

Xenacoelomorph Neuropeptidomes Reveal a Major Expansion of Neuropeptide Systems during Early Bilaterian Evolution

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Abstract

Neuropeptides are neurosecretory signaling molecules in protostomes and deuterostomes (together Nephrozoa). Little, however, is known about the neuropeptide complement of the sister group of Nephrozoa, the Xenacoelomorpha, which together form the Bilateria. Because members of the xenacoelomorph clades *Xenoturbella*, *Nemertodermatida*, and *Acoela* differ extensively in their central nervous system anatomy, the reconstruction of the xenacoelomorph and bilaterian neuropeptide complements may provide insights into the relationship between nervous system evolution and peptidergic signaling. Here, we analyzed transcriptomes of seven acoels, four nemertodermatids, and two *Xenoturbella* species using motif searches, similarity searches, mass spectrometry and phylogenetic analyses to characterize neuropeptide precursors and neuropeptide receptors. Our comparison of these repertoires with previously reported nephrozoan and cnidarian sequences shows that the majority of annotated neuropeptide GPCRs in cnidarians are not orthologs of specific bilaterian neuropeptide receptors, which suggests that most of the bilaterian neuropeptide systems evolved after the cnidarian–bilaterian evolutionary split. This expansion of more than 20 peptidergic systems in the stem leading to the Bilateria predates the evolution of complex nephrozoan organs and nervous system architectures. From this ancient set of neuropeptides, acoels show frequent losses that correlate with their divergent central nervous system anatomy. We furthermore detected the emergence of novel neuropeptides in xenacoelomorphs and their expansion along the nemertodermatid and acoel lineages, the two clades that evolved nervous system condensations. Together, our study provides fundamental insights into the early evolution of the bilaterian peptidergic systems, which will guide future functional and comparative studies of bilaterian nervous systems.

Key words: neuropeptides, G protein-coupled receptors, Xenacoelomorpha, Cnidaria, CNS evolution, Bilateria.

Introduction

Neuropeptides are a diverse group of signaling molecules that play a crucial role in the function of the nervous system in most metazoan animals (Hökfelt et al. 2000; Grimmelikhuijzen et al. 2002; Jékely 2013; Mirabeau and Joly 2013; Roch and Sherwood 2014). These signaling molecules do not only mediate by direct synaptic transmission but mostly transfer signals by volume transmission as neurohormones or neuromodulators and, thus, play important roles in the modulation of neural circuits (Christie et al. 1995; Nassel and Winther 2010; Catak et al. 2014; Diao et al. 2017; Senatore et al. 2017; Williams et al. 2017; Jékely et al. 2018). Most neuropeptides are about 3–20 amino acids long and transfer signals via conserved G protein-coupled receptors (GPCRs) (Frooninckx et al. 2012; Jékely 2013; Mirabeau and Joly 2013; Stevens et al. 2013). Comparison of neuropeptides and neuropeptide GPCRs of different animals has shown that the last common ancestor of the Deuterostomia and Protostomia (together Nephrozoa, fig. 1a) had at least 30 different peptidergic systems (Janssen et al. 2008; Grimmelikhuijzen and Hauser 2012; Jékely 2013; Mirabeau and Joly 2013; Semmens

et al. 2015; Tian et al. 2016; Van Sinay et al. 2017; Zandawala et al. 2017; Elphick et al. 2018). These peptidergic systems are involved in various physiological and behavioral processes such as osmoregulation and water balance (Salzet et al. 1994; Fujino et al. 1999; De Mota et al. 2004), muscle activity (Semmens et al. 2013; Dickinson et al. 2015), metabolism and growth (Mizoguchi and Okamoto 2013; Van Sinay et al. 2017), feeding and defecation (Sakurai et al. 1998; Wang et al. 2013; Williams et al. 2015; Chung et al. 2017), reproduction (Lindemans et al. 2009; Beets et al. 2013), and stress tolerance (Schank et al. 2012; Terhzaz et al. 2015; Cannell et al. 2016). In contrast to the large number of plesiomorphic peptidergic systems of nephrozoans, the only peptidergic systems that are shared between Nephrozoa and Cnidaria are insulin-like peptides (ILPs), glycoprotein hormone (GPH)-related peptides, and prokineticin-related peptides (Jékely 2013; Roch and Sherwood 2014; Elphick et al. 2018). The existence of a few putative cnidarian orthologs of nephrozoan neuropeptides or neuropeptide GPCRs has been proposed, but their actual phylogenetic relationship is inconclusive (Ancil 2009;

