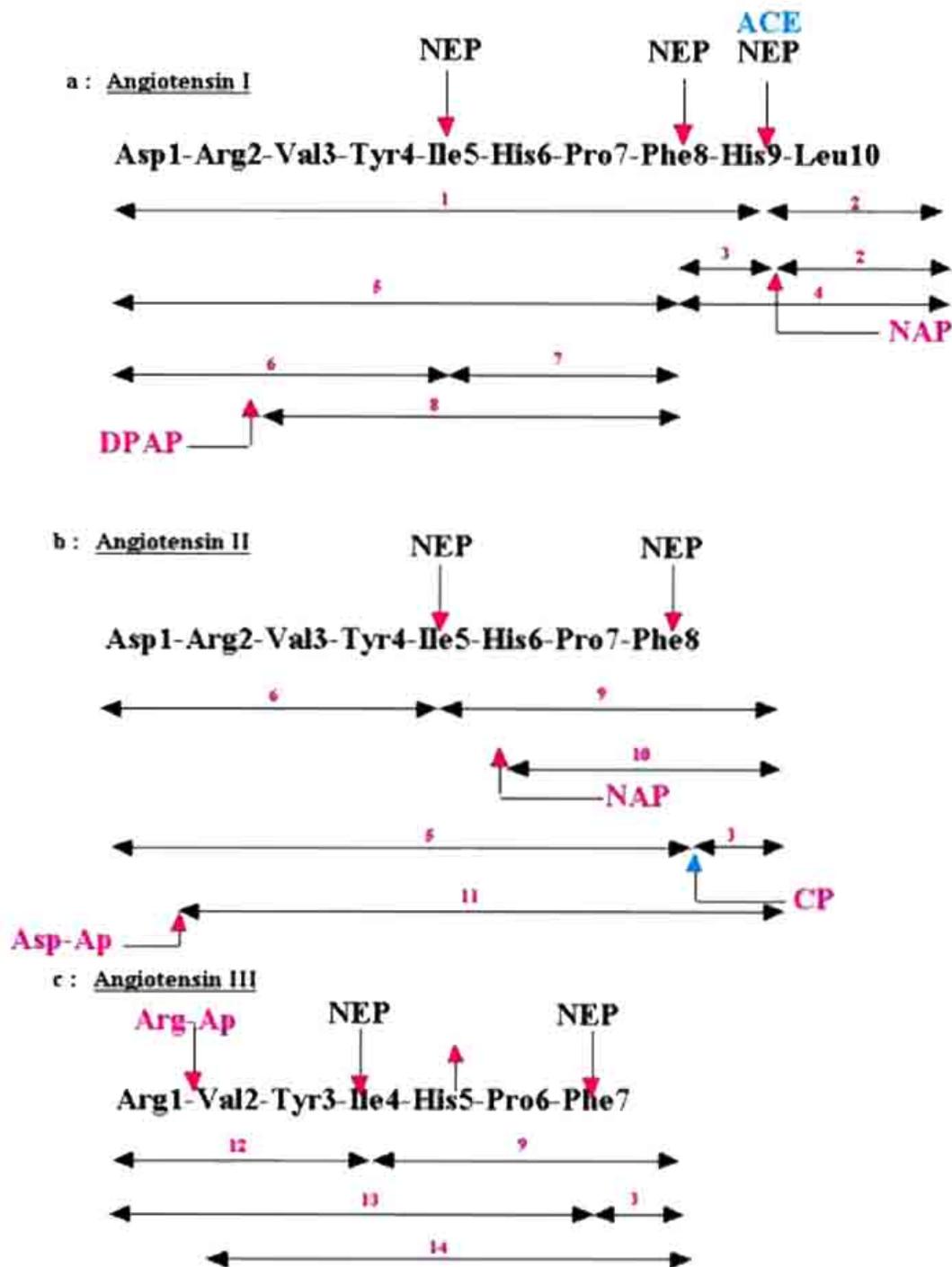
Biosynthesis study of leech AII revealed the existence of a renin- [22] and angiotensin-converting like [23-25] enzymes as well as the enzyme implicated in its catabolism [Fig. 1, 25]. The complete molecular cloning of leech ACE confirmed its homology with vertebrate ACE (unpublished data). Experiments conducted on the biological activity of the AIIamide established that this peptide is involved in the control of leech's hydric balance exerting a diuretic effect

(Table 3; [26]). Because AIIamide injections at different doses in *T. tessulatum* suggest the existence of two different types of receptors, one at high and the other one at low affinities towards AIIamide [6], we focused our interest on the identification of the leech AIIamide receptors. Binding experiments on *T. tessulatum* brain membranes with mono [ $^{125}$ I]AIIamide reveals 70% specific binding and a  $Ic_{50}$  of 10 nM [20]. In addition, biochemical studies using commercial anti-AT1 receptor reveal the existence of a specific protein at a molecular weight of 140 kDa [20]. Finally, immunocytochemical studies performed at the level of the brain confirmed the presence of labeling in neurons and glial cells to anti-AII, anti-leech renin, anti-leech ACE and anti-AT1 [8, 20]. Taken together these data strongly suggest the existence of a complete renin-angiotensin system in leeches.

