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Loss of DNA: A plausible molecular level explanation for crustacean neuropeptide gene evolution

Francisco Martínez-Pérez [a,b], *Darinka Durán-Gutiérrez* [a], *Luis Delaye* [c], *Arturo Becerra* [c], *Guadalupe Aguilar* [a], *Samuel Zinker* [a,*]

[a] *Departamento de Genética y Biología Molecular, Centro de Investigación y de Estudios Avanzados del I.P.N., Apartado Postal 14-740, México D.F. 07000, México*

[b] *Posgraduado en Ciencias Biológicas, Facultad de Ciencias, UNAM, México D.F. 04510, México*

[c] *Facultad de Ciencias, UNAM, México D.F. 04510, México*

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ABSTRACT

Alignment of nucleotides of APGWamide, RPCH and AKH genes gives region stretches (common regions) present in all family member variants. Common regions were separated by gap sections in the larger variants of family members. Consensus sequences for single polynucleotides from virtual hybrid molecules of DNA were obtained by joining the common regions of DNA and deleting the extra DNA nucleotides. Conceptual translation of these virtual hybrids resulted in polypeptides similar to APGWamide, RPCH and the AKH pre-propeptide. Virtual polypeptides were also similar to LWamide and RFamide along hydras to mammals. DNA loss probably explains the origin of neuropeptides.

1. Introduction

Neuropeptide synthesis takes place in neurons and endocrinal cells. These signal molecules function as hormones, neurotransmitters or neuromodulators on excitable cells. Neuropeptide molecules have low molecular weights, ranging in length from 4 to 30 amino acid residues and rarely larger. The biosynthetic pathway for these peptides is similar to what is known for other proteins, namely, the encoded genes are transcribed into mRNA templates which are translated by ribosomes of the rough endoplasmic reticulum. The immediate product of translation is the pre-pro-peptide, the precursor of the active peptide. Three different structural components are identified in the precursor. Component one is the 16–30 amino acid residue N-terminal signal sequence. It functions as guide for the newly formed polypeptide's internalization into the lumen of the endoplasmic reticulum and splits off rapidly after translation. Component two is one pair or more of basic amino acid sites (Arg–Lys, Lys–Arg, Lys–Lys and Arg–Arg) adjoining the polypeptide. They are potential targets of proteolysis. Component three are spacers between each neuropeptide molecule. Spacers lengthen the polypeptide by either a few amino acids or by as much as 10 times the length of the final active product [10].

* *Corresponding author*. Tel.: +55 5061 3800x5322; fax: +55 5061 3931.
E-mail address: szinker@cinvestav.mx (S. Zinker).

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Several animal species share a given neuropeptidergic physiological activity. The diversity of physiological activities allow to group neuropeptides into different families. For example, the adipokinetic hormone (AKH) that stimulates lipid metabolism in insects during flight [7,20]; the red pigment concentrating hormone (RPCH), involved in rhythmic circadian movements of pigments located in the retina or in chromatophoral cells of crustaceans [11,16]; the APGWamide hormone that takes part in mollusks' reproductive behavior [4,12]; the LWamide neuropeptides that control metamorphosis in hydrazoa [8] and the family of RFamide peptides that regulates feeding behavior in insects [5,9]. Differences in the primary amino acid sequence of neuropeptide members show that some molecular structures are more conserved than others. Indeed, 90% similitude in RPCH, AKH and APGWamide C-terminal fragments have been reported [15].

At the molecular level, several mechanisms have been postulated to explain the origin of new gene structures. Examples include the emergence of melanin concentrating hormone genes by retro transposition and acquisition of exons from single non-coding regions [2]. The origin of AKH/RPCH and APGWamide from an ancestral gene and its subsequent process of evolution, since portions of the pre-pro-peptide from APGWamide might have given rise to the RPCH pre-pro-peptide [15]. Other mechanisms include exon shuffling, DNA duplication, incorporation of mobile elements, lateral gene transfer and gene fusion [10,14]. None of these explains how the pre-pro-peptide for APGWamide could be converted to the AKH/RPCH neuropeptides.