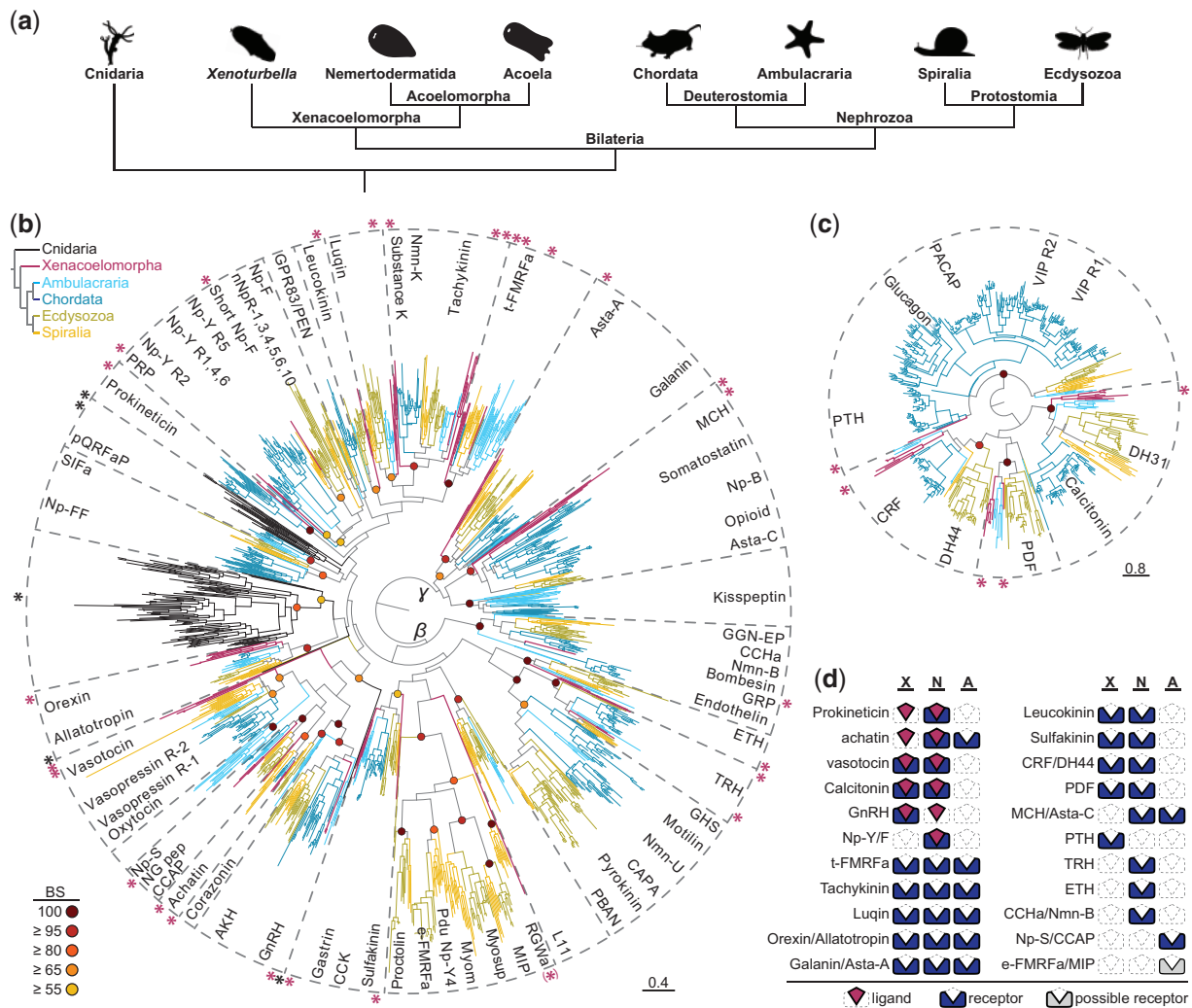


Fig. 1. Phylogenetic analysis of neuropeptide GPCRs. (a) Simplified phylogeny of Bilateria with Cnidaria as their sister group. (b) RAXML analysis of rhodopsin type neuropeptide GPCRs. Rhodopsin β GPCRs are rooted against rhodopsin γ GPCRs. Bootstrap values of the particular nodes are represented as red-to-yellow circles as shown on the lower left. The scale bar on the lower right indicates amino acids substitution rate per site. Magenta asterisks indicate the position of xenacoelomorph sequences, and black asterisks indicate the position of cnidarian sequences. If more than one monophyletic cluster of xenacoelomorph sequences is present within one receptor type, it is indicated by the corresponding number of asterisks. Dashed lines demarcate orthologous receptor types of different animal groups. Color coding of tree branches is shown on the upper left: Magenta = xenacoelomorphs, blue = chordates, light blue = ambulacrarians, olive = ecdysozoans, yellow = spirilians, black = cnidarians. (c) RAXML analysis of secretin-type neuropeptide GPCRs. Bootstrap support, substitution rate per site, and taxa color coding are depicted as in figure (b). (d) Distribution of conserved bilaterian neuropeptide receptors and ligands in Xenacoelomorpha. Blue boxes show the presence of conserved receptors, whereas the gray boxes display the presence of a possible conserved receptor (bootstrap support < 50%). Magenta diamonds show presence of conserved ligand. A, Acoela; a, amide; AKH, adipokinetic hormone; Asta, allatostatin; BS, bootstrap support; CCAP, crustacean cardioacceleratory peptide; CCK, cholecystokinin; CRF, corticotropin releasing factor; DH, diuretic hormone; GGN-EP, GGN excitatory peptide; e-, ecdysozoan; ETH, ecdysis triggering hormone; GHS, growth hormone segretagogue; GnRH, gonadotropin releasing hormone; GRP, gastrin releasing peptide; N, Nemertodermatida; Np, neuropeptide; nNpR, nematode neuropeptide receptor; Nmn, neuromedin; L11 = elevenin; MCH, melanin concentrating hormone; MIP, myoinhibitory peptide; Myosup, myosuppressin; PACAP, pituitary adenylate cyclase-activating polypeptide; PBAN, pheromone biosynthesis activating neuropeptide; PDF, pigment dispersing factor; Pdu, *Platynereis dumerilii*; NG pep, NG peptide; pQRFaP, pyroglutaminated RFamide peptide; PTH, parathyroid hormone; PRP, prolactin releasing peptide; R, receptor; t-, trochozoan; TRH, thyrotropin releasing hormone; VIP, vasoactive intestinal peptide; X, *Xenoturbella*. Silhouette pictures are downloaded from www.phylopic.org under the Public Domain Dedication 1.0 (CC0 1.0) or Public Domain Mark 1.0 license without any copyright restrictions or were drawn by one of the authors.

Alzugaray et al. 2013; Jékely 2013; Krishnan and Schioth 2015; Alzugaray et al. 2016). Therefore, the available data suggest that the major radiation of the nephrozoan peptidergic systems occurred sometime after the cnidarian–bilaterian

evolutionary split (Jékely 2013; Elphick et al. 2018). Compared with cnidarians, nephrozoans have several evolutionary novelties, such as a coelom, a through gut, an excretory system, and a circulatory system, which need to be

controlled by defined neural circuits (Holzer and Guth 1991; Zhang et al. 2014; Halberg et al. 2015; Cropper et al. 2018), and importantly, nephrozoans typically have highly condensed centralized nervous systems (CNS) (Hejnol and Lowe 2015). Many of the nephrozoan neuropeptides are also expressed in brains. Thus, orthologous neuropeptides have been used to compare brains and other parts of the nervous system between different animal species (Tessmar-Raible et al. 2007; Conzelmann and Jékely 2012; Tosches and Arendt 2013; Arendt et al. 2016; Kerbl et al. 2017). This led to the hypothesis that the origin of the bilaterian peptidergic systems is tightly connected to the origin of the different nephrozoan organ systems, such as their complex, condensed CNS (Jékely 2013). Such a hypothesis, however, has been difficult to test.

An important animal group for understanding the evolution of nervous systems is the Xenacoelomorpha. Recent phylogenomic evidence supports an earlier hypothesis that places the Xenacoelomorpha as the sistergroup to the Nephrozoa (Ruiz-Trillo et al. 1999; Hejnol et al. 2009; Srivastava et al. 2014; Cannon et al. 2016; Rouse et al. 2016). This placement has been challenged in a different phylogenomic analysis (Philippe et al. 2011) that places this clade as a sistergroup to Ambulacraria instead (see Discussion). Xenacoelomorphs lack several nephrozoan features, including coeloms, excretory organs, a circulatory system, and a through gut, but share their bilateral symmetry with clearly defined body-axes and the mesodermal germ layer as synapomorphic characters with nephrozoans (Jondelius et al. 2011; Hejnol 2015a, 2015b; Haszprunar 2016; Hejnol and Pang 2016). Furthermore, xenacoelomorphs display highly diverse neuroanatomies and seem to have evolved clade-specific CNS with multiple nerve cords and brain-like structures, which have evolved convergently to those of other bilaterians (Achatz and Martinez 2012; Hejnol 2015a; Perea-Atienza et al. 2015; Gavilan et al. 2016; Haszprunar 2016; Hejnol and Pang 2016; Raikova et al. 2016; Martin-Duran et al. 2018). Their divergent neuroanatomies provide a unique case to investigate general mechanisms regarding the early nervous system evolution in bilaterians. Xenacoelomorpha comprises three major clades: *Xenoturbella*, Nemertodermatida, and Acoela, with the latter two forming the clade Acoelomorpha (fig. 1a). The nervous system of *Xenoturbella* species is often considered to reflect a more ancestral form, as it only consists of a basiepidermal—somewhat cnidarian-like—nerve net without any considerable condensations (Raikova et al. 2000a; Hejnol and Rentzsch 2015; Stach 2015; Gavilan et al. 2016; Haszprunar 2016; Hejnol and Pang 2016). Nemertodermatids possess additional condensed basiepidermal nerve cords that can be located at different places along the dorsoventral axis, and additional basiepidermal anterior brain-like condensations are observed in many species (Raikova et al. 2004, 2016; Børve and Hejnol 2014; Martínez et al. 2017). The nervous system of acoels is considered as more derived with several novelties, including internalized anterior brains and multiple subepidermal pairs of longitudinal nerve cords (Achatz and Martinez 2012; Hejnol 2015a; Gavilan et al. 2016; Hejnol and Pang 2016;

Raikova et al. 2016; Martínez et al. 2017). Immunohistochemical studies have demonstrated reactivity of antibodies that were raised against neuropeptides of nephrozoan animals, like different RFamides and SALMFamides (Raikova et al. 2000a, 2004; Reuter et al. 2001; Stach et al. 2005; Kotikova and Raikova 2008; Semmler et al. 2010; Achatz and Martinez 2012; Børve and Hejnol 2014; Dittmann et al. 2018). However, not much is known about the actual xenacoelomorph neuropeptide repertoire, except for the presence of GPCRs that are related to FMRFamide, luqin, tachykinin, and neuropeptide F receptors (Thiel et al. 2017).

Due to the phylogenetic position of Xenacoelomorpha, investigations on these animals can be informative for the reconstruction of the evolutionary origin of nephrozoan peptidergic systems, with the potential to provide a deeper understanding of the connection between the emergence of neural morphological novelties and changes in peptidergic systems. Here, we conducted a detailed bioinformatic survey for neuropeptides and neuropeptide GPCRs in transcriptomes of 13 xenacoelomorph species of varying relatedness. Our *in silico* approach included a survey for neuropeptide precursors using sequence similarity and sequence motif searches that were complemented by a mass spectrometric analysis of peptide extracts from three acoel species. This nested survey allowed not only comparisons between different xenacoelomorph neuropeptide complements but also comparisons with other bilaterians and cnidarians. Together, we provide novel insights into the early diversification of bilaterian neuropeptide signaling systems and the evolution of peptidergic signaling in Xenacoelomorpha.

Results

We identified various types of peptidergic systems in xenacoelomorphs that have previously been reported from other animal lineages and found that most of the annotated cnidarian neuropeptide GPCRs are not orthologs of the proposed nephrozoan neuropeptide GPCRs. The three types of ancient metazoan peptidergic systems that have orthologs in nephrozoans as well as some nonbilaterians are also present in xenacoelomorphs (supplementary fig. 1, Supplementary Material online). Furthermore, we detected that 21 out of the 28 peptidergic systems that have previously been characterized in nephrozoans are present in xenacoelomorphs (figs. 1 and 2). In addition, we detected 14 types of multicopy peptides (MCPs) that are specific to all xenacoelomorphs or particular subclades (fig. 3) and several MCPs that seem specific to single xenacoelomorph species (supplementary fig. 2, Supplementary Material online). A comparison of the peptidergic complements of *Xenoturbella*, Nemertodermatida, and Acoela shows great differences in the representation of ancestral bilaterian systems and novel neuropeptides (fig. 4a; see supplementary tables 1 and 2, Supplementary Material online, for a detailed distribution), indicating multiple changes in the different xenacoelomorph clades.

