Evolutionary and behavioral analysis of neuropeptides in bilaterians

Daniel Thiel

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Author: Daniel Thiel

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Author contributions

I hereby declare that I have written this thesis by myself, with help of other people in form of comments and formal corrections (except for manuscript IV, for which my contribution is stated separately at the end of this section).

I was involved in the design of the studies, performed most of the experiments and wrote the different drafts and the final versions of manuscript I, manuscript II and manuscript III. Direct contributions of other people to manuscript I, II and III are as follows:

Philipp Bauknecht (Max Planck Institute for Developmental Biology, Tübingen, Germany) supervised me during the receptor deorphanizations. He also conducted one experimental replicate of the *L. longissimus* EP receptor dose-response curves and the *T. transversa* DFLRFamide receptor dose-response curve by himself and commented on manuscript I.

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Gáspár Jékely (Max Planck Institute for Developmental Biology, Tübingen, Germany) was involved in the design of the studies for manuscript I & II, interpreted the results and commented on manuscript I.

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Manuscript IV was written by Chiara Sinigaglia and my contribution was the adaption of the hybridization protocol for *Terebratalia transversa*, *Novocrania anomala* and *Priapulus caudatus,* and I commented on the manuscript draft.

Abbreviations

ABRM	Anterior byssus retractor muscle
ACP	AKH/CRZ-related peptide
AKH	Adipokinetic hormone
CCAP	Crustacean cardioactive peptide
CK	Cholecystokinin
CKH	Cystine knot hormone
CRZ	Corazonin
EC50	Half maximal effective concentration
EP	Excitatory peptide
FaRP	FMRFamide-related peptides
FLP	FMRFamide-like peptides
GnRH	Gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
ILP	Insulin-like peptide
MIP	Myo-inhibitory peptide
NpF	Neuropeptide F
NpY	Neuropeptide Y
sNpF	Short Neuropeptide F
TRH	Thyrotropin-releasing hormone

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1. Abstract

Neuropeptides are a diverse group of neurosecretory signaling molecules that are utilized by the nervous system of all bilaterian animals. These signaling molecules are involved in most physiological processes and can play major roles in animal behavior. Most of our knowledge about neuropeptides – whether in regards to neuropeptide diversity or the influence of neuropeptides on animal behavioral – originates from a collection of species that can be grouped into a few bilaterian clades. In this thesis, I investigate two different aspects of neuropeptide signaling, that involve animal species from clades that haven't been investigated before: the neuropeptide repertoire of xenacoelomorphs and the influence of neuropeptides on the behavior of planktonic larvae from a brachiopod and a nemertean species.

A major bilaterian clade where nothing is known about the neuropeptide repertoire are the Xenacoelomorpha. According to recent phylogenetic analysis Xenacoelomorpha are the sister group to all remaining Bilateria (Deuterostomia + Protostomia) and therefore hold an important phylogenetic position for understanding bilaterian neuropeptide evolution.

We identified the neuropeptide and neuropeptide receptor repertoire of xenacoelomorphs, by combining an *in silico* analysis of transcriptomes from 13 xenacoelomorph species with the mass spectrometric analysis of peptide extracts from 3 of these species. Our findings show the presence of several bilaterian neuropeptides as well as the presence of novel, xenacoelomorph specific neuropeptides and their diversification during xenacoelomorph evolution.

Only a few functional studies have shown the influence of neuropeptides on the behavior of trochozoan larvae, and those used annelid or mollusc larvae. The only knowledge that we have about neuropeptidergic signaling in brachiopod or nemertean larvae originates from a few immunohistochemical studies.

We investigated the excitatory peptide (EP) that has previously been identified in annelids and molluscs, where it showed myo-excitatory properties on tissue preparations of adult animals. We show that EP and the EP receptor are also present in brachiopods and nemerteans. We deorphanized the *Lineus longissimus* (Nemertea) EP receptor and show that EP can shift the swimming distribution of *L. longissimus* pilidium larvae in a water column upwards by increasing the beat frequency of the larval locomotory cilia.

Another neuropeptide that we investigated is FLRFamide and its influence on the behavior of *Terebratalia transversa* (Brachiopoda) larvae. When mechanically disturbed, *Terebratalia transversa* larvae protrude their stiff and pointy chaetae in a defensive manner and sink down slowly. Both of these reactions can be induced simultaneously by FLRFamide. We deorphanized the *T. transversa* FLRFamide receptor and found its expression at the apical prototroch of the larvae and in the trunk musculature, which are the tissues that are responsible to perform the two sub-reactions. Customized antibodies against FLRFamide revealed FLRFamidergic nerves in and around the apical neuropil as well as FLRFamidergic nerves that directly innervate the trunk musculature.

In this thesis, I present a substantial dataset of neuropeptides and neuropeptide receptors from different species of the Xenacoelomorpha, an animal clade where nothing has known about their neuropeptide repertoire before. I also present two studies where I show how neuropeptides can influence the behavior of brachiopod and nemertean planktonic larvae.

2. Introduction

2.1 Neuropeptides are signaling molecules of the nervous system

Animal nervous systems are composed of connected nerve cells that transfer electric signals between each other. Depending on the animal, these nerve cells can have different degrees of condensation and form nets, nerve bundles, ganglia or complex brains (Schmidt-Rhaesa, 2007). The direction of nerve signals is mainly provided by the physical wiring via synapses, the different properties of their dendrites and axons and the use of different transmitters. Low molecular weight neurotransmitters like acetylcholine, amino acids (e.g. aspartate, glutamate, D-serine) or monoamines (e.g. adrenaline, dopamine, serotonin) are released from synaptic vesicles and ensure a fast and efficient signal transmission between neighboring cells. Another class of neurotransmitters that can transfer or modify signals, are neuropeptides. Most neuropeptides are less than 50 amino acids long and the smallest neuropeptides consist of only 3 amino acids, such as the thyrotropin-releasing hormone (Hokfelt et al., 2000; Jékely, 2013; Liu et al., 2006b, 2008). Neuropeptides are secreted by neurons and neurosecretory cells, in which usually one or more kind of neuropeptides are present in addition to low molecular weight transmitters (Merighi, 2002; Salio et al., 2006). Neuropeptides do not only act as direct signal transmitters, but can also act as co-transmitters to modify signal transmission (Baraban and Tallent, 2004; Nusbaum et al., 2001; Salio et al., 2006), for example, by inhibiting the presynaptic release of other transmitters (Cherubini and North, 1985; Choi et al., 2015; Whittaker et al., 1999). Neuropeptides can also be released at non-synaptic sites along axons, dendrites or even the cell soma, and can act on distant targets, distributed as neurohormones through extracellular fluids, blood or hemolymph (Cuadras, 1989; Landgraf and Neumann, 2004; Ludwig and Leng, 2006; Nassel, 2002). The specificity of the target activation is ensured by a selective peptide-receptor activation and the existence of a high variety of peptide-receptor pairs (Frooninckx et al., 2012; Jékely, 2013; Mirabeau and Joly, 2013).

2.2 Neuropeptides emerged early during metazoan evolution

Neuropeptides emerged early during metazoan evolution and are considered to be the oldest neural signaling transmitters (Jékely, 2013; Moroz and Kohn, 2016). Processing enzymes for neuropeptide precursors and potential neuropeptides are present in all major metazoan clades. For example, glycoprotein hormone/bursicon-related peptides of a cystine knot hormone like family (CKH) and enzymes that are necessary to process proneuropeptides and prohormones have been identified in sponges (Roch and Sherwood, 2014; Srivastava et al., 2010). Glycoprotein hormones, bursicons and CKHs usually consist of two subunits (alpha and beta) that are encoded on two separate precursors, both of which are larger peptides with conserved cysteine residues (Fig.1) and an overall low sequence similarity between distantly related taxa (Luo et al., 2005; Roch and Sherwood, 2014). Ctenophores possess several neuropeptides (Moroz et al., 2014), but the only neuropeptide homologous to other metazoan neuropeptides is a CKH that is similar to the CKH of sponges (Roch and Sherwood, 2014). Some of the other ctenophore neuropeptide candidates are short peptides, where the predicted bioactive peptides are encoded multiple times (also called paracopies) on the peptide precursor (Fig.1).

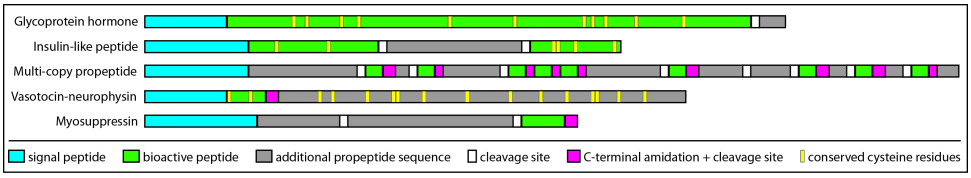

Fig.1: Simplified neuropeptide precursor structures of glycoprotein hormone, insulin-like peptide, short repetitive peptides, vasotocin-neurophysin and myosuppressin.

Even placozoans, a group of animals that have no neurons at all (Smith et al., 2014), have CKH-like peptides, several short peptides with multiple paracopies on their precursors and insulin-like peptides (ILPs) (Jékely, 2013; Nikitin, 2015). ILPs consist of two small subunits with conserved cysteine residues, which are encoded on a single peptide precursor (Fig.1) (Chan and Steiner, 2000; Claeys et al., 2002; Floyd et al., 1999). The position of the two subunits on the propeptide is relatively conserved, but the overall sequence similarity between ILPs of distantly related taxa

is very low (Anctil, 2009; Claeys et al., 2002; Floyd et al., 1999; Semmens et al., 2016). Cnidarians have ILPs, bursicon-related peptides and many different short peptides with multiple paracopies on the precursor (Anctil, 2009; Grimmelikhuijzen et al., 2002; Jékely, 2013; Roch and Sherwood, 2014). Bilaterians can have an enormous repertoire of different neuropeptides, including ILPs, glycoprotein hormones and many more that can be found either in all bilaterians or only in certain clades or subclades (Jékely, 2013; Mirabeau and Joly, 2013). The number of predicted peptide precursors published in studies of different bilaterian species varies, e.g. 30 in the hymenopteran *Nasonia vitripennis* (Hauser et al., 2010), 35 in the snail *Theba pisana* (Adamson et al., 2015), 40 in the starfish *Asterias rubens* (Semmens et al., 2016), 74 in the oyster *Crassostrea gigas* (Stewart et al., 2014), 98 in the annelid *Platynereis dumerilii* (Conzelmann et al., 2013a), 119 in *C. elegans* (Frooninckx et al., 2012) and 119 in *Drosophila melanogaster* (Liu et al., 2006a). It is however hard to compare these predicted numbers as they are based on partially different search strategies and different criteria to determine potential neuropeptide precursors. A similarly high variety of neuropeptides as in bilaterians might also exist in cnidarians or ctenophores, but is not yet known due to a comparatively low taxon sampling and the lack of functional studies.

