Large-scale deorphanization of G-protein coupled receptors from *Platynereis dumerilii*

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Abbreviations and Symbols

AADC aromatic amino acid decarboxylase

G5A green fluorescent protein – 5 repeats of linker sequence – aequorin

GDP guanosine diphosphate

GEF guanine nucleotide exchange factor

GFP green fluorescent protein

GTP guanosine triphosphate

GPCR G-protein coupled receptor

TDC tyrosine decarboxylase

TRH thyrotropin-releasing hormone

Zusammenfassung

G-Protein-gekoppelte Rezeptoren (GPCRs) sind ein wichtiger Rezeptor-Typ mit vielen unterschiedlichen Arten von Liganden, zu denen unter anderem Neuropeptide und biogene Amine gehören. Viele GPCR-Familien sind in allen Tieren konserviert, was ihre Bedeutung in der neuronalen und hormonellen Signalweiterleitung unterstreicht. Während die Liganden vieler GPCRs des Menschen und populärer Modellorganismen bekannt sind, ist für die meisten GPCRs anderer Tiere kein Ligand bekannt ("orphan GPCRs") oder der Ligand wurde nur aufgrund von Sequenzähnlichkeit zu bekannten GPCRs vorhergesagt. Besonders innerhalb des wenig erforschten Phylums der Lophotrochozoa wurden bisher nur wenige GPCRs biochemisch untersucht.

In dieser Dissertation stelle ich eine Herangehensweise zur Deorphanisierung von GPCRs im großen Maßstab vor, sowie einen großen Datensatz an deorphanisierten Rezeptoren aus dem Lophotrochohzoen *Platynereis dumerilii* und weiteren marinen Invertebraten. Darunter befinden sich Rezeptoren für Neuropeptide und biogene Amine.

Ich habe 87 GPCRs gegen 126 Neuropeptide getestet und konnte dadurch 19 neuropeptiderge GPCRs deorphanisieren. Darunter befinden sich GPCRs aus bisher noch nicht beschriebenen Familien. Einige davon, nämlich die FMRFamid-Rezeptoren, Achatin-Rezeptoren und Elevenin-Rezeptoren, sind in Bilateriern konserviert. Andere kommen nur in Lophotrochozoen vor. Ich habe außerdem einen Liganden für den Thyreotropin Releasing Hormon-Rezeptor identifiziert.

In einer zweiten Studie habe ich mich auf Rezeptoren von biogenen Aminen konzentriert. Ich habe adrenerge, octopaminerge und tyraminerge Rezeptoren aus *Platynereis* sowie dem Ecdysozoen *Priapulus caudatus* und dem Deuterostomier *Saccoglossus kovalewskii*, einem Hemichordaten, deorphanisiert. Dadurch konnte ich zeigen, dass alle drei Rezeptorfamilien in Bilateriern konserviert sind. Sie sind also viel älter als bisher angenommen wurde. Außerdem beweist dies, dass Octopamin eindeutig nicht das Äquivalent zu Noradrenalin ist, wie dies von Forschern in diesem Feld häufig behauptet worden war.

Zusammenfassend beleuchtet mein Datensatz die Evolution der GPCRs in Bilateriern. Wichtige Aspekte der Evolution von GPCRs, die bisher übersehen worden waren, da nur Stichproben aus wenigen Taxa untersucht wurden, sind

nun aufgeklärt. Außerdem wird dieser Datensatz eine wichtige Ressource für die Deorphanisierung von weiteren GPCRs darstellen.

Summary

G-protein coupled receptors (GPCRs) are an important receptor class that can have various types of ligands, including neuropeptides and biogenic amines. Many GPCR families are conserved throughout animals, highlighting their importance in neuronal and hormonal signaling. While the ligands of many human GPCRs and GPCRs from popular model organisms are known, most of the GPCRs of other animals remain without a known ligand ("orphan GPCRs") or their ligands have only been predicted based on sequence similarity to known GPCRs. Especially within the understudied phylum of the lophotrochozoa, few GPCRs have been biochemically characterized.

In this thesis, I present an approach for the large-scale deorphanization of G-protein coupled receptors and a large dataset of deorphanized receptors from the lophotrochozoan *Platynereis dumerilii* and other marine invertebrates. Among these are neuropeptide receptors and biogenic amine receptors.

By testing 87 GPCRs against 126 neuropeptides, I could deorphanize 19 neuropeptide GPCRs. Among them are GPCRs that belong to hitherto undescribed families. Some of these, namely the FMRFamide receptors, achatin receptors, and elevenin receptors, are conserved across bilateria. Others are restricted to the lophotrochozoa. I also identified a ligand for the *Platynereis* thyrotropin-releasing hormone receptor.

In a second study, I concentrated on biogenic amine receptors. I deorphanized adrenergic, octopaminergic and tyraminergic receptors from *Platynereis* as well as the ecdysozoan *Priapulus caudatus* and the deuterostome *Saccoglossus kovalewskii*, a hemichordate. This way I could show that all three receptor families are conserved across bilaterians. They are therefore much older than was previously appreciated. Also, this is proof that octopamine is clearly not an equivalent of norepinephrine, as was often suggested by scientists in the field.

Taken together, my dataset sheds light on the evolution of GPCRs in bilateral animals. Important aspects of GPCR evolution that had been overlooked because

of limited taxon sampling were cleared up. Also, the dataset presented here will be an important resource for future GPCR deorphanizations.

Publications incorporated into this thesis

P. Bauknecht, G. Jékely, Large-Scale Combinatorial Deorphanization of *Platynereis* Neuropeptide GPCRs. Cell Rep. 12, 684–693 (2015).

P. Bauknecht, G. Jékely, Ancient coexistence of norepinephrine, tyramine, and octopamine signaling in bilaterians. BMC Biology 15:6 (2017).

Contributions to the publications

P. Bauknecht, G. Jékely, Large-Scale Combinatorial Deorphanization of *Platynereis* Neuropeptide GPCRs:
Design of the study was shared with GJ. I performed all experiments and analyzed all data. The manuscript was written by GJ with some contributions by me. I prepared all figures except Figure 1 (shared with GJ) and figures S4 and S6 (by GJ).

P. Bauknecht, G. Jékely, Ancient coexistence of norepinephrine, tyramine, and octopamine signaling in bilaterians:
Design of the study was shared with GJ. I performed all experiments and analyzed all experimental data. Preparation and analysis of the phylogenetic trees and cluster maps was shared with GJ. The manuscript was written by GJ with some contributions by me. I prepared figures 2-4, as well as the additional files 9 and 10. Preparation of all other figures was shared with GJ.

Introduction

G-protein coupled receptors

G-protein coupled receptors (GPCRs) are a large class of receptors with hundreds of members present in most animals (Bradford et al. 2013). They are commonly made up of seven transmembrane helices connected by short loops. Their name derives from the fact that they couple to heterotrimeric G proteins with αβγ subunits (Dohlman et al. 1987). GPCRs can be activated by a wide range of ligands, including biogenic amines, peptides, chemokines, lipids, and light. Usually, the binding of an activating ligand will slightly change the relative position of the transmembrane helices (Manglik and Kobilka 2014; Rasmussen et al. 2011), which turns the GPCR into a guanine nucleotide exchange factor (GEF) for the Gα subunit of the coupled G protein (Gilman 1987), facilitating the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP). The GTP-loaded Gα will then dissociate and elicit further downstream signaling, potentially involving a plethora of second messengers and other factors. These downstream signaling components evolved independently, only later acquiring the need of GPCR activation to function (Bradford et al. 2013).

Generally, GPCRs are classified using the GRAFS system (Fredriksson et al. 2003), in which GRAFS is an acronym for the names of the five main classes Glutamate, Rhodopsin, Adhesion, Frizzled and Secretin. At least the first four classes are ancient to animals (Krishnan et al. 2014).

Interesting insights into GPCR function come from protein crystal structures, which have only recently become available. They show the sequence of conformational changes that a GPCR undergoes upon activation (Choe et al. 2011; Rasmussen et al. 2011).

Other recent developments include the finding that many GPCRs across the animal kingdom dimerize (Kasai and Kusumi 2014; Sakai et al. 2012), which potentially allows for additional means of manipulating GPCR signaling with drugs (Fujita et al. 2014). Another hot topic in GPCR research is functional selectivity (also called biased agonism), the fact that some ligands activate their receptors in a way that primarily triggers one of several potential downstream signaling pathways (Wisler et al. 2014; Nobles et al. 2011).

Deorphanization of G-protein coupled receptors

GPCRs without known ligands are called orphan. Hence, the process of establishing a ligand-receptor relationship is called deorphanization. Historically, bioactive ligands were purified from animal tissue extracts and were used as a starting point to find a receptor that would be activated by them (Caers et al. 2012). This approach is known as forward pharmacology. With the advent of genomics, the approach has changed drastically: cloned GPCRs are now expressed in cell culture, coupled to a readout format like luminescence (Civelli et al. 2013) and synthetic potential ligands are tested, usually one by one. This more modern approach is known as reverse pharmacology (Civelli et al. 2013).

Many human GPCRs, as well as GPCRs from rat and mouse were already deorphanized in the last decades of the 20th century. Also, the GPCR complements of popular models like *Drosophila* (Blenau and Baumann 2001; Caers et al. 2012) and *C. elegans* (Frooninckx et al. 2012) are rather well studied. However, some GPCRs still remain orphan to date, with the human GPCRs drawing most attention because of their potential use as drug targets (Tang et al. 2012).

Beside the pharmacological exploitation of orphan GPCRs, there are also gaps in the knowledge of GPCR evolution, mostly owing to limited taxon sampling. Only few select species have been studied in regard to GPCR deorphanization (Frooninckx et al. 2012; Caers et al. 2012; Civelli et al. 2013), and often even species with annotated genomes have no GPCRs that were biochemically tested for their specific ligands. Specifically, many neuropeptide GPCRs have been identified in vertebrate species like human, rat and mouse (Pawson et al. 2014), as well as *Drosophila melanogaster* (Caers et al. 2012; Hewes and Taghert 2001) and *Caenorhabditis elegans* (Frooninckx et al. 2012), but only very few in nonmodel species (Tensen, Cox, Smit, et al. 1998; Tensen, Cox, Burke, et al. 1998; Cox et al. 1997; Kim et al. 2010; Conzelmann, Williams, Tunaru, et al. 2013; Bigot et al. 2014; Lee et al. 2016; Sekiguchi et al. 2015). This gap in knowledge is what the present thesis is about.

Neuropeptides

Neuropeptides are a diverse class of signaling molecules that can function as neurotransmitters or hormones. They form the largest group of

neurotransmitters (Liu et al. 2008). Generally, peptides are generated from longer precursor molecules, the prepropeptides, which can contain multiple copies of the peptide sequence. The prepropeptides are cleaved at conserved sites that often are made up of two basic amino acids (Hook et al. 2008). Biochemical modifications, like conversion of a C-terminal glycine to an amide group or conversion of an N-terminal glutamate to pyroglutamate, make the peptide mature (Eipper et al. 1992). The mature peptides are stored in dense core vesicles, from which they are secreted upon a signal.

Many peptide families are conserved throughout animals (Mirabeau and Joly 2013; Jékely 2013). Often, however, the relatedness of peptides is unclear, because their short amino acid sequences can quickly mutate beyond recognition (Semmens et al. 2015). In these cases, it is often better to rely on GPCR deorphanization: the peptide ligands and their receptors often coevolve (Kim et al. 2012; Mirabeau and Joly 2013; Jékely 2013). The fact that GPCR sequences are usually better conserved than neuropeptide sequences makes it possible to use the receptor identity to assign a neuropeptide to its family (Semmens et al. 2015).

The monoamines norepinephrine, octopamine and tyramine

Norepinephrine (also called noradrenaline), octopamine and tyramine are signaling molecules that belong to the class of monoamines. They are synthesized from tyrosine by decarboxylation and hydroxylation. Their structures are therefore quite similar. Also, the enzymes that catalyse their synthesis are similar enough to accept slightly different substrates and make both dopamine and tyramine (in the case of aromatic amino acid decarboxylase, (Lindemann and Hoener 2005)) or both octopamine and noradrenaline (in the case of dopamine β-hydroxylase and tyramine β-hydroxylase (Monastirioti et al. 1996; Wallace 1976; Kaufman et al. 1968)).

Noradrenaline signaling is well-studied in vertebrates, where it has roles in autonomic functions like blood pressure and heart beat (Kim et al. 2002), as well as behavioral functions like wakefulness/arousal (Singh et al. 2015) and aggression (Marino et al. 2005). In invertebrates, norepinephrine is usually considered to be absent. Instead, it seems that octopamine has the analogous role

(Roeder 2005), at least in insects (Zhou et al. 2008; Crocker and Sehgal 2008; Florey and Rathmayer 1978; Crisp et al. 2010).

The receptors for norepinephrine, octopamine and tyramine are generally G-protein coupled receptors (Verlinden et al. 2010), with the exception of a tyramine-gated ion channel that has been identified in *C. elegans* (Ringstad et al. 2009; Pirri et al. 2009). For norepinephrine, three major receptor families exist in vertebrates: α1-adrenergic, α2-adrenergic and β-adrenergic receptors. In invertebrates, two classes of octopamine receptors have been found, with one showing a lot of sequence similarity to α-adrenergic receptors, while the other class is more similar to β-adrenergic receptors. Hence, these families are called octopamine-α and octopamine-β receptors, respectively (Verlinden et al. 2010). Tyramine receptors also form two distinct classes in invertebrates, termed tyramine type 1 and type 2 receptors (Verlinden et al. 2010; Cazzamali et al. 2005), which are not to be confused with the more distantly related trace amine associated receptors found in vertebrates (Borowsky et al. 2001; Eyun et al. 2016). All of these receptors show sequence similarity and can be partially cross-activated by their respective ligands (Verlinden et al. 2010), making it sometimes difficult to assign a GPCR to its correct class if not all potential ligands are tested (Arakawa et al. 1990; Saudou et al. 1990).

These manifold similarities (biogenic amine structure and synthesis, receptor structure and function) have led to the notion that norepinephrine signaling in vertebrates and octopamine signaling in invertebrates are equivalent counterparts (Roeder 2005; El-Kholy et al. 2015).

Overview of bilaterian evolution

Animals with a bilateral body plan are considered to be monophyletic, with the so-called urbilaterian as their last common ancestor. About 550 million years ago, the bilaterian lineage is traditionally considered to have split into deuterostomes and protostomes. This view has recently been challenged by the fact that the phyletic placement of chaetognaths and xenacoelomorphs is uncertain (Cannon et al. 2016). Xenacoelomorphs habe been proposed to be either part of the deuterostomes or a sister group to all other bilaterians (Bourlat et al. 2006; Philippe et al. 2011; Cannon et al. 2016). Here, the classical protostome-

deuterostome categories will be kept for simplicity. From the protostome-deuterostome split, several deuterostomian lineages have emerged, with chordates being the most well-studied one, since they include the vertebrates. Other deuterostomian lineages, like the ambulacrarians, including the hemichordates and echinoderms, have received less attention. In this thesis, the hemichordate *Saccoglossus kovalewskii* will be of importance.

Among the protostomian phyla are the ecdysozoans and lophotrochozoans. Ecdysozoans comprise, among others, the insects, with *Drosophila melanogaster* as one of the most popular model organisms, and the nematodes, among which *Caenorhabditis elegans* has been studied in detail. Among the lophotrochozoa, which include mollusks and annelids, not many species have been studied, leading to an undersampling of this superphylum. The marine annelid *Platynereis dumerilii* is therefore getting the most attention in this study.

The marine ragworm *Platynereis dumerilii*

The annelid *Platynereis dumerilii* is a marine ragworm with a pelagic-benthic biphasic life cycle (Fischer et al. 2010). It is slowly evolving, and therefore has an ancient genomic complement (Raible et al. 2005). This puts *Platynereis* in contrast to popular protostomian models like *Drosophila* or *C. elegans*, which both have a shorter generation time and a faster mutation rate. The fact that *Platynereis* can be bred in the lab with freshly hatched larvae available all year round makes it a powerful emerging lab model, with the possibility of behavioral studies (Conzelmann et al. 2011), pharmacological interference (Tosches et al. 2014), the use of genetics tools (Zantke et al. 2014; Gühmann et al. 2015) and the study of neuroanatomy (Asadulina et al. 2012) and connectomics (Randel et al. 2014; Shahidi et al. 2015).

The neuropeptide complement of *Platynereis* has been studied by a combination of mass-spectrometric and bioinformatics approaches (Conzelmann, Williams, Krug, et al. 2013). However, a receptor was found only for one of its neuropeptides (Conzelmann, Williams, Tunaru, et al. 2013).

Other species used in this study

Saccoglossus kovalewskii

The acorn worm *Saccoglossus kovalewskii* is an invertebrate belonging to the hemichordates, a subphylum of the deuterostomes. It lives in U-shaped burrows in the seabed, where it filter feeds. Hemichordates are closely related to echinoderms (Cannon et al. 2014) with which they form the ambulacrarians. The genome of *Saccoglossus* has been sequenced (Simakov et al. 2015) and serves as a reference of deuterostome development before the split of the chordates about 535 million years ago. Its repertoire of GPCRs is very similar to that of vertebrates (Krishnan et al. 2013).

Priapulus caudatus

The penis worm, *Priapulus caudatus*, is an early-branching Ecdysozoan and therefore belongs to the protostomes (Dunn et al. 2008) despite its deuterostome-like development (Martín-Durán et al. 2012). Archaeal priapulids have been identified in the Burgess Shale, showing their presence in the Middle Cambrian about 505 million years ago (Vannier et al. 2010). They live on the sea floor where they dig into the sediment so that the mouth just stays at the surface. There they feed on slow-mowing invertebrates like polychaetes. Because of its ancient features (Martín-Durán et al. 2012; Webster et al. 2006), *Priapulus caudatus* here serves as a reference of early ecdysozoa.

Branchiostoma floridae

The lancelet *Branchiostoma floridae* is a fish-like marine chordate. It belongs to the cephalochordates, the most basal subphylum of the chordates (Gee 2008). The genome of *Branchiostome floridae* has been sequenced (Putnam et al. 2008), making it an important model of vertebrate ancestors (Holland et al. 2004; Garcia-Fernàndez and Benito-Gutiérrez 2009).

Aplysia californica

The sea hare *Aplysia californica* is a giant sea snail that can become up to 75 cm long. Belonging to the mollusks, it is a representative of the lophotrochozoa. It is one of the few lophotrochozoan species that have served as a model in biology.

Because of its easily identifiable giant neurons, it has been popular for studies in neurobiology (Kandel et al. 2014). Its genome has been sequenced.

Objectives of this thesis

Many G-protein coupled receptors from model species are deorphanized and described. However, to clarify the evolutionary history of many GPCR families, data from non-model species is lacking. Limited taxon sampling, especially in the superphylum of the lophotrochozoa, leads to an undersampling of GPCRs. Even more importantly, some GPCR families only present in invertebrate animals have not been described yet at all.

The goal of this thesis is the deorphanization of GPCRs from the marine annelid *Platynereis dumerilii*. GPCR deorphanization is necessary to unequivocally establish the nature of a given GPCR, because bioinformatic methods, especially the automated annotation of genomes, are not very reliable in doing so.

Obtaining a large dataset of deorphanized *Platynereis* GPCRs is useful for the confirmation of the origins of ancient GPCR families, providing support for their conservation across large evolutionary distances. These GPCRs will also aide in the understanding of neuropeptide evolution, since the sequence conservation of peptides is often limited and the establishment of neuropeptide homology across large evolutionary distances needs additional support from coevolved receptor sequences. Furthermore, a large-scale approach in deorphanization will help to discover previously undescribed GPCR families, thus helping to drive our knowledge of GPCR families toward completion.

A rich dataset of deorphanized GPCRs can also be used to test hypothesis about peptide-receptor coevolution and generate more such hypotheses.

In the long term, ligand-receptor pairs will be useful to complement neuroanatomical data from connectomics studies, when molecular identities can be assigned to individual neurons and synapses.

The GPCRs from this dataset might also serve as targets to study neurotransmitter function, for example by knockout, knockdown, or pharmacological interference with the receptor.

A specific goal of this thesis is also to clear up the evolutionary history of adrenergic, tyraminergic and octopaminergic receptors. To this end, additional species were sampled for these receptors.