Most of the Acoel Species Lack Otherwise Conserved Metazoan Neuropeptides

The three types of neuropeptides that are conserved between nephrozoans and nonbilaterians, the GPH-related peptides, the ILPs and the prokineticin-related peptides, are also found in the xenacoelomorph lineages ([supplementary fig. 1, Supplementary Material](#) online). GPH alpha precursors are found in both *Xenoturbella* species (i.e., *X. bocki* and *X. profunda*) and in the nemertodermatid *Nemertoderma westbladi*. In both *Xenoturbella* species we also identified GPH beta, and in *X. bocki* the GPH-related bursicon beta precursor. In acoels, we detected a single sequence in *Isodiametra pulchra*, which shows no clear similarity with a specific type of GPH-related peptide in our phylogenetic analysis ([supplementary fig. 1a, Supplementary Material](#) online), but this protein sequence has the typical cysteine arrangement of a bursicon (see [Roch and Sherwood 2014](#)).

ILPs were found in the nemertodermatids *N. westbladi*, *Meara stichopi* and *Ascoparia* sp., as well as in both *Xenoturbella* species. The only identified acoel ILP was detected in the transcriptome of *Diopisthoporus gymnopharyngeus*, and this sequence shows an unusually long C-chain between the predicted bioactive A- and B-chains ([supplementary fig. 1b, Supplementary Material](#) online). Interestingly, the nemertodermatid *Ascoparia* sp. has a large expansion of 23 potential ILPs, compared with the one, two or three paralogs of the other xenacoelomorphs ([supplementary fig. 1b, Supplementary Material](#) online). Although many bilaterians possess more than one ILP, such an expansion is only known from a few species ([Pierce et al. 2001](#); [Mizoguchi and Okamoto 2013](#)).

Prokineticin precursors were found in *X. bocki*, and in the nemertodermatids *M. stichopi* and *N. westbladi* ([supplementary fig. 1c, Supplementary Material](#) online). We could not detect a prokineticin-related peptide in any of the acoel transcriptomes. While receptors of GPH-related peptides and ILPs are also known to exist outside bilaterians ([Steele et al. 1996](#); [Jékely 2013](#); [Roch and Sherwood 2014](#)), prokineticin receptors are so far only known from deuterostomes ([Jékely 2013](#); [Mirabeau and Joly 2013](#)). However, we identified an ortholog of the deuterostome prokineticin GPCR in the nemertodermatid *N. westbladi* ([figs. 1b and d](#)), which is the first evidence that the prokineticin GPCR already existed at the dawn of bilaterians.

The Analysis of Cnidarian GPCRs Confirms That the Major Radiation of Peptidergic Systems Occurred after the Cnidarian–Bilaterian Evolutionary Split

While a previous large analysis of cnidarian and bilaterian taxa showed that only relaxin-like and GPH-related GPCRs are plesiomorphic to cnidarians and bilaterians ([Jékely 2013](#)), other smaller, but rather inconclusive studies, proposed the presence of additional cnidarian orthologs of bilaterian neuropeptide GPCRs ([Ancil 2009](#); [Alzugaray et al. 2013, 2016](#); [Krishnan and Schioth 2015](#)). In addition, various sequences deposited into NCBI database, which were predicted by the NCBI eukaryotic genome annotation pipeline as part of the

genome projects from the cnidarians *Exaiptasia pallida* ([Baumgarten et al. 2015](#)), *Acropora digitifera* ([Shinzato et al. 2011](#)), and *Orbicella faveolata* (<http://montastraea.psu.edu/genome/>; last accessed August 23, 2018), were also annotated as bilaterian neuropeptide GPCRs. These neuropeptides include neuropeptide FF, neuropeptide Y, orexin, cholecystokinin-, qRFamide-, RYamide, substance-K-, bombesin-, gastrin-, galanin-, and somatostatin-GPCRs. In order to resolve the inconsistencies between previous studies and to better reconstruct the ancestral cnidarian–bilaterian neuropeptide complement, we reanalyzed the automatically annotated sequences, as well as GPCR sequences that were predicted from the genome of *Nematostella vectensis* as galanin-like, tachykinin/SIFamide-like, RFamide/neuropeptide FF/GnIH/neuropeptide Y-like, and GnRH/vasopressin-like receptors ([Ancil 2009](#)). Our analysis shows that most of these cnidarian GPCRs are closely related to each other, rather than to specific nephrozoan receptors ([fig. 1b](#) and [supplementary figs. 3a and 4a, Supplementary Material](#) online). Only the *Nema. vectensis* sequences showed some degree of diversity, with four to five types of GPCRs that clustered separately from each other ([fig. 1b](#) and [supplementary figs. 3a and 4a, Supplementary Material](#) online). One group of *Nema. vectensis* receptors showed similarity to the pQRFamide peptide receptors, while others had a more basal position to several different bilaterian GPCR types ([fig. 1b](#) and [supplementary fig. 3a, Supplementary Material](#) online). One of these basal sequences was originally predicted as a GnRH/vasotocin-like receptor by [Ancil \(2009\)](#). This sequence was also grouped in both phylogenetic analyses as a sister group to the “superclade” that consists of the bilaterian vasotocin, CCAP, GnRH, corazonin, and achatin GPCRs ([fig. 1b](#) and [supplementary fig. 3a, Supplementary Material](#) online). This result indicates that a single ancestral receptor might have been present in the last common ancestor of cnidarians and bilaterians, which later diversified along the stem leading to bilaterians into five different receptor types. The presence of several *Nema. vectensis* neuropeptide GPCR types also suggests that the last common ancestor of cnidarians and bilaterians possessed a few different neuropeptide GPCRs (four or five in our analysis, in addition to the relaxin-like and GPH-related GPCRs), while the main diversification into the different bilaterian GPCR groups occurred after the cnidarian–bilaterian evolutionary split, with a parallel diversification in the cnidarian lineage.

Xenacoelomorph Orthologs of Nephrozoan Neuropeptide GPCRs Reveal the Early Diversification of Peptidergic Systems in Bilaterians

In our GPCR survey, we detected potential orthologs of 22 of the 29 previously characterized nephrozoan GPCR types, including the first nondeuterostome prokineticin GPCR ([figs. 1b–d](#), and [supplementary fig. 3, Supplementary Material](#) online). Therefore, our analysis indicates the presence of these receptors in the last common ancestor of all bilaterians. Eighteen of these receptors were detected in nemertodermatids and 13 in *Xenoturbella* species, whereas only nine homologs were identified in Acoela ([fig. 1d](#) and

supplementary table 1, Supplementary Material online). The different types of rhodopsin- and secretin-type GPCRs were confirmed in both the phylogenetic analysis (figs. 1b and c, and supplementary figs. 3a and b, Supplementary Material online) and the cluster analysis (supplementary figs. 4a and b, Supplementary Material online). We identified GPCRs related to trochozoan FMRFamide, tachykinin, luquin, allatotropin, and allatostatin A signaling in all three xenacoelomorph clades. Vasotocin, leukokinin, sulfakinin, calcitonin, DH44, and PDF type GPCRs were only found in *Xenoturbella* and Nemertodermatida. GPCRs that were only found in *Xenoturbella* but not in Acoelomorpha (Nemertodermatida + Acoela) were those related to GnRH and PTH signaling, whereas GPCRs related to achatin and allatostatin C signaling were found in both Acoelomorpha clades, but not in *Xenoturbella*. Receptors related to prokineticin, neuropeptide Y/F, TRH, ETH, and CCHamide signaling were restricted to Nemertodermatida. The only GPCRs exclusively detected in acoels were receptors that are related to CCAP signaling, and a single sequence that shows similarity to a large cluster that diversified greatly in protostomes into proctolin, arthropod FMRFamide, myomodulin, RGWamide, and myoinhibitory peptide receptors (fig. 1b and supplementary fig. 3a, Supplementary Material online). These results indicate an extensive loss of conserved bilaterian neuropeptide GPCRs in acoels. We did not include uncharacterized receptors in our survey (see Elphick et al. [2018] for more details about those receptors). The only receptor types that we could not identify in xenacoelomorphs, but that are known to be pleiomorphic to nephrozoans, are GPCRs related to corazonin, GPR83/PEN, neuromedin U/pyrokinin, neuropeptide FF/SIFamide, elevenin, pQRFamide peptide, and Kisspeptin signaling. The neuropeptide FF/SIFamide receptors and the pQRFamide receptors show in both phylogenetic analyses and in the cluster map an affinity to each other. The other missing receptors show no putative relationship, indicating that most receptor types without clear orthologs in xenacoelomorphs evolved as independent novelties in the nephrozoan lineage or were lost independently in the xenacoelomorph lineage. The pQRFamide receptors, however, show potential cnidarian orthologs (fig. 1b, supplementary figs. 3a and 4a, Supplementary Material online), which might indicate that this group of receptors could belong to the ancestral repertoire of bilaterian receptors and was lost in xenacoelomorphs.