2.3 Neuropeptides regulate physiological processes and behavior

The function of neuropeptides is highly diverse and they are involved in most physiological processes and influence important aspects of animal life, such as feeding (Dockray, 2004; Williams et al., 2015), locomotion (Choi et al., 2015; Willows et al., 1997), reproduction (Gorbman et al., 2003; Terakado, 2001), life stage transitions (Conzelmann et al., 2013b; Erwin and Szmant, 2010), stress-tolerance (Terhzaz et al., 2015; Zhang et al., 2015) or social interaction and bonding (Kosfeld et al., 2005; Lukas et al., 2011). Some neuropeptides influence simple behavioral modifications such as changes in ciliary beating that can, for example, cause changes in cilia-based locomotion (Braubach et al., 2006; Conzelmann et al., 2011; Penniman et al., 2013; Willows et al., 1997). Other neuropeptides activate whole cascades of different processes and subreactions in separate tissues, like the

ecdysis triggering hormone and eclosion hormone during insect ecdysis (Kim et al., 2006; Taghert and Nitabach, 2012; Truman, 2005). It has been shown that some neuropeptide orthologs of distantly related animals can trigger comparable behaviors, like gonadotropin-releasing hormone (GnRH) in deuterostomes and some protostomes: GnRHs stimulate the synthesis and release of gonadotropins, luteinizing hormone and follicle-stimulating hormone in vertebrates, which ultimately leads to gametogenesis and steroid production in the gonads (Okubo and Nagahama, 2008). Endogenous GnRH of *Ciona intestinalis* induces gamete release (Terakado, 2001). Lamprey and tunicate GnRHs were even able to induce gamete release in the mollusc *Mopalia* sp. (Gorbman et al., 2003) and the GnRH-related peptide of *Patinopecten yessoensis* stimulated spermatogonia mitosis in testicular cells of *P. yessoensis* (Treen et al., 2012). The GnRH ortholog of *Aplysia californica*, however, induced parapodial opening and inhibited food consumption, but had no influence on reproduction-related parameters (Tsai et al., 2010). The GnRH-related adipokinetic hormone (AKH) is also involved in reproduction of *C. elegans*, indicated by a decrease in the number of eggs that were laid by animals with peptide and receptor knockdowns (Lindemans et al., 2009). In insects, however, AKHs are often involved in energy mobilization from lipids or carbohydrates during energy-intensive tasks like flying (Gade and Auerswald, 2003).

Different neuropeptides can act on the same system and evoke similar, complementary or opposite effects. In the snail *Aplysia californica*, for example, two structurally related peptides with different receptors (FMRFamide and FRFamide), can work in complementary ways towards an egestive behavior (protraction of the radula): FMRFamide seems to work primarily presynaptic to depresses motor neuron-elicited contraction of the radula closer muscle, while FRFamide seems to work primarily postsynaptic and activates a contraction-depressing mechanism (Vilim et al., 2010). An example for opposite effects of different neuropeptides on the same system is the effect of two neuropeptides on the cardiac stomach of *Asterias* starfishes (Asteroidea). Starfishes can eat by everting their cardiac stomach over their prey and ingesting it slowly before they retract their stomach later on (Anderson, 1954). This eversion happens due to a muscle relaxation and can be induced by the neuropeptide SALMFamide (Elphick et al., 1995; Melarange et al.,

1999), whereas the retraction of the cardiac stomach can be induced by the neuropeptide NGFFFamide (Semmens et al., 2013).

The situation-dependent release of specific neuropeptides can influence autonomous behaviors like radula protraction during eating, metabolic processes like energy mobilization during insect flight or reproduction-related processes like gamete release. However, these are only a few examples of processes in which neuropeptides are involved.

2.4 Neuropeptides are expressed as preproneuropeptides, that get processed and modified after translation

Neuropeptides are first expressed as longer preproneuropeptides, where the actual neuropeptide is accompanied by additional, often less- or non-conserved peptide sequences (Fig.1) (Hook et al., 2008; Rholam and Fahy, 2009). The preproneuropeptide has a N-terminal hydrophobic signal peptide sequence, that facilitates its transport to the secretory pathway of the endoplasmatic reticulum, where the signal peptide gets cleaved off by a signal peptidase (Ng and Walter, 1994). The resulting propeptide gets sorted into a secretory dense core vesicle, where it is cleaved into smaller peptides at characteristic basic cleavage sites [lysine/arginine] by amino-peptidases and carboxy-peptidases (Hook et al., 2008; Rholam and Fahy, 2009). In some animals, alternative cleavage sites with different amino acid residues have been suggested (Hummon et al., 2002; Leviev et al., 1997), but these seem to be exceptions. Many neuropeptides are further modified, for example, by N-terminal pyroglutamic acid formation, sulfation of tyrosine, or C-terminal amidation (Varro, 2001). These modifications can decrease peptide degradation (Marks et al., 1976) and are also often necessary for efficient receptor activation (Eipper et al., 1992; Kubiak et al., 2002; Merkler, 1994). Such peptide modifications can be ancient characteristics that are conserved in most homologous neuropeptides, like C-terminal amidation and N-terminal pyroglutamination of GnRH/AKH-related peptides (Gade, 2009; Hauser and Grimmelikhuijzen, 2014), or tyrosine sulfation and C-terminal amidation of sulfakinins (Nichols, 2007; Predel et al., 1999). A few examples, however, show that peptide-specific modifications can

sometimes be lost in certain taxa, like the lack of C-terminal amidation in some oligochaete GGNG excitatory peptides (Minakata et al., 1997), in the nematode AKH/corazonin homolog (Lindemans et al., 2011; Lindemans et al., 2009) or in the *Ciona intestinalis* vasotocin homolog (Kawada et al., 2008). A proneuropeptide can encode a single neuropeptide or multiple, often similar or identical neuropeptides (Fig.1). The conservation of the overall propeptide structure, with respect to position, length and number of bioactive peptides, can thereby vary depending on the neuropeptides. Vasotocin and related neuropeptides for example have a single copy of the bioactive peptide between the N-terminal signal peptide and the C-terminal neurophysin on the peptide precursor (Fig.1) in all bilaterians (Mirabeau and Joly, 2013; Stafflinger et al., 2008), and the arthropod specific myosuppressins usually have a precursor where the bioactive peptide is located at the C-terminus (Fig.1) (Bendena et al., 1997; Vilaplana et al., 2004). Other neuropeptide precursors, like FMRFamide, have multiple paracopies of identical or similar neuropeptides in variable positions (Appendix) (Adamson et al., 2015; Walker et al., 2009; Zatylny-Gaudin and Favrel, 2014). An extreme case showed up to 37 copies of very similar peptides on a single preproneuropeptide (Leviev and Grimmelikhuijzen, 1995).

In summary, neuropeptides are expressed as longer prepropeptides that get cleaved and modified into the actual bioactive peptides. The arrangement of the bioactive neuropeptides on the prepropeptide and certain posttranslational modifications of the neuropeptides can be conserved in homologous neuropeptides of different animals, which can be a criterion to infer homology.

2.5 Neuropeptides and their receptors coevolve

Most neuropeptide receptors are G-protein coupled receptors (GPCR) from the rhodopsin or secretin GPCR family (Fredriksson et al., 2003; Jékely, 2013; Mirabeau and Joly, 2013). Exceptions are peptide-gated ion channels that have been identified in molluscs (Cottrell, 1997; Cottrell et al., 1990; Lingueglia et al., 1995) and cnidarians (Durrnagel et al., 2010; Golubovic et al., 2007; Grunder and Assmann, 2015), a guanylyl cyclase that is activated by the eclosion hormone (Chang et al., 2009), a class I cytokine receptor that is activated by leptin (Shpilman

et al., 2014; Tartaglia et al., 1995) or the insulin/ILP receptors that belong to the tyrosine kinase receptor family (Fernandez et al., 1995; Hernandez-Sanchez et al., 2008; Pashmforoush et al., 1996; Ullrich et al., 1985).

The ligand receptor activation is in most cases very specific and homologous peptides of distantly related animal clades usually activate the corresponding homologous receptors (Grimmelikhuijzen and Hauser, 2012; Jékely, 2013; Mirabeau and Joly, 2013; Park et al., 2002). Neuropeptide receptors are usually several hundred amino acids long and therefore carry more phylogenetic information than their respective shorter ligands (Hauser and Grimmelikhuijzen, 2014; Jékely, 2013; Mirabeau and Joly, 2013). This co-evolution of ligand-receptor pairs can be used to test homology hypotheses of strongly diverged ligands by testing and comparing their receptor (Janssen et al., 2008; Kawada et al., 2008; Semmens et al., 2015). This homologous conservation of ligand-receptor pairs has been demonstrated for many cases. Examples with overall well-conserved ligands and receptors are the oxytocin/vasopressin/vasotocin ligand-receptor pairs of chordates (Kawada et al., 2008; Kimura et al., 1992), trochozoans (Bauknecht and Jekely, 2015; Kanda et al., 2003; Levoye et al., 2005; van Kesteren et al., 1996) and ecdysozoans (Beets et al., 2012; Beets et al., 2013; Stafflinger et al., 2008) or the achatin ligand-receptor pairs of trochozoans, ambulacrarians and cephalochordates (Bauknecht and Jekely, 2015). In other cases, the ligands diverged to a larger extend during evolution, and the identification of the corresponding receptors helped to reconstruct the diversifications (Semmens et al., 2015) and/or clade specific duplications (Hauser and Grimmelikhuijzen, 2014). Examples of stronger diverging ligands that activate conserved receptors are the protostome paralogs corazonin (CRZ), adipokinetic hormone (AKH) and AKH/CRZ-related peptide (ACP) and their deuterostome ortholog the gonadotropin-releasing hormone (Hansen et al., 2010; Hauser and Grimmelikhuijzen, 2014; Lindemans et al., 2011; Lindemans et al., 2009) or the nematode cholecystokinin/sulfakinin ortholog (CK) of arthropod sulfakinin and deuterostome cholecystokinin and gastrin (Janssen et al., 2008; Staljanssens et al., 2011).