Similarly, in the Polychaeta *Nereis diversicolor*, injections of polyclonal antisera against AII provoked a partial inhibition of the increase in body weight in animals exposed to hypo-osmotic medium. In a subsequent test, injections of synthetic AII-amide and, to a lesser extent AII [27] enhanced the increase in body weight and, therefore, strengthened the importance of these peptides in the neuroendocrine control of *Nereis* osmoregulation.

#### 4.2. Oxytocin/Vasopressin Peptides

The peptides of the vasopressin/oxytocin family have been discovered throughout the animal kingdom. They are alike, sharing at least five of nine residues and a disulfide-linked ring structure, which puts severe constraints on conformational flexibility [28]. In vertebrates, gene duplication gave rise to two distinct families i.e. the vasopressin (VP) one and the oxytocin (OT) one [28]. The differential binding of VP and OT to their respective receptors is largely due to the amino acid residue in position



**Fig. (1). Angiotensins catabolism**

Identities of the products formed by incubation of angiotensins with *T. tessulatum* membranes.

The numbered peptides correspond to the HPLC peaks and were identified by coelution with peptide markers in HPLC, antisera recognition and spectral scanning comparison. (1) : DRVYIHPF; (2) : HL; (3) : F; (4) : FHL; (5) : DRVYIHP; (6) : DRVY; (7) : IHP; (8) : VYIHP; (9) : IHPF; (10) : HPF; (11) : RVYIHPF; (12) : RVY; (13) RVYIHP; (14) : VYIHPF.

The enzymes acting on the angiotensins metabolism are the following: ACE : angiotensin-converting enzyme; AP : aminopeptidase; Asp-AP : aspartyl-aminopeptidase; Arg-AP : arginyl-aminopeptidase; CP : carboxypeptidase; DPAP : dipeptidyl aminopeptidase; NAP : neutral aminopeptidase; NEP : neuropeptide-degrading endopeptidase.

8 *i.e.* a basic one in VP-related peptides and a neutral one in OT-related peptides. In the leech *Erpobdella octoculata*, a VP related peptide; the lysine-conopressin has been isolated [7]. Recently, we have cloned in the gut leech *T. tessulatum*, a novel OT-VP receptor (unpublished data). Oumit *et al.* [29] confirm existence in oligochaeta annelids of the OT-VP peptide by the purification of annetocin (Table 2), a peptide having 7 residues on 9 common with the leech lysine-conopressin. This peptide, like in vertebrates, is associated to a neurophysine having 45.6% sequence identity to vertebrate neurophysin [30]. These data confirm the hypothesis of an ancestral common gene to both OT and vasopressin peptide families since 500 million years ago. Annetocin like lysine-conopressin acts, like in annelids, on osmoregulation *via* nephridia [29-31]. Lysine-conopressin inhibits the Na<sup>+</sup> amiloride dependent transitory current before to highly stimulate it on *Hirudo medicinalis* stomach or tegument preparation (Table 3). Moreover, lysine-conopressin induces egg laying in earthworm like OT does in vertebrates [29, 30]. This confirms that through the course of evolution the OT/VP peptides family has conserved his function on both osmoregulation and reproduction

#### 4.3. RF-Amide Peptides

In annelids, authentic FMRFamide is present in the polychaetes *Nereis virens* and *Nereis diversicolor* [31, 32]. In this last species, two other RFamide peptides (FM(O)RFamide and FTRFamide) have been isolated.

Pharmacological data suggest that RFamide peptides are involved in the control of heartbeat and body wall tone in the polychaete *Sabellastarte magnifica* [33].

In the earthworm *Eisenia fetida*, FMRF-like peptides are co-localized with serotonin, suggesting a role as neuromodulators influencing serotonergic neurons [34]. In *Lumbricus terrestris*, FMRF-like peptides seem to be involved in both central integratory processes, neuromuscular regulation and sensory processes [34].