Results

Summary of publication 1

Introduction

Neuropeptides commonly signal via G-protein coupled receptors. Neuropeptide GPCRs have been deorphanized in vertebrate models (Civelli et al. 2013; Pawson et al. 2014), as well as *Drosophila melanogaster* (Caers et al. 2012; Hewes and Taghert 2001) and *Caenorhabditis elegans* (Frooninckx et al. 2012), but only very few other invertebrates. To remedy the lack of deorphanized neuropeptide GPCRs especially in the lophotrochozoan phylum, I undertook the effort of a large-scale deorphanization of *Platynereis* GPCRs.

Results

I identified putative *Platynereis* GPCR sequences in a mixed stages transcriptome (Conzelmann, Williams, Krug, et al. 2013). I selected GPCRs that were predicted to be neuropeptidergic by the best BLAST result in SwissProt and subcloned them into the mammalian expression vector pcDNA3.1 to express them in CHO-G5A cells (Tunaru et al. 2005). This cell line features a reporter construct called G5A (for GFP, 5 repeats of linker sequence, aequorin (Baubet et al. 2000)) that allowed me to test for GPCR activation by recording calcium-induced luminescence. To do this, I cotransfected the promiscuous Gα protein Gα-16 (Offermanns and Simon 1995) that couples many GPCRs to the phospholipase C pathway that eventually leads to calcium release.

To identify many new GPCR-neuropeptide ligand pairs in a short time, I developed a combinatorial screening strategy in which I tested 87 GPCRs against 126 synthetic neuropeptides (10,962 combinations). Synthetic *Platynereis* neuropeptides were obtained as they were predicted in (Conzelmann, Williams, Krug, et al. 2013). To save time, I applied three complex peptide mixtures to the GPCR-expressing cells as a first screening step. Each mixture contained up to 52 peptides. Only a subset of the GPCRs was activated by each mixture, which indicated that these signals were the result of specific GPCR activation.

The GPCRs that were activated by these mixtures were then further tested in a second step with submixtures that were designed by using the rows and columns of a matrix containing all peptides from the original mixture. If two submixtures

representing one row and one column activated a GPCR, I considered the peptide at the intersection as the top candidate to be a specific ligand.

For some GPCRs, it was also possible to predict the ligand based on sequence similarity to deorphanized GPCRs from other species. In the third experimental step, I tested the predictions from the submixture experiments as well as from sequence similarity comparisons. For this, I used single synthetic peptides at a fixed concentration of 1 μM to try to activate a GPCR.

The fourth step was to validate the identified ligands from the fixed-concentration experiments. To do this, I recorded dose-response curves for all receptor-ligand pairs. This way, I was able to confirm 25 receptor-ligand pairs for 19 GPCRs. I found the following receptors: an FLamide receptor (which was also activated by prokineticin short peptide 1), two allatotropin receptors, a neuropeptide KY receptor (activated by both neuropeptide KY versions and cross-activated by FMRFamide), a luqin receptor, two elevenin receptors, an RGWamide receptor, an FMRFamide receptor, an excitatory peptide receptor, a neuropeptide-Y-4 receptor (also activated by neuropeptide-Y-1 and 3), an EFLGamide receptor, an allatostatin-A receptor (which was only activated by a specific copy of allatostatin-A, called allatostatin-A-2-2), an achatin receptor, a myomodulin receptor (activated by at least two different myomodulins), two diuretic hormone 31 receptors, a vasotocin receptor, and an allatostatin-C receptor.

To learn more about the evolution of these 19 GPCRs, I analyzed metazoan neuropeptide GPCR sequences by clustering. I collected sequences of deorphanized GPCRs from the literature (Conzelmann, Williams, Tunaru, et al. 2013; Caers et al. 2012; Frooninckx et al. 2012; Pawson et al. 2014; Bigot et al. 2014; Cox et al. 1997; Tensen, Cox, Burke, et al. 1998; Kim et al. 2010) and used the sequences of my 19 newly deorphanized GPCRs to seed BLASTP searches in metazoan genomes. All collected sequences were used for similarity-based clustering with the program CLANS2 (Frickey and Lupas 2004). This method had previously been used to recover orthologous groups of GPCRs (Jékely 2013). I could assign all 19 *Platynereis* GPCRs to well-resolved sequence groups.

Eleven of the *Platynereis* GPCRs clustered to groups that contained characterized sequences from other animal models. For eight of these, their ligand had been previously recognized as an ortholog of other known peptides: this was the case

for the allatostatins A and C, vasotocin (an ortholog of both vasopressin and oxytocin), allatotropin (an orexin ortholog), luqin (orthologous to insect RYamide), and diuretic hormone 31 (an ortholog of calcitonin).

In some cases, the clustering confirmed hypotheses of orthology among receptor-ligand pairs that had previously been suggested (Jékely 2013): the myomodulin receptor clustered with arthropod myosuppressin receptors, confirming the orthology of myomodulins and myosuppressin. Also, a suggested relationship of excitatory peptide with CCHamide and neuromedin-B peptides (Roller et al. 2008; Jékely 2013) was confirmed this way. Similarly, neuropeptide KY receptors clustered with neuropeptide Y and neuropeptide F receptors, confirming that the sequence similarity of the respective peptides is a sign of their homology.

I also found several new receptor families that had not been described previously. In these cases, the *Platynereis* GPCR was the only deorphanized member of a cluster. These families are the FMRFamide receptors, elevenin receptors, achatin receptors, RGWamide receptors, FLamide receptors (surprisingly also containing a receptor for diuretic hormone 31), and the neuropeptide-Y-4 receptors.

Orthologous sequences from different phyla indicate that three of these receptors were present in the urbilaterian, namely the FMRFamide, elevenin, and achatin receptors. Sequences from the deuterostomes *Saccoglossus kovalewskii* and *Branchiostoma floridae* cluster with the respective *Platynereis* sequences. Interestingly, for the FMRFamide receptor there is even an ortholog in the cartilaginous fish *Callorhinchus milii*, which has a particularly slowly evolving genome (Venkatesh et al. 2014). Notably, the FMRFamide receptors cluster does not contain a previously described FMRFamide receptor from *Drosophila* (Cazzamali and Grimmelikhuijzen 2002), which instead forms a distinct cluster with other arthropod sequences.

The clusters for RGWamide receptor, FLamide receptor/diuretic hormone 31 receptor 2, and neuropeptide-Y-4 receptor only contained lophotrochozoan sequences, indicating that these families developed after the split of ecdysozoa and lophotrochozoa.

One of the *Platynereis* GPCRs, the EFLGamide receptor, clustered with vertebrate thyrotropin-releasing hormone (TRH) receptors. Previously, no ligand for invertebrate TRH receptors had been known. This was due to the fact that TRH is

a very short peptide (pyroGlu-His-Pro-NH_2) whose primary sequence is not well conserved across the superphyla. Only the combination of GPCR deorphanization and clustering analysis revealed the orthology of *Platynereis* EFLGamide and vertebrate TRH. A neuropeptide that shows limited sequence similarity with both vertebrate TRH and *Platynereis* EFLGamide has been identified in the sea urchin *Strongylocentrotus purpuratus* (Rowe and Elphick 2012), which further argues for the orthology of these peptides.

I found that achatin, a peptide containing a D-amino acid (Gly-**D-Phe**-Gly-Asp) (Kamatani et al. 1989), but not its all-L-counterpart, activates the *Platynereis* achatin receptor. To test if this mode of signaling is conserved, I examined the GPCR sequences in the achatin receptor cluster. This cluster contained lophotrochozoan and deuterostome sequences, but no sequences from ecdysozoa. To test conservation of achatin signaling across a large evolutionary distance, I chose GPCRs from *Aplysia californica*, *Saccoglossus kovalevskii* and *Branchiostoma floridae* for additional tests. I expressed these GPCRs from synthetic DNA and tested the D- and L-versions of the achatin peptides found in each organism. Only D-amino acid containing achatins activated their receptors. Thus, I could show that achatin receptors are activated by D-peptides and this was already true for the urbilaterian.

Discussion

In this publication, I presented the deorphanization of 19 *Platynereis* neuropeptide GPCRs. A combinatorial screening strategy enabled me to quickly test 126 neuropeptides on 87 GPCRs, which makes a total of 10,962 possible combinations. Only few receptors got activated by the application of complex peptide mixtures, which highlights the specificity of their activation. Using combinations of smaller mixtures, I could readily identify specific GPCR ligands. By this unbiased approach, I was able to identify new GPCR families whose ligands could not have been predicted based on available data.

Among the newly discovered families are the FMRFamide receptors. FMRFamides form one of the oldest and most conserved neuropeptide families (Jékely 2013; Mirabeau and Joly 2013), but the FMRFamide receptors have long remained elusive. An FMRFamide receptor was identified in *Drosophila* (Cazzamali and Grimmelikhuijzen 2002), but it only has homologs in arthropods. The newly

discovered *Platynereis* FMRFamide receptor has different orthologous animal GPCRs, mainly from invertebrate deuterostomes and lophotrochozoans. I also identified an orthologous receptor in the hemipteran brown planthopper *Nilaparvata lugens*, which shows that this type of FMRFamide receptor was retained for a while in ecdysozoans before it was lost and replaced with a different receptor.

Another discovery that was only possible by unbiased combinatorial testing of ligands was that *Platynereis* EFLGamide is the ligand of *Platynereis* TRH receptor. An interesting question is whether the biological functions of EFLGamide resemble those of vertebrate TRH (Laudet 2011), particularly the regulation of thyroid hormone synthesis. Thyroid hormones are present in *Aplysia* (Heyland et al. 2006) and could therefore be an ancient bilaterian feature.

The identification of new GPCR families makes the prediction of some new GPCR-ligand pairs easier. To demonstrate this, I tested putative achatin receptors from *Aplysia californica*, *Saccoglossus kovalevskii* and *Branchiostoma floridae* based on my predictions by sequence clustering. These receptors could not have been predicted correctly before, as they had been annotated as crustacean cardioactive peptide receptors by automatic genome annotation tools. I confirmed their identity as achatin receptors and thereby showed that the deorphanized *Platynereis* GPCRs can be used to predict further neuropeptide GPCRs across phyla.

In general, my results add to our current knowledge of deorphanized GPCRs by exploring lophotrochozoan receptors. Only very few of these had been biochemically characterized so far. The new data will be a valuable resource for future GPCR and ligand discoveries and the study of neuropeptide signaling in general, especially in invertebrates. By demonstrating the broad phylectic distribution of some conserved neuropeptides, it also highlights the complexity of the neuropeptide complement of the urbilaterian.

Summary of publication 2

Introduction

Norepinephrine is an important monoamine that controls autonomic responses in vertebrates via its GPCRs, the adrenergic receptors (Kim et al. 2002).

Norepinephrine signaling has traditionally been considered absent from invertebrates. Octopamine, a monoamine that shows many similarities with noradrenaline, including similar receptors, is a well-described transmitter found in insects and other invertebrates (Roeder 2005). It is often considered the invertebrate equivalent of noradrenaline. The precursor of octopamine, called tyramine, also has functions as a neurotransmitter, working via its own dedicated receptors (reviewed in (Lange 2009)).

In this study, I show that adrenergic, octopaminergic and tyraminergic signaling are much older and more widespread than previously thought by demonstrating the coexistence of dedicated receptors in the protostomes *Platynereis dumerilii* and *Priapulus caudatus* as well as in the deuterostome *Saccoglossus kovalewskii*.

Results

I analyzed monoamine receptor sequences from bilateral animals by clustering with CLANS2 (Frickey and Lupas 2004). Doing this, I found GPCRs from lophotrochozoa and ecdysozoa that clustered with α1-adrenergic and α2-adrenergic receptors, among them sequences from *Platynereis dumerilii* and *Priapulus caudatus*. A cluster of β-adrenergic receptors only contained sequences from deuterostomes and xenacoelomorphs. I also found sequences from the deuterostome *Saccoglossus kovalewskii* that clustered with octopamine and tyramine receptors.

To confirm the phylogenetic placement of these GPCRs, I constructed a maximum likelihood tree based on multiple alignments of a representative collection of GPCR sequences, involving many deorphanized octopamine and tyramine receptors. I could resolve the following well supported clades: Tyramine class I and II receptors, octopamine-α and -β receptors, α1-adrenergic receptors, α2-adrenergic receptors and β-adrenergic receptors. Serotonin receptors served as an outgroup. All clades except the β-adrenergic receptors contained sequences from *Platynereis, Priapulus* and *Saccoglossus*. Representatives of α1- and α2-adrenergic receptor orthologs were also present in other ecdysozoans and lophotrochozoans, including *Daphnia pulex* and *Aplysia californica*. A receptor from the moth *Chilo suppressalis* that had previously been described as a new type

of octopamine receptor (Wu et al. 2014) clustered with α2-adrenergic receptors. This cluster also contained some nematode sequences.
Regarding β-adrenergic receptors, the cluster only contained sequences from vertebrates, cyclostomes, tunicates, and the xenacoelomorph *Meara stichopi*. If the xenacoelomorpha are indeed a sister group to all other bilaterians, this would mean that the β-adrenergic receptors are an ancient bilaterian feature that was lost early in the protostomian lineage.

To confirm the identity of some of the monoamine receptors from different phyla, I cloned selected GPCRs from *Platynereis, Priapulus* and *Saccoglossus*. Since monoamine receptors tend to be cross-reactive, which can lead to misidentifications (Saudou et al. 1990; Arakawa et al. 1990), I figured that only the recording of dose-response curves for all relevant monoamines on each receptor could be a fail-safe way to characterize them biochemically. For this, I used the calcium mobilization assay from my previous publication.
I tested representatives of putative α1- and α2-adrenergic receptors from all three species. In my assays, all of these were activated by norepinephrine in small concentrations. Most of them were also activated by epinephrine at similarly small concentrations, with the exception of a *Priapulus* α1-adrenergic receptor on which epinephrine was inactive. The agonist clonidine activated all α2-adrenergic receptors. In contrast, octopamine, tyramine and dopamine were either inactive on the candidate adrenergic receptors or only activated them at higher concentrations. These results confirm the tested receptors as *bona fide* adrenergic receptors. Adrenergic signaling is therefore probably present in protostomes and not confined to deuterostomes as previously thought.
I also tested some putative octopamine and tyramine receptors. Since octopamine and tyramine signaling is already well-described in ecdysozoa, especially *C. elegans* (Rex and Komuniecki 2002; Alkema et al. 2005; Pirri et al. 2009) and *Drosophila* (Saraswati et al. 2004; Nagaya et al. 2002; Saudou et al. 1990) , I did not clone the *Priapulus* octopamine and tyramine receptors. Instead, I focused on receptors from *Platynereis*, because no tyramine receptors had been described in lophotrochozoans yet, and from *Saccoglossus*, for neither octopamine nor tyramine signaling had so far been described in any deuterostome. Two

Platynereis receptors were activated by tyramine at about 100-fold smaller concentrations than by octopamine or other monoamines. In contrast, a third *Platynereis* receptor was more readily activated by octopamine. This confirms that *Platynereis* has at least one receptor each of the tyramine type 1, tyramine type 2 and octopamine-α families. A putative octopamine-β receptor was not active in my assays. These results show that adrenergic signaling coexists with octopaminergic and tyraminergic signaling in *Platynereis* and probably also in *Priapulus*. These monoamine signaling systems can therefore not be functionally equivalent counterparts or even homologs, as had previously been assumed (Roeder 2005; Schwaerzel et al. 2003; Nall and Sehgal 2014; Shakiryanova et al. 2011; El-Kholy et al. 2015).

Of the *Saccoglossus* receptors tested, three were preferentially activated by tyramine and two by octopamine. Other substances tested only activated the receptors at higher concentrations or failed to activate them altogether. Thus, I could confirm an octopamine-α and an octopamine-β receptor from *Saccoglossus*, as well as two tyramine type 2 receptors and a tyramine type 1 receptor. Another putative tyramine type 1 receptor is present in the phylogeny, but I was not able to clone it.

A hallmark of tyramine and octopamine signaling, apart from the presence of dedicated receptors, is the presence of the enzyme tyrosine decarboxylase (TDC), which decarboxylates tyrosine to yield tyramine. In contrast to other aromatic amino acid decarboxylases (AADCs), this enzyme is specifically used for the production of tyramine and octopamine in neuronal signaling (Alkema et al. 2005). By constructing a maximum likelihood tree, I could show that TDC is not only present in *Platynereis* and *Priapulus*, but also in *Saccoglossus* and can be distinguished from other, more general purpose AADCs. These results confirm that tyramine and octopamine signaling are not an exclusively protostomian feature, but also exist in the deuterostome *Saccoglossus*.

To further characterize the cloned receptors pharmacologically, I tested the inhibitors yohimbine and mianserin on them. Both substances are known to inhibit α-adrenergic receptors, but are not specific to them. I found that many of the cloned receptors were inhibited by yohimbine and/or mianserin to some

extent. The lack of specificity presents a *caveat* regarding the use of these two substances to study the neurobiology of invertebrates.

Discussion

To unequivocally establish a substance as a neurotransmitter, a lot of criteria have to be met (Lange 2009): it must be present in a signaling cell, released upon stimulation of that cell, removed from the extracellular space by reuptake or degradation, specific receptors have to be present on a target cell, application of the substance to the target cell has to mimic the effect of signaling from the signaling cell, and inhibition of the receptor must cancel the activity of the substance. Showing that all of these criteria are met for norepinephrine, octopamine and tyramine in the three representative species *Platynereis*, *Priapulus* and *Saccoglossus* would by far go beyond the scope of this thesis. Instead, I reasoned that the presence of specific monoamine receptors is a good indicator for the use of this monoamine in neuronal signaling. In contrast, monoamines for which no specific receptors are present might still occur in an animal in trace amounts without being used in signaling. Also, the presence or absence of biosynthetic enzymes might be misleading, as some of these enzymes are not very substrate specific (Monastirioti et al. 1996; Wallace 1976; Kaufman et al. 1968; Lindemann and Hoener 2005) and might be used by different organisms in different ways.

I identified and biochemically characterized two families of adrenergic receptors in the lophotrochozoan *Platynereis* and the ecdysozoan *Priapulus* as well as in the hemichordate *Saccoglossus*. Furthermore, I showed that two families each of tyraminergic and octopaminergic receptors coexist with the adrenergic receptors in these animals. The presence of specific receptors across the three animal phyla shows the ancient bilaterian origin of adrenergic, octopaminergic and tyraminergic signaling. These signaling systems are therefore much older and more widespread than was previously acknowledged. Also, they cannot be functionally equivalent (although some degree of functional redundancy cannot be excluded). This misconception came about because tyramine and octopamine receptors were lost from vertebrates, and adrenergic receptors were lost from many ecdysozoans, including the popular model species *Drosophila* and *C. elegans*.

An α2-adrenergic receptor from *Chilo suppresalis* has already been described (Wu et al. 2014), but misidentified as a novel type of octopamine receptor. This highlights the danger of misidentifying receptors because of their cross-reactivity to related monoamines (Arakawa et al. 1990; Saudou et al. 1990). I circumvented this by testing several monoamines on each receptor, including norepinephrine, octopamine, tyramine and dopamine.

In my study, I could not identify any tyramine or octopamine receptors in chordates. In contrast, β-adrenergic receptors seem to be present in chordates and xenacoelomorphs only. They might have taken over functions previously occupied by tyramine or octopamine receptors.

What the ancestral functions of adrenergic, tyraminergic and octopaminergic signaling systems were remains unclear. To gain insights into this, their function must be studied in organisms in which all three signaling systems still coexist. We can, however, rule out that they have equivalent functions, as was assumed so far. Studies comparing octopamine signaling in invertebrates with norepinephrine signaling in vertebrates will have to be interpreted carefully against this background.

General Discussion

In this study, I deorphanized a total of 24 *Platynereis* GPCRs and 12 GPCRs from other marine invertebrates. These proved very informative regarding the evolution of GPCRs and their ligands. Several of my findings, like the identification of novel neuropeptide GPCR families and the identification of EFLGamide as a ligand for *Platynereis* TRH receptor, highlight the importance to characterize GPCRs biochemically. *In silico* analysis of these GPCRs by multiple sequence alignments could not have led to these results. Apart from that, the monoamine receptors from *Saccoglossus* and *Priapulus* that I analyzed had been correctly labeled by automatic genome annotation, but had been overlooked so far. My experiments confirmed the identities of these receptors.