In this work probable molecular events that lead to the origin of AKH/RPCH and APGWamide members are proposed, based on specific nucleotide or codon elimination from spacer regions. Therefore, functional translational constrictions are promoted in favor of the pre-pro-peptide. A putative hybrid gene, and its variants, is postulated which eventually would generate the genes for APGWamide, RPCH and AKH from the LWamide gene.

2. Materials and methods

2.1. cDNA sequences

The sequences of the cDNA pre-pro-peptide were obtained either from the report of the original paper or from sequences reported in the GenBank (accession numbers are indicated).

2.2. Organisms and neuropeptides

The neuropeptide members and organisms selected was as follows: AKH from *Drosophila melanogaster* [NM_079194], *Manduca sexta* [J04972], *Locusta migratoria* [X86799; X86800; X86801], *Shistoserca gregaria* [6,19], *Shistocerca nitans* [J05170; J05171] and *Blabelus discoidales* [U35277]; RPCH from *Cherax quadricarinatus* [AY642684], *Callinectes sapidus* [L36824] and *Carcinus maenans* [S65357]; APGWamide from *Limnea stagnalis* [18], *Metilus edulis* [X92372] and *Aplisia californica* [U85585]; LWamide from *Hydrachtinia magnipapillata* [8], *Anthopleura elegantísima* [U34781] and *Hydrachtina echinata* [CAA61886]; RFamide from *Hidrachtina echinata* [X97413]; *Hydra magnipapillata* [Y11680]; *Podicornea carnea* [X82896]; *Anthopleura elegantissima* [M98269]; *Polyorchis penicillatus* [L14777]; *Calliactis parasitica* [M59166]; *Renilla koellikeri* [Z25484]; *Loligo opalescens* [AF303160]; *A. californica* [P08021]; *Lymnaea stagnalis* [P19802]; *Sepia officinalis* [CAA72116]; *Procambarus clarkii* [BAE06263 and BAE06262]; *D. melanogaster* [P10552]; *Drosophila virilis* [A60918]; *Drosophila pseudoobscura* [EAL25813]; *Periplaneta americana* [AY333435].

2.3. Selection of nucleotides, primary sequence for polynucleotides, segment alignments and conceptual translation

Details of the primary sequences for stretches of polynucleotide segments were previously reported [15]. Cluster of numbers for open reading frames (ORF) of newly selected polynucleotides as they appear in GenBank were as follows. For the model of mollusk–crustacean-insects consensus hybrids of DNA (Fig. 2): *A. californica* [cds-U85585], 2–6; 11–22; 59–72; 77–85; 98–106; 133–156; 216–228; 273–279; 306–346; 356–363; 374–478; 487–517; 534–544; 660–690; 705–729. *L. stagnalis* [cds-18], 58–82; 129–132; 137–145; 158–166; 193–216; 276–288; 333–339; 366–406; 416–424; 440–538; 547–577; 594–604; 718–750; 766–789. *M. edulis* [cds X92372], 1–60; 74–82; 119–132; 137–145; 158–166; 193–216; 276–288; 333–339; 366–406; 416–424; 441–453; 456–538; 547–577; 594–604; 718–750; 766–792; 793–810.

For the model of mollusk–crustacean consensus hybrids of DNA (Fig. 3): *A. californica* [cds-U85585]: 1–91; 138–160; 204–224; 227–232; 274–280; 375–406; 429–523; 547–553; 566; 572–580; 621–662; 668–671; 680–689; 701–719; 730–732; 737–753; 760–821. *L. stagnalis* [cds-18]: 1–91; 138–160; 204–224; 227–232; 274–280; 375–406; 429–523; 547–553; 566; 572–580; 621–662; 668–671; 680–689; 701–719; 730–732; 737–753; 760–771. *M. edulis* [cds-X92372]: 1–91; 137–160; 223–224; 227–232; 274–280; 375–406; 428–510; 547–553; 566; 572–580; 621–662; 668–671; 680–689; 701–719; 730–732; 737–753; 760–771.