In Hirudinae, anti-FMRFamide immunoreactivity is found in cell bodies and neuronal processes of the central nervous system [35, 36]. In the segmental ganglia of the ventral nerve cord, this immunoreactivity is localized in heart excitatory (HE) motor neurons, heart accessory (HA) modulatory neurons and several motor neurons innervating the longitudinal and medio-dorso ventral muscles [35, 36]. Among the 21 segmental ganglia (SG1-SG21) of the ventral nerve cord of leeches, SG5 and SG6 that innervate the sexual organs are designated as sex SG. These sex ganglia contain, as compared to the non-sex ganglia [8], an additional population of neurons immunostained with anti-FMRFamide in *Hirudo medicinalis* [8]. Furthermore, two RFamide peptides (FMRFamide and FLRFamide) were identified in *Hirudo medicinalis* [37]. These peptides increases the strength and accelerates the rate of myogenic contractions as well as inducing myogenic contractions in quiescent hearts [35, 36]. Besides, these tetrapeptides we characterized an extended form of FLRFamide, the

**Table 2. Characterized Annelids Neuropeptides other than Leeches**

Species	Sequence	Name
<i>Perinereis Vancaurica</i> <i>Nereis diversicolor</i> <i>Nereis virens</i> <i>Eisenia Foetida</i>	AMGMLRMamide WVGDVQ ATWLDT WMVGDVQ FYEGDVPI FMRF-amide, FM(O)RF-amide FTRF-amide FMRF-amide FLRF-amide CFVRNCPTGamide APKCSGRWAIHSCGGNG GKCAGQWAIHACAGGNG RPKCAGRWAIHSCGGNG	Myomodulin-CARP Esophagus regulation peptides FMRF-amide FTRF-amide FMRF-amide FLRF-amide Annetocin GGNG1 GGNG2 GGNG3

**Table 3. Percental Changes of I<sub>SC</sub> and I<sub>amil</sub> caused by Serosal Application of the Leech Peptides Conopressin, Oxytocin-like Peptide and Angiotensin-Amide (5 μM) and two forms of Vertebrate Vasopressin (20 mU/ml)**

Peptide	Percental changes		n
	I <sub>SC</sub>	Amiloride-sensitive Na <sup>+</sup> current	
Lysine-Conopressin	- 27.8 ± 7.6 *	- 26.8 ± 9.6 *	3
LORF	- 18.2 ± 0.9 *	- 28.2 ± 2.7 *	3
Angiotensin-amide	- 28.6 ± 5.8 *	- 44.8 ± 11.5 *	3
8-Arg-vasopressin	+ 24.0 ± 9.6 *	- 44.7 ± 10.4 *	4
8-Lys-vasopressin	- 31.1 ± 7.6 *	- 49.5 ± 10.1 *	5

GDPFLRFamide from sex ganglia extracts of *E. octoculata* [9]. In *T. tessulatum* presence of RFamide peptides in neurosecretory granules in fibers of the neurohaemal area suggests that at least one of the characterized peptides is secreted into the dorsal vessel. The brain could exert a neuroendocrine control of certain functions via RFamide peptides. Taking into account a previous study showing a loss of weight of *T. tessulatum* after a GDPFLRFamide injection and an increase of weight after a FMRFamide injection [37], we surmise that GDPFLRFamide may act as a diuretic hormone and FMRFamide as an anti-diuretic hormone. Electrophysiological experiments confirmed our speculation [Table 3, 9]. The anti-diuretic effect of FMRFamide seemed not due to a direct action on the caecal epithelium. Nevertheless, the control of the hydric balance might be also exerted directly on the nephridia. Indeed, Zerbst-Boroffka *et al.* [38] demonstrated that the nephridial nerve cells, which innervate the nephridia and contact the urine forming cells, contain RFamide peptide(s) in *H. medicinalis*. Furthermore, Wenning and Calabrese [39] showed that FMRFamide leads the hyperpolarization and decreases the rate of firing of the nephridial nerve cells, suggesting autoregulation of peptide release.