Conserved Bilaterian Neuropeptide Precursors Are Found in Nemertodermatida and *Xenoturbella*, but Not in Acoela

We identified neuropeptide precursors related to five nephrozoan neuropeptides: Vasotocin, neuropeptide Y/F, calcitonin, GnRH/corazonin, and achatin, which were only found in nemertodermatid and *Xenoturbella* species but not in any of the acoel transcriptomes (figs. 1c and 2). The presence of these neuropeptide precursors in xenacoelomorphs and the high similarity to their nephrozoan orthologs show that these ligands have diverged less when compared with many other

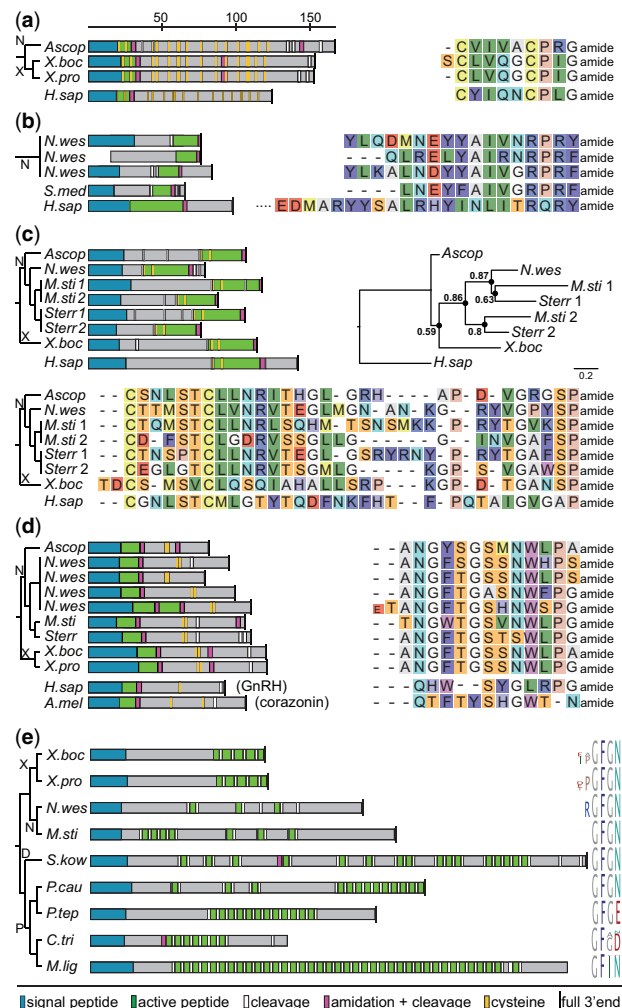


FIG. 2. Xenacoelomorph orthologs of bilaterian neuropeptides. (a) Vasotocin-neurophysin precursor schemes and vasotocin sequences. (b) Neuropeptide Y/F precursor diagrams and active peptide sequences. (c) Calcitonin precursor schemes, phylogenetic FastTree analysis of predicted active ligands and alignment of predicted active peptides. (d) GnRH/corazonin related peptide precursor diagrams and predicted active ligands. (e) Achatin precursor schemes and peptide sequence logo of xenacoelomorph and nephrozoan sequences. D, Deuterostomia; N, Nemertodermatida; P, Protostomia; X, Xenoturbella; A.mel, *Apis mellifera*; Ascop, *Ascoparia* sp.; C.tri, *Charonia tritonis*; H.sap, *Homo sapiens*; M.lig, *Macrostromum lignano*; M.sti, *Meara stichopi*; N.wes, *Nemertoderma westbladi*; P.tep, *Parasteatoda tepidariorum*; P.cau, *Priapululus caudatus*; S.kow, *Saccoglossus kowalevskii*; S.med, *Schmidtea mediterranea*; Sterr, *Sterreria* sp.; X.boc, *Xenoturbella bocki*; X.pro, *Xenoturbella profunda*. Scale bar on the top indicates length of neuropeptide precursors in number of amino acids. Color coding of amino acid residues of the sequences is according to their biochemical properties (i.e., Rasmol coloring).

bilaterian neuropeptides. Vasotocin–neurophysin precursors were found in both *Xenoturbella* species and in the nemertodermatid *Ascoparia* sp. (fig. 2a). Neuropeptide Y/F precursors were only found in the nemertodermatid *N. westbladi*, where we detected three potential paralogs (fig. 2b). Interestingly, the C-terminal YYAIVGRPRFamide motif of one of the

N. westbladi paralogs is similar to the C-terminus of the neuropeptide Y of different protostome species (McVeigh et al. 2009; Veenstra 2010, 2011; Nassel and Wegener 2011; Conzelmann et al. 2013), which might be an indication for a similar ancestral neuropeptide Y/F. Calcitonin precursors were found in all four nemertodermatid species and in *X. bocki* (fig. 2c). The two more closely related species *M. stichopi* and *Sterreria* sp. possess two calcitonin paralogs that likely arose from a single duplication event (fig. 2c). GnRH/AKH/corazonin-like peptides were found in all four nemertodermatids and in both *Xenoturbella* species (fig. 2d). The predicted active ligands lack an N-terminal glutamine, which is characteristic for GnRH/CRZ-related peptides (Hansen et al. 2010; Lindemans et al. 2011; Hauser and Grimmelikhuijzen 2014; Roch et al. 2014; Li et al. 2016; Zandawala et al. 2017) and only absent in a few cases (Tian et al. 2016). It seems like one of the *N. westbladi* paralogs underwent a modification where the peptide precursor encodes two, instead of one ligand, which is typical for GnRH/AKH/CRZ peptides. Achatins were found in both *Xenoturbella* species and in the nemertodermatids *M. stichopi* and *N. westbladi* (fig. 2e). All predicted ligands share the GFGN sequence known from the achatin of the hemichordate *Saccoglossus kowalevskii*, the cephalochordate *Branchiostoma floridae*, and the ecdysozoans *Priapulius caudatus* and *Halicryptus spinulosus* (fig. 2e and supplementary precursor sequences, Supplementary Material online). The presence of identical GFGN sequences in deuterostome, protostome, and xenacoelomorph taxa could be due to a similar ancestral achatin.

Acoelomorphs Show a Larger Expansion of Clade-Specific Novel Neuropeptides Compared with *Xenoturbella*

Beside the neuropeptides that are known from other animals, we identified 14 types of MCPs that seem to be specific to Xenacoelomorpha or particular xenacoelomorph clades (fig. 3). Three of these MCP types are symplesiomorphic for all Xenacoelomorpha (figs. 3a–c and 4a), three types seem to have emerged in the last common ancestor of acoelomorphs (figs. 3d–f and 4a), four types of MCPs are only shared among acoel species (figs. 3g–j and 4a), three types are only present in nemertodermatid species (figs. 3k–m and 4a), and one type of novel MCP seems to have appeared in the last common ancestor of *Xenoturbella* (figs. 3n and 4a). In addition, we found 11 full-length neuropeptide precursors that were only identified in single acoel species and five full-length precursors that were restricted to single nemertodermatid species (fig. 4a and supplementary fig. 2, Supplementary Material online).

Three Types of Novel MCPs Are Plesiomorphic to Xenacoelomorpha

The three types of ancestral xenacoelomorph MCPs are SFxNamides, LxFamides, and PxFVamides, with “x” standing for a variable amino acid position. SFxNamide peptides are 4–5 amino acids long and show a high conservation of the paracopies within each precursor (fig. 3a). LxFamide peptides

show a higher variability between the paracopies of the precursors and partially great variability between the different species (fig. 3b). Some of the LxFamides show similarity to echinoderm L-type SALMFamide (fig. 4b). PxFVamides were only found in *N. westbladi* and *X. bocki* but were entirely absent in acoel species (fig. 3c). These PxFVamide peptides show similarity to neuropeptides with a similar motif that are known from mollusk species (fig. 4c).

Three Types of Novel MCP Are Plesiomorphic to Acoelomorpha

In acoel as well as nemertodermatid species, we discovered peptides that share the motifs AWDF, LWDY, and FxxxFamide (figs. 3d–f). The *Ascoparia* sp. FxxxFamide can also be grouped into the LxFamide peptides, as some of the paracopies possess a leucine in their third to last position (fig. 3f). In contrast to other xenacoelomorph peptides, most AWDF and LWDY orthologs have precursors on which the paracopies are very evenly distributed (figs. 3d and e). These two types of peptides also share a Trp-Asp sequence that is followed by an aromatic amino acid. This could be an indication that the AWDF and LWDY peptides might have evolved from a common ancestral sequence that split into two paralogs early in the stem leading to acoelomorphs.