Polynucleotide stretches were aligned using the ClustalW program [21] with a 0.05 gap extension and a gap distance of 9. Conceptual translation of polynucleotides was performed with The Expert Protein Analysis System programs (ExPASy; http://au.expasy.org).

2.4. Putative neuropeptides

Polynucleotides for the pre-pro-neuropeptide had to include, sequentially, a sequence signal for the endoplasmic reticulum, two adjacent codons for basic amino acids, a GGN codon for glycine and a sequence for the residual peptide. DNA sequences not found in all members of the families were eliminated, since selection most likely would not exert pressure on them (Fig. 1). The search was done by BLAST program of gene sequences and by the reported cDNAs in GenBank.

3. Results

We reasoned that ortologus genes would likely maintain clusters of nucleotides, which might create alternative open reading frames (ORF) under selective pressure conditions. Therefore, homologous polynucleotide regions should translate for a virtual polypeptide containing the main structural

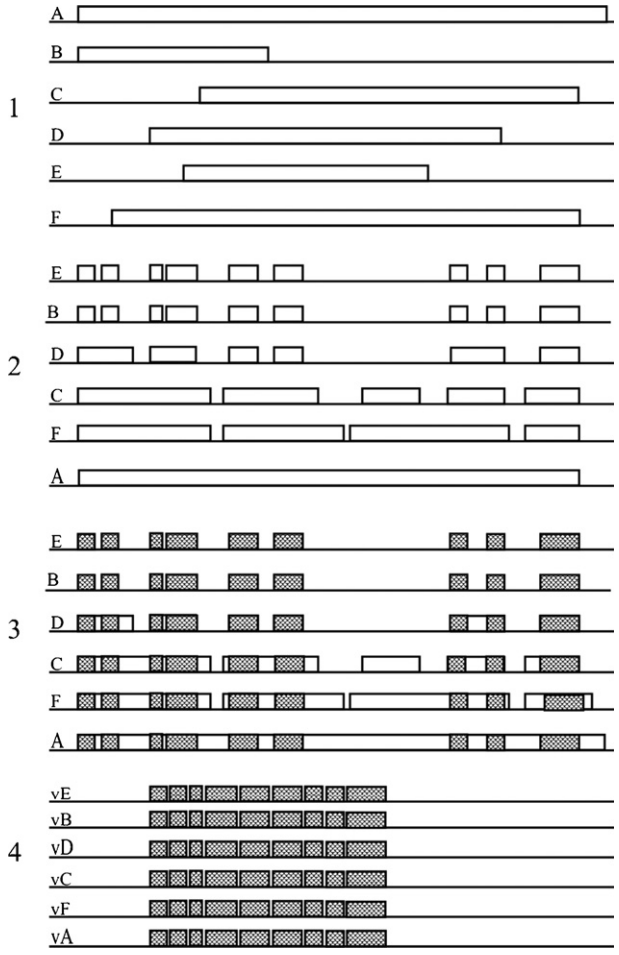

Fig. 1 – Outline for obtaining virtual open reading frames (vORF). (1) Selection of polynucleotide stretches from homologous genes (A–F). (2) Polynucleotide alignment (empty boxes). (3) Selection of polynucleotide sequences shared by all species (full boxes). (4) Joining of the largest common sequences (vC, vF and vA).

features of a pre-pro-peptide, namely: (a) reticulum endoplasmic signal; (b) two adjacent codons for basic amino acids flanking the active neuropeptide; (c) an indispensable GGN codon as the C-terminal glycine that eventually will be amidated; (d) the presence or absence of an extra amino acid sequence for the related peptide.