2.6 Evidence for a change in ligand-receptor pairing during evolution

Even though the conservation of ligand-receptor pairing in distantly related taxa has been shown for many peptides, few cases indicate that some ligands might have changed their receptor during evolution. For example, there are two very different classes of FMRFamide receptors in trochozoans: a peptide-gated amiloride-sensitive Na^+ channel that was identified in the mollusc *Helix aspersa* (Cottrell et al., 1990; Lingueglia et al., 1995) and a GPCR that was identified in the annelid *P. dumerilii* (Bauknecht and Jekely, 2015). Electrophysiological experiments indicated that *H. aspersa* has 2 different FMRFamide receptors with different concentration thresholds, one mediating an increase in Na^+-conductance and another one mediating an increase in K^+-conductance (Cottrell and Davies, 1987). Although further experiments suggested that a GPCR is not involved in the *H. aspersa* FMRFamide-sensitivity, they supported the hypothesis of the existence of two different FMRFamide sensitive receptors (Green et al., 1994). Electrophysiological experiments on *Aplysia californica* neurons also showed that they possess FMRFamide-sensitive ion channels (Ruben et al., 1986), and *A. californica* has at least one homolog of the *P. dumerilii* FMRFamide GPCR as well (Bauknecht and Jekely, 2015). In addition to these two receptors in trochozoans, a third FMRFamide receptor has been identified in *Drosophila melanogaster* (Cazzamali and Grimmelikhuijzen, 2002; Meeusen et al., 2002), that is a GPCR, but is not a homolog of the *P. dumerilii* FMRFamide GPCR (Bauknecht and Jekely, 2015). Another example of the presence of two different receptors for one neuropeptide is the DH31 receptors. The calcitonin-related neuropeptide DH31 (Furuya et al., 2000) activates a homolog of the secretin type calcitonin GPCR in the fruit fly *D. melanogaster* (Johnson et al., 2005) and the annelid *P. dumerilii* (Bauknecht and Jekely, 2015). However, there is a second type of DH31 receptor in *P. dumerilii,* which belongs to the rhodopsin type GPCRs and has orphan homologs in other trochozoans as well (Bauknecht and Jekely, 2015).

In several studies where receptor activation was tested in transfected cell cultures, it has been shown that different neuropeptides can be able to trigger the same receptor (Bauknecht and Jekely, 2015; Mertens et al., 2006; Peymen et al., 2014). The studies also revealed that some neuropeptides are able to trigger several

unrelated receptors in one animal (Bauknecht and Jekely, 2015; Peymen et al., 2014). The EC_{50} values (half maximal effective concentration) for these cross-activation, however, were often in a lower micro-molar range, whereas the EC_{50} values of more specific peptides were in a nano-molar range (Bauknecht and Jekely, 2015; Mertens et al., 2006; Peymen et al., 2014). The fact that some neuropeptides can cross-activate other receptors and the fact that there are cases with very different receptors for a single ligand suggests a general possibility for evolutionary changes in ligand-receptor binding.

2.7 Neuropeptides in Trochozoa

Several phylogenetic studies proposed different relationships of several protostomian clades within the Spiralia, which have been referred to as Lophotrochozoa (Aguinaldo et al., 1997; Dunn et al., 2008; Laumer et al., 2015; Struck et al., 2014) or Lophophorata (Halanych et al., 1995; Nesnidal et al., 2013). A common result of most studies, however, showed that the taxa Annelida, Mollusca, Brachiopoda, Nemertea, Phoronida and likely Ectoprocta (Bryozoa) are closely related and form the clade Trochozoa (Fig.2 - without Phoronida and Bryozoa) (Dunn et al., 2014; Edgecombe et al., 2011; Giribet, 2008; Hejnol et al., 2009). Trochozoans are mainly aquatic organisms that have a biphasic life cycle with ciliated planktonic larvae and benthic adults (with a few exceptions, e.g. terrestrial annelids). Several functional, immunohistochemical and molecular studies mainly focused on neuropeptides in annelids and molluscs. Bioinformatic studies described the neuropeptide complement of the annelids *Platynereis dumerilii* (Conzelmann et al., 2013a)*, Capitella teleta* (Veenstra, 2011) and *Helobdella robusta* (Veenstra, 2011) and the molluscs *Theba pisana* (Adamson et al., 2015), *Pictata fucata* (Stewart et al., 2014), Charonia tritonis (Bose et al., 2007), *Crassostrea gigas* (Stewart et al., 2014), *Lottia gigantea* (Veenstra, 2010) and *Sepia officinalis* (Zatylny-Gaudin et al., 2016). Other studies included publicly available sequence data of annelids and molluscs to analyze neuropeptide evolution and conserved patterns on a broader phylogenetic scale (Elphick and Mirabeau, 2014; Jékely, 2013; Liu et al., 2006b, 2008; Mirabeau and Joly, 2013).

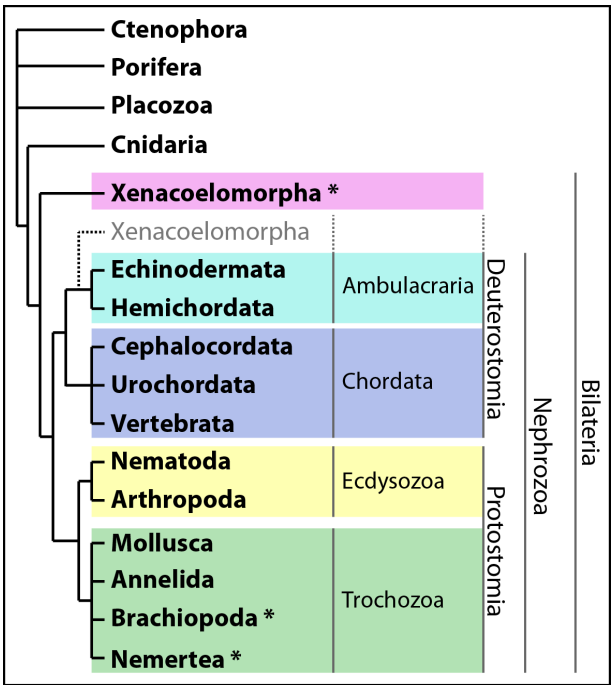

Fig.2: Simplified phylogenetic tree of different metazoan clades with emphasis on Bilateria.

Studies on neuropeptides were greatly influenced by the sequence identification of FMRFamide that has been extracted from the bivalve *Macrocallista nimbosa* (Price and Greenberg, 1977). FMRFamide has been shown to have excitatory effects on certain muscular tissues and since its identification it has been the subject of many research articles. Antibodies that were raised against FMRFamide were tested in a variety of animals, and showed positive immunoreactivity in species belonging to Protostomia (Boer et al., 1980; Dickinson et al., 1999; Hayschmidt, 1990c; Krajniak and Greenberg, 1992), Deuterostomia (Boer et al., 1980; D'Aniello et al., 2016; Dockray et al., 1983), Cnidaria (Eichinger and Satterlie, 2014; Grimmelikhuijzen, 1983; Satterlie and Eichinger, 2014), Ctenophora (Grimmelikhuijzen and Graff, 1985; Grimmelikhuijzen, 1983) and Placozoa (Schuchert, 1993; Smith et al., 2014). FMRFamide immunoreactivity is still used in immunohistochemical studies to investigate the neuroanatomy of invertebrates (Helm et al., 2014; Helm et al., 2016; Satterlie and Eichinger, 2014; Schmidt-Rhaesa et al., 2016) and vertebrates (D'Aniello et al., 2015; D'Aniello et al., 2016). FMRFamide antibodies, however, are known to cross-react with other neuropeptides ending in RFamide, which can make it difficult or impossible to distinguish between different RFamide peptides in a single

species (Peymen et al., 2014; Vilim et al., 2010). This cross-reactivity and the search for FMRFamide in animals of different clades have also led to the discovery of other peptides (Dockray et al., 1983; Price and Greenberg, 1989). FMRFamide is encoded in multiple copies on its precursor and many peptides with a repetitive propeptide structure or the C-terminal ending RFamide, have been referred to as FMRFamide-like peptides (FLPs) or FMRFamide-related peptides (FaRPs) and reviewed in several publications (Espinoza et al., 2000; Lopez-Vera et al., 2008; Mousley et al., 2005; Orchard et al., 2001; Peymen et al., 2014; Walker et al., 2009; Zatylny-Gaudin and Favrel, 2014). FMRFamide is one of the best-studied neuropeptides in trochozoans and many studies investigated the influence of FMRFamide on various annelid and mollusc tissues. Single studies have shown, that FMRFamide can be involved in different physiological functions, like osmoregulation in *Erpobdella octoculata* (Salzet et al., 1994), chromatophore expansion in *Sepia officinalis* (Loi and Tublitz, 1997), salivary gland activity in *Helisoma* sp. (Bulloch et al., 1988) or coordination of feeding behavior in *Aplysia californica* (Vilim et al., 2010). Many studies show an excitatory effect of FMRFamide on various muscle tissues (e.g. (Krajniak and Greenberg, 1992; Lehman and Greenberg, 1987; Moulis, 2006; Moulis and Huddart, 2004; Muneoka and Saitoh, 1986)). The type of muscle that is sensitive to FMRFamide, but also the effect itself, can differ between animal species. Tests with 50 bivalve species, for example, showed that even though FMRFamide had an excitatory effect on the heart in the majority of the investigated species, in some species it had an inhibitory effect instead (Painter and Greenberg, 1982). Some studies showed that FMRFamide is able to modify the signal transmission of other neurotransmitters. Prolonged exposure of longitudinal muscles of the leech *Hirudo medicinalis* to FMRFamide potentiated the contractile response of the muscles to acetylcholine-induced contractions if FMRFamide was washed out before the acetylcholine exposure (Norris and Calabrese, 1987). In *Mytilus edulis*, the presence of FMRFamide enhanced contractile responses of the anterior byssus retractor muscle (ABRM) to low concentrations of acetylcholine (Raffa and Bianchi, 1986). Interestingly, another study showed that low concentrations of FMRFamide (0.1 μmol/l or lower) were able to relax acetylcholine-induced contraction of the ABRM of

M. edulis and higher concentrations induced contractions (Muneoka and Saitoh, 1986). The authors suggest that this phenomenon of the concentration dependent opposite effects could be explained by two different FMRFamide receptors - one located at the muscle fiber membranes which is involved in contractile responses and the second one might be located at relaxing nerve terminals.

Most functional studies on trochozoans that include neuropeptides, were conducted on adult or juvenile specimens, whereas the influence of neuropeptides on their larval forms have only been investigated in a few cases. One of these studies showed that the neuropeptide MIP (myo-inhibitory peptide) is involved in the settlement of *Platynereis dumerilii* larvae (Conzelmann et al., 2013b). Other studies have shown that neuropeptides can regulate beating of larval locomotory ciliary bands and thereby influence the swimming height in a water column (Braubach et al., 2006; Conzelmann et al., 2011; Penniman et al., 2013). It was shown that in fact a variety of neuropeptides is able to influence the swimming height of a single species, by influencing the beat frequency of cilia and/or the length and frequency of ciliary arrests (Conzelmann et al., 2011). The only myogenic effect of FMRFamide that is described for larvae, is that it induces rhythmic contraction in the velum of *Tritia obsoleta* larvae, which are accompanied by ciliary arrests (Braubach et al., 2006).