The fact that I discovered GPCR families in some phyla where they were not expected shows that these families are older and more widespread than previously thought. This study adds to taxon sampling mainly by presenting data

from *Platynereis*, a member of the understudied lophotrochozoan phylum. But also other marine invertebrates proved to have interesting GPCR complements, as exemplified by *Saccoglossus kovalewskii, Branchiostoma floridae, Priapulus caudatus,* and *Aplysia californica.* This highlights the usefulness of studying slowly evolving marine species, since their genomes retained genes of ancient ancestry.

In my neuropeptide GPCR study, I was able to show that the D-amino acid in the neuropeptide achatin is conserved. This raises the question of the function of this D-amino acid, because it is costly for an animal to make. The achatin precursor is first produced as a standard, all L-amino acid containing prepropeptide from a regular mRNA transcript (Conzelmann, Williams, Krug, et al. 2013) and cleaved into small propeptides (Bai et al. 2013). Then, presumably, a dedicated L-D-convertase enzyme converts the phenylalanine at position 2 of the peptide to the D-form (Jilek et al. 2005). The production of this dedicated enzyme is costly for the organism, therefore the D-amino acid probably has an important role. What this role might be is, however, unclear. It could make the achatin peptide more stable than other peptides, because peptidases are usually specific to L-amino acid containing peptides (Kreil 1997). Another possibility is that the unique three-dimensional structure that is caused be D-amino acid (Ishida et al. 1992) is needed for receptor activation and that there is no easy way to evolve a different receptor specificity.

The dataset of deorphanized GPCRs I have created is a valuable resource for further neuropeptide and GPCR research. Based on my data, putative achatin receptors were identified in two chelicerates: the scorpion *Mesobuthus martensii* and the African social velvet spider *Stegodyphus mimosarum* (Veenstra 2016).

Orthologs of the elevenin receptors that I identified are present in arthropods, including insects (Veenstra 2014). The distribution of these receptors matches the distribution of the elevenin peptides well.

FMRFamides are probably orthologs of the FIRFamide peptides that occur in chelicerates, who are early-branching ecdysozoans. An interesting feature of some chelicerates is that they have both "*Platynereis*-type" and "*Drosophila*-type" FMRFamide receptors (Veenstra 2016). It will be interesting to find out what these receptors do, which might hint at why the "*Platynereis*-type" receptors were lost from most hexapods.

I identified EFLGamide as the probable ortholog to vertebrate TRH via the orthology of their receptors. A peptide with intermediate sequence characteristics was known from the sea urchin *Strongylocentrotus purpuratus* (Rowe and Elphick 2012). A similar TRH-type peptide sequence was since then found in the transcriptome of the starfish *Asterias rubens* with the predicted structure pQWYT-NH2 (Semmens et al. 2016), providing another example of the sequence variation of TRH-type peptides.

It is hypothesized that the arthropod EFLamide genes might be orthologs to *Platynereis* EFLGamide (Veenstra 2016). It is, however, notable that EFLamide seems to be absent from insects, although a TRH receptor is present in a few of them, namely *Nilaparvata lugens*, *Rhodnius prolixus* and *Diaphorina citri.* It will be interesting to find out what the TRH receptor ligand is in these species.

Recently, siGOLD, an immunogold labeling technique to identify peptidergic neurons in transmission electron microscopy, was developed (Shahidi et al. 2015). The combination of my GPCR dataset with mapping of peptidergic neurons in electron microscopy makes it possible to study neuropeptide signaling at cellular level. Since neuropeptide signaling can happen without synapses over large distances, it represents an alternative mode of signaling connectivity between neurons. In this case, the target cells are defined by their receptor expression profile rather than their synaptic connections. Using the information about neurotransmitter-receptor pairs from my present work, together with expression data and anatomical information at the single-neuron level, it is possible to define chemical neuronal signaling networks with sparse and specific connectivity.

Conclusion

Here I present a large dataset of deorphanized GPCRs from *Platynereis dumerilii* and other marine invertebrates. This data is an important resource that reduces gaps in GPCR sampling. By identifying many new receptors, I contribute to our understanding of the evolution of neuropeptide receptors and monoamine receptors. Highlights of my work are the evidence that many receptor families are more broadly conserved across phyla and of more ancient origin than was appreciated before, as well as the discovery of hitherto undescribed receptor families.

References

Alkema, M.J., Hunter-Ensor, M., Ringstad, N. and Horvitz, H.R. 2005. Tyramine Functions independently of octopamine in the Caenorhabditis elegans nervous system. *Neuron* 46(2), pp. 247–260.

Arakawa, S., Gocayne, J.D., McCombie, W.R., Urquhart, D.A., Hall, L.M., Fraser, C.M. and Venter, J.C. 1990. Cloning, localization, and permanent expression of a Drosophila octopamine receptor. *Neuron* 4(3), pp. 343–354.

Asadulina, A., Panzera, A., Verasztó, C., Liebig, C. and Jékely, G. 2012. Whole-body gene expression pattern registration in Platynereis larvae. *EvoDevo* 3(1), p. 27.

Bai, L., Livnat, I., Romanova, E.V., Alexeeva, V., Yau, P.M., Vilim, F.S., Weiss, K.R., Jing, J. and Sweedler, J.V. 2013. Characterization of GdFFD, a D-Amino Acid-Containing Neuropeptide that Functions as an Extrinsic Modulator of the Aplysia Feeding Circuit. *The Journal of Biological Chemistry*.

Baubet, V., Le Mouellic, H., Campbell, A.K., Lucas-Meunier, E., Fossier, P. and Brúlet, P. 2000. Chimeric green fluorescent protein-aequorin as bioluminescent Ca2+ reporters at the single-cell level. *Proceedings of the National Academy of Sciences of the United States of America* 97(13), pp. 7260–7265.

Bigot, L., Beets, I., Dubos, M.P., Boudry, P., Schoofs, L. and Favrel, P. 2014. Functional characterization of a short neuropeptide F-related receptor in a lophotrochozoan, the mollusk Crassostrea gigas. *The Journal of Experimental Biology* 217(Pt 16), pp. 2974–2982.

Blenau, W. and Baumann, A. 2001. Molecular and pharmacological properties of insect biogenic amine receptors: lessons from Drosophila melanogaster and Apis mellifera. *Archives of Insect Biochemistry and Physiology* 48(1), pp. 13–38.

Borowsky, B., Adham, N., Jones, K.A., Raddatz, R., Artymyshyn, R., Ogozalek, K.L., Durkin, M.M., Lakhlani, P.P., Bonini, J.A., Pathirana, S., Boyle, N., Pu, X., Kouranova, E., Lichtblau, H., Ochoa, F.Y., Branchek, T.A. and Gerald, C. 2001. Trace amines: identification of a family of mammalian G protein-coupled receptors. *Proceedings of the National Academy of Sciences of the United States of America* 98(16), pp. 8966–8971.

Bourlat, S.J., Juliusdottir, T., Lowe, C.J., Freeman, R., Aronowicz, J., Kirschner, M., Lander, E.S., Thorndyke, M., Nakano, H., Kohn, A.B., Heyland, A., Moroz, L.L., Copley, R.R. and Telford, M.J. 2006. Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida. *Nature* 444(7115), pp. 85–88.

Bradford, W., Buckholz, A., Morton, J., Price, C., Jones, A.M. and Urano, D. 2013. Eukaryotic G protein signaling evolved to require G protein-coupled receptors for activation. *Science Signaling* 6(276), p. ra37.

Caers, J., Verlinden, H., Zels, S., Vandersmissen, H.P., Vuerinckx, K. and Schoofs, L. 2012. More than two decades of research on insect neuropeptide GPCRs: an overview. *Frontiers in endocrinology* 3, p. 151.

Cannon, J.T., Kocot, K.M., Waits, D.S., Weese, D.A., Swalla, B.J., Santos, S.R. and Halanych, K.M. 2014. Phylogenomic resolution of the hemichordate and echinoderm clade. *Current Biology* 24(23), pp. 2827–2832.

Cannon, J.T., Vellutini, B.C., Smith, J., Ronquist, F., Jondelius, U. and Hejnol, A. 2016. Xenacoelomorpha is the sister group to Nephrozoa. *Nature* 530(7588), pp. 89–93.

Cazzamali, G. and Grimmelikhuijzen, C.J. 2002. Molecular cloning and functional expression of the first insect FMRFamide receptor. *Proceedings of the National Academy of Sciences of the United States of America* 99(19), pp. 12073–12078.

Cazzamali, G., Klaerke, D.A. and Grimmelikhuijzen, C.J. 2005. A new family of insect tyramine receptors. *Biochemical and Biophysical Research Communications* 338(2), pp. 1189–1196.

Choe, H.W., Kim, Y.J., Park, J.H., Morizumi, T., Pai, E.F., Krauss, N., Hofmann, K.P., Scheerer, P. and Ernst, O.P. 2011. Crystal structure of metarhodopsin II. *Nature* 471(7340), pp. 651–655.

Civelli, O., Reinscheid, R.K., Zhang, Y., Wang, Z., Fredriksson, R. and Schiöth, H.B. 2013. G protein-coupled receptor deorphanizations. *Annual Review of Pharmacology and Toxicology* 53, pp. 127–146.

Conzelmann, M., Offenburger, S.L., Asadulina, A., Keller, T., Münch, T.A. and Jékely, G. 2011. Neuropeptides regulate swimming depth of Platynereis larvae. *Proceedings of the National Academy of Sciences of the United States of America* 108(46), pp. E1174–E1183.

Conzelmann, M., Williams, E.A., Krug, K., Franz-Wachtel, M., Macek, B. and Jékely, G. 2013. The neuropeptide complement of the marine annelid Platynereis dumerilii. *BMC Genomics* 14, p. 906.

Conzelmann, M., Williams, E.A., Tunaru, S., Randel, N., Shahidi, R., Asadulina, A., Berger, J., Offermanns, S. and Jékely, G. 2013. Conserved MIP receptor-ligand pair regulates Platynereis larval settlement. *Proceedings of the National Academy of Sciences of the United States of America* 110(20), pp. 8224–8229.

Cox, K.J., Tensen, C.P., Van der Schors, R.C., Li, K.W., van Heerikhuizen, H., Vreugdenhil, E., Geraerts, W.P. and Burke, J.F. 1997. Cloning, characterization, and expression of a G-protein-coupled receptor from Lymnaea stagnalis and identification of a leucokinin-like peptide, PSFHSWSamide, as its endogenous ligand. *The Journal of Neuroscience* 17(4), pp. 1197–1205.

Crisp, K.M., Grupe, R.E., Lobsang, T.T. and Yang, X. 2010. Biogenic amines modulate pulse rate in the dorsal blood vessel of Lumbriculus variegatus. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology* 151(4), pp. 467–472.

Crocker, A. and Sehgal, A. 2008. Octopamine regulates sleep in drosophila through protein kinase A-dependent mechanisms. *The Journal of Neuroscience* 28(38), pp. 9377–9385.

Dohlman, H.G., Caron, M.G. and Lefkowitz, R.J. 1987. A family of receptors coupled to guanine nucleotide regulatory proteins. *Biochemistry* 26(10), pp. 2657–2664.

Dunn, C.W., Hejnol, A., Matus, D.Q., Pang, K., Browne, W.E., Smith, S.A., Seaver, E., Rouse, G.W., Obst, M., Edgecombe, G.D., Sørensen, M.V., Haddock, S.H., Schmidt-Rhaesa, A., Okusu, A., Kristensen, R.M., Wheeler, W.C., Martindale, M.Q. and Giribet, G. 2008. Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature* 452(7188), pp. 745–749.

Eipper, B.A., Stoffers, D.A. and Mains, R.E. 1992. The biosynthesis of neuropeptides: peptide alpha-amidation. *Annual Review of Neuroscience* 15, pp. 57–85.

El-Kholy, S., Stephano, F., Li, Y., Bhandari, A., Fink, C. and Roeder, T. 2015. Expression analysis of octopamine and tyramine receptors in Drosophila. *Cell and Tissue Research* 361(3), pp. 669–684.

Eyun, S.-I., Moriyama, H., Hoffmann, F.G. and Moriyama, E.N. 2016. Molecular Evolution and Functional Divergence of Trace Amine-Associated Receptors. *Plos One* 11(3), p. e0151023.

Fischer, A.H., Henrich, T. and Arendt, D. 2010. The normal development of Platynereis dumerilii (Nereididae, Annelida). *Frontiers in zoology* 7, p. 31.

Florey, E. and Rathmayer, M. 1978. The effects of octopamine and other amines on the heart and on neuromuscular transmission in decapod crustaceans: Further evidence for a role as neurohormone. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 61(1), pp. 229–237.

Fredriksson, R., Lagerström, M.C., Lundin, L.G. and Schiöth, H.B. 2003. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Molecular Pharmacology* 63(6), pp. 1256–1272.

Frickey, T. and Lupas, A. 2004. CLANS: a Java application for visualizing protein families based on pairwise similarity. *Bioinformatics* 20(18), pp. 3702–3704.

Frooninckx, L., Van Rompay, L., Temmerman, L., Van Sinay, E., Beets, I., Janssen, T., Husson, S.J. and Schoofs, L. 2012. Neuropeptide GPCRs in C. elegans. *Frontiers in endocrinology* 3, p. 167.

Fujita, W., Gomes, I. and Devi, L.A. 2014. Revolution in GPCR signalling: opioid receptor heteromers as novel therapeutic targets: IUPHAR review 10. *British Journal of Pharmacology* 171(18), pp. 4155–4176.

Garcia-Fernàndez, J. and Benito-Gutiérrez, E. 2009. It's a long way from amphioxus: descendants of the earliest chordate. *Bioessays: News and Reviews in Molecular, Cellular and Developmental Biology* 31(6), pp. 665–675.

Gee, H. 2008. Evolutionary biology: the amphioxus unleashed. *Nature* 453(7198), pp. 999–1000.

Gilman, A.G. 1987. G proteins: transducers of receptor-generated signals. *Annual Review of Biochemistry* 56, pp. 615–649.

Gühmann, M., Jia, H., Randel, N., Verasztó, C., Bezares-Calderón, L.A., Michiels, N.K., Yokoyama, S. and Jékely, G. 2015. Spectral Tuning of Phototaxis by a Go-Opsin in the Rhabdomeric Eyes of Platynereis. *Current Biology* 25(17), pp. 2265–2271.

Hewes, R.S. and Taghert, P.H. 2001. Neuropeptides and neuropeptide receptors in the Drosophila melanogaster genome. *Genome Research* 11(6), pp. 1126–1142.

Heyland, A., Price, D.A., Bodnarova-Buganova, M. and Moroz, L.L. 2006. Thyroid hormone metabolism and peroxidase function in two non-chordate animals. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution* 306(6), pp. 551–566.

Holland, L.Z., Laudet, V. and Schubert, M. 2004. The chordate amphioxus: an emerging model organism for developmental biology. *Cellular and Molecular Life Sciences* 61(18), pp. 2290–2308.

Hook, V., Funkelstein, L., Lu, D., Bark, S., Wegrzyn, J. and Hwang, S.R. 2008. Proteases for processing proneuropeptides into peptide neurotransmitters and hormones. *Annual Review of Pharmacology and Toxicology* 48, pp. 393–423.

Ishida, T., In, Y., Doi, M., Inoue, M., Yasuda-Kamatani, Y., Minakata, H., Iwashita, T. and Nomoto, K. 1992. Crystal structure and molecular conformation of achatin-I (H-Gly-D-Phe-Ala-Asp-OH), an endogenous neuropeptide containing a D-amino acid residue. *International Journal of Peptide and Protein Research* 39(3), pp. 258–264.

Jékely, G. 2013. Global view of the evolution and diversity of metazoan neuropeptide signaling. *Proceedings of the National Academy of Sciences of the United States of America* 110(21), pp. 8702–8707.

Jilek, A., Mollay, C., Tippelt, C., Grassi, J., Mignogna, G., Müllegger, J., Sander, V., Fehrer, C., Barra, D. and Kreil, G. 2005. Biosynthesis of a D-amino acid in peptide linkage by an enzyme from frog skin secretions. *Proceedings of the National Academy of Sciences of the United States of America* 102(12), pp. 4235–4239.

Kamatani, Y., Minakata, H., Kenny, P.T., Iwashita, T., Watanabe, K., Funase, K., Sun, X.P., Yongsiri, A., Kim, K.H. and Novales-Li, P. 1989. Achatin-I, an endogenous neuroexcitatory tetrapeptide from Achatina fulica Férussac containing a D-amino acid residue. *Biochemical and Biophysical Research Communications* 160(3), pp. 1015–1020.

Kandel, E.R., Dudai, Y. and Mayford, M.R. 2014. The molecular and systems biology of memory. *Cell* 157(1), pp. 163–186.

Kasai, R.S. and Kusumi, A. 2014. Single-molecule imaging revealed dynamic GPCR dimerization. *Current Opinion in Cell Biology* 27, pp. 78–86.

Kaufman, S., Bridgers, W.F. and Baron, J. 1968. The Mechanism of Action of Dopamine β-Hydroxylase. In: Mayo, F. R. ed. *Oxidation of Organic Compounds*. WASHINGTON, D.C.: AMERICAN CHEMICAL SOCIETY, pp. 172–176.

Kim, C.H., Zabetian, C.P., Cubells, J.F., Cho, S., Biaggioni, I., Cohen, B.M., Robertson, D. and Kim, K.S. 2002. Mutations in the dopamine beta-hydroxylase gene are associated with human norepinephrine deficiency. *American Journal of Medical Genetics* 108(2), pp. 140–147.

Kim, D.K., Cho, E.B., Moon, M.J., Park, S., Hwang, J.I., Do Rego, J.L., Vaudry, H. and Seong, J.Y. 2012. Molecular Coevolution of Neuropeptides Gonadotropin-Releasing Hormone and Kisspeptin with their Cognate G Protein-Coupled Receptors. *Frontiers in Neuroscience* 6, p. 3.

Kim, Y.J., Bartalska, K., Audsley, N., Yamanaka, N., Yapici, N., Lee, J.Y., Kim, Y.C., Markovic, M., Isaac, E., Tanaka, Y. and Dickson, B.J. 2010. MIPs are ancestral ligands for the sex peptide receptor. *Proceedings of the National Academy of Sciences of the United States of America* 107(14), pp. 6520–6525.

Kreil, G. 1997. D-amino acids in animal peptides. *Annual Review of Biochemistry* 66, pp. 337–345.

Krishnan, A., Almén, M.S., Fredriksson, R. and Schiöth, H.B. 2013. Remarkable similarities between the hemichordate (Saccoglossus kowalevskii) and vertebrate GPCR repertoire. *Gene* 526(2), pp. 122–133.

Krishnan, A., Dnyansagar, R., Almén, M., Williams, M.J., Fredriksson, R., Manoj, N. and Schiöth, H.B. 2014. The GPCR repertoire in the demosponge Amphimedon queenslandica : insights into the GPCR system at the early divergence of animals. *BMC Evolutionary Biology* 14(1), p. 270.

Lange, A.B. 2009. Tyramine: from octopamine precursor to neuroactive chemical in insects. *General and Comparative Endocrinology* 162(1), pp. 18–26.

Laudet, V. 2011. The origins and evolution of vertebrate metamorphosis. *Current Biology* 21(18), pp. R726–R737.

Lee, H.R., Zandawala, M., Lange, A.B. and Orchard, I. 2016. Isolation and characterization of the corticotropin-releasing factor-related diuretic hormone receptor in Rhodnius prolixus. *Cellular Signalling* 28(9), pp. 1152–1162.

Lindemann, L. and Hoener, M.C. 2005. A renaissance in trace amines inspired by a novel GPCR family. *Trends in Pharmacological Sciences* 26(5), pp. 274–281.

Liu, F., Baggerman, G., Schoofs, L. and Wets, G. 2008. The construction of a bioactive peptide database in Metazoa. *Journal of Proteome Research* 7(9), pp. 4119–4131.

Manglik, A. and Kobilka, B. 2014. The role of protein dynamics in GPCR function: insights from the β2AR and rhodopsin. *Current Opinion in Cell Biology* 27, pp. 136–143.