Alignment of the nucleotides for AKH, RPCH, and APGWamide members gave eight adjacent regions as demonstrated previously [15]. In this work these adjacent regions were joined into a continuous polynucleotide, and we asked if the product obtained served as a virtual open reading frame (vORF). The answer was affirmative. The oligonucleotides for the virtual consensus primary sequences for the mollusks *A. californica*, *L. stagnalis* and *M. edulis*, in parallel to their conceptual translation products, are shown in Fig. 2. For *A. californica* a virtual protein of 108 amino acids was obtained. The first 81 nucleotides would code for a signal peptide of 27 amino acid residues in length. A putative RPCH sequence followed, with a Ser to Leu change at position 2 (amino acid residue 29). A four time repeat of the sequence for the basic amino acid dipeptide was present at 37–38, 59–60, 71–72 and 90–91. Before the third repeat, a virtual sequence of APGamide was present. The structural equivalent of a possible related peptide sequence could not be identified (Fig. 2A).

For *L. stagnalis* the vORF was 98 amino acids in length (Fig. 2B). The first 28 amino acid residues would correspond to the signal peptide. The RPCH sequence with a Lys–Gln insertion between amino acids 1 and 2 (amino acid position 30–31) would go next, followed by the sequence Lys–Arg as a putative proteolitic site. This basic amino acid dipeptide sequence is repeated three more times at amino acid positions 57–58, 73–74 and 80–81. Just before the second and right after the third repeat, two copies of the putative APGamide structure were located (Fig. 2B). For *M. edulis*, the vORF had 137 amino acids, where the first 48 residues would correspond to the signal peptide (Fig. 2C). The cleavage of the basic Arg–Arg dipeptide sequence would produce five polypeptides, one of them similar to APGWamide but with Ala instead of Arg at position 1 (amino acid position 87) (Fig. 2C).

3.1. *The DNA loss model holds for mollusks and crustaceans*

Having shown that loss of DNA segments joining the resulting stretches from the AKH, RPCH and APGWamide generated RPCH and APGWamide virtual neuropeptides, we asked how the model would behave when limits of restriction of polynucleotide hybrid consensus sequences were to APGW amide and RPCH precursors (mollusk and crustacean) only. The results are shown in Fig. 3. Both *A. californica* and *L. stagnalis* virtual products' structural organization were similar. The length of their polypeptides was 113 and 109 amino acid residues, respectively (Fig. 3A and B). The signal peptides were 26 and 29 amino acid residues in length for *A. californica* and *L. stagnalis*, respectively. A stretch of APGWamide sequences, two basic amino acids, and a putative RPCH structure followed. Forty amino acid residues later, toward the C-terminal end, three copies of APGW amide in tandem were located, separated by pairs of basic amino acids. In *M. edulis* two stop codons at positions 4 and 41 were present (Fig. 3C). The difference between the real RPCH from crustacean and the putative virtual RPCH in *A. californica* and *M. edulis* was, again, a Leu to Ser or Phe change, respectively.

3.2. *The APGWamide is related to LWamide and RFamide from Phylum Cnidaria*

The hybrid vORF between mollusks and crustaceans gave possible evidence for pre-pro-peptides for *A. californica*, *L. stagnalis* and *M. edulis*. The next question was whether mollusk–crustacean hybrid like peptides (vp) would be present in species other than mollusks and crustaceans. BLAST alignments were performed and the sequences were looked for in GeneBank. Each vp was compared in an independent way to the real peptides (rp) of *A. californica*, *L. stagnalis* and *M. edulis*. The results were as follows: both the (vp)-*A. californica* and (vp)-*L. stagnalis* were 48%, 43% and 40% similar to the rp-APGWamide from *A. californica*, *L. stagnalis* and *M. edulis*, respectively. Surprisingly, both (vp)-*A. californica* and (vp)-*L. stagnalis* showed 46% and 39% similarity to the (rp)-LWamide