#### 4.4. Opioids

Leech proenkephalin (Fig. 2) demonstrates considerable amino acid sequence similarity with amphibian

proenkephalin (26.2 %) and it contains Met- and Leu-enkephalins in a ratio of 1/2. It also possesses Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe that are flanked by dibasic amino acid residues, which are targets for proteolytic enzymes such like prohormone convertases present in leeches (unpublished data). Specific receptors for these peptides have been characterized in invertebrates, both in neural and in immune systems and these have been reviewed extensively elsewhere [14, 40]. These studies also demonstrate that these receptors have been particularly well conserved during evolution. In the case of the prodynorphin, alpha-Neo-endorphin, exhibiting 100% sequence identity with the mammalian material, was firstly purified from *T. tessulatum* central nervous system (CNS) and suckers [41], suggesting the presence of a larger precursor polypeptide similar to pro-Dyn of vertebrates. Subsequent characterization of the entire pro-dynorphin opioid precursor revealed that it exhibits a 28.8% sequence identity to rat, and 22% to the human and pig [40]. Although the  $\alpha$  Neo endorphin is identical to the one found in vertebrates, the dynorphins are slightly shorter. A POMC-like molecule has also been demonstrated in leeches [15]. Of the six peptides, three showed high sequence similarity to their vertebrate counterparts, namely, met-enkephalin,  $\alpha$ -MSH and ACTH (100, 84.6 and 70% respectively) whereas  $\gamma$ -MSH,  $\beta$ -endorphin and  $\gamma$ -LPH exhibited only 45, 20 and 10% sequence identity. No dibasic amino acid residues were found at the C-terminus of the  $\gamma$ - and  $\beta$ -MSH peptides suggesting that they are not produced in the leech or that

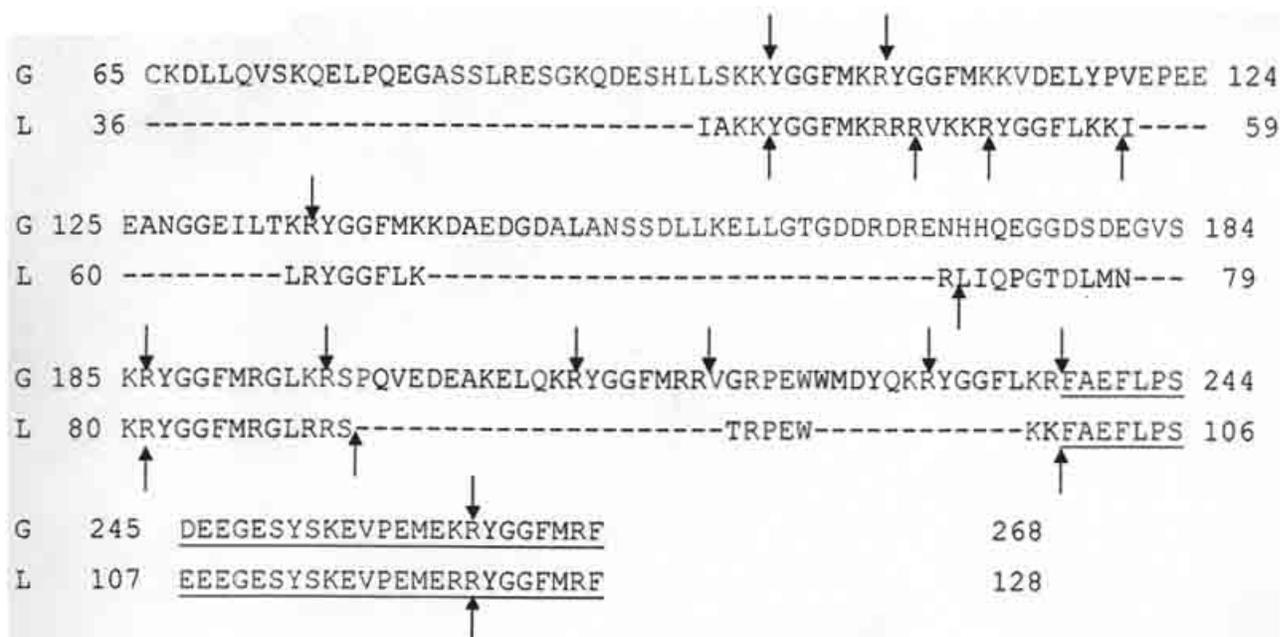


Fig. (2). Opioid precursors structure of the leech *Theromyzon tessulatum*

The Proenkephalin generates after its processing opioid peptides like leucine-enkephalin (Leu Enk), methionine-enkephalin (Met Enk) and other active molecules like methionine enkephalin Arginine Glycine Leucine (Enk-MRGL) and the methionine enkephalin Arginine Phenylalanine (Enk-MRF). POMC maturation also releases opioids like Met Enk and the  $\alpha$ -endorphin ( $\alpha$ -end). The structure comparison of the two species shows differences in the precursor structures. By contrast, the yielding active peptides are similar. Methionine enkephalin (Met-Enk); Leucine enkephalin (Leu-Enk); Methionine enkephalin Arginine Glycine Leucine (Enk-MRGL); Methionine enkephalin Arginine Phenylalanine (Enk-MRF); Melanocyte Stimulating Hormone (MSH); Adrenocorticotropin Hormone (ACTH); Corticotropin Like Intermediate Lobe Peptide (CLIP); Lipotropin Hormone (LPH) and  $\alpha$ -endorphine ( $\alpha$ -end.). The size of the precursor is expressed in amino acid residues.

they could be synthesized via an alternate biosynthetic pathway. In contrast, the leech  $\alpha$ -MSH was flanked at its C-terminus by the Gly-Arg-Lys amidation signal.