Marino, M.D., Bourdélat-Parks, B.N., Cameron Liles, L. and Weinshenker, D. 2005. Genetic reduction of noradrenergic function alters social memory and reduces aggression in mice. *Behavioural Brain Research* 161(2), pp. 197–203.

Martín-Durán, J.M., Janssen, R., Wennberg, S., Budd, G.E. and Hejnol, A. 2012. Deuterostomic development in the protostome Priapulus caudatus. *Current Biology* 22(22), pp. 2161–2166.

Mirabeau, O. and Joly, J.S. 2013. Molecular evolution of peptidergic signaling systems in bilaterians. *Proceedings of the National Academy of Sciences of the United States of America* 110(22), pp. E2028–E2037.

Monastirioti, M., Linn, C.E. and White, K. 1996. Characterization of Drosophila tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. *The Journal of Neuroscience* 16(12), pp. 3900–3911.

Nagaya, Y., Kutsukake, M., Chigusa, S.I. and Komatsu, A. 2002. A trace amine, tyramine, functions as a neuromodulator in Drosophila melanogaster. *Neuroscience Letters* 329(3), pp. 324–328.

Nall, A. and Sehgal, A. 2014. Monoamines and sleep in Drosophila. *Behavioral Neuroscience* 128(3), pp. 264–272.

Nobles, K.N., Xiao, K., Ahn, S., Shukla, A.K., Lam, C.M., Rajagopal, S., Strachan, R.T., Huang, T.Y., Bressler, E.A., Hara, M.R., Shenoy, S.K., Gygi, S.P. and Lefkowitz, R.J. 2011. Distinct phosphorylation sites on the β(2)-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. *Science Signaling* 4(185), p. ra51.

Offermanns, S. and Simon, M.I. 1995. G alpha 15 and G alpha 16 couple a wide variety of receptors to phospholipase C. *The Journal of Biological Chemistry* 270(25), pp. 15175–15180.

Pawson, A.J., Sharman, J.L., Benson, H.E., Faccenda, E., Alexander, S.P., Buneman, O.P., Davenport, A.P., McGrath, J.C., Peters, J.A., Southan, C., Spedding, M., Yu, W., Harmar, A.J. and NC-IUPHAR 2014. The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. *Nucleic Acids Research* 42(Database issue), pp. D1098–D1106.

Philippe, H., Brinkmann, H., Copley, R.R., Moroz, L.L., Nakano, H., Poustka, A.J., Wallberg, A., Peterson, K.J. and Telford, M.J. 2011. Acoelomorph flatworms are deuterostomes related to Xenoturbella. *Nature* 470(7333), pp. 255–258.

Pirri, J.K., McPherson, A.D., Donnelly, J.L., Francis, M.M. and Alkema, M.J. 2009. A tyramine-gated chloride channel coordinates distinct motor programs of a Caenorhabditis elegans escape response. *Neuron* 62(4), pp. 526–538.

Putnam, N.H., Butts, T., Ferrier, D.E., Furlong, R.F., Hellsten, U., Kawashima, T., Robinson-Rechavi, M., Shoguchi, E., Terry, A., Yu, J.K., Benito-Gutiérrez, E.L., Dubchak, I., Garcia-Fernàndez, J., Gibson-Brown, J.J., Grigoriev, I.V., Horton, A.C., de Jong, P.J., Jurka, J., Kapitonov, V.V., Kohara, Y., Kuroki, Y., Lindquist, E., Lucas, S., Osoegawa, K., Pennacchio, L.A., Salamov, A.A., Satou, Y., Sauka-Spengler, T., Schmutz, J., Shin-I, T., Toyoda, A., Bronner-Fraser, M., Fujiyama, A., Holland, L.Z., Holland, P.W., Satoh, N. and Rokhsar, D.S. 2008. The amphioxus genome and the evolution of the chordate karyotype. *Nature* 453(7198), pp. 1064–1071.

Raible, F., Tessmar-Raible, K., Osoegawa, K., Wincker, P., Jubin, C., Balavoine, G., Ferrier, D., Benes, V., de Jong, P., Weissenbach, J., Bork, P. and Arendt, D. 2005. Vertebrate-type intron-rich genes in the marine annelid Platynereis dumerilii. *Science (New York)* 310(5752), pp. 1325–1326.

Randel, N., Asadulina, A., Bezares-Calderón, L.A., Verasztó, C., Williams, E.A., Conzelmann, M., Shahidi, R. and Jékely, G. 2014. Neuronal connectome of a sensory-motor circuit for visual navigation. *eLife* 3.

Rasmussen, S.G., DeVree, B.T., Zou, Y., Kruse, A.C., Chung, K.Y., Kobilka, T.S., Thian, F.S., Chae, P.S., Pardon, E., Calinski, D., Mathiesen, J.M., Shah, S.T., Lyons, J.A., Caffrey, M., Gellman, S.H., Steyaert, J., Skiniotis, G., Weis, W.I., Sunahara, R.K. and Kobilka, B.K. 2011. Crystal structure of the β2 adrenergic receptor-Gs protein complex. *Nature* 477(7366), pp. 549–555.

Rex, E. and Komuniecki, R.W. 2002. Characterization of a tyramine receptor from Caenorhabditis elegans. *Journal of Neurochemistry* 82(6), pp. 1352–1359.

Ringstad, N., Abe, N. and Horvitz, H.R. 2009. Ligand-gated chloride channels are receptors for biogenic amines in C. elegans. *Science (New York)* 325(5936), pp. 96–100.

Roeder, T. 2005. Tyramine and octopamine: ruling behavior and metabolism. *Annual Review of Entomology* 50, pp. 447–477.

Roller, L., Yamanaka, N., Watanabe, K., Daubnerová, I., Žitňan, D., Kataoka, H. and Tanaka, Y. 2008. The unique evolution of neuropeptide genes in the silkworm Bombyx mori☆. *Insect Biochemistry and Molecular Biology* 38(12), pp. 1147-1157.

Rowe, M.L. and Elphick, M.R. 2012. The neuropeptide transcriptome of a model echinoderm, the sea urchin Strongylocentrotus purpuratus. *General and Comparative Endocrinology* 179(3), pp. 331–344.

Sakai, T., Aoyama, M., Kawada, T., Kusakabe, T., Tsuda, M. and Satake, H. 2012. Evidence for differential regulation of GnRH signaling via heterodimerization among GnRH receptor paralogs in the protochordate, Ciona intestinalis. *Endocrinology* 153(4), pp. 1841–1849.

Saraswati, S., Fox, L.E., Soll, D.R. and Wu, C.F. 2004. Tyramine and octopamine have opposite effects on the locomotion of Drosophila larvae. *Journal of Neurobiology* 58(4), pp. 425–441.

Saudou, F., Amlaiky, N., Plassat, J.L., Borrelli, E. and Hen, R. 1990. Cloning and characterization of a Drosophila tyramine receptor. *The EMBO Journal* 9(11), pp. 3611–3617.

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S. and Heisenberg, M. 2003. Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. *The Journal of Neuroscience* 23(33), pp. 10495–10502.

Sekiguchi, T., Kuwasako, K., Ogasawara, M., Takahashi, H., Matsubara, S., Osugi, T., Muramatsu, I., Sasayama, Y., Suzuki, N. and Satake, H. 2015. Evidence for conservation of the calcitonin superfamily and activity-regulating mechanisms in the basal chordate Branchiostoma floridae: insight into the molecular and functional evolution in chordates. *The Journal of Biological Chemistry*.

Semmens, D.C., Beets, I., Rowe, M.L., Blowes, L.M., Oliveri, P. and Elphick, M.R. 2015. Discovery of sea urchin NGFFFamide receptor unites a bilaterian neuropeptide family. *Open biology* 5(4), p. 150030.

Semmens, D.C., Mirabeau, O., Moghul, I., Pancholi, M.R., Wurm, Y. and Elphick, M.R. 2016. Transcriptomic identification of starfish neuropeptide precursors yields new insights into neuropeptide evolution. *Open biology* 6(2), p. 150224.

Shahidi, R., Williams, E.A., Conzelmann, M., Asadulina, A., Verasztó, C., Jasek, S., Bezares-Calderón, L.A. and Jékely, G. 2015. A serial multiplex immunogold labeling method for identifying peptidergic neurons in connectomes. *eLife* 4.

Shakiryanova, D., Zettel, G.M., Gu, T., Hewes, R.S. and Levitan, E.S. 2011. Synaptic neuropeptide release induced by octopamine without Ca2+ entry into the nerve terminal. *Proceedings of the National Academy of Sciences of the United States of America* 108(11), pp. 4477–4481.

Simakov, O., Kawashima, T., Marlétaz, F., Jenkins, J., Koyanagi, R., et al. 2015. Hemichordate genomes and deuterostome origins. *Nature* 527(7579), pp. 459–465.

Singh, C., Oikonomou, G. and Prober, D.A. 2015. Norepinephrine is required to promote wakefulness and for hypocretin-induced arousal in zebrafish. *eLife* 4, p. e07000.

Tang, X.L., Wang, Y., Li, D.L., Luo, J. and Liu, M.Y. 2012. Orphan G protein-coupled receptors (GPCRs): biological functions and potential drug targets. *Acta Pharmacologica Sinica* 33(3), pp. 363–371.

Tensen, C.P., Cox, K.J., Burke, J.F., Leurs, R., van der Schors, R.C., Geraerts, W.P., Vreugdenhil, E. and Heerikhuizen, H. 1998. Molecular cloning and characterization of an invertebrate homologue of a neuropeptide Y receptor. *The European Journal of Neuroscience* 10(11), pp. 3409–3416.

Tensen, C.P., Cox, K.J., Smit, A.B., van der Schors, R.C., Meyerhof, W., Richter, D., Planta, R.J., Hermann, P.M., van Minnen, J., Geraerts, W.P., Knol, J.C., Burke, J.F., Vreugdenhil, E. and van Heerikhuizen, H. 1998. The lymnaea cardioexcitatory peptide (LyCEP) receptor: a G-protein-coupled receptor for a novel member of the RFamide neuropeptide family. *The Journal of Neuroscience* 18(23), pp. 9812–9821.

Tosches, M.A., Bucher, D., Vopalensky, P. and Arendt, D. 2014. Melatonin signaling controls circadian swimming behavior in marine zooplankton. *Cell* 159(1), pp. 46–57.

Tunaru, S., Lättig, J., Kero, J., Krause, G. and Offermanns, S. 2005. Characterization of determinants of ligand binding to the nicotinic acid receptor GPR109A (HM74A/PUMA-G). *Molecular Pharmacology* 68(5), pp. 1271–1280.

Vannier, J., Calandra, I., Gaillard, C. and Zylinska, A. 2010. Priapulid worms: Pioneer horizontal burrowers at the Precambrian-Cambrian boundary. *Geology* 38(8), pp. 711–714.

Veenstra, J.A. 2016. Neuropeptide evolution: Chelicerate neurohormone and neuropeptide genes may reflect one or more whole genome duplications. *General and Comparative Endocrinology* 229, pp. 41–55.

Veenstra, J.A. 2014. The contribution of the genomes of a termite and a locust to our understanding of insect neuropeptides and neurohormones. *Frontiers in physiology* 5, p. 454.

Venkatesh, B., Lee, A.P., Ravi, V., Maurya, A.K., Lian, M.M., Swann, J.B., Ohta, Y., Flajnik, M.F., Sutoh, Y., Kasahara, M., Hoon, S., Gangu, V., Roy, S.W., Irimia, M., Korzh, V., Kondrychyn, I., Lim, Z.W., Tay, B.H., Tohari, S., Kong, K.W., Ho, S., Lorente-Galdos, B., Quilez, J., Marques-Bonet, T., Raney, B.J., Ingham, P.W., Tay, A., Hillier, L.W., Minx, P., Boehm, T., Wilson, R.K., Brenner, S. and Warren, W.C. 2014. Elephant shark genome provides unique insights into gnathostome evolution. *Nature* 505(7482), pp. 174–179.

Verlinden, H., Vleugels, R., Marchal, E., Badisco, L., Pflüger, H.J., Blenau, W. and Broeck, J.V. 2010. The role of octopamine in locusts and other arthropods. *Journal of Insect Physiology* 56(8), pp. 854–867.

Wallace, B.G. 1976. The biosynthesis of octopamine--characterization of lobster tyramine beta-hydroxylase. *Journal of Neurochemistry* 26(4), pp. 761–770.

Webster, B.L., Copley, R.R., Jenner, R.A., Mackenzie-Dodds, J.A., Bourlat, S.J., Rota-Stabelli, O., Littlewood, D.T. and Telford, M.J. 2006. Mitogenomics and phylogenomics reveal priapulid worms as extant models of the ancestral Ecdysozoan. *Evolution & Development* 8(6), pp. 502–510.

Wisler, J.W., Xiao, K., Thomsen, A.R. and Lefkowitz, R.J. 2014. Recent developments in biased agonism. *Current Opinion in Cell Biology* 27, pp. 18–24.

Wu, S.F., Xu, G., Qi, Y.X., Xia, R.Y., Huang, J. and Ye, G.Y. 2014. Two splicing variants of a novel family of octopamine receptors with different signaling properties. *Journal of Neurochemistry* 129(1), pp. 37–47.

Zantke, J., Bannister, S., Rajan, V.B., Raible, F. and Tessmar-Raible, K. 2014. Genetic and genomic tools for the marine annelid Platynereis dumerilii. *Genetics* 197(1), pp. 19–31.

Zhou, C., Rao, Y. and Rao, Y. 2008. A subset of octopaminergic neurons are important for Drosophila aggression. *Nature Neuroscience* 11(9), pp. 1059–1067.

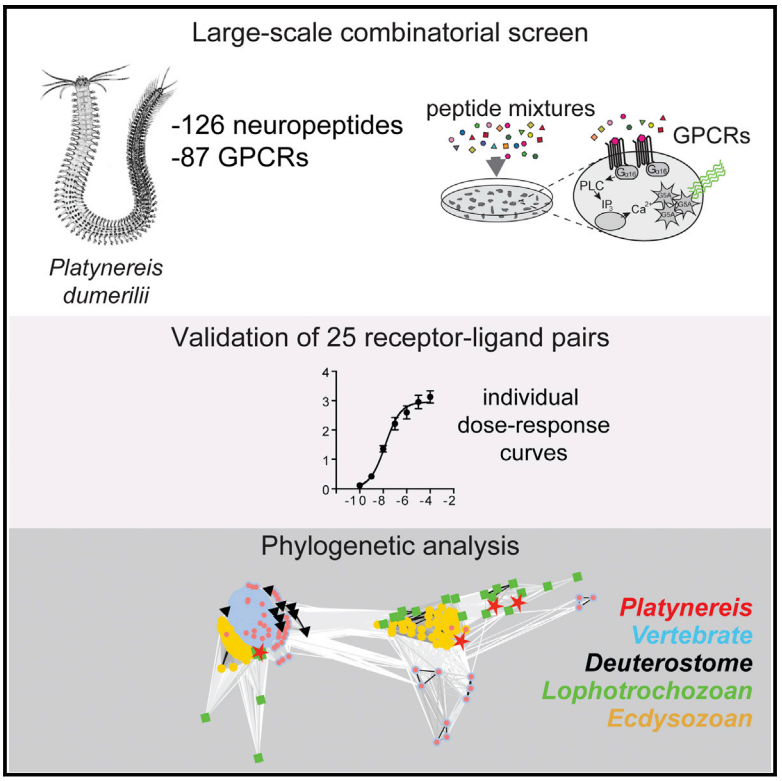
Appendix: Publications

Cell Reports

Resource

Large-Scale Combinatorial Deorphanization of *Platynereis* Neuropeptide GPCRs

Graphical Abstract



Highlights

- 19 GPCRs from *Platynereis* were deorphanized
- Ligands for previously uncharacterized GPCR families were found
- A *Platynereis* ortholog of thyrotropin-releasing hormone was identified
- Conserved D-peptides activate an ancient bilaterian family of achatin receptors

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In Brief

Bauknecht and Jékely report the deorphanization of 19 neuropeptide G-protein-coupled receptors from the marine annelid *Platynereis*. Among them are members of previously uncharacterized families. This work provides information about the evolution of peptidergic systems and neuropeptide signaling in bilaterians.

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KP420212–KP420214

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Resource

Large-Scale Combinatorial Deorphanization of *Platynereis* Neuropeptide GPCRs

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SUMMARY

Neuropeptides, representing the largest class of neuromodulators, commonly signal by G-protein-coupled receptors (GPCRs). While the neuropeptide repertoire of several metazoans has been characterized, many GPCRs are orphans. Here, we develop a strategy to identify GPCR-peptide pairs using combinatorial screening with complex peptide mixtures. We screened 126 neuropeptides against 87 GPCRs of the annelid *Platynereis* and identified ligands for 19 receptors. We assigned many GPCRs to known families and identified conserved families of achatin, FMRFamide, RGWamide, FLamide, and elevenin receptors. We also identified a ligand for the *Platynereis* ortholog of vertebrate thyrotropin-releasing hormone (TRH) receptors, revealing the ancient origin of TRH-receptor signaling. We predicted ligands for several metazoan GPCRs and tested predicted achatin receptors. These receptors were specifically activated by an achatin D-peptide, revealing a conserved mode of activation. Our work establishes an important resource and provides information about the complexity of peptidergic signaling in the urbilaterian.

INTRODUCTION

Neuropeptides represent the largest and most diverse class of neuron-secreted signaling molecules. These peptides can have neuromodulatory, neurotransmitter, or hormonal functions and can affect development, physiology, and the activity in neural circuits. The majority of neuropeptides signal by G-protein-coupled receptors (GPCRs), with some exceptions (Chang et al., 2009; Leung et al., 1987; Lowe et al., 1989; Rechler and Nissley, 1985). While the neuropeptide repertoire of an animal can be determined using a combination of sequencing and mass-spectrometry approaches (Collins et al., 2010; Conzelmann et al., 2013a; Dircksen et al., 2011; Hauser et al., 2010; Li et al., 2008; Xie et al., 2010), the determination of neuropeptide receptors is more difficult and is usually carried out using in vitro experiments with individual peptide-receptor pairs.

Several years of effort have led to the identification of ~35 neuropeptide GPCRs in *Drosophila melanogaster* (Caers et al., 2012; Hewes and Taghert, 2001), 23 in *Caenorhabditis elegans* (Frooninckx et al., 2012), 50 in human and mouse, and only a few in non-model organisms (Bigot et al., 2014; Conzelmann et al., 2013b; Cox et al., 1997; Kim et al., 2010; Tensen et al., 1998a, 1998b).

Among the lophotrochozoans, an animal superphylum that with ecdysozoans and deuterostomes forms the Bilateria (Telford and Copley, 2011), peptidergic neuromodulation has been extensively studied in several species (Cropper et al., 1987; Kamatani et al., 1989; Rajpara et al., 1992; Hoek et al., 2005; Willows et al., 1997). However, the lack of information regarding neuropeptide receptors hinders the identification of the downstream signaling mechanisms underlying neuromodulation.

The annelid *Platynereis* has emerged in recent years as a powerful lophotrochozoan laboratory animal for the study of development, neuronal circuits, and zooplankton behavior (Jékely et al., 2008; Randel et al., 2014; Tosches et al., 2014; Zantke et al., 2014). Its larval stages represent accessible models for studying the role of neuropeptides in behavior, development, and physiology at the whole-organism level (Conzelmann et al., 2011, 2013b; Williams et al., 2015). *Platynereis* has an ancestral neuropeptide repertoire, including 30 ancestral bilaterian pro-neuropeptide families (Conzelmann et al., 2013a); however, only one neuropeptide receptor has been identified so far (Conzelmann et al., 2013b).

Here, building on established transcriptomic and peptidomic resources (Conzelmann et al., 2013a), we present a large-scale deorphanization screen of *Platynereis* neuropeptide GPCRs. We identified the peptide ligand of 19 *Platynereis* receptors. We also perform a phylogenetic analysis of *Platynereis* and other metazoan neuropeptide GPCRs to gain insights into the evolution of peptidergic signaling in bilaterians.

RESULTS

Combinatorial Screening for *Platynereis* GPCR-Neuropeptide Ligand Pairs

To facilitate the rapid identification of neuropeptide GPCRs, we developed a combinatorial cell-culture-based screening strategy (Figure 1).