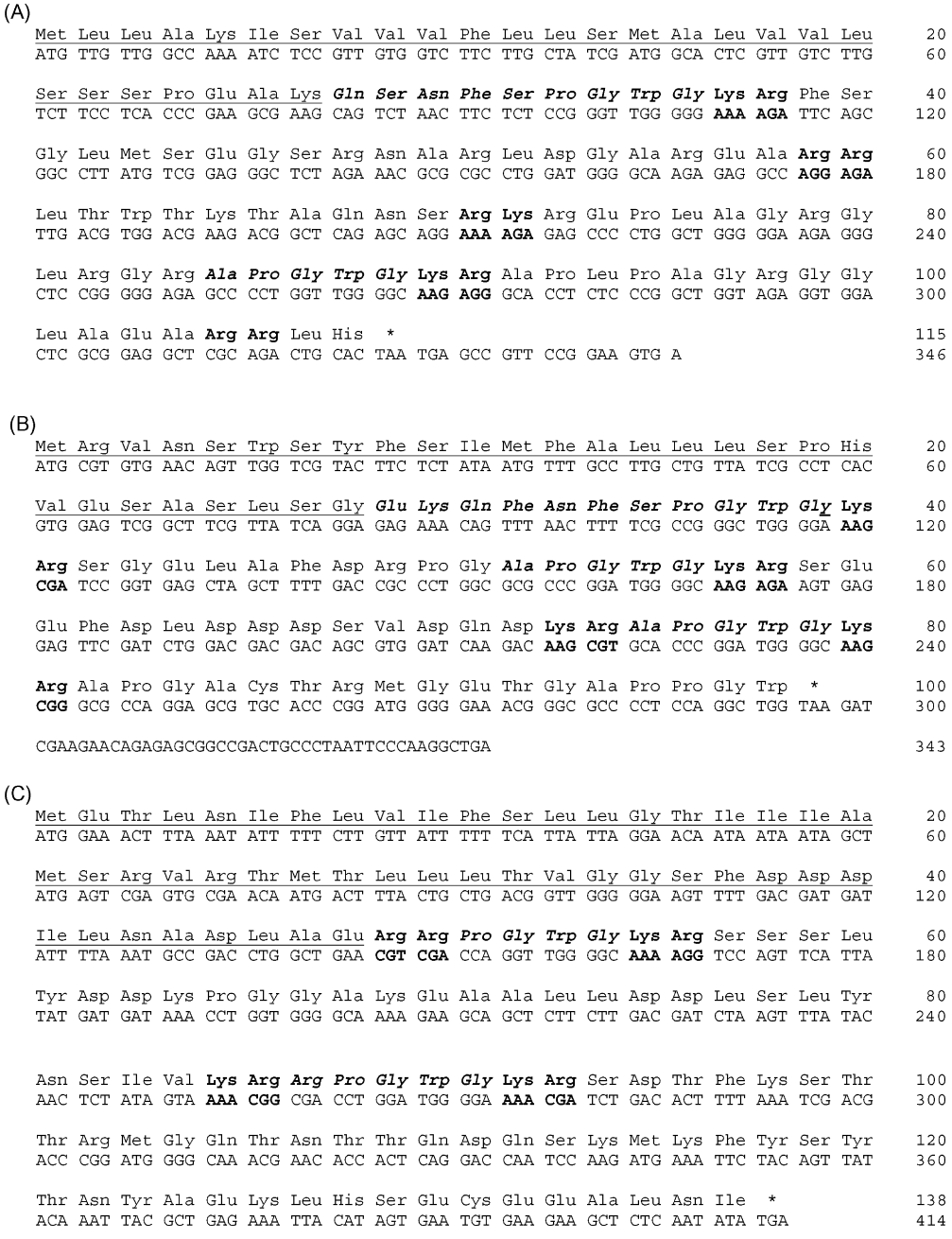

Fig. 2 – vORF detected for *A. californica* (A), *L. stagnalis* (B) and *M. edulis* (C), mollusk species. The virtual genes were obtained after selection of common nucleotide sequences and virtual hybridization between mollusks, crustaceans and insects (see Section 1). The virtual and conceptual signal peptide sequence is underlined. The virtual RPCH sequence is in bold and italics. The virtual basic amino acid dipeptides, as potential cleavage sites, are in bold and the virtual APGWamide is in bold and italics.

precursor from *Anthopleura elegantisima* and *H. echinata*. (vp)-*M. edulis* was 49%, 47% and 37% similar to the (rp)-APGWamide of *A. californica*, *M. edulis* and *L. stagnalis*, respectively, and 47% similar to (rp)-RFamide family members.