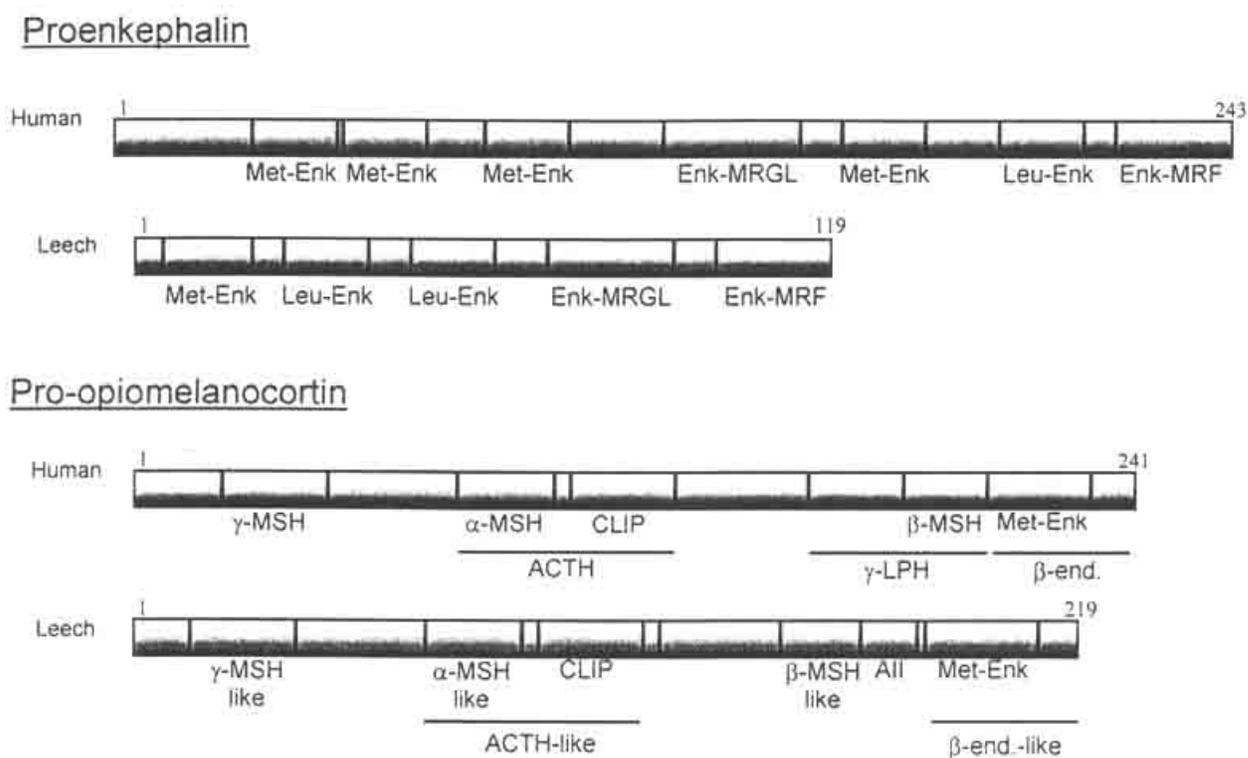
Taken together, the results from leeches now demonstrate, those opioid precursors and many of the derived bioactive peptides, *i.e.*,  $\alpha$ -MSH and ACTH, important in mammalian neuroendocrine signaling are present in invertebrates. This adds to the growing body of evidence that a neuroendocrine apparatus is also present in simple animals.

## 5. ARE SOME NEUROENDOCRINE SIGNALING MOLECULES SPECIFIC TO ANNELIDS?

The history of neurobiology demonstrates the significance of the invertebrate nervous system as a valuable model. The giant axon of the squid and crayfish neuromuscular junction stands out in this regard. This field of scientific endeavor also stands out for its demonstration of the conservation of signaling molecules and their functions during evolution. Probably the first peptide found in invertebrates that later was also found in mammals is the *Hydra* head activator peptide [42]. In *Hydra* this peptide modulates morphogenesis, cellular growth and differentiation. In mammals, this peptide sequence proved to be unique in that it differed from that of any known peptide. Furthermore, the investigators surmise that this peptide may have the same functions in mammals. We propose here to extend this several families specifically found in annelids and not net found in mammals.