Studies that investigated neuropeptides in larvae of trochozoan species outside Annelida and Mollusca are restricted to a few immunohistochemical descriptions. These studies describe FMRFamide immunoreactivity in the nervous system of brachiopod (Hayschmidt, 1992), nemertean (Hayschmidt, 1990a; Hindinger et al., 2013), phoronid (Hayschmidt, 1990b, c) and bryozoan (Gruhl, 2009; Shun'kina et al., 2013) larvae. FMRFamide immunoreactivity in trochozoan larvae can generally be found in sensory neurons, interneurons or motor neurons and are often associated with one or several of the following structures: muscles (Braubach et al., 2006; Dyachuk and Odintsova, 2009; Dyachuk et al., 2012; Gruhl, 2009; Hayschmidt, 1995; Helm et al., 2016; Hindinger et al., 2013; Shun'kina et al., 2013), apical organ (Dyachuk and Odintsova, 2009; Dyachuk et al., 2012; Gruhl, 2009; Hayschmidt, 1990a; Helm et al., 2016; Nezlin, 2010), neuropile/apical ganglion (Dyachuk et al., 2012; Hayschmidt, 1990b, c, 1992, 1995; Nezlin, 2010; Shun'kina et

al., 2013) or ciliary bands (Braubach et al., 2006; Gruhl, 2009; Hayschmidt, 1990b, c, 1995; Hindinger et al., 2013; Nezlin, 2010; Penniman et al., 2013).

In summary, neuropeptide signaling has been well studied in adult or juvenile molluscs and annelids. The identification of FMRFamide clearly influenced neuropeptide-related research and FMRFamide antibody staining was used to investigate nervous system of trochozoan larvae, also outside Annelida and Mollusca. Functional studies on trochozoan larvae are rare and were done in few species, which showed an involvement of neuropeptides in cilia-based locomotion and larval settlement.

2.8 Neuropeptides in Xenacoelomorpha

Xenacoelomorpha are bilaterian animals with a relatively simple body plan and a flatworm like appearance (Fig.3 a-d). Historically, they were thought to belong to the Platyhelminthes, but this placement was revised with the advance of molecular phylogeny (Ruiz-Trillo et al., 1999; Telford et al., 2003). More recent studies placed Xenacoelomorpha as the sister group to all other Bilateria (Cannon et al., 2016; Hejnol et al., 2009; Rouse et al., 2016; Srivastava et al., 2014), which stands in contrast to a proposed placement as a sister group to ambulacrarians (Fig.2) (Bourlat et al., 2006; Bourlat et al., 2003; Philippe et al., 2011). Xenacoelomorpha consist of the three major clades - *Xenoturbella*, Nemertodermatida and Acoela - with the latter two forming the clade Acoelomorpha as a sister group to *Xenoturbella* (Cannon et al., 2016; Edgecombe et al., 2011).

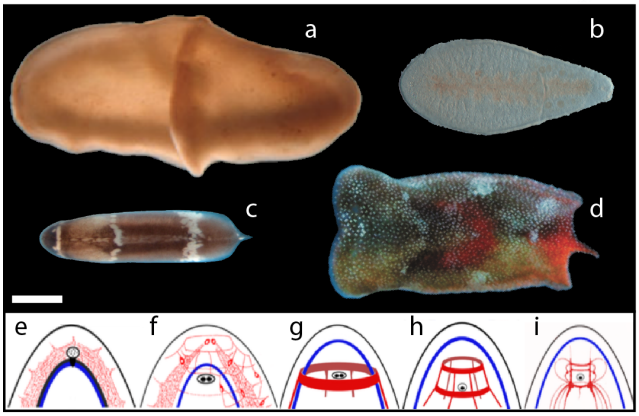

Fig.3: Light-microscopic pictures of Xenacoelomorphs and schematic representation of xenacoelomorph anterior nervous system. **a-d** lightmicroscopic pictures. **a** *Xenoturbella bocki*. **b** *Meara stichopi* (Nemertodermatida). **c** *Hofstenia miamia* (Acoela). **d** *Convolutriloba longifissura* (Acoela). **e-i** Schematic drawing of xenacoelomorph anterior nervous system (red) in relation to body wall musculature (blue) and statocysts (black circles in the center). **e** *Xenoturbella westbladi*. **f** *Meara stichopi* (Nemertodermatida). **g** *Nemertoderma westbladi* (Nemertodermatida). **h** *Diopisthoporus longitubus* (Acoela). **i** *Childia crassum* (Acoela). Pictures taken and modified from (Rouse et al., 2016) (a), (Hejnol, 2015) (b,d), (Srivastava et al., 2014) (c), (Raikova et al., 2016) (e-i). Scalebar: 250 *µ*m

Xenacoelomorphs have simple nervous systems with strong differences between the clades and species (Gavilan et al., 2016; Hejnol, 2015; Hejnol and Pang, 2016; Raikova et al., 2016). *Xenoturbella* have a basiepithelial nerve net, without prominent nerve condensations or ganglion formations (Fig.3 e) (Raikova et al., 2000b; Stach, 2015). Nemertodermatida have basiepithelial or internalized nervous systems with nerve condensations in form of longitudinal nerves and some have brain-like anterior ring nerves (Fig.3 f,g) (Hejnol, 2015; Raikova et al., 2016; Raikova et al., 2000a). Acoela have mainly internalized nerve nets with longitudinal nerve cord condensations and usually a ganglionic brain (Fig.3 h,i) (Hejnol, 2015; Raikova et al., 2016). Studies about the existence of neuropeptides in xenacoelomorphs are restricted to immunohistochemical research with antibodies that were raised against neuropeptides of other animals. These studies show binding of antibodies that were raised against RFamide (Semmler et al., 2010), FMRFamide (Achatz and Martinez, 2012; Børve and Hejnol, 2014; Raikova et al., 2016; Raikova et al., 2000a, b), GYIRFamide (Raikova et al., 2004) and echinoderm SALMFamide 1 and 2 (Stach et al., 2005) (with the C-termal ending SALMFamide and SGLTFamide, respectively (Elphick et al., 1991a; Elphick et al., 1991b)). The amount and position of peptidergic nerves varied strongly between different taxa. FMRFamide-like immunoreactivity in nemertodermatids for example, is restricted to a few nerve cells in the anterior brain ring of *N. westbladi* (Raikova et al., 2000a), is more prominent in the whole anterior region of *Sterreria* sp. (Raikova et al., 2016) and shows strong staining of brain and longitudinal nerves that run along the whole anterior-posterior body axis of *Meara stichopi* (Børve and Hejnol, 2014). Beside several studies that

show antibody reactivity, nothing is known about the neuropeptides that are actually present in Xenacoelomorpha.

3. Aim of this study

The general aim of this study is to investigate neuropeptide evolution in animals and how they can influence animal behavior.

We first aimed to test how neuropeptides can influence the behavior of planktonic trochozoan larvae from clades other than molluscs and annelids. We therefore chose larvae of the brachiopod *Terebratalia transversa* and the nemertean *Lineus longissimus*. After an initial screening, where we exposed larvae to different synthetic neuropeptides, we focused on characterizing the influence of FLRFamide on the behavior of *T. transversa* larvae and the influence of the excitatory peptide on the behavior of *L. longissimus* larvae.

To investigate neuropeptide evolution, we chose to study the neuropeptide repertoire of xenacoelomorphs since this animal clade is considered to be the sister group of all remaining bilaterians, but nothing is known about their neuropeptide signaling systems. We therefore investigated the transcriptomes of 13 xenacoelomorph species in parallel and combined this search with mass spectrometric analysis of peptide extracts from 3 of these species. This way we were able to investigate the clade specific diversification of neuropeptides within Xenacoelomorpha and compare the neuropeptides present in xenacoelomorphs with those present in other bilaterians.

4. Results

4.1 An ancient FMRFamide-related peptide-receptor pair induces defense behavior in a brachiopod larva.

When we exposed larvae of the brachiopod *T. transversa* to the neuropeptide FLRFamide, they contracted, spread out their chaetae and sank down slowly. This behavior is comparable to the startle or defense behavior that the larvae show, when they get mechanically disturbed. DFLRFamide and AFLRFamide, which are endogenously expressed by *T. transversa* larvae, were able to induce this reaction, whereas structurally unrelated neuropeptides did not elicit this response. To test how specific this reaction is, we deorphanized the FLRFamide receptor and investigated its spatial expression. We found that the T. transversa FLRFamide receptor is an ortholog of the previously deorphanized *Platynereis dumerilii* (Annelida) FMRFamide receptor, and that it is expressed at the ciliated apical prototroch and in musculature of trunk and chaetae sacks. These expression domains coincide with the tissues that we would expect would be involved in this behavior: contraction of the musculature of the trunk and the chaetae sacks leads to contraction of the larvae and protrusion of their chaetae, and the ciliated cells of the apical prototroch are used for larval locomotion. We also used in situ hybridization and customized antibodies against FLRFamide to visualize FLRFamide expressing cells in the larvae. We found FLRFamide expression in nerves directly adjacent to the musculature of the trunk with projections towards the chaetae sacks and in nerves in the apical neuropil with some neurons projecting underneath the prototroch. We therefore hypothesize that FLRFamide specifically induces this startle-like behavior by directly activating the receptor in the musculature of trunk and chaetae sacks and underneath the ciliated prototroch.

4.2 The trochozoan specific myo-excitatory GGNG peptide (EP) increases the ciliary beat frequency of *Lineus longissimus* pilidium larvae.

The excitatory peptide (EP) was only known from annelids and molluscs before, where it showed to have an excitatory effect on muscle preparations. Through

homology searches, we identified the excitatory peptide in the two brachiopod species *Lingula anatina* and *Novocrania anomala* and the two nemertean species *Lineus longissimus* and *Lineus* cf. *ruber*, showing its presence in other trochozoan clades as well. The nemertean EP differs from the EP of molluscs, annelids and brachiopods in having a C-terminal GNGamide instead of the otherwise conserved GGNamide. We deorphanized the *L. longissimus* EP receptor and showed that it is an ortholog to the previously deorphanized annelid EP receptor with further orthologs in brachiopods and molluscs. We tested the influence of EP on the behavior of *L. longissimus* larvae and discovered that it does not induce any myogenic activity. Our results showed that EP increases the beat frequency of the ciliary bands of the apical and lateral lobes of the *L. longissimus* pilidium larvae. This increase of the ciliary beat frequency ultimately shifts the vertical swimming distribution of the larvae in a water column upwards.