A broad range of members spanning from class *Hydrozoa* to class *Mammalian* are included in the LWamide and RFamide group of neuropeptides. In invertebrates, LWamide and RFamide precursors include several copies in tandem of the active peptide, varying in number depending on the species (Table 1). The vORF homology between the amino acid sequence from APGWamide, LWamide and RFamide families suggests that the corresponding genes of these neuropetides could be related. These results suggest that stretches of nucleotides were structurally and functionally conserved. Confirmation came from codon alignment of active peptides from each species (Table 2). The sequence PGWG (amino acids in position 6–9) from RPCH and AKH was similar to APGWamide from *A. californica* and *M. edulis* (amino acids in

(A) *Aca* MLL---AKISVVVLLLAIDGTCCLVFLTR***APGWG*KR*QSNFSPGWG*KR**GQEIDVDEDGSEQ 57
(B) *Lst* MRVNSWSYFSIMFAQLAVILSRGVGFVIR***APGWG*KR*QFNFSPGWG*KR**SEEFDLDDDSVDQ 60
(C) *Med* MET**X**-----NIFLVIFSLLGTIIIASSYDTNNDFLRGLKF**X**PGWGKRSDMLKRRPGWGKR 55

(A) *Aca* E**KR***APGWG*KR*APGWG*KR*APGWG*KGSTCTCGDYCETLEKMVDAFIE-VDSRRLADCVAREL 116
(B) *Lst* D**KR***APGWG*KR*APGWG*KR*APGWG*KSSTGASSDYCETLKEVADGLVR-SKNRERPTA----- 114
(C) *Med* SSSLYDDE**KR**KPGWGKRSSLFDNSNGDLDGENDLTLLKFRMGQDGESAQVHFQDEILYKL 115

(A) *Aca* ISGSD**KR**G-------- 124
(B) *Lst* ----------
(C) *Med* LNEAEKLHSECEALNI 131

Fig. 3 – vORF detected for *A. californica* (A), *L. stagnalis* (B) and *M. edulis* (C), mollusk species. Virtual gene selection and virtual hybridization between mollusks and crustaceans (see Section 1). The virtual and conceptual signal peptide sequence is underlined. The virtual APGWamide is in bold, italics and underlined. The virtual basic amino acid dipeptides, as potential cleavage sites, are in bold. The virtual RPCH sequence is in bold and italics. Stop signal is an X in bold.

position 2–5) respectively, and APGW from *L. stagnalis* was similar to LWamide from *H. magnipapillata* and *C. elegans*.

4. Discussion

In this work we propose a theoretical model of DNA loss to explain the generation of new neuropeptide genes in invertebrates. The homologous DNA sequences would give virtual hybrids genes that by rearrangement and loses of nucleotides, dispensable for the final neuropeptide function, would generate new vORFs coding for the genes of APGWamide, RPCH and AKH.

The rationale behind the model relies on two premises. First, in mollusks and crustaceans, AKH, RPCH and APGWamide have stretches of non-contiguous nucleotides that contain the sequences for APGWamide, RPCH and AKH [15]. Second, APGWamide, RPCH and AKH have functional cross-characteristics. Namely, AKH and APGWamide (insect and crustacean) concentrate pigments in crustaceans [1,17], whereas RPCH (crustacean) stimulates lipid metabolism in insects [3,22]; the last three amino acids $PGWNH_2$ from RPCH and APGWamide (crustacean and mollusk) are identical to the last amino acid sequence of Taa–AKH, Psi AKH and Lem–HrTH (insect) from orders Blataria, Diptera and Odonata, respectively (Table 2). These three amino acids by themselves are necessary [1,13,23] but not sufficient to produce physiological activity of RPCH and AKH in target cells.