## 5.1. Rxamides Peptides

Several related Rxamides have been found in annelids. A myomodulin-like peptide, GMGALRLamide, has been purified and sequenced from the medicinal leech nerve cords [43]. Myomodulin-like immunoreactivity has recently been found to be present in a set of leech neurons, including Leydig neurons [43]. The glial responses to Leydig neuron stimulation persisted in a high-divalent cation saline, when polysynaptic pathway are suppressed, indicating that the effects on the glial cell were direct. The glial responses to myomodulin A application persisted in high-Mg<sup>2+</sup>/low-Ca<sup>2+</sup> saline, when chemical synaptic transmission is suppressed, indicating a direct effect of myomodulin A on the glial membrane. The glial hyperpolarization evoked by myomodulin A was dose dependent (EC<sub>50</sub> = 50 nM) and accompanied by a membrane conductance increase of approximately 25%. Ion substitution experiments indicated that myomodulin A triggered a Ca<sup>2+</sup>-independent K<sup>+</sup> conductance [43]. Moreover, synthetic leech myomodulin-like peptide showed identical neuronal modulation effect on the giant leech Retzius cell compare to that by the synthetic *Aplysia* myomodulin A PMGMLRLamide [44, 45]. This neural and muscular modulation has been shown to be important for shaping and modifying behavior. Experiments focused on the Retzius cell (R) revealed that the myomodulin-like peptide increased the excitability of the R cell such that the cell fires more action potentials with a shorter latency to the first action potential. This effect is mediated by the activation of a Na<sup>+</sup>-mediated inward current near the cell resting membrane potential [44, 45].



**Fig. (3).** Leech and guinea pig Proenkephalins sequence comparison

G : Guinea Pig; L: Leech.

↑: Putative cleavage site

In polychaete annelids a heptapeptide, AMGMLRMamide, termed Pev-myomodulin, was isolated from *Perinereis vancaurica* using the esophagus of the animal as the bioassay system [46]. The sequence of the annelid peptide is highly homologous with those of the myomodulin-CARP-family peptides found in molluscs. The annelid peptide is regarded as a member of the myomodulin-CARP family, though all the molluscan peptides have a Leu-NH<sub>2</sub> at their C-termini. The annelid peptide showed a potent contractile action on the esophagus of the annelid. The peptide may be an excitatory neuromediator involved in the regulation of the esophagus. Among various myomodulin-CARP-family peptides and their analogues, the annelid peptide showed the most potent contractile action on the esophagus [46]. Replacement of the C-terminal Met-NH<sub>2</sub> of the annelid peptide with a Leu-NH<sub>2</sub> decreased its contractile potency, while replacement of the C-terminal Leu-NH<sub>2</sub> of myomodulin and CARP with a Met-NH<sub>2</sub> increased their potency. The C-terminal Met-NH<sub>2</sub> of the annelid peptide seems to be important, but not essential, for exhibiting its contractile activity on the esophagus [46].

## 5.2. Leech Osmoregulator Factor

In early reports we found that LORF (IPEPYVWD) is a peptidergic-signaling molecule involved in osmoregulation [47]. Furthermore, electrophysiological experiments conducted in the leech *H. medicinalis* revealed an inhibition of the efficacy of Na<sup>+</sup> conductance in leech skin [Table 3; 11, 31]. Immunocytochemical studies with an antiserum against synthetic LORF found a great amount of positive immunoreactive neurons in all ganglia. This material was present in a single type of electron-dense secretory granules of a size of 80-100 nm [32]. However, we recently showed its presence in rat tissues, including discrete brain areas [10], which demonstrate that this peptide, originally isolated from the leech, is also present in mammals. Furthermore, in both the leech central nervous system (CNS) and the rat brain, e.g., hypothalamus, LORF is coupled to nitric oxide (NO) release [10]. It is also capable of stimulating NO release from human saphenous vein fragments [10]. These results showed that LORF is not specific to annelids but they also demonstrated that Leech represents a suitable model to isolate novel peptides also conserved in vertebrates.