Four Types of Novel MCPs Are Plesiomorphic to Acoela

Four types of MCPs are only present in acoel species. These peptides possess the motifs MxGFG(amide) (fig. 3g), SSxxxF(amide) (fig. 3h), YAFNMamide (fig. 3i), and MR(F) (fig. 3j). The MR(F), SSxxxF(amide), and MxGFG(amide) peptides are in some species amidated, in some species nonamidated, and in other species the precursor encodes both types. A mixture of otherwise structurally similar amidated and nonamidated peptides on the same precursor is rather uncommon for MCPs. The SSxxxFamides might be paralogs of the LxFamides, as they share several residues, particularly with the LxFamides of *D. longitubus*, *D. gymnopharyngeus* and *Hofstenia miamia*, indicating a possible duplication and diversification of an ancestral LxFamide peptide. The MxGFG peptides share the GFG motif with achatin peptides, but they do however vary between species and between paracopies in terms of length and amino acid sequence, whereas bilaterian achatins are usually tetrapeptides.

Three Types of Novel MCPs Are Plesiomorphic to Nemertodermatida

The three types of peptides that are present in several nemertodermatid species have the motifs LRIGamide (fig. 3k), ELamide (fig. 3l), and WDL(G)amide (fig. 3m). The *N. westbladi* ELamide peptides possess aromatic amino acids on position 3 and 5 from the C-terminus, which makes them somewhat similar to arthropod allatostatin A peptides (fig. 4d). The *N. westbladi* MCP complement differs from the other nemertodermatid species in having three LRIG paralogs (fig. 3k), two ELamide paralogs (fig. 3l), and a WDL peptide that ends in WDLGamide, instead of WDLamide (fig. 3m).

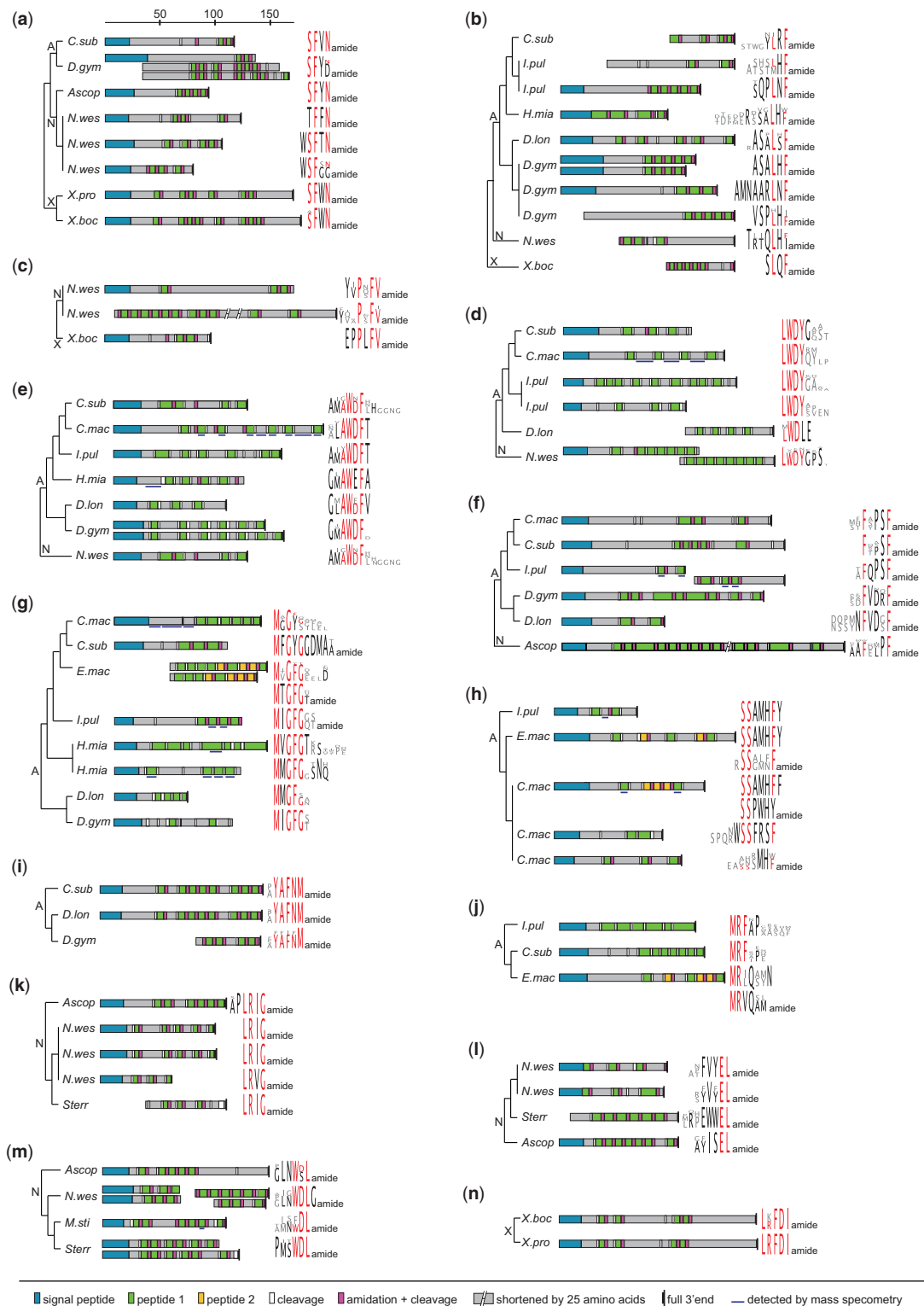


Fig. 3. Neuropeptide precursors and their peptide sequence logo representations of xenacoelomorph-specific MCPs. (a) SFxNamides. (b) LxFamides. (c) PxFVamides. (d) LWDY peptides. (e) AWDF peptides. (f) FxxxFamides. (g) MxGFG peptides. (h) SSxxxF peptides. (i) YAFNMamides. (j) MRF peptides. (k) LRIGamides. (l) ELamides. (m) WDLamides. (n) LRFDlamides. Peptide sequence logo representations are created using all aligned peptide sequences of the corresponding precursor. Conserved amino acid residues between species are highlighted in red. A, Acoela; N, Nemertodermatida; X, Xenoturbella; Ascop, Ascoparia sp.; C.sub, Childia submaculatum; C.mac, Convolutriloba macropyga; D.gym, Diopisthoporus gymnopharyngeus; D.lon, Diopisthoporus longitubus; E.mac, Eumecynostomum macrobursarium; H.mia, Hofstenia miamia; I.pul, Isodiametra pulchra; M.sti, Mearia stichopi; N.wes, Nemertoderma westbladi; Sterr, Sterreria sp.; X.boc, Xenoturbella bocki; X.pro, Xenoturbella profunda.