BLAST analyses showed that the vORF for AKH, RPCH and APGWamide are homologous to LWamide and RFamide that could be traced back in time as early as organisms from Phylum *Cnidaria* (Table 1). The number of copies for the putative

Table 1 – Invertebrate neuropeptide members

Period	Mya[a]	Phylum	Class	Organism	Nueropeptide	ORF[b]	NpC[c]	GenBank
Devonian	396–407	Artropoda	Insecta	*S. gregaria*	AKH II	186	1	[6]
Devonian	396–407	Artropoda	Insecta	*S. nitans*	AKH II	186	1	J05171
Devonian	396–407	Artropoda	Insecta	*L. migratoria*	AKH II	186	1	X86800
Devonian	396–407	Artropoda	Insecta	*S. gregaria*	AKH	192	1	[21]
Devonian	396–407	Artropoda	Insecta	*S. nitans*	AKH	192	1	J05170
Devonian	396–407	Artropoda	Insecta	*M. sexta*	AKH	198	1	J04972
Devonian	396–407	Artropoda	Insecta	*L. migratoria*	AKH	204	1	X86799
Devonian	396–407	Artropoda	Insecta	*B. discoidalis*	HTH	219	1	U35277
Devonian	396–407	Artropoda	Insecta	*L. migratoria*	AKH III	234	1	X86801
Devonian	396–407	Artropoda	Insecta	*D. melanogaster*	AKH	240	1	NM_079194
Lower-Cambrian	511	Artropoda	Malacostraca	*C. quadricarinatus*	RPCH	273	1	AY642684
Lower-Cambrian	511	Artropoda	Malacostraca	*C. sapidus*	RPCH	330	1	L36824
Lower-Cambrian	511	Artropoda	Malacostraca	*C. maenans*	RPCH	333	1	S65357
Lower-Cambrian	570–507	Mollusca	Bivalvia	*M. edulis*	APGWamide	591	7	X92372
Lower-Cambrian	570-507	Mollusca	Gastropoda	*A. californica*	APGWamide	651	9	U85585
Lower-Cambrian	570–507	Mollusca	Gastropoda	*L. stagnalis*	APGWamide	660	10	[20]
Late-Precambrian	600-570	Cnidaria	Hydrozoa	*H. magnipapillata*	LWamide	1212	12	U53444
Late-Precambrian	600–570	Cnidaria	Hydrozoa	*H. echinata*	LWamide	1260	18	X89734
Late-Precambrian	600–570	Cnidaria	Anthozoa	*A. elegantisima*	LWamide	1545	40	U34781

[a] Millon years to today.
[b] Open reading frame in base pairs.
[c] Neuropeptide copies in the pre-pro-peptide.

Table 2 – Relationship between position of amino acids and codons in LWamide, APGWamide, RPCH, and AKH