## 5.3. Leech Egg-Laying Hormone

In leeches, egg laying may be under the control of a leech egg laying hormone (L-ELH) [8]. In *Eisenia fetida*, although that the OT-VP related peptide, annetocin, is known to potentiate the pulsatory contractions in bladder-shaking movement of the nephridia, indicating an involved of osmoregulation though nephridial function, this peptide is also implicated in egg laying behaviors [48]. In fact, annetocin, induced a series of egg-laying-related behaviors in the earthworms. These stereotyped behaviors consisted of well-defined rotatory movements, characteristic body-shape changes, and mucous secretion from the clitellum. Each of these behaviors is known to be associated with formation of the cocoon in which eggs are deposited. In fact, some of the earthworms injected with annetocin (> 5 nmol) laid eggs.

Such egg-laying-related behaviors except for oviposition were also induced by oxytocin, but not by Arg-vasopressin. Furthermore, annetocin also induced these egg-laying-like behaviors in the leech *Whitmania pingra*, but not in the polychaete *Perinereis vancaurica*. These results suggest that annetocin plays some key role in triggering stereotyped egg-laying behaviors in terrestrial or fresh-water annelids that have the clitella [48].

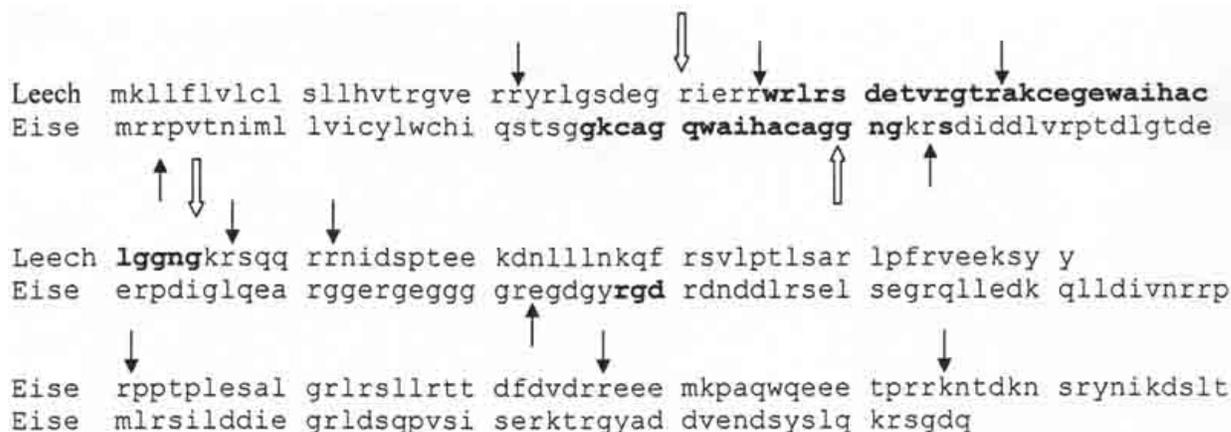
## 5.4. GGNG Peptides

The most specific peptides presently isolated in annelids are the ones related to the GGNG, excitatory peptides (Fig. 4). In leeches, these peptides elicit muscular contractions of isolated preparation of penis and intestine, suggesting that they may play a role in reproduction behavior like the PLGWamide in mollusks [49]. In earthworm, they elicit gut and esophagus muscular contractions. No peptides homologous to GGNG peptides have been isolated so far in any living organisms.

## 6. ARE NEUROPEPTIDE PROCESSING ENZYMES PRESENT IN INVERTEBRATES?

The biologically active neuropeptides are produced via the proteolytic processing of precursor molecules. This post-translational step involves many types of enzymes that have been identified both in vertebrates and in invertebrates. Between these enzymes, neutral endopeptidase (NEP) [19, 50, 51], ACE [23-25], renin [22], aminopeptidases [52], and serine proteases belonging to the subtilisin family [45] play an important role in neuropeptide precursors processing.