We reasoned that complex peptide mixtures could be used to identify receptors activated by specific peptides present in these mixtures. Mixtures of subsets of peptides (submixtures) would

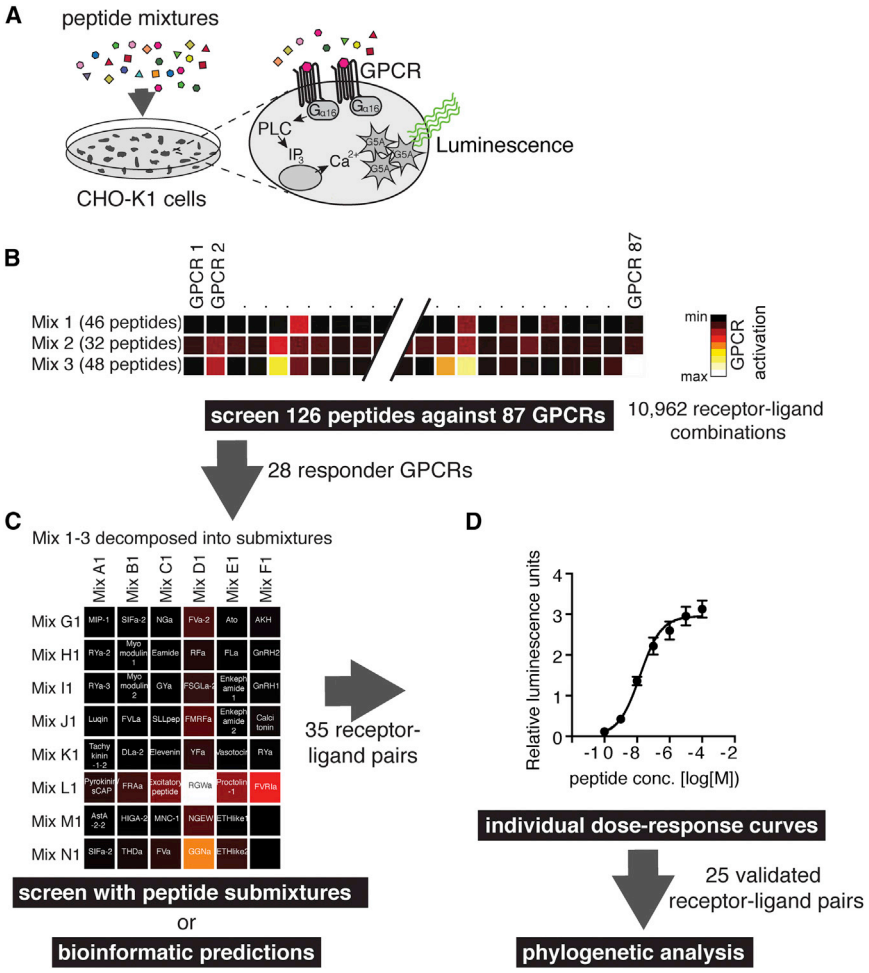

Figure 1. Schematic of the Combinatorial Screen for *Platynereis* Neuropeptide GPCRs

(A) We used a cell-culture assay with CHO-K1 cells stably transfected with a calcium-sensitive bioluminescent GFP-aequorin fusion protein (G5A).

(B) In the primary screen, 87 *Platynereis* GPCRs were tested against three complex neuropeptide mixtures. This screen identified 28 responder GPCRs.

(C) A secondary screen based on peptide submixtures and bioinformatic predictions identified 35 receptor-ligand pairs. See also Figure S1.

(D) Individual peptide-receptor dose-response measurements validated 25 receptor-ligand pairs. A phylogenetic analysis provided information about GPCR-ligand coevolution across bilaterians. See also Figure S1 and Table S1.

then allow the unambiguous identification of a single active peptide.

We screened 87 orphan *Platynereis* GPCRs (Table S1) against 126 *Platynereis* neuropeptides, pooled into three mixtures based on peptide pI and solubility (Table S2). We used a Ca^{2+}-mobilization assay with CHO-K1 cells stably transfected with a calcium-sensitive bioluminescent GFP-aequorin fusion protein (Tunaru et al., 2005). This screen identified 28 GPCRs that responded reproducibly to at least one of the peptide mixtures (Figure 2; Table S3).

We focused on these receptors and tested them with peptide mixtures derived by decomposing the original active mixture into the rows and columns of a matrix (Tables S2 and S3). If two mixtures representing a row and a column in the matrix activate the receptor, the peptide at the intersect likely represents a specific ligand. We defined an activation value for each peptide in the matrix as the square root of the product of the measured values of two intersecting mixtures (Figure 1; Figure S1). Using this approach, we found candidate receptors for the neuropeptides FLa (also activated by prokineticin short peptide 1 [SP-1]), allatotropin, neuropeptide KY (NKY), luqin, elevenin (two receptors), RGWa, FMRFa, excitatory peptide, neuropeptide-Y-4 (NPY-4), achatin, EFLGamide (EFLGa), and diuretic hormone 31 (DH31) (Figure S1; Table S3).

For six receptors, we were able to predict the ligands based on orthology relationships (see below). These included candidate receptors for vasotocin, allatostatin-A, allatotropin, myomodulin, allatostatin-C, and DH31 (Table S3). The GPCR-ligand pairs identified either by combinatorial screening or bioinformatic prediction were further tested in individual receptor ligand assays (Table S3).

Validation of 25 GPCR-Ligand Pairs

To confirm that the identified peptides are indeed specific ligands to the respective GPCRs, we recorded dose-response curves for each of the identified receptor-ligand pairs. After excluding a few receptors with inconsistent activation in replicate experiments, we identified 25 GPCR-ligand pairs with half maximal effective concentration (EC_{50}) values in the nanomolar or low-micromolar range (Table 1; Figure 3).

We recorded two sets of dose-response curves with different normalizations, using responses of either the *Platynereis* MIP receptor or an endogenously expressed histamine receptor as reference (Figure 3; Figure S2). For three receptors, we identified two or three ligands derived from distinct precursors (FLamide, NKY, and NPY-4 receptors), and for four peptides (allatotropin, FMRFamide, elevenin, and DH31), we identified two receptors. Overall, we established neuropeptide ligands for 19 *Platynereis* GPCRs (Table 1).

To partially characterize the signaling mechanism of these receptors, we tested them in the same assay, but without co-transfecting the Gα-16 construct. If receptor activation leads to Ca^{2+} increase without the promiscuous G protein, this indicates that the GPCR couples to the endogenous Gq-α to activate the phospholipase C (PLC)/inositol trisphosphate (IP_3)/Ca^{2+} release pathway. Ligand stimulation of ten receptors (allatotropin-1 and -2, FLamide, FMRFamide, luqin, elevenin-1 and -2, DH31-2, and vasotocin receptors) produced Ca^{2+} signals without Gα-16,

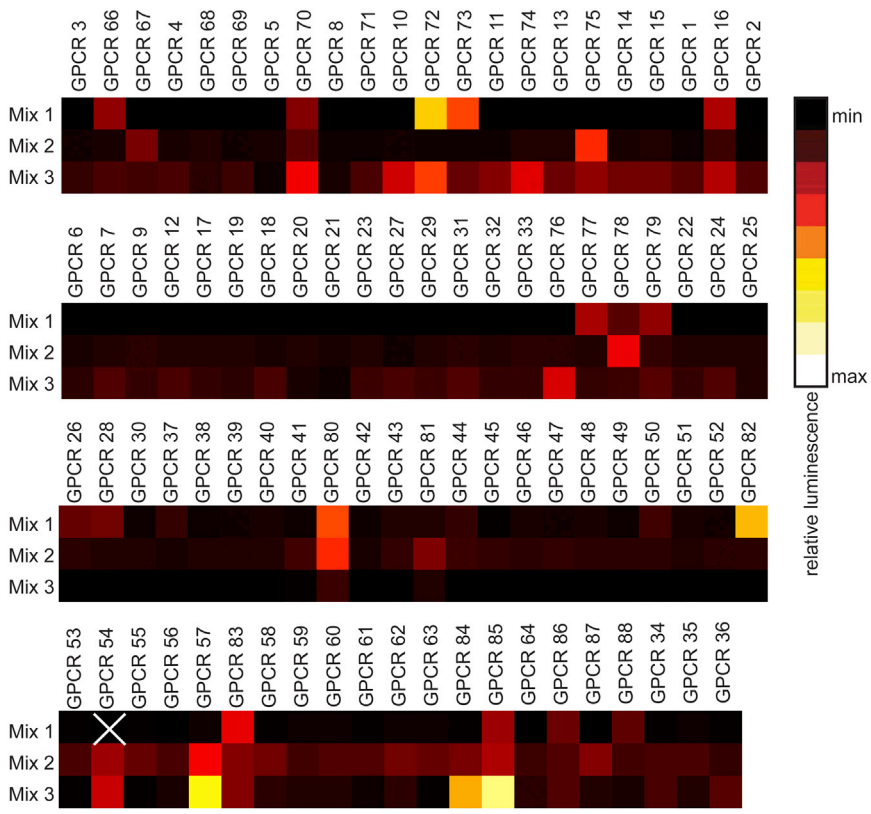

Figure 2. Primary Screen of 87 *Platynereis* GPCRs against Three Complex Peptide Mixtures

28 responder GPCRs that showed consistent activation in three replicate experiments were studied further. Mean relative luminescence values from three replicates are shown with color-coding. GPCR54 was not tested with Mix1. See also Table S3.

suggesting that these receptors couple to Gq-α (Table 1; Table S3). Given the limitations of the GFP-aequorin assay, we did not test Gi-α and Go-α signaling.

Phylogenetic Analysis of *Platynereis* Neuropeptide GPCRs

Recent bioinformatic analyses found strong support for the long-term stability of GPCR-neuropeptide ligand pairs across animal phyla (Janssen et al., 2010; Jékely, 2013; Mirabeau and Joly, 2013; Park et al., 2002). However, given the limited knowledge of GPCR ligands among the lophotrochozoans, it has not been possible to rigorously assess receptor-ligand coevolution across all three superphyla of Bilateria. Our deorphanized GPCR resource provides a large-scale dataset to test the generality of inter-phyletic receptor-ligand coevolution.

To identify orthologs of the 87 *Platynereis* GPCRs used in the screen, we performed similarity-based clustering, a method previously shown to be an efficient means of recovering orthologous groups of GPCRs (Jékely, 2013), with results similar to tree-based molecular phylogenetic analyses (Mirabeau and Joly, 2013). First, we seeded BLASTP searches with the *Platynereis* GPCR sequences in metazoan genomes. We also collected further representative neuropeptide GPCRs, including an annotated list of GPCRs whose peptide ligands have been experimentally characterized. We then separately clustered the 68 orphan *Platynereis* receptors and the 19 deorphanized *Platynereis* receptors with their respective BLASTP hits and further GPCR representatives (Figure 4; Figure S3). Clustering analysis of the 68 orphan receptors identified orthologs for 20 of them with a known peptide ligand in another species (Figure S3; Table S1). For 14 of these GPCRs, the corresponding *Platynereis* peptides were present in our mixtures. There can be several reasons why we did not see activation for these receptor-peptide pairs: (1) GPCR expressions may have failed, (2) the ligands may not have been dissolved or were unstable, (3) the ligand changed during evolution, or (4) some clusters may contain closely related paralogous receptors with different ligands.

Clustering analysis of the 19 deorphanized *Platynereis* GPCRs allowed us to assign all of them to well-resolved sequence groups (Figure 4; Figure S3). Many of these sequence clusters represented established orthology groups of metazoan GPCRs (Jékely, 2013; Mirabeau and Joly, 2013) containing already-characterized GPCRs. In most cases, the *Platynereis* receptor was activated by a peptide that was a previously recognized ortholog of known peptide ligands in that cluster (allatostatin-A, allatostatin-C, vasopressin/oxytocin, allatotropin/orexin, luqin/insect-RYamide, and DH31/calcitonin). These *Platynereis* receptors, together with previously identified mollusk receptors (Bigot et al., 2014; Tensen et al., 1998a, 1998b), represent deorphanized lophotrochozoan members of their respective families. These examples provide further evidence for receptor-ligand conservation during evolution.

Furthermore, our GPCR analysis could confirm the orthology relationships of some annelid neuropeptide families that had previously been proposed based on peptide-sequence similarity alone. Annelid and mollusk myomodulins (Cropper et al., 1987; Veenstra, 2011) were suggested to be orthologs of arthropod myosuppressins (Holman et al., 1986) based on limited peptide similarity (Jékely, 2013). The orthology of the *Platynereis* myomodulin receptor to the arthropod myosuppressin receptor confirms this (Figure 4).

Similarly, annelid excitatory peptides (Oumi et al., 1995) were suggested to belong to the bilaterian CCHamide/neuromedin-B family (Jékely, 2013; Roller et al., 2008). The *Platynereis* excitatory peptide receptor clusters with CCHamide/neuromedin-B receptors, confirming this (Figure 4).

The NKY receptors we identified are related to bilaterian NPY/NPF receptors (Bigot et al., 2014; Mertens et al., 2002) and their paralogs, the short neuropeptide F (sNPF) receptors from

Table 1. Summary of Validated Receptor-Ligand Pairs

Receptor Name	Ligand Name	Sequence of Tested Ligand	EC_{50} Normalized to Histamine Receptor Response	EC_{50} Normalized to MIP Receptor Response	Activation without Gα-16
Allatotropin receptor1	allatotropin	GFRTGAYDRFSHGF-NH2	116 nM	840 nM	yes
Allatotropin receptor2	allatotropin	GFRTGAYDRFSHGF-NH2	7.8 nM	6.0 nM	yes
EFLGa receptor1	EFLGa	FSEFLG-NH2	350 nM	5.4 nM	no
FLamide receptor1	FLamide	AKYFL-NH2	8.9 nM	2.0 nM	yes
FLamide receptor1	prokineticin-short peptide1	GRSRPLFV-NH2	47 nM	390 nM	yes
FMRFamide receptor1	FMRFamide	FMRF-NH2	78 nM	1.5 nM	yes
NKY receptor1	NKY-1	KAFWQPMMGGPLPVETRLASFGS RIEPDRTEPGSGPNGIKAMRY-NH2	120 nM	120 nM	no
NKY receptor1	NKY-2	NNGIWIWMPAQGYVSVPHQQEGG AADEGKPGKIMRY-NH2	410 nM	390 nM	no
NKY receptor1	FMRFamide	FMRF-NH2	1.4 μM	840 nM	no
NPY-4 receptor1	NPY-4	DPSFISSGPPVRPSSFKSPEELMEY LQKVRAYYNVMSRPRF-NH2	350 nM	110 nM	no
NPY-4 receptor1	NPY-3	pGluNMEGPPPRPAIFRTPQELRDY LSDLNEYFMIVGRPRF-NH2	630 nM	1.0 μM	no
NPY-4 receptor1	NPY-1	KVLEEMPTLQQIPLKPVRPNRFRNK DELHSYLQSLRDYYSVIGRPRF-NH2	420 nM	3.7 μM	no
Luqin receptor1	luqin	WRPQGRF-NH2	5.2 nM	0.86 nM	yes
RGWamide receptor1	RGWamide	RGW-NH2	2.9 nM	10 nM	no
Excitatory peptide receptor1	excitatory peptide	KCSGQWAIHACAGGN-NH2	7.9 nM	15 nM	no
Allatostatin-A receptor1	allatostatin-A-2-2	NDALKFSGL-NH2	12 μM	15 μM	no
Elevenin receptor1	elevenin (L11)	PDCTRFVFHPSCRGVAA	62 nM	120 nM	yes
Elevenin receptor2	elevenin (L11)	PDCTRFVFHPSCRGVAA	1.3 nM	2.3 nM	yes
Achatin receptor1	D-Achatin	G{dF}GD	120 nM	150 nM	no
Achatin receptor1	L-Achatin	GFGD	not available	11 μM	no
Myomodulin receptor1	myomodulin-2	AMGMLRM-NH2	26 nM	9.6 nM	no
Myomodulin receptor1	myomodulin-1	AMSMLRM-NH2	10 nM	~10 nM	no
DH31 receptor1	DH31	RIDAGYGSRYAAGASVGSKLRALK QAADWNGP-NH2	180 nM	87 nM	no
DH31 receptor2	DH31	RIDAGYGSRYAAGASVGSKLRALK QAADWNGP-NH2	34 nM	15 nM	yes
Vasotocin receptor1	vasotocin	CFVRNCPPG-NH2	1.1 μM	920 nM	yes
Allatostatin-C receptor1	allatostatin-C	pGluPVQCLVNIVSCW-NH2	1.0 μM	1.2 μM	no
Aplysia achatin receptor	*Aplysia* D-achatin	G{d-F}FD	14 nM	62 nM	no
Aplysia achatin receptor	*Aplysia* L-achatin	GFFD	not available	190 μM	no
Branchiostoma Achatin receptor	*Branchiostoma* D-Achatin	G{d-F}GN	0.87 nM	2.4 nM	yes
Branchiostoma achatin receptor	*Branchiostoma* L-Achatin	GFGN	not available	>1 M	not available
Saccoglossus achatin receptor	*Saccoglossus* D-Achatin	G{d-F}GN	16 nM	27 nM	no
Saccoglossus achatin receptor	*Saccoglossus* L-Achatin	GFGN	15 μM	13 μM	no

Name and sequence of the tested ligands are shown. pGlu indicates N-terminal pyroglutamylation. -NH2 indicates C-terminal amidation. Cys residues that form disulfide bonds are underlined. EC_{50} values of dose-response curves are shown for the two different normalizations. All receptors were also tested without cotransfecting the promiscuous Gα-16. Activation in the absence of Gα-16 in the GFP-aequorin assay indicates that the receptor couples to the Gq-α protein endogenously present in the CHO cells.

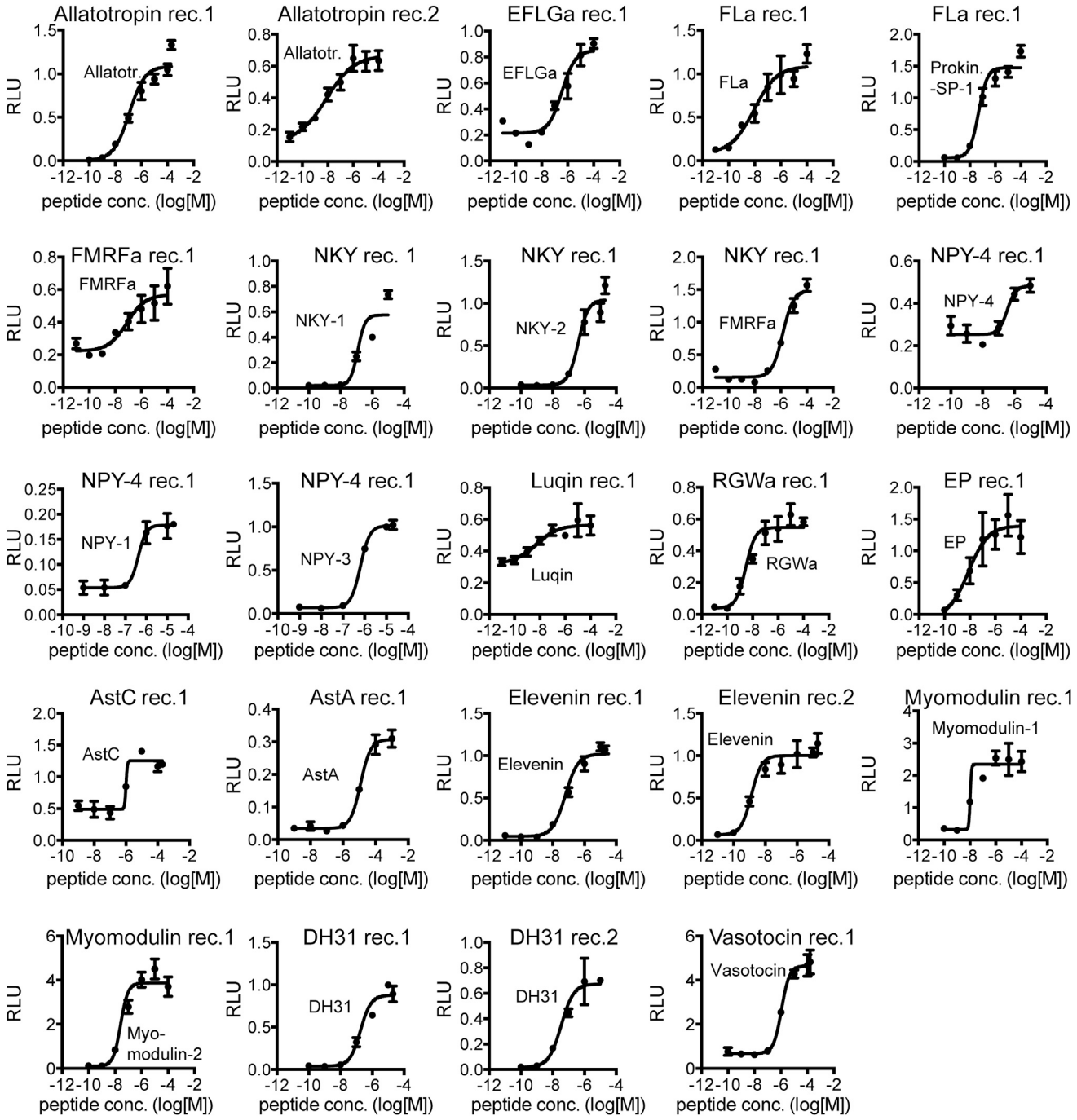

Figure 3. Dose-Response Curves of *Platynereis* Deorphanized GPCRs Treated with Varying Concentrations of Peptides

Data, representing luminescence units relative to the control response (1 mM histamine), are shown as mean ± SEM (n = 3). Dose-response curves fitted to the data are shown. Ligand names are shown beside the curves. EC_{50} values are listed in Table 1. RLU, relative luminescence unit; AstC, allatostatin-C; AstA, allatostatin-A; EP, excitatory peptide. See also Figure S2.

mollusks and insects (Bigot et al., 2014). The NKY peptides of annelids and mollusks show similarity to NPY/NPF peptides, including the RF/Yamide motif, a proline-rich stretch, and an acidic stretch (Conzelmann et al., 2013a) (Figure S4A). These results establish NKY and NPY/NPF peptides as paralogs.