Peptide	Amino acid no.[a]														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
[2]LWH*m*				Gly	Pro	Met	Thr	Gly	Leu	**Trp**			**Gly**	Lys	Lys
				GGA	CCA	ATG	ACA	GGA	CTT	TGG			GGA	AAA	CGC
[2]LWH*m*				Gly	Pro	Pro	**Pro**	**Gly**	Leu	**Trp**			**Gly**	Lys	Arg
				GGT	CCC	CCA	CAT	GGA	CTT	TGG			GGA	AAA	CGC
[1]LWH*m*			Pro	Pro	Trp	Arg	Gly	**Gly**	Met	**Trp**			**Gly**	Arg	Ser
			CCT	CCA	TGG	AGA	GGA	GGT	ATG	TGG			GGT	AGA	AGC
[1]LWC*e*	Val	Leu	Gly	Trp	Lys	**Ala**	His	**Gly**	Leu	**Trp**			**Gly**	Lys	Arg
	GTG	TGG	ATG	AAT	AAG	GCA	CAC	GGA	TTG	TGG			GGT	AAG	AGG
[3]APGW						**Ala**	**Pro**	**Gly**		**Trp**			**Gly**	Lys	Arg
						GCN	CCC	GGC		TGG			GGT	AAG	AGG
[3]RPCH		Glu	Leu	Asn	Phe	Ser	**Pro**	**Gly**		**Trp**			**Gly**	Lys	Arg
		CAG	CTT	AAC	TTC	TCC	CCC	GGC		TGG			GGT	AAG	AGG
[3]AKH2		Glu	Leu	Asn	Phe	Ser	Thr	**Gly**		**Trp**			**Gly**	Arg	Arg
							Ala								
		CAG	CTC	AAC	TTC	TCA	ACC	GGT		TGG			GGT	CGG	GCG
						TCG	GCG	GGG					GGG		
[1]AKH*Lm*3		Glu	Leu	Asn	Phe		Thr	Pro		**Trp**		Trp	**Gly**	Lys	Arg
		CAG	CTC	AAC	TTC		ACG	CCG		TGG		TGG	GGC	AAG	AGG
[1]AKH*Ms*		Glu	Leu	Thr	Phe	Thr	Ser	Ser		**Trp**		Gly	**Gly**	Arg	Lys
		CAG	CTC	ACC	TTC	ACC	TCG	AGC		TGG		GGA	GGA	AAG	AGG
[4]AKH		Glu	Leu	Asn	Phe	Thr	**Pro**	**Gly**		**Trp**	Gly	Thr	**Gly**	Lys	Arg
			Val			Ser		Asn							
		CAG	CTC	AAC	TTC	ACC	CCC	AAC		TGG	GGC	ACC	GGC	AAA	CGG
			GTG			TCA	CCT	GGC			GGG	ACT		AAG	CGG
											GGT				

Super index number = number of species that have the same codon and amino acid. The GenBank access numbers are indicated in Section 2.
[a] Amino acids in bold are essential for activity.

LWamide and RFamide peptides was highest. Thereafter numbers decreased for APGWamide, present in about seven copies in tandem, and for AKH and RPCH that were present as mono-copies (Table 1). Likewise, among different neuropeptides the length of the polynucleotide changed for each virtual precursor. APGWamide from mollusk was the largest with an average of 600 bp. The number of nucleotides for RPCH from crustaceans and AKH from insects was 300 and 190 bp average, respectively (Table 1).

It should be point out that although the model of DNA loss was applied to organisms of the group molluska and crustacean, analysis showed that the model could be sustained up to the group of insecta (Table 2). Our interpretation of this result is that RFamide and LWamide genes may generate APGWamide by duplication and DNA loss, which under the influence of similar mechanisms would give rise to RPCH and AKH. In support of this hypothesis, the loss of the codon for Leu in LWamide of *H. magnipapillata* gave rise to APGWamide, and the loss of some regions of APGWamide gave origin to RPCH and AKH (Fig. 4 and Table 2). Loss of DNA in LWamide, APGWamide, RPCH and AKH genes would suggest that neuropeptides have had a tendency to shorten both their gene size and their copy number down to minimal gene structures that retain a variety of functions.

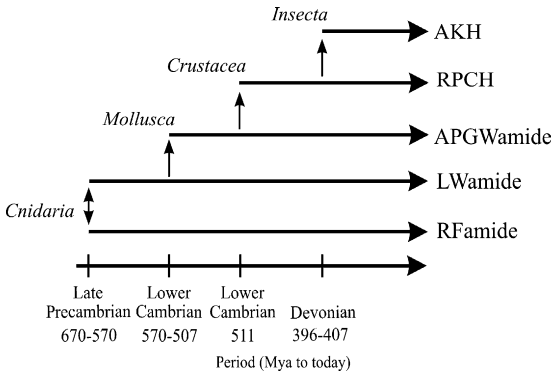

Fig. 4 – Appearance of neuropeptides in different *Phyla* along evolution.

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