4.3 Evolution and diversity of neuropeptide signaling in Xenacoelomorpha.

We searched the transcriptomes of 13 xenacoelomorph species for neuropeptide precursor sequences and neuropeptide GPCRs by using a variety of query sequences from animals of different deuterostome and protostome clades in a BLAST search. To find hidden orthologs, we used the few positive results as new query sequences and blasted them against all 13 transcriptomes again. We also extracted peptides from three of these xenacoelomorph species and analyzed them by mass spectroscopy. In our results, we identified glycoprotein hormones and insulin-like peptides, (which are also present in non-bilaterian animals) as well as homologs of neuropeptides and their potential receptors, that are only known from deuterostomes and protostomes, like vasotocin, calcitonin, achatin, neuropeptide Y/F and gonadotropin/corazonin. We also identified different short neuropeptides with multiple-copy precursors that are specific to xenacoelomorph and analyzed their peptide-precursor structures. This way we were able to provide a substantial dataset of xenacoelomorph neuropeptides and xenacoelomorph neuropeptide GPCRs and gained insights into neuropeptide evolution of xenacoelomorphs.

4.4 A safer, urea-based *in situ* hybridization method improves detection of gene expression in diverse animal species.

The detection of the FLRFamide receptor expression in *Terebratalia transversa* showed to be rather difficult with our standard *in situ* hybridization protocol. Background staining and probe trapping lead to no detection of a specific signal, despite troubleshooting. I learned from a friend, Gonzalo Quiroga Artigas, who works in the marine station "Observatoire Océanologique de Villefranche sur Mer", of a urea-based *in situ* hybridization protocol that they use for the jelly fish *Clytia hemisphaerica.* With some crucial additions, it gave a specific signal without unspecific staining even though the signal development of the receptor took several days. Due to these positive results, we decided to try this hybridization buffer in more species and added my results to this manuscript.

5. Discussion

I described in the manuscripts the neuropeptide repertoire of xenacoelomorpha and how specific neuropeptides can influence trochozoan larval behavior. The results of these studies are discussed in each of the manuscripts separately. Here I will discuss some aspects that caught my attention during my research for these manuscripts. One discussion point is the phylogenetic significance of multicopy propeptides and single-copy propeptides in regards of how usefull the propeptide structure is to infer homology. The second discussion point addresses the phylogenetic relationship of insect and trochozoan FMRFamide in the light of ligand-receptor pairs of related receptor groups. The third discussion point will address the different effects and mechanisms, of how FMRFamide influences trochozoan larval swimming.

5.1 Conservation of multi-copy propeptide structures and single-copy propeptide structures

Many neuropeptides have propeptide sequences with multiple copies (also called paracopies) of identical or similar short peptides. Such precursors with multiple neuropeptide copies are known from placozoans, ctenophores, cnidarians and bilaterians (Grimmelikhuijzen et al., 2002; Jékely, 2013; Moroz et al., 2014; Nikitin, 2015)(manuscript I & III). Orthologous neuropeptides have usually the same kind of propeptide-structure in related species, with regards to multi-copy or single-copy propeptides (Jékely, 2013; Wegener and Gorbashov, 2008). But how conserved is the presence of multi-copy or single-copy propeptides in orthologous neuropeptides between more distantly related animals? This question is especially relevant in regards to how useful the precursor structure is to assign homology hypothesis of neuropeptides between distantly related species, when the receptors are unknown.

Achatin is an example of a multi-copy peptide with very conserved ligands across Bilateria, which activate orthologous receptors in deuterostomes and protostomes (Bauknecht and Jekely, 2015). Achatin is at least present in trochozoans (Adamson et al., 2015; Conzelmann et al., 2013a; Jékely, 2013; Kamatani et al., 1989; Stewart

et al., 2014; Veenstra, 2010), arthropods (XP_015908358.1, KFM77812.1), priapulids (XP_014662331.1), hemichordates (Bauknecht and Jekely, 2015; Jékely, 2013), cephalochordates (Bauknecht and Jekely, 2015; Jékely, 2013) and xenacoelomorphs (manuscript III). It has a conserved length of 4 amino acids that share the sequence GF[G/A][N/E/D], with a D-phenylalanine in position 2 (Bauknecht and Jekely, 2015; Kamatani et al., 1989). The number of copies on the precursors can vary from 2 in *C. gigas* (Stewart et al., 2014) to at least 16 in *Saccoglossus kowalevskii* (Jékely, 2013) (XP_002732147.1). The achatin receptor group shows sequence similarity to the neuropeptide S/crustacean cardioactive peptide (CCAP) receptor group, the GnRH/AKH/corazonin receptor group and the vasotocin/vasopressin/oxytocin receptor group (manuscript III). These different groups were already present in the last common ancestor of all bilaterians (manuscript III) (Bauknecht and Jekely, 2015; Jékely, 2013; Mirabeau and Joly, 2013; Roch et al., 2011). In contrast to achatins, the propeptides of GnRH and related neuropeptides, as well as the propeptides of vasotocin and related peptides, have only a single copy of the respective bioactive peptide (manuscript III) (Beets et al., 2012; Hauser and Grimmelikhuijzen, 2014; Lindemans et al., 2011; Mirabeau and Joly, 2013). One of the GnRH-like paralogs in *N. westbladi* has 2 copies of the predicted peptide, but since all other xenacoelomorph GnRH-like peptides have a single copy, this seems to be an exception (manuscript III). Vertebrate neuropeptide S, non-vertebrate deuterostome NG peptides and protostome CCAPs have diverged more strongly after the split into deuterostomes and protostomes and differ in their propeptide structures, but also strongly in their ligand sequence (Semmens et al., 2015). Neuropeptide S is present as a single copy on the precursor of vertebrates (Reinscheid, 2009; Xu et al., 2004), while the orthologous NG peptide of non-vertebrate deuterostomes has two or more copies on the precursor (Semmens et al., 2015; Semmens et al., 2013). The protostome CCAPs have only a single copy of the two-cysteine-containing CCAP in insects (Derst et al., 2016; Hauser et al., 2010; Li et al., 2008; Wegener and Gorbashov, 2008) and crustaceans (Christie et al., 2010; Chung et al., 2006), whereas trochozoans are found to have one (Conzelmann et al., 2013a) or more copies (Adamson et al., 2015; Stewart et al., 2014; Veenstra, 2010). In this whole cluster of four different related receptor groups,

one multi-copy peptide is consistent throughout Bilateria (achatin), two different single copy peptides are consistent throughout Bilateria (GnRH-related and vasotocin-related) and one group has independently evolved different precursor structures in protostomes (arthropod CCAPs and trochozoan CCAPS) as well as in deuterostomes (NG peptides and neuropeptide S).

Similarly variable are propeptides of ligands with receptors that show sequence similarity to the trochozoan FMRFamide receptor group (Bauknecht and Jekely, 2015) (manuscript I & III). Receptor groups that are related to the trochozoan FMRFamide receptor group include the leucokinin receptors of arthropods (Radford et al., 2002) and trochozoans (Cox et al., 1997), the trochozoan luqin receptors (Bauknecht and Jekely, 2015; Tensen et al., 1998b), the arthropod RYamide receptors (Collin et al., 2011) and the tachykinin receptors of arthropods (Li et al., 1991; Monnier et al., 1992), trochozoans (Kanda et al., 2007), urochordates (Satake et al., 2004) and vertebrates (Buell et al., 1992; Sundelin et al., 1992; Takeda et al., 1991). Most leucokinin peptides are between 6-15 amino acids long, share the C-terminal ending FxxWxamide and have multi-copy propeptides in ecdysozoans (Cox et al., 1997; Hayes et al., 1989; McVeigh et al., 2008; Radford et al., 2002) and trochozoans (Conzelmann et al., 2013a; Cox et al., 1997; Veenstra, 2010, 2011). The trochozoan luqins are 7-13 amino acid long peptides that share the C-terminal sequence RFamide, and are encoded a single time on the propeptide (Adamson et al., 2015; Conzelmann et al., 2013a; Elphick and Mirabeau, 2014; Rowe et al., 2014; Stewart et al., 2014; Tensen et al., 1998b; Veenstra, 2010, 2011). The trochozoan luqin receptors are orthologs of the arthropod RYamide receptor (Bauknecht and Jekely, 2015; Elphick and Mirabeau, 2014; Mirabeau and Joly, 2013) (manuscript I & III). RYamide precursors have 2 or multiple copies of peptides with variable length, that share the C-terminal sequence FFxxxRYamide (Collin et al., 2011; Hauser et al., 2010). Tachykinins are about 7-14 amino acids long and have multiple copies of peptides that end in Fx[G/A]x[R/M]amide in trochozoan (Conzelmann et al., 2013a; Kanda et al., 2007; Nassel, 1999; Satake et al., 2003; Van Loy et al., 2010; Veenstra, 2010, 2011) and arthropod precursors (Broeck, 2001; Hauser et al., 2010; Nassel, 2002; Satake et al., 2003; Schoofs et al., 1993; Severini et al., 2002; Van Loy et al., 2010; Wegener and Gorbashov, 2008), while

vertebrate and urochordate tachykinin precursors encode only one or two peptides with the C-terminal ending FxGLMamide (Erspamer, 1981; Kimura et al., 1983; Pennefather et al., 2004; Satake et al., 2003; Severini et al., 2002). The activating peptides of receptors from this cluster also show differences in the occurrence of single copy propeptides and multi-copy propeptides of orthologous groups, like the difference between tachykinins of deuterostomes and protostomes or the difference between RYamide of arthropods and luqins of trochozoans. Other related receptor groups are those of short neuropeptide F (sNpF), neuropeptide Y (NpY) and neuropeptide F (NpF), which are described in more detail in the next section.

In summary, these results show that the evolutionary distance over which the precursor structure of orthologous neuropeptides is conserved, depends entirely on the neuropeptide. The fact that the propeptide structure of orthologous peptides can vary in some cases and is more consistent in others can make the identification of homologous neuropeptides in distantly related animals, like xenacoelomorphs, very difficult when the receptors are not yet known and when an outgroup is missing.