Ligand Discoveries for Uncharacterized GPCR Families

We also identified six GPCR clusters where the only member with a known ligand was one of the deorphanized *Platynereis* GPCRs. These included clusters of sequences orthologous to *Platynereis* FMRFamide, L11/elevenin, achatin, RGWamide,

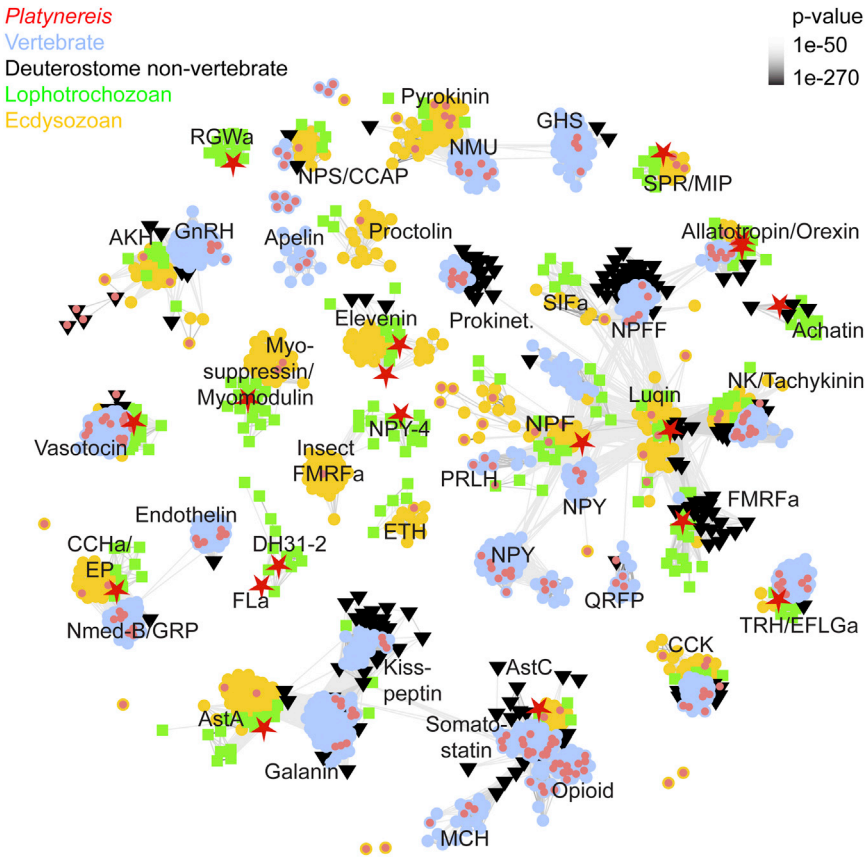

Figure 4. Sequence-Similarity-Based Clustering of Neuropeptide Class A GPCRs

Nodes represent sequences, and edges represent BLASTP connections. Edges are colored according to BLASTP p values. Nodes are colored based on taxonomy. Only deorphanized *Platynereis* GPCRs were included and are indicated as red stars. The *Platynereis* MIP receptor was described previously (Conzelmann et al., 2013b). Deorphanized receptors from other species are marked with a small red dot. Clusters are named according to the name of the deorphanized family members. DH31-receptor1 and other class B GPCRs were clustered separately and are shown in Figure S3A. AKH, adipokinetic hormone; AstC, allatostatin-C; AstA, allatostatin-A; CCK, cholecystokinin; EP, excitatory peptide; ETH, ecdysis triggering hormone; GnRH, gonadotropin-releasing hormone; MCH, melanin-concentrating hormone; MIP, myoinhibitory peptide; Nmed-B, neuromedin-B; NMU, neuromedin-U; NPF/Y, neuropeptide F/Y; NPFF, neuropeptide FF; PRLH, prolactin releasing hormone; QRFP, pyroglutamylated RFamide peptide; SK, substance-K; SPR, sex peptide receptor; TRH, thyrotropin releasing hormone. The Clans file is available at https://github.com/JekelyLab/GPCR_Clans_Maps. See also Figures S3, S4, and S6.

FLamide, DH31-rec2 and NPY-4 receptors (Figure 4). These clusters represent conserved neuropeptide GPCR families with an identified ligand in *Platynereis*.

Three of the identified families (FMRFamide, elevenin, and achatin) have both protostome and deuterostome orthologs, representing ancient bilaterian orthology groups (Figure 4). In contrast, the GPCR clusters containing the *Platynereis* RGWamide and FLamide receptors are restricted to lophotrochozoans (Figure 4).

The FMRFamide receptor cluster we identified is related to luqin receptors and contains several mollusk and annelid sequences, as well as sequences from the non-vertebrate deuterostomes *Branchiostoma* and *Saccoglossus* (Figure 4). Interestingly, we also identified a GPCR belonging to this group from the cartilaginous fish, the elephant shark *Callorhinchus milii*. The elephant shark genome represents the slowest evolving vertebrate genome thus far identified (Venkatesh et al., 2014). We also identified FMRFamide-receptor orthologs from a hemipteran insect, but no other arthropods (Table S4). The FMRFamide-receptor family thus represents a conserved bilaterian family that has been lost in most vertebrates and arthropods but is retained in lophotrochozoans and non-vertebrate deuterostomes. An FMRFamide receptor has also been identified in *Drosophila* (Cazzamali and Grimmelikhuijzen, 2002), but this sequence belongs to an arthropod-specific group and is not closely related to the FMRFamide receptors we describe here (Figure 4). In mollusks, FaNaCs, members of the DEG/ENaC family, have been identified as FMRFamide receptors (Linggueglia et al., 1995), but no FMRFamide GPCR has yet been found.

The receptors for *Platynereis* L11/elevenin peptide also belonged to a conserved bilaterian family with members in nematodes, insects, annelids, mollusks, *Branchiostoma*, and *Saccoglossus* (Figure 4; Table S4). Elevenin orthologs are known from annelids (Veenstra, 2011), mollusks (Veenstra, 2010), and nematodes and arthropods (Jékely, 2013), but we predict that they are also present in some non-vertebrate deuterostomes.

The receptor for *Platynereis* achatin clustered with orthologs from mollusks, annelids and non-vertebrate deuterostomes (Figure 4). Achatin was described from mollusks (Veenstra, 2010), annelids (Veenstra, 2011), and *Branchiostoma* and *Saccoglossus* (Jékely, 2013), but has been lost from vertebrates and most ecdysozoans (it is present in the chelicerate *Stegodyphus mimosarum*) (Figure S4B). The identification of a receptor family showing the same phyletic distribution establishes the achatin receptor-ligand pair as an ancient bilaterian system.

RGWamide and FLamide peptides are known from annelids and mollusks (Conzelmann et al., 2013a), and we identified distinct receptor clusters containing the *Platynereis* receptors and other annelid and mollusk sequences (Figure 4; Table S4). We also identified a second DH31 peptide receptor, closely related to FLamide receptor. We could not identify members of these receptor families outside the lophotrochozoans.

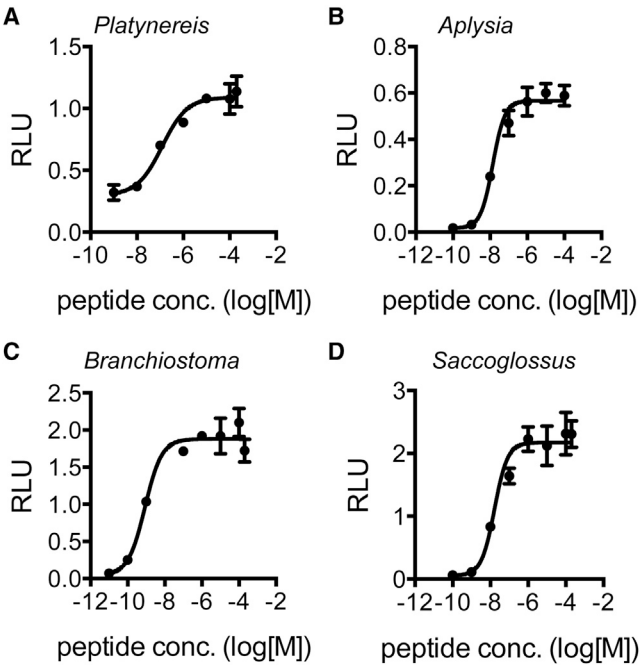

Figure 5. Dose-Response Curves of Bilaterian Achatin Receptors Treated with Varying Concentrations of D-achatin Peptides

The *Platynereis* (A), *Aplysia* (B), *Branchiostoma* (C), and *Saccoglossus* (D) receptors were tested with the species-specific achatin peptide containing a D-Phe. Data, representing luminescence units relative to the control response (1 mM histamine), are shown as mean ± SEM (n = 3). Dose-response curves fitted to the data are shown. EC_{50} values are listed in Table 1. Responses to L-achatin peptides and a separate set of measurements normalized to MIP receptor are shown in Figure S5.

A *Platynereis* Ortholog of Thyrotropin-Releasing Hormone

The identification of the ligand for the annelid ortholog of vertebrate thyrotropin-releasing hormone receptors sheds light on the evolution of this family. Thyrotropin-releasing hormones (TRHs) have so far only been identified in deuterostomes. However, the presence of GPCRs in some protostomes showing orthology to deuterostome TRH receptors suggested that TRH orthologs are present in some protostomes (Jékely, 2013; Mirabeau and Joly, 2013). We identified EFLGa as the ligand of the *Platynereis* TRH receptor ortholog (Figure 4; Table 1). EFLGa has already been described in *Platynereis*, other annelids, and mollusks (Conzelmann et al., 2013a), but its identity as a potential TRH ortholog was not recognized, since the sequence of the mature peptide (FSEFLGamide) is not similar to vertebrate TRH (pQHPamide, with pQ indicating pyroglutamate). Intriguingly, however, *Platynereis* EFLGa shows some similarity to the TRH ortholog of the sea urchin *Strongylocentrotus purpuratus* (Rowe and Elphick, 2012) (Figure S4C). Uniquely among the deuterostomes, the sea urchin peptide (Q[W/Y]PGamide) is a Gamide. This sea urchin sequence shows intermediate characteristics and bridges the gap between the protostome and deuterostome families, further suggesting the orthology of *Platynereis* EFLGa and deuterostome TRH.

An Ancient Bilaterian Family of Achatin Receptors Activated by a D-peptide

Our sequence analyses provide additional support for the widespread conservation of neuropeptide-GPCR signaling pairs. This allows us to predict ligands for several lophotrochozoan GPCRs, including receptors from *Capitella teleta*, *Aplysia californica*, and *Crassostrea gigas*, and deuterostome receptors from *Branchiostoma* and *Saccoglossus* (Table S4).

To test our predictions, we focused in more detail on the achatin family. Achatin receptors represent one of the ancient bilaterian families we identified (Figure 4), allowing us to test the feasibility of ligand predictions across Bilateria. We performed activation assays with putative achatin receptors from the sea slug *A. californica*, and the deuterostomes *S. kowalewskii* and *B. floridae* (Figure 5; Figure S5).

Achatins are 4-amino-acid peptides that share the G[FYM][GAF][DNG] motif (Figure S4B). Achatin was identified in the giant snail Achatina fulica (Kamatani et al., 1989) and was shown to contain a D-amino acid (Gly-D-Phe-Ala-Asp). Achatin is a potent neuroexcitatory peptide, and this activity is specific to the D-form. We therefore also tested species-specific achatin ligands synthesized with a D-Phe. We found that D-achatins activated all receptors with EC_{50} values in the nanomolar range, but L-achatins were poor agonists (in the high micromolar, millimolar range) (Figure 5; Figure S5; Table1).

These results show that the D-form of achatin has been conserved throughout evolution as a ligand for the bilaterian orthology group of achatin GPCRs. This indicates that our receptor-ligand predictions (Table S4) are reliable and can be used to predict receptor-ligand pairs across Bilateria.

DISCUSSION

Here, we described a large-scale screen for neuropeptide GPCRs in *Platynereis*. Our combinatorial strategy allowed us to quickly screen 10,962 receptor-ligand combinations without the need to assay all combinations individually. We could identify specific receptor-ligand pairs and study them in individual assays. However, measurements with peptide mixtures also revealed the high specificity of the interactions. We screened each receptor against 126 neuropeptides, but we found strong activation by only one or two related peptides. This strategy is generally applicable for GPCR ligand screens and could speed up ligand discovery. Here, we reported 19 deorphanized receptors and 25 validated receptor-ligand pairs from *Platynereis*. Based on these results, we now provide an updated overview (Jékely, 2013) of the phyletic distribution of peptides and peptide receptors in metazoans (Figure S6).

Importantly, many of the receptors we found represent GPCR families for which the ligand could not have been predicted based on available data. As more ligand-receptor pairs are discovered, however, ligand predictions will become increasingly straightforward.

Our results illuminate large, poorly studied areas of the GPCR sequence space within the lophotrochozoans, where only few receptors have been characterized biochemically.

The *Platynereis* GPCR-ligand pairs and our bioinformatic analyses provide further evidence for the long-term coevolution of

neuropeptides and their receptors across bilaterians. One exception may be the FMRFamide receptors of arthropods (Cazzamali and Grimmelikhuijzen, 2002). These GPCRs evolved in stem arthropods but respond to FMRFamides derived from an older FMRFamide precursor. The presence of the ancestral bilaterian FMRFamide receptor in hemipterans indicates that the two receptors coexisted for some time, but the ancestral receptor was lost from most arthropod genomes.

Receptor-ligand conservation allowed us to predict the peptide ligand for many yet uncharacterized receptors from lophotrochozoans and non-vertebrate deuterostomes. Using achatin as an example, we demonstrated that ligand predictions work across phyla.

Our results also provide information about the complexity of neuroendocrine signaling in the urbilaterian. We present receptor or ligand evidence for urbilaterian peptidergic systems, including TRH, elevenin, FMRFamide, and achatin signaling. The presence of TRH orthologs in annelids and mollusks is particularly interesting and begs the question whether TRH signaling regulates thyroid hormone synthesis in these animals, similar to its function in some vertebrates (Laudet, 2011). Thyroid hormones have been described from *Aplysia* (Heyland et al., 2006) and may have ancestrally regulated postembryonic developmental transitions in bilaterians (Laudet, 2011). This possibility is supported by the parallel loss of TRH and thyroid hormone receptors from the ecdysozoans that use ecdysone to orchestrate life-cycle transitions (Laudet, 2011).

The deorphanized GPCR dataset we describe here represents a valuable resource for the study of neuropeptide signaling in invertebrates, including annelids and mollusks. Similar combinatorial screening strategies could also be used for other species and receptor classes and could speed up GPCR ligand discovery.

EXPERIMENTAL PROCEDURES

Gene Identification and Receptor Cloning

Platynereis genes were identified from a *Platynereis* mixed-stages transcriptome assembly (Conzelmann et al., 2013a). GPCRs were cloned from cDNA or expressed sequence tag clones into pcDNA3.1(+) (Thermo Fisher Scientific). Forward primers consisted of a spacer (5′-ACAATA-3′) followed by a BamHI or EcoRI restriction site, the Kozak consensus sequence (5′-CGCCACC-3′), the start codon (5′-ATG-3′) and a sequence corresponding to the target sequence. Reverse primers consisted of a spacer (5′-ACAATA-3′), a NotI restriction site, a STOP codon, and reverse complementary sequence to the target sequence. Primers were designed to end with a C or G with 72°C melting temperature. PCR was performed using Phusion polymerase (New England Biolabs GmbH).

Open reading frames coding for achatin GPCRs from *Aplysia* (XP_005106606.1), *Branchiostoma* (XM_002600016.1), and *Saccoglossus* (XM_006815704.1) were generated by gene synthesis (GenScript). The sequence of the *Branchiostoma* receptor was complemented based on information from Metazome v3.0.

Cell Culture and Receptor Deorphanization

CHO-K1 cells were kept in Ham's F12 Nut Mix medium (Thermo Fisher Scientific) with 10% fetal bovine serum and PenStrep. We used a stable cell line expressing a luminescent reporter apoaequorin-GFP fusion protein (G5A) that has been shown to emit more light than apoaequorin alone (Baubet et al., 2000). Cells were seeded in 96-well plates (Thermo Fisher Scientific) at ~10,000 cells/well. After 1 day, cells were transfected with plasmids encoding a GPCR and the promiscuous Gα-16 protein (60 ng each) using 1.5 μl of the transfection reagent TurboFect (Thermo Fisher Scientific). To measure dose-response curves, cells were also cotransfected with the G5A construct to increase the expression of the reporter. After 2 days of expression, the medium was removed and replaced with Hank's balanced salt solution (HBSS) supplemented with 1.8 mM Ca^{2+}, 10 mM glucose, and 1 mM coelenterazine h (Promega). After incubation at 37°C for 2 hr, cells were tested by adding synthetic peptides (GenScript) in HBSS supplemented with 1.8 mM Ca^{2+} and 10 mM glucose. Luminescence was recorded for 45–60 s in a plate reader (BioTek Synergy Mx or Synergy H4, BioTek). Data during the screen were normalized using the response of *Platynereis* MIP receptor to 10 nM MIP-7 (Conzelmann et al., 2013b). The final dose-response curves were normalized using the response of the same well to 1 mM histamine that was recorded following the peptide treatment. To record the second set of dose-response curves, data were normalized using the response of the *Platynereis* MIP receptor to 10 nM MIP-7. The MIP control values were recorded from three separate wells on each plate.

Deorphanization Strategy

All *Platynereis* GPCRs were first tested with three peptide mixtures containing up to 48 synthetic peptides (Table S2) at 1 μM each. Measurements were done in triplicate. Those GPCRs that showed a response compared to the negative control (empty pcDNA3.1) were tested further. We tried to predict the specific ligand from the active mixture based on receptor clustering using CLANS2 (Frickey and Lupas, 2004) and tested individual peptides where deorphanized orthologs were identified. Alternatively, GPCRs were tested with submixtures of synthetic peptides arranged in three matrices, corresponding to the decomposition of mixtures 1–3 (Table S2). The combination of mixtures that elicited a response pointed to the active ligand. These measurements were done in single wells or in duplicate. Using this information, individual ligands were tested. After identification of a bona fide ligand, dose-response curves were recorded using concentrations between 0.01 nM and 200 μM. Data for dose-response curves were recorded in triplicate for each concentration. Dose-response curves were fitted with a four-parameter curve using Prism 6 (GraphPad).