Leech brain and immune tissues also contain RAS involving renin and ACE enzymes in angiotensins biosynthesis and ACE, NEP and aminopeptidase in the catabolism of the angiotensins [25]. Leech renin has been detected in immune cells with other aspartyl proteases that would be involved in ACTH (1-39) cleavage to ACTH (1-24) [20]. Interestingly, differential processing was revealed by opiate stimulation of ACTH processing by triggering ACE and NEP actions [4, 19, 40, 53]. As in invertebrates, it is important to note that RAS, *i.e.*, renin gene, has been detected in mammalian macrophage/monocyte cells [53] as was ACE [54] and AII [55]. In man, AII may be implicated in the recruitment of monocytes into the blood vessel wall through the activation of NFκB and the induction of the monocyte chemoattractant protein-1 [54]. Angiotensin II also increases monocyte binding to endothelial cells [55]. The ACE gene is expressed in macrophage-like U937 histiocytic lymphoma cells [53, 56]. In contrast, in leeches, AII has, in preliminary experiments, no immunocyte chemotaxis effect. It also exerts an inhibitory effect *via* nitric oxide release during an inflammatory response [20]. Immunocytochemical studies performed at the level of the immunocytes revealed a co-localization with AT1-like, AII, renin-like and ACE-like enzymes in leech immunocytes [20]. These data were confirmed with binding experiments on immunocytes with <sup>125</sup>I-saralazine. Thus, these data suggest an inhibitory effect of the RAS on the invertebrate immune response. This is opposite to what was recently found in man; nevertheless



**Fig. (4).** Annelids Excitatory peptide Precursor sequences



↓ : Cleavage sites; ↓ : Putative cleavage site

this shown that RAS is an ancient evolutionary conserved hormonal system.

Among the enzymes involved in neuropeptide precursors processing, the serine proteases belonging to the subtilisin/kexin family and known as the proprotein convertases (PCs) are playing, at least in mammals, a crucial role. In mammals, this family includes, so far, seven members (PC1, PC2, furin, PC4, PACE4, PC5 and PC7) which are differentially involved in the processing, generally at single or dibasic residues, of a large number of protein precursors including neuropeptides, growth factors, receptors, enzymes and viral envelope glycoproteins. Between these enzymes, only PC1 and PC2, which are abundantly expressed in neurons and endocrine cells, play an important role in the neuropeptide precursors processing. Although these latter enzymes have been cloned in numerous species including mammals, amphibians, molluscs, insects and nematods, they have not yet been characterized in annelids [50]. Preliminary results obtained in our laboratory (vieu *et al.*, unpublished results) indicate that leech PC1 and PC2 are synthesized in leech brain. Future experiments will be necessary to evaluate the exact role(s) of these different enzymes i.e ACE, NEP and PCs into the generation of biologically active neuropeptides. Moreover, specific natural antagonists of these enzymes have also been discovered in annelids *e.g.* LORF for ACE [57].

## 7. ARE NATURALLY OCCURRING ENZYME INHIBITORS IMPORTANT IN THESE PROCESSES?

Leeches produce a variety of serine protease inhibitors [58]. These enzymes are involved in either preventing coagulation during blood feeding or as inhibitors of leukocytes enzymes. We have isolated in *T. tessulatum* both types of inhibitors [58]. Further, these enzymes are also involved in immune response modulation, by inhibiting inflammation through serine proteases inhibition. Moreover, some of these inhibitors can also act on some PCs such like furin, which has also been discovered in most of all

invertebrates' phyla. We also discovered an endogenous ACE inhibitor, the LORF and hemoglobin-derived peptides, the hemorphins [56].

Clearly, again, the significance of these actions becomes quite clear. Opioid neuropeptides are important for "awakening" the immune and neuroendocrine systems [40]. However, they are, for the most part, "trapped" in their respective precursors. Immunocyte activation initiates this precursor processing [40]. Besides the arguments made in the Stefano and Salzet [40] report, new evidence come from the vast numbers of serine protease inhibitors found in this external parasite. We surmise, in taking its lengthy blood meal, the leech injects these enzyme inhibitors into the host to prevent immune neuropeptide processing. Thus, eliminating the "early" alert system, simultaneously also preventing a general neuroendocrine stress response [40]. This hypothesis also takes into account the ability of this strategy to down regulate the host endothelial surface since it too responds to these common signaling molecules [4, 59, 60].

Recently, man has used this strategy with aprotinin, a serine protease inhibitor. Using this compound diminishes the diffuse inflammatory response associated with surgery [2, 61-63], indirectly demonstrating the significance of processing enzymes. In patients ready to undergo major heart surgery, we found that just before surgery, plasma ACTH levels dropped below the level of detection [64], indicating the activation or inhibition of the processing enzymes [53]. In this regard, it is widely known that various immune and neural-type signaling molecules can up-regulate enzymes such as NEP [51, 60, 65]. Taken together, it is imperative that more studies emerge to examine the critical significance of peptide processing in various processes.

## 8. CONCLUSION

In conclusion, given the wealth of information now emerging on these mammalian-like neuroendocrine processes found in invertebrates, it would appear that this system, in

all probability, originated in “simple” animals. Therefore, aside from its historical origin, it may be more appropriate to speak of the mammalian neuroendocrine system as Annelid-like.

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