5.2 Are arthropod FMRFamides and trochozoan FMRFamides related?

Many neuropeptides that are called FMRFamide-related peptide (FaRP) or FMRFamide-like peptide (FLP), are not real homologs of the FMRFamide that was originally discovered in the mollusc *Macrocallista nimbosa* by Price and Greenberg in 1977 (Price and Greenberg, 1977). The term FMRFamide-related peptide was often used to show a possible homology to the actual FMRFamide, whereas FLPs often only have the ending RFamide and might even be known to not be homologous to the above mentioned FMRFamide (Espinoza et al., 2000; Walker et al., 2009). Some arthropod FMRFamides, however, show strong similarity with "actual" trochozoan FMRFamides, including the C-terminal ending and the highly repetitive propeptide structure (see Appendix) (Li et al., 2008; Wegener and Gorbashov, 2008), but they activate different receptors Fig.4 (manuscript I) (Bauknecht and Jekely, 2015; Cazzamali and Grimmelikhuijzen, 2002; Meeusen et al., 2002). We deorphanized the *T. transversa* FLRFamide receptor (manuscript I) and showed that it is related to the *P. dumerilii* FMRFamide receptor (Bauknecht

and Jekely, 2015) and not to the *D. melanogaster* FMRFamide receptor (Cazzamali and Grimmelikhuijzen, 2002; Meeusen et al., 2002) (manuscript I).

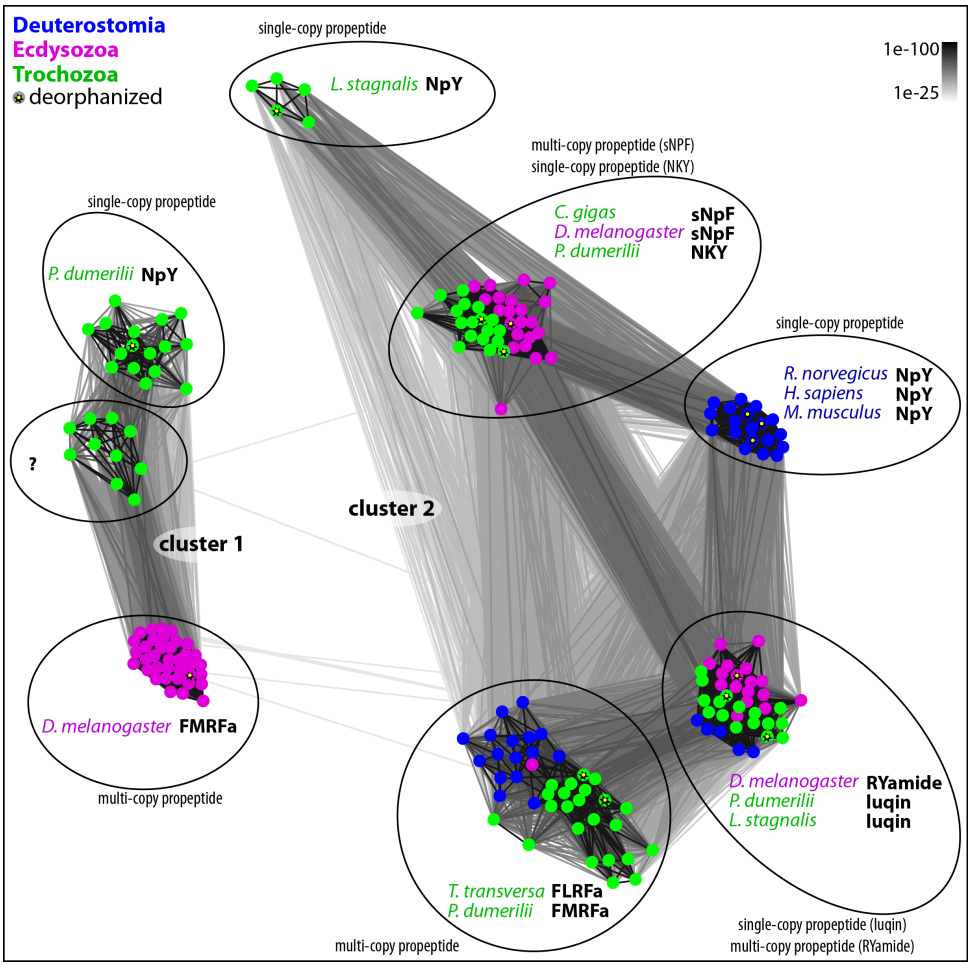

Fig.4: Clustermap of neuropeptide GPCR groups. Dots represent receptors. Deorphanized receptors are indicated by a star. Lines show connections based on blast similarities <1e-25. Species names followed by ligands are given for the deorphanized receptors in the encircled groups.

Many trochozoan FMRFamide-related peptides are 4-5 amino acids long, share the C-terminal motif [F/Y][L/M]RFamide, have a highly repetitive propeptide (see Appendix), and most likely activate receptors of the *T. transversa* FLRFamide/*P. dumerilii* FMRFamide receptor group, which show high sequence homology. The group of arthropod FMRFamide-related peptides that likely activate receptor

orthologs of the *D. melanogaster* FMRFamide receptor, have a propeptide with multiple copies of 6-11 amino acid long peptides that all share the C-terminal sequence [F/Y][M/L/I]RFamide (Appendix) (Li et al., 2008; Wegener and Gorbashov, 2008). But are the arthropod FMRFamides and the trochozoan FMRFamides orthologs? The fact that they activate unrelated receptors would suggest not. However, when the two different FMRFamide receptors and their relationship to other neuropeptide GPCRs is studied more closely, some interesting observations indicate that these peptides might be orthologs anyway.

The arthropod FMRFamide receptors show sequence similarity with a group of trochozoan orphan receptors and a group of receptors that includes the *P. dumerilii* neuropeptide Y (NpY) receptor (Fig.4 - cluster 1) (manuscript I) (Bauknecht and Jekely, 2015). The *P. dumerilii* NpY is similar to other protostome NpYs* and neuropeptide F (NpF), but those activate other receptors, as specified further below in the text** (Bauknecht and Jekely, 2015; Conzelmann et al., 2013a). The relationship of these arthropod FMRFamide and *P. dumerilii* NpY-related receptors to other neuropeptide receptors is not well known, but they seem to be a protostome specific group that shares some similarity to arthropod proctolin receptors (Jékely, 2013). When either of these receptors is queried for BLAST on NCBI, the most similar sequences of receptors from deuterostomes are thyrotropin-releasing hormone (TRH) receptors and GPCR 139. TRH receptors and GPCR 139, however, show little sequence similarity with these receptors (BLAST E-values >1e-19 and >1e-23, respectively) and both belong into two different receptor groups. While GPCR 139 shares more sequence similarity with protostome allatostatin B and proctolin receptors, TRH receptors share more sequence similarity with neuromedin U /pyrokinin and motilin/grehlin receptors (Elphick and Mirabeau, 2014; Mirabeau and Joly, 2013), all of which have ligands that are different from FMRFamide (Jékely, 2013; Mirabeau and Joly, 2013).

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* Many trochozoan NpYs should actually be named NpF due to their C-terminal phenylalanine residue and their similarity to arthropod NpFs, as it has also been suggested in Veenstra 2010 and in Nässel and Wegener 2011. ** The high EC_{50} values of the *P. dumerilii* NpY receptor activation by the *P. dumerilii* NpY orthologs (120 nmol/l - 3.4 μmol/l) (Bauknecht and Jekely 2015) could indicate that this receptor has a more specific ligand. In this case, the more specific ligand however, still has to be structurally related to NpY.

The trochozoan FMRFamide receptors have closely related orphan receptors in xenacoelomorphs, non-vertebrate deuterostomes, the elephant shark Callorhinchus milii, the arthropod *Nilaparvata lugens* and the priapulid *H. spinulosa* (Bauknecht and Jekely, 2015) (manuscript I & III). Deuterostomes and xenacoelomorphs, however, don't possess short peptides that end in FMRFamide or at least they have not been identified yet (Elphick and Mirabeau, 2014) (manuscript III) and ligands of non-trochozoan GPCRs that are related to the trochozoan FMRFamide receptors are yet unknown. Interestingly, a 6-12 amino acid long luqin-like peptide in ambulacrarians has the C-terminal ending F[M/L]RWamide, but the receptor has not yet been identified (Elphick and Mirabeau, 2014; Rowe et al., 2014; Semmens et al., 2016). Receptor groups that are related to trochozoan FMRFamide GPCRs are the leucokinin, RYamide/luqin and tachykinin receptors, as mentioned in the section before. Other receptors which show sequence similarity and belong to this cluster, are different groups of NpY/F receptors, and a group that includes receptors of short neuropeptide F (sNpF) and NKY (Fig.4 - cluster 2) (Bauknecht and Jekely, 2015)(manuscript I & III). One group includes the NpY receptor of *Lymnea stagnalis* (Tensen et al., 1998a), and another group includes the sNpF receptors of *L. stagnalis* (Bigot et al., 2014), *D. melanogaster* (Feng et al., 2003; Mertens et al., 2002) and the NKY receptor of *P. dumerilii* (Bauknecht and Jekely, 2015). NpYs and NpFs of arthropods (Feng et al., 2003; Garczynski et al., 2002; Hauser et al., 2010; Nassel and Wegener, 2011), trochozoans (Conzelmann et al., 2013a; Feng et al., 2003; Nassel and Wegener, 2011; Tensen et al., 1998a; Veenstra, 2010), platyhelminths (Feng et al., 2003; McVeigh et al., 2005; McVeigh et al., 2009; Nassel and Wegener, 2011) and vertebrates (Feng et al., 2003; Nassel and Wegener, 2011; Tensen et al., 1998a) are 30-50 amino acids long, share the C-terminal sequence RxR[F/Y]amide and are usually encoded in single copy on the peptide precursor. sNpF of arthropods (Broeck, 2001; Dillen et al., 2013; Li et al., 2008) and trochozoan (Bigot et al., 2014; Stewart et al., 2014) are 6-15 aa long, have the C-terminal ending [L/R]xR[F/W]amide and are encoded once (Li et al., 2008) or a few times (Broeck, 2001; Dillen et al., 2013; Wegener and Gorbashov, 2008) on arthropod precursors, and multiple times on trochozoan precursors (Bigot et al., 2014; Stewart et al., 2014). NKYs are only known from trochozoans, are

about 40 amino acids long, have the C-terminal ending (F/M)RYamide and have a propeptide with a single copy of the bioactive peptide (Adamson et al., 2015; Conzelmann et al., 2013a; Stewart et al., 2014; Veenstra, 2010). NKYs generally show sequence similarities to NpYs and NpFs and are likely paralogs (Bauknecht and Jekely, 2015).

Interestingly, we identified a sequence in the priapulid *Halicryptus spinulosa* that shows some similarity to the insect FMRFamide-receptors and a second sequence that shows some similarity to the trochozoan FMRFamide receptors. (These GPCRs cluster slightly apart from the rest of both FMRFamide groups on a clustermap (Fig. 3A in manuscript I). *H. spinulosa* also has a propeptide that encodes 3 peptides ending in FLRFamide (Appendix).