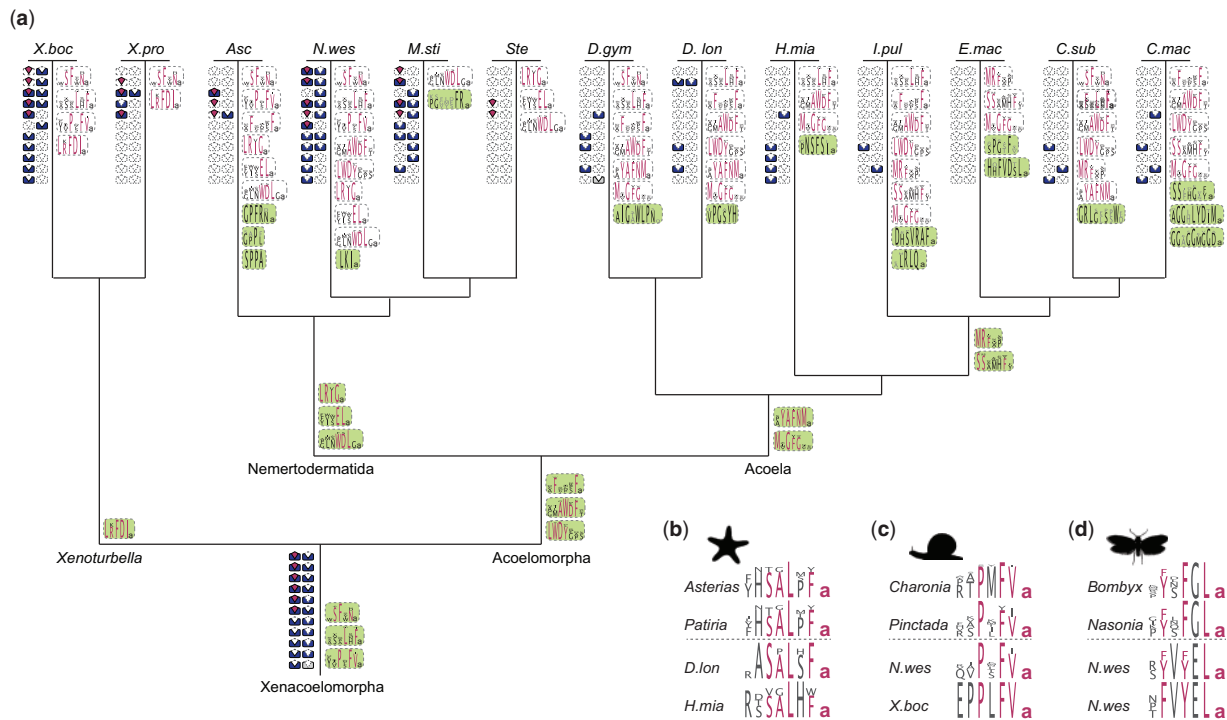


FIG. 4. Distribution of peptidergic systems in Xenacoelomorpha and comparison of xenacoelomorph MCPs. (a) Distribution of detected ancestral bilaterian systems (left side of a branch) and novel MCPs (right side of a branch) in the 13 xenacoelomorph species. Symbols and order of bilaterian systems are similar to figure 1. Newly evolved MCPs are shown with a green background, next to the branch where they evolved. (b) Comparison of two xenacoelomorph LxFamides with echinoderm L-type SALMFamides of *Asterias rubens* and *Patiria miniata*. (c) Comparison of two xenacoelomorph PxFVamides with molluskan PxFVamides of *Charonia tritonis* and *Pinctada fucata*. (d) Comparison of two xenacoelomorph ELamides with insect allatostatin A of *Bombyx mori* and *Nasonia vitripennis*. Residues of peptide sequence logos that are conserved across species are highlighted in magenta.

One Type of Novel MCP Is Plesiomorphic to *Xenoturbella*

The only potential MCP type that is specific to the two *Xenoturbella* species is the LRFDLamide (fig. 3n). The precursors of both species are very similar with four copies of the peptide in the same arrangement and an overall high conservation of the nonrepetitive peptides.

Full-Length MCPs without Orthologs Were Only Detected in Acoelomorph Species

We detected several putative neuropeptide precursors that were only identified in single acoelomorph species, without any orthologous sequences in other xenacoelomorph transcriptomes (fig. 4a and supplementary fig. 2, Supplementary Material online). A few partial sequences with a repetitive structure were found in *Xenoturbella* species, but all of them are missing the 5'-end; therefore, they could not be tested for the presence of a signal peptide. Such single repetitive sequences with a missing 5'-end were also found in acoel and nemertodermatid species. Full-length precursors with a signal peptide belonged exclusively to acoel and nemertodermatid species.

The Mass Spectrometric Analysis Confirms Several Types of MCPs in Acoel Species

Our mass spectrometric analysis of peptide extracts from three acoel species (*Convolutriloba macropyga*, *H. miamia*,

and *I. pulchra*) was mainly used to confirm our bioinformatic predictions of the neuropeptide precursors. From the predicted peptide precursors, the presence of processed LWDY peptides, AWDF peptides, SSxxxF peptides, FxxxFamide peptides, and amidated as well as nonamidated MxGFG peptides was confirmed by LC-MS/MS in at least one species (see fig. 3d–h, supplementary precursor sequences and supporting mass spectrometric data files, Supplementary Material online). In addition, our LC-MS/MS analysis showed evidence for peptide candidates that either had no repetition of similar peptides or were lacking an N-terminal signal peptide; however, these peptides are not listed here (see additional pre-neuropeptide candidates in the supplementary precursor sequences and the supporting mass spectrometric data, Supplementary Material online).

Discussion

In our analyses, we found that a high number of peptidergic systems that are plesiomorphic to Nephrozoa are also present in Xenacoelomorpha, whereas most cnidarian neuropeptide GPCRs are not directly orthologous to these bilaterian GPCRs. In addition, we detected not only many MCPs that seem to be restricted to Xenacoelomorpha but also some MCPs that show a sequence similarity to neuropeptides that have so far only been reported in specific deuterostome or protostome clades. When we compare the peptidergic systems of *Xenoturbella*, *Nemertodermatida*, and *Acoela*, we observed

differences that might reflect the differences in their nervous system anatomy.

The phylogenetic placement of the Xenacoelomorpha has been controversial in the past, and contradicting phylogenetic characteristics of different genes are, for example, also reflected by the varying affinities of the xenacoelomorph neuropeptide GPCRs to deuterostome and protostome sequences in our phylogenetic GPCR analysis (fig. 1b, supplementary fig. 3a and table 3, Supplementary Material online). Here, we discuss our results on the background of recent phylogenomic analyses which place Xenacoelomorpha as the sister group to Nephrozoa (Deuterostomia + Protostomia) (Srivastava et al. 2014; Cannon et al. 2016; Rouse et al. 2016). This position has previously been challenged by a phylogenomic study that instead suggests a sister-group relationship to Ambulacraria (Echinodermata + Hemichordata) (Philippe et al. 2011), albeit with low support (see also Giribet [2016] and Cannon et al. [2016] for a critical assessment and reanalysis). The alternative placements would have generally different implications on our understanding of bilaterian evolution, some of which are, for example, discussed in Lowe and Pani (2011) and Telford and Copley (2016). Also smaller analyses that only use up to 13 mitochondrial genes place xenacoelomorphs as either sister group to Chordata (Rouse et al. 2016) or sister group to all remaining deuterostomes (Philippe et al. 2011; Robertson et al. 2017). However, the general utility of such small mitochondrial data sets for resolving deep phylogenetic nodes has been questioned before (Bernt et al. 2013; Rouse et al. 2016). Even though we interpret our findings on the background of the position of Xenacoelomorpha as a sister group to all remaining Bilateria (Srivastava et al. 2014; Cannon et al. 2016; Rouse et al. 2016), we want to encourage the reader to also see the presented data from the view of an alternative placement of xenacoelomorphs as a deuterostome clade.

The Early Expansion of the Bilaterian Peptidergic Systems Is Independent from the Origin of Complex Bilaterian Organ Systems

Our GPCR survey and phylogenetic analysis revealed a novel expansion into more than 20 different types of neuropeptide GPCRs in the bilaterian stem lineage. This is surprising, because it suggests that many of the extant nephrozoan peptidergic systems evolved and diverged before the split of Nephrozoa and Xenacoelomorpha. Because organ systems such as the circulatory system, excretory organs, or a through gut are absent from xenacoelomorphs (Hejnal and Martindale 2008; Haszprunar 2016), the major diversification of the bilaterian peptidergic system is not directly related to the origin of organ systems that characterize more complex animals. Neuropeptides in bilaterians are very divergent in their function and can trigger simple reactions to complex behaviors in animals. The early bilaterian neuropeptides thus likely were involved in different roles, as orthologous neuropeptides are not tightly associated with the same structures, functions, or behaviors in different clades. GnRH, for example, controls mammalian reproduction by stimulating the

synthesis and release of follicle stimulating hormone and luteinizing hormone from the anterior pituitary (Okubo and Nagahama 2008), whereas its insect ortholog, the neuropeptide AKH, is involved in energy mobilization in different insects (Gade and Auerswald 2003). Ancestral peptides can also be related to clade- or species-specific behaviors, such as FMRFamide-related peptides to the startle behavior of the brachiopod *Terebratalia transversa* (Thiel et al. 2017), to chromatophore expansions in the cuttlefish *Sepia officinalis* (Loi and Tublitz 1997), or to the feeding behavior of the sea hare *Aplysia californica* (Vilim et al. 2010). Some studies that suggest a homology of deuterostomian and protostomian brains include overlapping expression of homologous neuropeptides in their line of evidence (Tessmar-Raible et al. 2007; Tosches and Arendt 2013; Arendt et al. 2016). An immunohistochemical study using antibodies against various neuropeptides in three dinophilid species (Annelida), however, shows that orthologous neuropeptides can be expressed in different brain regions, even in closely related species with morphologically similar brains (Kerbl et al. 2017). This indicates that a general long-term conservation of strictly defined local peptidergic expression is rather unlikely. Furthermore, nephrozoans that lack condensed brains, such as ambulacrarians, possess basically all bilaterian peptidergic systems (Jékely 2013; Mirabeau and Joly 2013; Semmens et al. 2016; Tian et al. 2016; Suwansa-Ard et al. 2018). The integration of existing neuropeptides into novel circuits and roles can, therefore, be rather plastic, and the ancestral peptidergic systems were later integrated into the complex circuits and behaviors that are known from deuterostomes and protostomes. Closer investigations of the function of neuropeptides in xenacoelomorphs and their implementation in neural circuits might, therefore, help to understand early roles of neuropeptides in Bilateria.