Bioinformatics

For clustering, a previous collection of GPCRs (Jékely, 2013) was complemented with deorphanized *Platynereis* sequences and deorphanized GPCR sequences from human, mouse, and rat retrieved from the IUPHAR database (Pawson et al., 2014). Deorphanized GPCRs from *D. melanogaster* and other insects (Caers et al., 2012), *C. elegans* (Frooninckx et al., 2012), and other organisms (Bigot et al., 2014; Conzelmann et al., 2013b; Cox et al., 1997; Kim et al., 2010; Tensen et al., 1998a, 1998b) were also included. Furthermore, the sequences of all *Platynereis* GPCRs tested in the screen were used to initiate BLAST searches at NCBI with an e-value cutoff of 1e-50, and all hits were downloaded and added to the collection. Deorphanized sequences were tagged "deorphanized." All sequences were complemented with taxonomic information based on the NCBI taxonomy identifier (taxid) using a bioperl script (https://github.com/JekelyLab/GPCR_Clans_Maps), or taxonomy information was added manually. Redundant sequences were removed from the collection using CD-HIT (Li and Godzik, 2006). Clustering analysis was done using CLANS2 (Frickey and Lupas, 2004) with a BLOSUM62 matrix and a p value cutoff of 1.e-50. Deorphanized and orphan *Platynereis* receptors were clustered separately with their respective orthologs. Clusters that contained no *Platynereis* sequences were removed from the map (including relaxin, melanocortin, bradykinin, urotensins, and neurotensin receptors).

ACCESSION NUMBERS

The accession numbers of all *Platynereis* GPCRs tested here are GenBank: KP293941–KP294026 and KP420212–KP420214.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.06.052.

AUTHOR CONTRIBUTIONS

G.J. conceived the experiments; G.J. and P.B. designed methodology; P.B. performed all investigations and conducted the formal analysis; G.J. wrote the first draft of the manuscript; G.J. and P.B. reviewed and edited the manuscript; and G.J. supervised the study and acquired funding.

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REFERENCES

Baubet, V., Le Mouellic, H., Campbell, A.K., Lucas-Meunier, E., Fossier, P., and Brúlet, P. (2000). Chimeric green fluorescent protein-aequorin as bioluminescent Ca2+ reporters at the single-cell level. Proc. Natl. Acad. Sci. USA *97*, 7260–7265.

Bigot, L., Beets, I., Dubos, M.-P., Boudry, P., Schoofs, L., and Favrel, P. (2014). Functional characterization of a short neuropeptide F-related receptor in a lophotrochozoan, the mollusk Crassostrea gigas. J. Exp. Biol. *217*, 2974–2982.

Caers, J., Verlinden, H., Zels, S., Vandersmissen, H.P., Vuerinckx, K., and Schoofs, L. (2012). More than two decades of research on insect neuropeptide GPCRs: an overview. Front. Endocrinol. (Lausanne) *3*, 151.

Cazzamali, G., and Grimmelikhuijzen, C.J.P. (2002). Molecular cloning and functional expression of the first insect FMRFamide receptor. Proc. Natl. Acad. Sci. USA *99*, 12073–12078.

Chang, J.C., Yang, R.B., Adams, M.E., and Lu, K.H. (2009). Receptor guanylyl cyclases in Inka cells targeted by eclosion hormone. Proc. Natl. Acad. Sci. USA *106*, 13371–13376.

Collins, J.J., 3rd, Hou, X., Romanova, E.V., Lambrus, B.G., Miller, C.M., Saberi, A., Sweedler, J.V., and Newmark, P.A. (2010). Genome-wide analyses reveal a role for peptide hormones in planarian germline development. PLoS Biol. *8*, e1000509.

Conzelmann, M., Offenburger, S.-L., Asadulina, A., Keller, T., Münch, T.A., and Jékely, G. (2011). Neuropeptides regulate swimming depth of *Platynereis* larvae. Proc. Natl. Acad. Sci. USA *108*, E1174–E1183.

Conzelmann, M., Williams, E.A., Krug, K., Franz-Wachtel, M., Macek, B., and Jékely, G. (2013a). The neuropeptide complement of the marine annelid *Platynereis dumerilii*. BMC Genomics *14*, 906.

Conzelmann, M., Williams, E.A., Tunaru, S., Randel, N., Shahidi, R., Asadulina, A., Berger, J., Offermanns, S., and Jékely, G. (2013b). Conserved MIP receptor-ligand pair regulates *Platynereis* larval settlement. Proc. Natl. Acad. Sci. USA *110*, 8224–8229.

Cox, K.J., Tensen, C.P., Van der Schors, R.C., Li, K.W., van Heerikhuizen, H., Vreugdenhil, E., Geraerts, W.P., and Burke, J.F. (1997). Cloning, characterization, and expression of a G-protein-coupled receptor from *Lymnaea stagnalis* and identification of a leucokinin-like peptide, PSFHSWSamide, as its endogenous ligand. J. Neurosci. *17*, 1197–1205.

Cropper, E.C., Tenenbaum, R., Kolks, M.A., Kupfermann, I., and Weiss, K.R. (1987). Myomodulin: a bioactive neuropeptide present in an identified cholinergic buccal motor neuron of *Aplysia*. Proc. Natl. Acad. Sci. USA *84*, 5483–5486.

Dircksen, H., Neupert, S., Predel, R., Verleyen, P., Huybrechts, J., Strauss, J., Hauser, F., Stafflinger, E., Schneider, M., Pauwels, K., et al. (2011). Genomics, transcriptomics, and peptidomics of *Daphnia pulex* neuropeptides and protein hormones. J. Proteome Res. *10*, 4478–4504.

Frickey, T., and Lupas, A. (2004). CLANS: a Java application for visualizing protein families based on pairwise similarity. Bioinformatics *20*, 3702–3704.

Frooninckx, L., Van Rompay, L., Temmerman, L., Van Sinay, E., Beets, I., Janssen, T., Husson, S.J., and Schoofs, L. (2012). Neuropeptide GPCRs in *C. elegans*. Front. Endocrinol. (Lausanne) *3*, 167.

Hauser, F., Neupert, S., Williamson, M., Predel, R., Tanaka, Y., and Grimmelikhuijzen, C.J.P. (2010). Genomics and peptidomics of neuropeptides and protein hormones present in the parasitic wasp *Nasonia vitripennis*. J. Proteome Res. *9*, 5296–5310.

Hewes, R.S., and Taghert, P.H. (2001). Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. Genome Res. *11*, 1126–1142.

Heyland, A., Price, D.A., Bodnarova-Buganova, M., and Moroz, L.L. (2006). Thyroid hormone metabolism and peroxidase function in two non-chordate animals. J. Exp. Zoolog. B Mol. Dev. Evol. *306*, 551–566.

Hoek, R.M., Li, K.W., van Minnen, J., Lodder, J.C., de Jong-Brink, M., Smit, A.B., and van Kesteren, R.E. (2005). LFRFamides: a novel family of parasitation-induced -RFamide neuropeptides that inhibit the activity of neuroendocrine cells in *Lymnaea stagnalis*. J. Neurochem. *92*, 1073–1080.

Holman, G.M., Cook, B.J., and Nachman, R.J. (1986). Isolation, primary structure and synthesis of leucomyosuppressin, an insect neuropeptide that inhibits spontaneous contractions of the cockroach hindgut. Comp. Biochem. Physiol. C Comp. Pharmacol. *85*, 329–333.

Janssen, T., Lindemans, M., Meelkop, E., Temmerman, L., and Schoofs, L. (2010). Coevolution of neuropeptidergic signaling systems: from worm to man. Ann. N Y Acad. Sci. *1200*, 1–14.

Jékely, G. (2013). Global view of the evolution and diversity of metazoan neuropeptide signaling. Proc. Natl. Acad. Sci. USA *110*, 8702–8707.

Jékely, G., Colombelli, J., Hausen, H., Guy, K., Stelzer, E., Nédélec, F., and Arendt, D. (2008). Mechanism of phototaxis in marine zooplankton. Nature *456*, 395–399.

Kamatani, Y., Minakata, H., Kenny, P.T., Iwashita, T., Watanabe, K., Funase, K., Sun, X.P., Yongsiri, A., Kim, K.H., Novales-Li, P., et al. (1989). Achatin-I, an endogenous neuroexcitatory tetrapeptide from *Achatina fulica Férussac* containing a D-amino acid residue. Biochem. Biophys. Res. Commun. *160*, 1015–1020.

Kim, Y.-J., Bartalska, K., Audsley, N., Yamanaka, N., Yapici, N., Lee, J.-Y., Kim, Y.-C., Markovic, M., Isaac, E., Tanaka, Y., and Dickson, B.J. (2010). MIPs are ancestral ligands for the sex peptide receptor. Proc. Natl. Acad. Sci. USA *107*, 6520–6525.

Laudet, V. (2011). The origins and evolution of vertebrate metamorphosis. Curr. Biol. *21*, R726–R737.

Leung, D.W., Spencer, S.A., Cachianes, G., Hammonds, R.G., Collins, C., Henzel, W.J., Barnard, R., Waters, M.J., and Wood, W.I. (1987). Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature *330*, 537–543.

Li, W., and Godzik, A. (2006). Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics *22*, 1658–1659.

Li, B., Predel, R., Neupert, S., Hauser, F., Tanaka, Y., Cazzamali, G., Williamson, M., Arakane, Y., Verleyen, P., Schoofs, L., et al. (2008). Genomics, transcriptomics, and peptidomics of neuropeptides and protein hormones in the red flour beetle *Tribolium castaneum*. Genome Res. *18*, 113–122.

Lingueglia, E., Champigny, G., Lazdunski, M., and Barbry, P. (1995). Cloning of the amiloride-sensitive FMRFamide peptide-gated sodium channel. Nature *378*, 730–733.

Lowe, D.G., Chang, M.S., Hellmiss, R., Chen, E., Singh, S., Garbers, D.L., and Goeddel, D.V. (1989). Human atrial natriuretic peptide receptor defines a new paradigm for second messenger signal transduction. EMBO J. *8*, 1377–1384.

Mertens, I., Meeusen, T., Huybrechts, R., De Loof, A., and Schoofs, L. (2002). Characterization of the short neuropeptide F receptor from *Drosophila melanogaster*. Biochem. Biophys. Res. Commun. *297*, 1140–1148.

Mirabeau, O., and Joly, J.-S. (2013). Molecular evolution of peptidergic signaling systems in bilaterians. Proc. Natl. Acad. Sci. USA *110*, E2028–E2037.

Oumi, T., Ukena, K., Matsushima, O., Ikeda, T., Fujita, T., Minakata, H., and Nomoto, K. (1995). The GGNG peptides: novel myoactive peptides isolated from the gut and the whole body of the earthworms. Biochem. Biophys. Res. Commun. *216*, 1072–1078.

Park, Y., Kim, Y.-J., and Adams, M.E. (2002). Identification of G protein-coupled receptors for Drosophila PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. Proc. Natl. Acad. Sci. USA *99*, 11423–11428.

Pawson, A.J., Sharman, J.L., Benson, H.E., Faccenda, E., Alexander, S.P.H., Buneman, O.P., Davenport, A.P., McGrath, J.C., Peters, J.A., Southan, C., et al.; NC-IUPHAR (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. Nucleic Acids Res. *42*, D1098–D1106.

Rajpara, S.M., Garcia, P.D., Roberts, R., Eliassen, J.C., Owens, D.F., Maltby, D., Myers, R.M., and Mayeri, E. (1992). Identification and molecular cloning of a neuropeptide Y homolog that produces prolonged inhibition in *Aplysia* neurons. Neuron *9*, 505–513.

Randel, N., Asadulina, A., Bezares-Calderón, L.A., Verasztó, C., Williams, E.A., Conzelmann, M., Shahidi, R., and Jékely, G. (2014). Neuronal connectome of a sensory-motor circuit for visual navigation. eLife *3*, e02730.

Rechler, M.M., and Nissley, S.P. (1985). The nature and regulation of the receptors for insulin-like growth factors. Annu. Rev. Physiol. *47*, 425–442.

Roller, L., Yamanaka, N., Watanabe, K., Daubnerová, I., Zitňan, D., Kataoka, H., and Tanaka, Y. (2008). The unique evolution of neuropeptide genes in the silkworm *Bombyx mori*. Insect Biochem. Mol. Biol. *38*, 1147–1157.

Rowe, M.L., and Elphick, M.R. (2012). The neuropeptide transcriptome of a model echinoderm, the sea urchin *Strongylocentrotus purpuratus*. Gen. Comp. Endocrinol. *179*, 331–344.

Telford, M.J., and Copley, R.R. (2011). Improving animal phylogenies with genomic data. Trends Genet. *27*, 186–195.

Tensen, C.P., Cox, K.J., Burke, J.F., Leurs, R., van der Schors, R.C., Geraerts, W.P., Vreugdenhil, E., and Heerikhuizen, H. (1998a). Molecular cloning and characterization of an invertebrate homologue of a neuropeptide Y receptor. Eur. J. Neurosci. *10*, 3409–3416.

Tensen, C.P., Cox, K.J., Smit, A.B., van der Schors, R.C., Meyerhof, W., Richter, D., Planta, R.J., Hermann, P.M., van Minnen, J., Geraerts, W.P., et al. (1998b). The lymnaea cardioexcitatory peptide (LyCEP) receptor: a G-protein-coupled receptor for a novel member of the RFamide neuropeptide family. J. Neurosci. *18*, 9812–9821.

Tosches, M.A., Bucher, D., Vopalensky, P., and Arendt, D. (2014). Melatonin signaling controls circadian swimming behavior in marine zooplankton. Cell *159*, 46–57.

Tunaru, S., Lättig, J., Kero, J., Krause, G., and Offermanns, S. (2005). Characterization of determinants of ligand binding to the nicotinic acid receptor GPR109A (HM74A/PUMA-G). Mol. Pharmacol. *68*, 1271–1280.

Veenstra, J.A. (2010). Neurohormones and neuropeptides encoded by the genome of *Lottia gigantea*, with reference to other mollusks and insects. Gen. Comp. Endocrinol. *167*, 86–103.

Veenstra, J.A. (2011). Neuropeptide evolution: neurohormones and neuropeptides predicted from the genomes of *Capitella teleta* and *Helobdella robusta*. Gen. Comp. Endocrinol. *171*, 160–175.

Venkatesh, B., Lee, A.P., Ravi, V., Maurya, A.K., Lian, M.M., Swann, J.B., Ohta, Y., Flajnik, M.F., Sutoh, Y., Kasahara, M., et al. (2014). Elephant shark genome provides unique insights into gnathostome evolution. Nature *505*, 174–179.

Williams, E.A., Conzelmann, M., and Jékely, G. (2015). Myoinhibitory peptide regulates feeding in the marine annelid Platynereis. Front. Zool. *12*, 1.

Willows, A.O., Pavlova, G.A., and Phillips, N.E. (1997). Modulation of ciliary beat frequency by neuropeptides from identified molluscan neurons. J. Exp. Biol. *200*, 1433–1439.

Xie, F., London, S.E., Southey, B.R., Annangudi, S.P., Amare, A., Rodriguez-Zas, S.L., Clayton, D.F., and Sweedler, J.V. (2010). The zebra finch neuropeptidome: prediction, detection and expression. BMC Biol. *8*, 28.

Zantke, J., Bannister, S., Rajan, V.B.V., Raible, F., and Tessmar-Raible, K. (2014). Genetic and genomic tools for the marine annelid *Platynereis dumerilii*. Genetics *197*, 19–31.

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RESEARCH ARTICLE Open Access

Ancient coexistence of norepinephrine, tyramine, and octopamine signaling in bilaterians

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Abstract

Background: Norepinephrine/noradrenaline is a neurotransmitter implicated in arousal and other aspects of vertebrate behavior and physiology. In invertebrates, adrenergic signaling is considered absent and analogous functions are performed by the biogenic amines octopamine and its precursor tyramine. These chemically similar transmitters signal by related families of G-protein-coupled receptors in vertebrates and invertebrates, suggesting that octopamine/tyramine are the invertebrate equivalents of vertebrate norepinephrine. However, the evolutionary relationships and origin of these transmitter systems remain unclear.

Results: Using phylogenetic analysis and receptor pharmacology, here we have established that norepinephrine, octopamine, and tyramine receptors coexist in some marine invertebrates. In the protostomes *Platynereis dumerilii* (an annelid) and *Priapulus caudatus* (a priapulid), we have identified and pharmacologically characterized adrenergic α1 and α2 receptors that coexist with octopamine α, octopamine β, tyramine type 1, and tyramine type 2 receptors. These receptors represent the first examples of adrenergic receptors in protostomes. In the deuterostome *Saccoglossus kowalevskii* (a hemichordate), we have identified and characterized octopamine α, octopamine β, tyramine type 1, and tyramine type 2 receptors, representing the first examples of these receptors in deuterostomes. *S. kowalevskii* also has adrenergic α1 and α2 receptors, indicating that all three signaling systems coexist in this animal. In phylogenetic analysis, we have also identified adrenergic and tyramine receptor orthologs in xenacoelomorphs.

Conclusions: Our results clarify the history of monoamine signaling in bilaterians. Given that all six receptor families (two each for octopamine, tyramine, and norepinephrine) can be found in representatives of the two major clades of Bilateria, the protostomes and the deuterostomes, all six receptors must have coexisted in the last common ancestor of the protostomes and deuterostomes. Adrenergic receptors were lost from most insects and nematodes, and tyramine and octopamine receptors were lost from most deuterostomes. This complex scenario of differential losses cautions that octopamine signaling in protostomes is not a good model for adrenergic signaling in deuterostomes, and that studies of marine animals where all three transmitter systems coexist will be needed for a better understanding of the origin and ancestral functions of these transmitters.

Keywords: Octopamine, Tyramine, Norepinephrine, Noradrenaline, GPCR evolution, Neurotransmitter, *Saccoglossus*, *Platynereis*, *Priapulus*, Xenacoelomorpha

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Background

Norepinephrine is a major neurotransmitter in vertebrates with a variety of functions, including roles in promoting wakefulness and arousal [1], regulating aggression [2], and autonomic functions such a heart beat [3]. Signaling by the monoamine octopamine in protostome invertebrates is often considered equivalent to vertebrate adrenergic signaling [4], with analogous roles in promoting aggression and wakefulness in flies [5, 6], and the regulation of heart rate in annelids and arthropods [7, 8]. Octopamine is synthesized from tyramine (Fig. 1a) which itself also acts as a neurotransmitter or neuromodulator in arthropods and nematodes [4, 9–15]. Octopamine and norepinephrine are chemically similar, are synthesized by homologous enzymes [16, 17], and signal by similar but not orthologous G-protein-coupled receptors (GPCRs) [4, 18].

Tyramine also signals via non-orthologous receptors in invertebrates and vertebrates. In insects and nematodes, tyramine signals via a GPCR that is related to octopamine receptors [12, 19]. In vertebrates, tyramine is only present at low levels and signals via the trace-amine receptors, a vertebrate-specific GPCR family only distantly related to the invertebrate tyramine receptors [20, 21]. Given these differences, the precise evolutionary relationships of these monoamine signaling systems are unclear.

The evolution of neurotransmitter systems has been analyzed by studying the distribution of monoamines or biosynthetic enzymes in different organisms [22]. This approach has limitations, however, because some of the biosynthetic enzymes are not specific to one substrate [16] and because trace amounts of several monoamines are found across many organisms, even if specific receptors are often absent [22]. For example, even if invertebrates can synthesize trace amounts of norepinephrine, these are not considered to be active neuronal signaling molecules, because the respective receptors are lacking. Consequently, the presence of specific monoamine receptors is the best indicator that a particular monoamine is used in neuronal signaling [11, 23].