Taken together, there are two separate clusters of neuropeptide receptors, both of which include a receptor group that gets activated by a FMRFamide and a receptor group that gets activated by a NpY (Fig.4). Cluster 1 shows no strong sequence similarity to other known neuropeptide receptors and includes the *D. melanogaster* FMRFamide receptor and the *P. dumerilii* NpY receptor. The second cluster includes the trochozoan FMRFamide receptors and different bilaterian NpY and NpF receptors. Several other neuropeptide receptor groups of this second cluster belong to protostomes and deuterostomes and many of them are activated by RFamides or RYamides of different length and with different propeptide structures (Elphick and Mirabeau, 2014). Notably, FMRFamide was able to cross-activate the *P. dumerilii* NKY receptor (EC_{50} values 840 nmol/l and 1.4 μmol/l) (Bauknecht and Jekely, 2015). If the arthropod FMRFamide and the trochozoan FMRFamide are orthologs, then an ancestral FMRFamide peptide was able to activate two different receptors, and at some point during evolution the main receptor changed in either the arthropod lineage or the trochozoan lineage. As long as the ligands of the non-trochozoan GPCRs that are related to the trochozoan FMRFamide receptors are unknown, no clear conclusions can be drawn, whether the ancestral FMRFamide of the protostome lineage activated an arthropod-like FMRFamide receptor or a trochozoan-like FMRFamide receptor. An alternative scenario is, that the arthropod FMRFamide peptides and the trochozoan FMRFamide peptides evolved convergently. The identification of the ligands of the corresponding ecdysozoan,

deuterostome and xenacoelomorph receptors that are related to the trochozoan FMRFamide receptor could provide insights. It might also be interesting to find the ligands from members of the trochozoan receptor group that connects the arthropod FMRFamide receptor and the *P. dumerilii* NpY receptor group (Fig.4) (manuscript I) to see if the whole cluster is generally activated by RFamides.

5.3 Similar behavioral influence by orthologous neuropeptides can be convergent

When the first physiological or behavioral effect of a certain neuropeptide is discovered, other animals are often tested for similar reactions. FMRFamide is a neuropeptide for which many studies have shown that it influences muscular contractions; the level of evolutionary conservation, however, is hard to determine when only few taxa are investigated. The actual effect on muscles is variable and even the same muscle tissues of closely related species showed opposite effects in response to FMRFamide, as it has been demonstrated in a survey of the effect of FMRFamide on the hearts of 50 different bivalve species (Painter and Greenberg, 1982). It was also shown that the effect of FMRFamide on the muscle of one single species can be contrary depending on the FMRFamide concentration (Muneoka and Saitoh, 1986). The authors suggested that the phenomenon of concentration-dependent opposite effects could be explained by the existence of two different FMRFamide receptors: one located at the muscle fiber membranes which is involved in contractile responses and the second one might be located at relaxing nerve terminals.

These opposing effects of FMRFamide on muscles of adult trochozoans is also reflected in the behavioral response of different trochozoan larvae. In larvae of four different trochozoan species, FMRFamide (or its ortholog) influences the cilia-based swimming [manuscript I](Braubach et al., 2006; Conzelmann et al., 2011; Penniman et al., 2013), but the effects can be opposite in regards to the direction of a vertical shift of their swimming level. In addition to altering the horizontal migration in different directions, the mechanisms of influencing the ciliary beating can also be different. Two mechanisms of ciliary movement can influence larval swimming: the

speed with which the cilia are beating (beat frequency) and the frequency and length of ciliary arrests during which cilia don't beat at all (Arkett et al., 1987; Braubach et al., 2006; Conzelmann et al., 2011; Penniman et al., 2013). In larvae of the annelid *P. dumerilii,* FMRFamide increases the ciliary beat frequency and decreases the amount of ciliary arrests, which ultimately shifts the vertical swimming distribution of the larvae upwards (Conzelmann et al., 2011). In the gastropod veliger larvae of *Crepidula fornicata*, FMRFamide decreases the ciliary beat frequency, which shifts the larval vertical distribution downwards, but FMRFamide doesn't influence the ciliary arrests significantly (Penniman et al., 2013). FMRFamide thus directly influences the ciliated cells or the nerves that innervate the ciliary band, in *P. dumerilii* and *C. fornicata*, and changes at least the ciliary beat frequency in opposite ways. For the veliger larvae of *Illyanasa obsoleta*, two different kinds of ciliary arrests have been described: contractile ciliary arrests and isolated ciliary arrests (Braubach et al., 2006). In contractile arrests the ciliated velar lobes of the larvae contract their musculature and this contraction is accompanied by a ciliary arrest. In isolated ciliary arrests, the cilia stop beating without any contraction of the velar lobes. In *I. obsoleta*, FMRFamide did not change the ciliary beat frequency or influence isolated ciliary arrests. Instead, FMRFamide led to an increase of contractile ciliary arrest of the *I. obsoleta* larvae (Braubach et al., 2006). Because the contractile arrests are induced by the contraction of the velum, FMRFamide acts either directly on the velar musculature of *I. obsoleta* larvae or on motor neurons that innervate the musculature, and therefore influences their cilia-based locomotion by a different mechanism. In the brachiopod larvae of *T. transversa*, FLRFamide induces, similar to what has been described in *C. fornicata*, a downward sinking. The FLRFamide receptor expression underneath the ciliary band shows that FLRFamide likely directly affects the ciliated cells (manuscript I). FLRFamide of *T. transversa* is the ortholog of FMRFamide in molluscs and annelids (manuscript I). FMRFamide/FLRFamide therefore influences the cilia-based swimming, by either directly acting on the ciliated cells, or by inducing muscle contractions that indirectly induce ciliary arrests.

In *T. transversa*, FLRFamide additionally induces a contraction of the trunk musculature that results in the typical defensive stance of brachiopod larvae

(manuscript I). Independent of the influence on the swimming behavior, in the cases in which FMRFamide acts on larval musculature, the effects are also divergent. While FMRFamide induces frequent contractions ("twitching") of the velar musculature in *I. obsoleta* larvae (Braubach et al., 2006), FLRFamide induces a sustained contraction in the trunk musculature of *T. transversa* larvae (manuscript I). Due to the different morphology of brachiopod and gastropod larvae, it is difficult to directly compare these muscles, but *I. obsoleta* larvae are able to sustain the contraction of the velum as well (Evans et al., 2009; Fuchs et al., 2004), which suggests different mechanisms for the effect of FMRFamide on the muscles of *T. transversa* and *I. obsoleta* larvae.

In summary, orthologous neuropeptides can influence seemingly similar behavioral traits in trochozoan larvae, but the mechanisms that cause the behavioral changes can differ between species.

6. Conclusions

The investigation of the neuropeptide repertoire of xenacoelomorphs shows the presence of several bilaterian neuropeptides in xenacoelomorphs as well as the presence of novel, lineage specific neuropeptides and their diversifications during xenacoelomorph evolution. The low presence of neuropeptides or neuropeptide receptors in some xenacoelomorph species shows the importance of a broad taxon sampling for the identification of neuropeptides and for understanding their evolution. This study represents a substantial dataset of xenacoelomorph neuropeptides and provides the foundation for further investigations about neuropeptide signaling in Xenacoelomorpha.

The investigation of the excitatory peptide (EP) shows that EP is also present in trochozoan clades other than Annelida and Mollusca. We show that the nemertean EP has no myogenic effect on *Lineus longissimus* larvae, but can, similar to several neuropeptides in other trochozoan larvae, modify the beat frequency of the larval locomotory cilia.

The study of the *Terebratalia transversa* FLRFamide shows that the brachiopod FLRFamide activates a receptor that is an ortholog of the annelid FMRFamide receptor, which belongs to an ancient group of neuropeptide GPCRs. The results show that the single neuropeptide FLRFamide can specifically induce the two coherent sub-reactions that are characteristic for the *T. transversa* larval startle/defensive behavior.

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8. Appendix

cleavage site

C-terminal amidation site (+ cleavage site)

Trochozoan FMRFamide prepropeptides

Mollusca

>lcl|ACD65487 [Haliotis asinina]
MRPWTSVALLAVVLIKWISCINGFSDFCNKPVNSRICAILSGGPPQNEEKRFLRFGRTLAGDSFLRFGRQFYRIGKDGDDMEKRFLRFGRSDP
DLDDVIRASLLAYSLDDSPNNRRRRSVATAPVEAKAVEAGNKDIEKRDADELTSEDKRFMRFGRSGEDEKRFMRFGKSGEEEKRFMRFGKSGD
AEKRFMRFGRAGEEEKRFMRFGRAGEDEKRFMRFGKSGEEEKRFMRFGRAGEDGEEIEDEDEGIEADKRFMRFGRDGEDEKRFMRFGKSGEDE
KRFMRFGKRFMRFGRDGQDKRFMRFGKRFMRFGKRDSGEGSSEKAETAES
> AAA63280 [Lymnaea stagnalis]
MYSPTLIVCLSFFHSAVTKRFLRFGRALDTTDPFIRLRRQFYRIGRGGYQPYQDKRFLRFGRSEQPDVDDYPRDVVLQSEEPLYRKRRSTEAG
GQSEEMTHRTARSAPEPAAENREIMKRETGAEDLDEEKRFMRFGRGDEEAEKRFMRFGKSFMRFGRDMSDVDKRFMRFGKRFMRFGREPGTDK
RFMRFGREPGADKRFMRFGKSFDGEEENDDDLYYNESDADSNDDVDKRFMRFGKSAEEKRFMRFGKSQDASRDKKEFLRIGKRESRSAEVENN
IQIAAKQS
>lcl|A25790 [Aplysia californica]
MRPWCQLALLACLSLKWLTSHVTAESFLCDDSELCENGYLRFGRSMSVEEPHFRLERRSYPPVVYHKRFLRFGRSQEPDIEDYARAIALIESE
EPLYRKRRSADADGQSEKVLHRARREAESEHKSLEEVSPDTKQDVEKRDADDVLDAEKRFMRFGKRFMRFGRGSSDDDESGDDDVQDLTDIGD
GLGGEGEVNKRFMRFGKRFMRFGKREDGEPDKRFMRFGKSMADNDLDKRFMRFGKRFMRFGKSLPDSEVDKRFMRFGKSVDGDVDKRFMRFGK
SVDGDVDKRFMRFGKSVDGDVDKRFMRFGKSVDGDVDKRFMRFGKSVDGDVDKRFMRFGKSVDDDVDKRFMRFGKSVDGDVDKRFMRFGKSVD
DAVDKRFMRFGKSVDSDLDKRFMRFGKSVGSDEVDKRFMRFGKSVGSDEVDKRFMRFGKSLGTDDVNKRFMRFGKSLGTDDVNKRFMRFGKSL
GTEDVDKRFMRFGKSLGTEDVDKRFMRFGKSLGTDDVDKRFMRFGKSLGTEDVDKRFMRFGKSLGTDDVDKRFMRFGKSLGTEDVDKRFMRFG
KRFMRFGRSVGDSKYRSASSESVMTTDSKQTTEQATNKS
>lcl|CAA10949 [Mytilus edulis]
MWTKSYATLIVAAIINWISVKVHADELSRWCLDNQEICSNLIQNLRDTDDTNAQKRNFLRFGRALAGDHFFRFGRSPYQTEDKRFLRFGRSGG
GGGFDNVGLADILKAALVKVESANQNGLKIRKRRSIDAVKDVPEKKSVTDNTEPEAEIKKRNVDNSYATNEDSADENLKAADKRFMRFGKRFM
RFGKRENADKRFMRFGKRGDEDFGEEEGDDTYGVEDKRFMRFGRGGTEDKRFMRFGRAGEDKRFMRFGKRADENFGEDEGDETYGVEDKRFMR
FGRGGTEDKRFMRFGKSMDNDDEKRFMRFGKSAEADKRFMRFGKSLEADKRFMRFGKSGDDEKRFMRFGKSVDGEDKEKRFMRFGKSTEDKRF
MRFGRDPAEKRFMRFGKSTTEDKRFMRFGRK
>lcl|ACP39631 [Idiosepius notoides]
MRCWSPCSLFVVIVLHCLSSHSSAAFDLAQACVESQRLSLLPICDTIFAVQDGAQQSSDDGLRSKRFIRFGRALSGDAFLRFGKNVPDLPFED
KRFLRFGRAAPQLDDLLKQALQRVESLQRADETSVRRKRSTDAAPQSSAEGGEQKNDSSATKITKRYVDDGEDTDVKRFMRFGKRFMRFGRNQ
GGVGISADLANRLGEKRFMRFGRDPEKRFMRFGKSDDKRFMRFGRNPEDDLEEEKRFMRFGRGDEFDEEDEEAEAEKRFMRFGRDPEKKFMRF
GKSGGEDKRFMRFGRNPEEQEADKRFMRFGRGGAEENELNSEDKRFMRFGRSADKCKGCLEG