Similarities of Xenacoelomorph MCPs with Clade-Specific Deuterostome and Protostome MCPs: Ancestral Peptides or Convergent Evolution?

In our analysis, we discovered MCPs that show similarities to MCPs that are only known from restricted deuterostome or protostome clades. The similarity to clade-specific neuropeptides might indicate that either these are homologous peptides with ancestral motifs or these motifs evolved convergently. Several acoel LxFamides that share the SxLHFamide motif and some of the SSxxxFamide peptides show sequence similarity to L-type SALMFamides from echinoderms (fig. 4b) (Elphick 2014; Elphick et al. 1991, 2015). It has also been reported that one of the antibodies that was raised against echinoderm SALMFamides shows immunoreactivity not only in different ambulacrarian species but also in *X. bocki* and three acoel species (Stach et al. 2005; Dittmann et al. 2018). These similarities would be in line with the previous notion of a phylogenetic affinity of Xenacoelomorpha to ambulacrarians (Philippe et al. 2011). Other xenacoelomorph MCPs, however, show similarities to neuropeptides that are only present in protostomes, such as the PxFVamide, which is similar to trochozoan PxFVamides

(fig. 4c). Indeed, PxFVamides have been described in various mollusk species (Fujisawa et al. 1991; Veenstra 2010; Stewart et al. 2014; Adamson et al. 2015; Zatylny-Gaudin et al. 2016; Bose et al. 2017), and in the annelid *Capitella teleta* (Veenstra 2011), which altogether are referred to as “PxFVamide” (Veenstra 2010; Adamson et al. 2015; Bose et al. 2017) “FVamide” (Veenstra 2011) or “Mytilus inhibitory peptide” (Fujisawa et al. 1991; Stewart et al. 2014). Other peptides with similarity to protostome peptides are the ELamides; particularly two sequences from *N. westbladi* show similarity to arthropod allatostatin A peptides (fig. 4d). Allatostatin A's are MCPs that are especially known from arthropods and share the C-terminal ending [Y/F]xFLGlamide (Woodhead et al. 1989, 1994; Pratt et al. 1991; Wegener and Gorbashov 2008; Hauser et al. 2010; Nassel and Winther 2010; Jékely 2013) with somewhat similar neuropeptides in trochozoans, such as buccalins (Veenstra 2010; Conzelmann et al. 2013; Bose et al. 2017). Orthologs of the allatostatin A receptors are present in Xenacoelomorpha, including *N. westbladi*. The receptors of the echinoderm SALMFamides and the trochozoan PxFVamides are to this date unknown, which makes it so far impossible to test the potential binding affinities. The general variability of the SALMFamides in echinoderms and the PxFVamides in trochozoans, however, shows that these peptides have a high evolutionary rate within those animal groups. Due to the short length and the high diversity of closely related orthologs of MCPs, the possibility of a convergent evolution of similar motifs in distantly related taxa is likely. RFamide-type neuropeptides, for example, share a similar C-terminal motif, but not all of them are homologous (Espinoza et al. 2000; Walker et al. 2009; Jékely 2013; Mirabeau and Joly 2013; Elphick and Mirabeau 2014; Peymen et al. 2014).

Changes in the Neuropeptide Complement Correlate with the Evolution of Nervous System Architectures in Xenacoelomorpha

The common notion is that the ancestral xenacoelomorph possessed a basiepidermal nerve net (Hejnal 2015a; Hejnal and Rentzsch 2015; Gavilan et al. 2016; Haszprunar 2016; Raikova et al. 2016; Martin-Duran et al. 2018). Because many of the ancestral bilaterian peptidergic systems are present in *Xenoturbella*, which only possess a basiepidermal nerve net (Raikova et al. 2000a; Stach 2015), it suggests that the presence of the bilaterian neuropeptide systems seems not to be correlated to the presence of a condensed CNS with brain-like structures. Brains and nerve cords are only present in acoels and nemertodermatids, and these structures seem to have evolved independently from the CNS of other bilaterians (Gavilan et al. 2016; Haszprunar 2016; Martin-Duran et al. 2018). While the nemertodermatid nerve condensations are usually situated in a basiepidermal position, the acoel nervous system components are generally internalized, which is considered to be a derived state in the Xenacoelomorpha (fig. 5) (Raikova et al. 2000b, 2004, 2016; Achatz and Martinez 2012; Hejnal 2015a; Gavilan et al. 2016; Haszprunar 2016; Hejnal and Pang 2016; Dittmann et al. 2018). Interestingly,

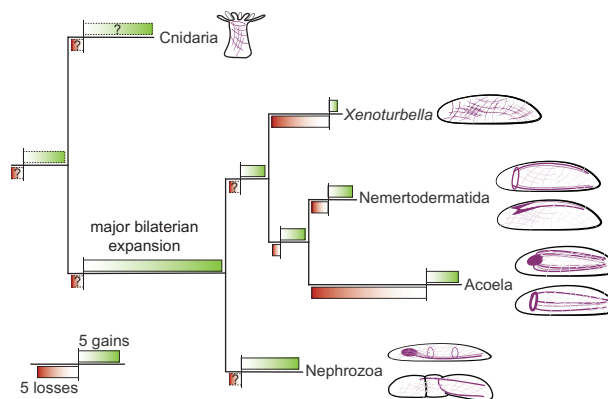


FIG. 5. Changes in the complement of peptidergic systems during bilaterian evolution. Tree shows major expansion of peptidergic systems in the ancestral bilaterian lineage followed by clade-specific changes in sublineages. Bars show number of changes as detected losses (red bars underneath the branches) and detected gains (green bars above the branches) of peptidergic systems in the corresponding lineage. Length of bars are estimated based on the results of this study, with 4–5 peptidergic systems in the cnidarian–bilaterian ancestor that gave rise to the main diversification in the bilaterian lineage (excluding relaxin GPCRs and GPH GPCRs). The two bars on the lower left side indicate the bar-length for five gains or five losses. Cartoons depict simplified examples of nervous system anatomies (purple) from representative taxa (top to bottom: anthozoan, *Xenoturbella bocki*, *Nemertoderma westbladi*, *Meara stichopi*, *Isodiametra pulchra*, *Dipisthoporus longitubus*, annelid, and hemichordate).

our results show that acoels have a great reduction of the conserved bilaterian peptidergic systems, which are still present in nemertodermatids. This observation is in line with a previous genomic survey that describes the presence of only a subset of conserved bilaterian GPCRs in the acoel *Symsagittifera roscoffensis* (Perea-Atienza et al. 2015). On the other hand, we also observe a gain of novel neuropeptides in the acoel lineages, similar to the nemertodermatid lineages. The expansion of MCPs in both acoelomorph lineages correlates with the formation of nervous system condensations in nemertodermatids and acoels, which could indicate a connection between a gain of morphological nervous system complexity and an expansion of the peptidergic complement. While it is difficult to correlate the origin of the bilaterian peptidergic system with the formation of a CNS, our study suggests that morphological novelties of the nervous system can be accompanied by a change in the peptidergic complement that comprises differential gains and losses. To further investigate this correlation and how it relates to the nervous system function (e.g., which circuits have been lost, changed or gained), more detailed characterizations of neuropeptides in xenacoelomorphs including their localization and function are necessary. By doing so, we would provide a better understanding of the origins of bilaterian nervous system evolution.

Conclusion

This study shows that the major diversification of the peptidergic signaling that is plesiomorphic to deuterostomes and

protostomes occurred after the cnidarian–bilaterian evolutionary split, along the stem leading to Nephrozoa and Xenacoelomorpha (fig. 5). This result, therefore, contradicts previous hypotheses that proposed the correlation of the evolution of the sophisticated bilaterian peptidergic systems with the origin of complex organ systems. The differential gains and losses of neuropeptide systems within xenacoelomorphs, however, do correlate with morphological novelties of their nervous systems, which mirrors the correlation of neuropeptide complement changes during the evolution of complex bilaterian brains (fig. 5). Therefore, our *in silico* analysis provides the basis for future research on neuropeptides within the nervous systems of xenacoelomorphs. This way, one might be able to further test a connection between the ancestral changes in peptidergic signaling and an increase in nervous system complexity.

Materials and Methods

Sequence Data

We investigated assembled transcriptomes from 13 Xenacoelomorpha species deposited into public databases. These species include: *Childia submaculatum* (Acoela), *C. macropyga* (Acoela), *D. gymnopharyngeus* (Acoela), *D. longitubus* (Acoela), *Eumecynostomum macrobursalium* (Acoela), *H. miamia* (Acoela), *I. pulchra* (Acoela), *Ascoparia* sp. (Nemertodermatida), *M. stichopi* (Nemertodermatida), *N. westbladi* (Nemertodermatida), *Sterreria* sp. (Nemertodermatida), *X. bocki* (*Xenoturbella*), *X. profunda* (*Xenoturbella*). Most of the transcriptomes were generated from several whole adults, except for *X. profunda*, and should thus include neuronal expressions (see [supplementary table 4, Supplementary Material](#) online, for more details). We collected neuropeptide precursor and neuropeptide receptor sequences from previous publications (Hauser et al. 2010; Veenstra 2011; Conzelmann et al. 2013; Jékely 2013; Mirabeau and Joly 2013; Adamson et al. 2015; Bauknecht and Jékely 2015; Semmens et al. 2016; Suwansa-Ard et al. 2018) and public databases (i.e., NCBI and UniProt) for further analyses. We aimed for a broad sampling across Bilateria and covered different clades of chordates (i.e., Craniata, Cephalochordata, and Urochordata), ambulacrarians (i.e., Echinodermata and Hemichordata), ecdysozoans (i.e., Nematoda and Arthropoda), and spiralian (i.e., Mollusca and Annelida). To increase the amount of ambulacrarian sequences in our phylogenetic analyses, we included additional echinoderm and hemichordate transcriptomes and genome-derived proteomes. The echinoderm and hemichordate species included *Astrotruma agassizii*, *Labidiaster annulatus*, *Leptosynapta clarki*, *Saccoglossus mereschkowskii*, *Ptychodera flava*, and *Acanthaster planci* (see [supplementary table 5, Supplementary Material](#) online, for more details).

To compare the bilaterian neuropeptide GPCRs with the cnidarian neuropeptide GPCRs, we used a diverse set of receptors that were predicted by the automated eukaryotic NCBI annotation pipeline from the genomes of the anthozoans *Exaiptasia pallida*, *Acropora digitifera* and *Orbicella*

faveolata, as well as receptor sequences of *Nema. vectensis* that were already predicted and published (Ancitil 2009).

Identification and Analysis of Neuropeptide GPCRs

GPCR sequences were clustered using CLANS2 (Frickey and Lupas 2004), and a subset of diverse sequences from each group was used as query sequences in TBlastN searching with an *e*-value cutoff of 1e-30. All resulting sequences from xenacoelomorphs and ambulacrarians were used as new query sequences for an additional search to find potential hidden orthologs (Martin-Duran et al. 2017). We analyzed all retrieved sequences in a cluster analysis using CLANS2 (Frickey and Lupas 2004) with the standard BlastP BLOSUM 62 scoring matrix. Sequences for phylogenetic trees were aligned with ClustalX v2.1 (Larkin et al. 2007), nonconserved regions were automatically removed with TrimAl (Capella-Gutierrez et al. 2009), and phylogenetic trees were generated with RAXML v8.2.9 (Stamatakis 2014) and FastTree v2.1 (Price et al. 2010) using the LG amino acid substitution model. Phylogenetic trees were visualized with FigTree v1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree>; last accessed August 23, 2018).

Identification of Neuropeptide Precursors

Preproneuropeptide sequences of related neuropeptides were compared using CLANS2, and a set of diverse sequences was used as query sequences. The reference sequences were used in TBlastN searching using BLOSUM62 and BLOSUM45 substitution matrices with an *e*-value cutoff of 1. In addition to sequence similarity search, we employed a Perl script that uses PROSITE pattern syntax and flat files to detect specific protein sequence motifs. We screened translated transcriptomes for the presence of recurrent cleavage and amidation sites that are commonly found in multicopy neuropeptide precursor sequences, including x(5, 200)-G-[KR]-[KR]-x(2, 35)-G-[KR]-[KR]-x(2, 35)-G-[KR](0, 1) and x(5, 200)-[KR]-[KR]-x(2, 35)-[KR]-[KR]-x(2, 35)-[KR]-[KR]-x(2, 35)-[KR]-[KR]. For possible RFamides with alternative monobasic cleavage sites, we searched for the following protein sequence motif: x(5, 200)-R-F-G-[KR]-x(2, 35)-R-F-G-[KR]-x(2, 35)-R-F-G-[KR](0, 1). This approach gave us candidate neuropeptides that were further analyzed manually for recurrent peptide sequences between the cleavage sites. All sequences from the motif and similarity search with complete 5'-ends were tested for the presence of a signal peptide with Signal-3L 2.0 (Zhang and Shen 2017) as well as with SignalP 4.1 (Petersen et al. 2011) using a *D*-cutoff value of 0.34. Cleavage sites were predicted at dibasic sites [R/K]-[R/K], and alternative monobasic cleavage sites were predicted with the online application of NeuroPred (<http://stagbeetle.animal.uiuc.edu>; last accessed August 23, 2018) (Southey et al. 2006). A C-terminal glycine residue in the predicted peptides was used as a signal for C-terminal amidation of the prior amino acid residue. Results were manually examined, and the positive hits were used for a reciprocal search in all transcriptomes to identify hidden orthologs, as described by Martin-Duran et al. (2017). Peptide sequence logo representations and precursor diagrams were created with CLC Main Workbench (Qiagen Bioinformatics). All figures were

created using Adobe Illustrator CS6 with the phylogenetic relationships among Xenacoelomorpha based on Cannon et al. (2016) and Jondelius et al. (2011).

Peptide Extraction and Mass Spectrometry (LC-MS/MS)

We extracted peptides from 5 to 10 specimens of whole animals of the acoels *C. macropyga*, *I. pulchra*, and *H. miamia* following the protocol described by Conzelmann et al. (2013). Collected specimens were rinsed with distilled water, transferred into the extraction buffer (90% methanol, 9% acetic acid, and 1% distilled water), grinded with a pestle, and vortexed vigorously. The suspension was centrifuged at maximum speed for 20 min at 4 °C. The supernatant was collected, completely evaporated in a vacuum concentrator, and dissolved in 200 µl of ultrapure water. Neuropeptide mixtures were reduced and alkylated as described by Borchert et al. (2010) and desalted with C₁₈ StageTips (Rappsilber et al. 2007).

LC-MS analysis was performed on an EasyLC nano-UHPLC (Thermo Scientific) coupled with a Q Exactive HF mass spectrometer (Thermo Scientific). Separations of the peptide mixtures were performed as previously described (Conzelmann et al. 2013) with slight modifications. Peptides were eluted with a 57-min segmented gradient of 10–20–50–90% HPLC solvent B (80% acetonitrile in 0.1% formic acid). The mass spectrometer was operated in the positive ion mode. Full scan was acquired in the mass range from *m/z* 300 to 1650 at a resolution of 120,000 followed by Higher energy collisional dissociation (HCD) fragmentation of the seven most intense precursor ions. High-resolution HCD MS/MS spectra were acquired with a resolution of 60,000. The target values for the MS scan and MS/MS fragmentation were 3×10^6 and 10^5 charges with a maximum fill time of 25 and 110 ms, respectively. Precursor ions were excluded from sequencing for 30 s after MS/MS. MS/MS on singly charged precursor ions was enabled. The acquired MS raw files were processed using the MaxQuant software suite v.1.5.2.8 (Cox and Mann 2008). Extracted peak lists were submitted to database search using the Andromeda search engine (Cox et al. 2011) to query target-decoy databases consisting of the predicted propeptides and the predicted active neuropeptides, commonly observed contaminants (285 entries), and the reversed complements of those sequences. Cleavage specificity for N- and C-terminal of arginine and lysine and no enzyme definition were defined. The minimal peptide length was set to four amino acids. The initial precursor mass tolerance was set to 4.5 ppm; for fragment ions, a mass tolerance of 20 ppm was used. Carbamidomethylation of cysteines was defined as fixed modification in the database search. A set of expected variable modifications was defined in the database search: Oxidation of methionine, acetylation of the peptide N-terminus, amidation of the peptide C-terminus, and sulfation of tyrosine. False discovery rates were set to 1% at peptide, modification site, and protein group level, estimated by the target/decoy approach (Elias and Gygi

2007). Spectra of peptides having scores below 100 were validated manually.

Data Accession

Amino acid sequences of xenacoelomorph neuropeptide precursors and neuropeptide GPCRs are available in the [supplementary material, Supplementary Material](#) online. Supporting files that include original alignments, RAXML and FastTree output files, CLANS files, amino acid sequences of GPCRs and precursors, and mass spectrometric data are available on figshare <https://doi.org/10.6084/m9.figshare.c.4064639.v1>; last accessed August 23, 2018 and <https://doi.org/10.6084/m9.figshare.6287759>; last accessed August 23, 2018. The script for the pattern searches is available on https://github.com/faguil/protein-motif-searching_neuropeptides; last accessed August 23, 2018.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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