Annelida

> AEE25641 [Platynereis dumerilii]
MRDQWLHLGALFFLAHAHSVISVTLESLCQSANEIHNEKLHIFCNAFKAYIEDLTESEDSSWDDGLAEKRGLKFGRRSDGNLGPMYFAIRHRR
SFDPSLYLQMRQGRGGGYIRFGRSVHLASDPSQAYLASFGNVDKRAGGHYMRFGRSVPNSESTSVVSVPAAEADAVVSQLTDESDKEKRFMRF
GKSDPEEELHEKDKKFMRFGRGDADDLEEAKRFMRFGRGGDEEKRFMRFGRGDEEAAKRFMRFGRGEKGFMRFGRDPQEMSDDKRFMRFGRDG
ADEDEVEKRFMRFGKRFMRFGRDPLKKRFMRFGKRDDDEDLAEEKRFMRFGKRDGENGFMRFGKRGDEEKRFMRFGKREDMDEEKRFMRFGKR
ESEDELDDEEQKRFMRFGKRDSEVMDEQKRFMRFGKRDGGEEEKRFMRFGKRGDGEEEEKRFMRFGKKDGGEEEKRFMRFGKKSETDDKRFMR
FGKKDNVEDAMDKRFMRFGKKDGAEDSADKRFMRFGRDPESKSEDDKRFMRFGKKDEVSE
>AAT72794.1 [Lumbriculus variegatus]
MCTVSVTSGLVFLAVSIAVLFPVNGAREEDIEEALDGHSKSSSQAAAAAAANRALLLTGMQYYNGRLGDDDMNQLEPSEDMAEAKRYMRFGRG
SQQWRLAPLYDPYYAMSKRYMRFGRGHDDGDYMPVDENEAAEKRYMRFGKRYMRFGRMVNNRGENV
>AAU20817.1 [Lumbriculus variegatus]
MTDFVDTRKIILCVLILESYLLEAAVDRSHDGSIEGAKASRFTHPASDGRGGADKEHTVLTRLRRSLKDDPDESHIDIGLSRNEYLRYSRNFM
RFGRDWRNKIKDEVACFPTESGFSVSREDFLRNEKGLGEQLSSNRVKNSEQFDKRFMRFGRQLFAPSVAEVKSLDSTIEEDKRFMRFGKRFMR
FGKREAKPMDVVCYQDVTEQRPSANFDS
>AAT72795.1 [Hirudo medicinalis]
MASQNIILMALALVLVLSDALSVRGGQTYAGEEYSDELIDLLDNEELRKVESGESLANEVSGRGPQKSRKFICFTARDVESANQVDKRFMRFGR
KRESLRKQSDYTYEDDAQSTIGGSPDKRFLRFGKRFMRFGKRSPSTTESKKYCVWL
>Dinophilus gyrociliatus_Locus_5034.0_Transcript_1
MKFLLLLLCLTFAAANLFELNDCGAAEGSILKRLCAIYQQGEDDDVPNLLRMRQRRDQGYIRFGRSMPISYMRFGRKKREAVDEKRYMRFGRG
NEKDDNDAIDVSKKYMRFGKRFQDDLQKKYMRFGKRYMRFGRGGSEDEAEKRYMRFGRNQLKPNEEGIENIDKKYMRFGKRSSSTTN*

Nemertea

>Lineus longissimus c40291_g1_i2
MYILYLVALLAGQAIATDFNALCSDPKLNSLKNFMVLCDAFRSFSDNLNDVSLDSKRHRATFIRYGRDASHVSQDNALETHLTTPSKRNTEGY
MRFGRSVKDPQPAQNVENVDVENTKKEEEKKAIPSTAVDSEQKQETTAEDDKAKRYMRFGRNNYIRFGRESEEADDDEIDDAKRYMRFGKKDD
AKRYMRFGKKNLGPVAEEEGEEQKRYMRFGKKDGSDDAEKRYMRFGKRYMRFGKREDDLEEDKRYMRFGKKSDDEKRYMRFGKKDGEEQDEKR
YMRFGKKSDEDEKRYMRFGRGDEDMDADKRYMRFGKRYMRFGKRYMRFGKRDEEDANEGGDDEKRYMRFGKRYMRFGKRDTPTDEVRKRNVRS
VDESKAEKAAHMRYRRSEGPDGFERDARYMRFGRNPNGATVDGYIRFGRLLDGEDSDETEETPAKRYMRFGRDYLRFGRLLDGEEESSNDNQN
SSPVQKRFIRLGRLFGGLGVSKEELQRRYLRNNRDRAGGYMRFGRSEPAGGYMRFGK*
>Lineus cf. ruber Lvir.rna.tri.19128.1
MYILYLVALLAGQAIATDFNALCSDQKLSSLKNFMVLCDAFRSFSDSLNDVSLDSKRHREASVRYGRDASHVSQENALETHLTTPSKKTREGY
MRFGRSVEDPQPAQNVENVDVEKSKKEEEKKAISSTAGDTKQKQETTAEDDKAKRYMRFGRNNYIRFGRESEEADDDEIDDAKRYMRFGKKDD
AKRYMRFGKKSLGPVAEEEGEEQKRYMRFGKKDGSEGGDDAEKRYMRFGKRYMRFGKREGDLDEDKRYMRFGKKSDEDEKRYMRFGKKDGDDQ
DEKRYMRFGKKSDEDEKRYMRFGRGDEEMEANKRYMRFGKRYMRFGKRDEEDATEGGDDEKRYMRFGKRYMRFGKRDTPTDEVRKRNVRSVDE
SKAEEATHMRYRRSEGRDEFERDARYMRFGRNPNGATVDGYIRFGRLLDGEDSDKTDETAEKRYMRFGRDYLRFGRLLDGEEESSNDNQNSSP
VQKRFIRLGRLFGGLGVSKEELQRRYLRNNRDRAGGYMRFGRSEPAGGYMRFGK*

Brachiopoda

>Terebratalia transversa FLRFamide
MNRSVLLVAVVASFLLDHSVGIYTSRYINPCSPWLKSTHFRKLCDESFWPSYGSQEKDMIPYKRGFFGGGGEYGEPEGLSSFVDEPYRGTIGK
RDFLRFFKRRYDDNERSDQLEALNRVTRDFLRFFKRDFLRFFSYNLRGYDSDNKRKHMRKRRSIKNESDKPSSIESNDVYNKGALLRSDKKS
MSAAKARDIKRAFLRFFSLQKEYQQQDRKRAFLRFFSNTGINLDNRPTSDTKTETNSETDNGSAKTVTNDHVIDNNVDSKRAFLRFFKKCASC
KRAFLRFFSSEKNLISSSNKSMKSSPLFSSEHTKRAFLRFFSVNRE*
>Novocrania anomala YMRFamide
MAKEAEMALLAAVLVIQLMVIPSGASQEKEAAKNMEFNQLRDDLSKLQQQLDDIKVLTAEVPEDKRFFDTPRYLTKRYYLRFGRGIVDPYPYE
HPSDAVDGGFFDKPHYGFNKRHYLRFGRGIIDSNPHGYPSNALEGAMADGFEKNKRYLRFGRSVDRYVETPETHHLSKRSVKQKTPENGKTKE
ISPPKKETIKDKRYMRFGKKSIEDEKRYMRFGKKSDEDAQKRYMRFGKKSDNNEEKRYMRFGKKSDDEKEKRYMRFGKKDESPDE
>XP_013379870.1 [Lingula anatina]
MKTSSRIFLLAFVALYQIQLIRCSGYGNFLCNYGQELNALCNRLQEFQDSADLQAPDDSNSMWRTKHRFQLRLGRREDEGDNEDDEEEFEHLV
GMLAPHLSRQRRAYLRFGRSAEWPGVDDIDENKRAYLRFGKRAYLRFGRSVDNKRTPNSGGMSNYMRFGRSAEPHHLSKRSVPVKQEATESKT
KENTMKSAAVPNSEKRYMRFGKKSSEGDEKRYMRFGKKSTEDSAPETHAKEEKRYMRFGKKSQPNGEDPEKRYMRFGKKSMEQTDEDKRYMRF
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Ecdysozoan FMRFamide prepropeptides

Hexapoda

> NP_523669 [Drosophila melanogaster]
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> XP_001986940 GH20248 [Drosophila grimshawi]
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>XP_011207181.1 [Bactrocera dorsalis]
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Crustacea

>ALQ28593.1 [Scylla paramamosain]
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Priapulida

>Halicryptus spinulosis Locus_23197.0_Transcript_1
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