To clarify the evolutionary history of adrenergic, octopamine, and tyramine signaling in animals, we undertook a comparative phylogenetic and pharmacological study of these receptor families in bilaterians. Bilaterians—animals with bilateral symmetry—comprise protostomes, deuterostomes, and xenacoelomorphs [24]. Deuterostomes include chordates and ambulacrarians (hemichordates and echinoderms), and protostomes are formed by the clades Ecdysozoa, Lophotrochozoa (Spiralia), and Chaetognatha. Ecdysozoa includes arthropods, nematodes, priapulids and other phyla. Lophotrochozoa includes annelids, mollusks, and other, mostly marine groups. Xenacoelomorpha, a group including acoel flatworms, nemertodermatids, and *Xenoturbella*, has been proposed to belong to the deuterostomes, or represent a sister group to all remaining bilaterians [25–27]. Here, we have attempted to establish the orthologous relationships of adrenergic, octopamine, and tyramine receptors across bilaterians. We found that six receptor families originated at the base of the bilaterian tree. We then pharmacologically characterized adrenergic receptors from an annelid and a priapulid, and octopamine and tyramine receptors from an annelid and a hemichordate. The broad phylogenetic sampling and comparative pharmacology paint a richer picture of the evolution of these receptors, characterized by ancestral coexistence and multiple independent losses.

Results

Using database searches, sequence-similarity-based clustering, and phylogenetic analysis, we reconstructed the phylogeny of α1, α2, and β adrenergic, octopamine α, octopamine β, and tyramine type-1 and type-2 receptors. Each family formed well-resolved clusters in a sequence-similarity-based clustering analysis and well-supported clades in molecular phylogenetic analysis (Fig. 1b, c and Additional file 1).

We identified several invertebrate GPCR sequences that were similar to vertebrate adrenergic α1 and α2 receptors (Fig. 1b, c). An adrenergic α1 receptor ortholog is present in the sea urchin *Strongylocentrotus purpuratus*. Adrenergic α1 and α2 receptors were both present in *Saccoglossus kowalevskii,* a hemichordate deuterostome (Fig. 1b, c and Additional files 1, 2, and 3), as previously reported [28]. We also identified adrenergic α1 and α2 receptor orthologs in annelids and mollusks (members of the Lophotrochozoa), including *Aplysia californica,* and in the priapulid worm *Priapulus caudatus* (member of the Ecdysozoa) (Fig. 1b, c and Additional files 1, 2, and 3). Adrenergic α receptors are also present in a few arthropods, including the crustacean *Daphnia pulex* and the moth *Chilo suppressalis* (the *Chilo* α2 receptor was first described as an octopamine receptor [29]), but are absent from most other insects (Additional files 1, 2, and 3). Adrenergic α2 receptors are also present in the xenacoelomorphs *Xenoturbella bocki* and *Meara stichopi. M. stichopi* also has two adrenergic α1 receptor orthologs (Fig. 1c and Additional files 1, 2, and 3).

The identification of adrenergic α1 and of α2 receptor orthologs in ambulacrarians, lophotrochozoans, ecdysozoans, and xenacoelomorphs indicates that both families were present in the bilaterian last common ancestor.

Adrenergic β receptors are found in chordates, including urochordates and cephalochordates. In addition, we identified an adrenergic β receptor ortholog in the

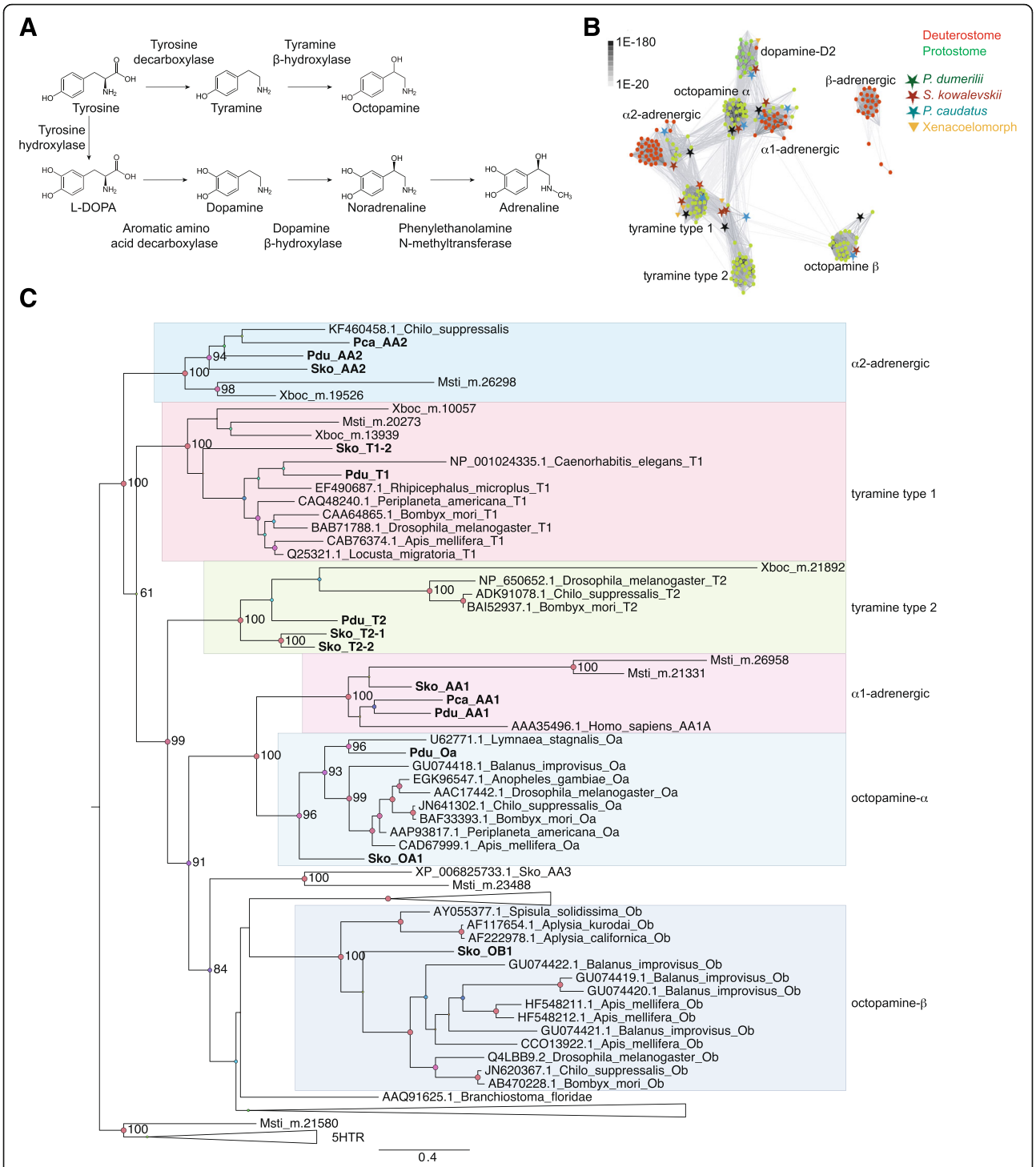

Fig. 1 Biosynthesis of monoamines and phylogeny of adrenergic, tyramine, and octopamine G-protein-coupled receptor (GPCR) sequences. **a** Biosynthesis of tyramine, octopamine, norepinephrine, and epinephrine from tyrosine. The enzymes catalyzing the reaction steps are indicated. **b** Sequence-similarity-based cluster map of bilaterian octopamine, tyramine, and adrenergic GPCRs. Nodes correspond to individual GPCRs and are colored based on taxonomy. Edges correspond to BLAST connections of *P* value >1e−70. **c** Simplified phylogenetic tree of bilaterian adrenergic, tyramine, and octopamine GPCR sequences. The tree is rooted on 5HT receptors (5HTR). *Abbreviations*: Pdu *P. dumerilii*, Pca *P. caudatus*, Sko *S. kowalevskii*, Msti *M. stichopi*, Xboc *X. bocki*

xenacoelomorph *M. stichopi* (Additional file 4). If xenacoelomorphs are sister to all remaining bilaterians, then this receptor family also originated at the base of Bilateria and was lost from all protostomes.

To characterize the ligand specificities of these putative invertebrate adrenergic receptors, we cloned them from *S. kowalevskii*, *Priapulus caudatus*, and the marine annelid *Platynereis dumerilii*. We performed in vitro GPCR activation experiments using a Ca^{2+}-mobilization assay [30, 31]. We found that norepinephrine and epinephrine activated both the adrenergic α1 and α2 receptors from all three species with half maximal effective concentration (EC_{50}) values in the high nanomolar range or lower. In contrast, tyramine, octopamine, and dopamine were either inactive or only activated the receptors at concentrations approximately two orders of magnitude higher (Fig. 2, Table 1). These phylogenetic and pharmacological results collectively establish these invertebrate receptors as bona fide adrenergic α receptors.

To investigate if adrenergic signaling coexists with octopamine and tyramine signaling in protostomes, we searched for octopamine and tyramine receptors in *Platynereis dumerilii* and *Priapulus caudatus*. In phylogenetic and clustering analyses, we identified orthologs for tyramine type 1 and type 2 and octopamine α and β receptors in both species (Fig. 1b, c and Additional files 5, 6, 7, and 8). We performed activation assays with the *Platynereis dumerilii* receptors. The tyramine type 1 and type 2 receptors orthologs were preferentially activated by tyramine with EC_{50} values in the nanomolar range (Fig. 3, Table 1). The *Platynereis dumerilii* octopamine α receptor was activated by octopamine at a lower concentration than by tyramine and dopamine (Fig. 4, Table 1). The *Platynereis dumerilii* octopamine β receptor was

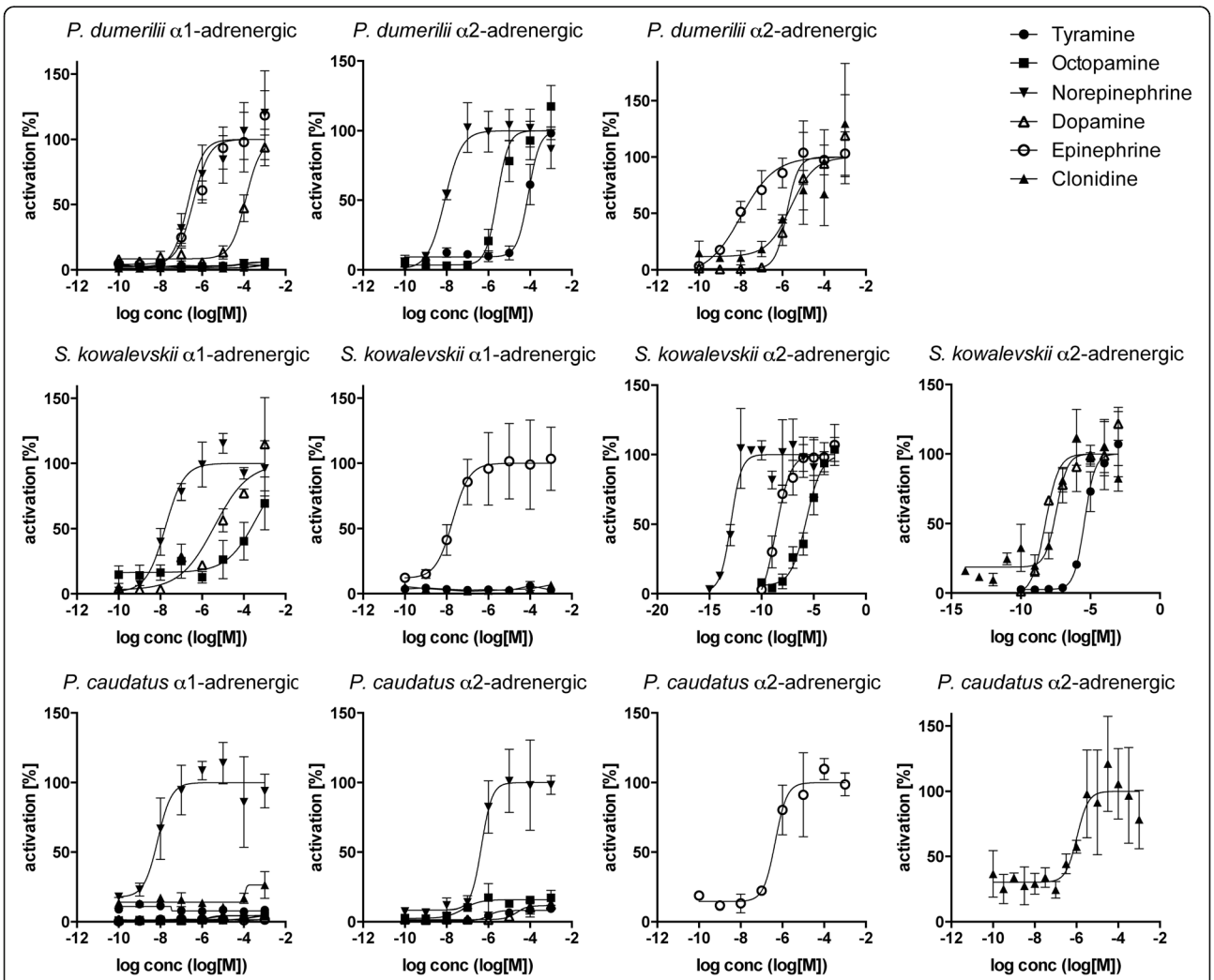

Fig. 2 Dose–response curves of adrenergic G-protein-coupled receptors from *Platynereis dumerilii*, *Priapulus caudatus*, and *Saccoglossus kowalevskii* treated with varying concentrations of ligand. Data, representing luminescence units relative to the maximum of the fitted dose–response curves, are shown as mean ± standard error of the mean (n = 3). Half maximal effective concentration (EC_{50}) values and significance values are listed in Table 1

Table 1 Half maximal effective concentration (EC_{50}) (M) and half maximal inhibitory concentration (IC_{50}) (M) values of all tested G-protein-coupled receptors with the indicated ligands or inhibitors

EC50 (M)/IC50 (M)	Tyramine	Octopamine	Clonidine	Norepinephrine	Dopamine	Epinephrine	Yohimbine	Mianserin
Platynereis dumerilii α1-adrenergic	inactive	inactive	inactive	**2.1E – 07*****	1.2E – 04***	3.7E – 07 ns	4.4E – 06	3.7E – 06
95% CI				1.0E – 007 to 4.2E – 007	2.7E – 005 to 0.00056	1.3E – 007 to 1.1E – 006	2.3E – 006 to 8.2E – 006	1.9E – 006 to 7.2E – 006
P. dumerilii α2-adrenergic	8.4E – 05	2.7E – 06***	2.6E – 06	**8.2E – 09*****	1.6E – 06	1.1E – 08 ns	5.7E – 06	2.5E – 05
95% CI	2.8E – 005 to 0.00024	6.683E – 007 to 1.0E – 005	2.4E – 007 to 2.7E – 005	5.7E – 009 to 1.1E – 008	8.3E – 007 to 3.2E – 006	5.0E – 009 to 2.2E – 008	3.5E – 006 to 9.1E – 006	1.2E – 005 to 5.1E – 005
S. kowalevskii α1-adrenergic	inactive	inactive	inactive	**1.7E – 08*****	3.8E – 06***	1.9E – 08 ns	1.3E – 05	4.5E – 06
95% CI				1.0E – 008 to 2.7E – 008	1.9E – 007 to 7.4E – 005	9.0E – 009 to 4.1E – 008	7.6E – 006 to 2.2E – 005	1.6E – 006 to 1.1E – 005
S. kowalevskii α2-adrenergic	3.7E – 06	1.9E – 06	3.6E – 08	**1.2E – 13*****	5.6E – 09	2.3E – 09***	3.3E – 07	inactive
95% CI	2.0E – 006 to 6.8E – 006	2.5E – 007 to 1.4E – 005	6.7E – 009 to 1.9E – 007	6.7E – 014 to 1.9E – 013	3.3E – 009 to 9.4E – 009	1.1E – 009 to 4.6E – 009	2.6E – 007 to 4.0E – 007	
Priapulus caudatus α1-adrenergic	inactive	inactive	inactive	**7.5E – 09**	inactive	inactive	inactive	inactive
95% CI				4.0E – 009 to 1.3E – 008				
P. caudatus α2-adrenergic	inactive	inactive	1.1E – 06 * p = 0.021	4.7E – 07*	inactive	**4.5E – 07** ns	inactive	9.8E – 07
95% CI			4.5E – 007 to 2.4E – 006	1.7E – 007 to 1.2E – 006		1.8E – 007 to 1.0E – 006		4.3E – 007 to 2.2E – 006
P. dumerilii Tyramine-1	**1.1E – 08*****	2.7E – 06***	2.1E – 06	1.7E – 05	7.8E – 06	3.1E – 05	2.1E – 06	4.7E – 05
95% CI	7.6E – 009 to 1.6E – 008	1.1E – 006 to 6.1E – 006	1.0E – 006 to 4.1E – 006	1.0E – 005 to 2.8E – 005	1.5E – 006 to 3.9E – 005	9.8E – 006 to 9.9E – 005	7.0E – 007 to 6.0E – 006	1.7E – 005 to 0.00012
P. dumerilii Tyramine-2	**7.0E – 09*****	7.8E – 07***	5.3E – 06	1.1E – 04	3.9E – 06	4.8E – 05	5.4E – 05	6.4E – 06
95% CI	3.0E – 009 to 1.6E – 008	3.8E – 007 to 1.5E – 006	2.1E – 006 to 1.3E – 005	2.9E – 005 to 0.00038	2.1E – 006 to 7.0E – 006	8.6E – 006 to 0.00026	3.6E – 005 to 7.9E – 005	3.9E – 006 to 1.0E – 005
S. kowalevskii Tyramine-1	**8.6E – 05** ns	inactive	2.9E – 04 n.s.	inactive	0.57	inactive	1.7E – 06	1.7E – 05
95% CI	2.8E – 005 to 0.00025		0.00013 to 0.00065		very wide	2.1E – 006 to 0.00017	7.1E – 007 to 3.9E – 006	7.7E – 006 to 3.7E – 005
S. kowalevskii Tyramine-2A	**1.0E – 09*****	8.6E – 08***	1.4E – 06	inactive	7.2E – 08	inactive	inactive	1.6E – 04
95% CI	6.6E – 010 to 1.5E – 009	4.0E – 008 to 1.8E – 007	7.4E – 007 to 2.6E – 006		1.4E – 008 to 3.5E – 007			5.4E – 008 to 0.47
S. kowalevskii Tyramine-2B	**5.9E – 09*****	1.6E – 06***	1.6E – 05	1.2E – 04	1.4E – 06	2.8E – 05	2.1E – 05	1.9E – 05
95% CI	2.4E – 009 to 1.4E – 008	6.5E – 007 to 3.7E – 006	6.2E – 006 to 4.0E – 005	3.6E – 005 to 0.00036	9.0E – 007 to 2.2E – 006	5.1E – 006 to 0.00015	1.1E – 005 to 3.6E – 005	1.1E – 005 to 3.0E – 005

Table 1 Half maximal effective concentration (EC_{50}) (M) and half maximal inhibitory concentration (IC_{50}) (M) values of all tested G-protein-coupled receptors with the indicated ligands or inhibitors *(Continued)*

P. dumerilii Octopamine α	1.3E – 05	**2.6E – 07***	1.4E – 07 n.s.	3.5E – 06* *P* = 0.003	inactive	8.8E – 06	9.0E – 09	1.6E – 06
95% CI	4.2E – 006 to 4.1E – 005	8.4E – 008 to 7.7E – 007	6.7E – 008 to 3.0E – 007	1.8E – 006 to 6.7E – 006		2.5E – 006 to 3.0E – 005	4.1E – 009 to 1.9E – 008	9.7E – 007 to 2.6E – 006
S. kowalevskii Octopamine α	1.7E – 05	**6.9E – 07***	1.6E – 07 * p = 0.048	5.3E – 05	2.6E – 04	1.8E – 05	7.8E – 06	2.2E – 05
95% CI	3.0E – 006 to 9.5E – 005	1.8E – 007 to 2.4E – 006	7.6E – 008 to 3.5E – 007	1.5E – 005 to 0.00018	3.4E – 006 to 0.02	7.1E – 006 to 4.7E – 005	3.1E – 006 to 1.8E – 005	1.2E – 005 to 3.6E – 005
S. kowalevskii Octopamine β	inactive	**6.4E – 08*****	inactive	3.5E – 06***	inactive	inactive	1.6E – 04	6.4E – 06
95% CI		4.0E – 008 to 1.0E – 007		1.4E – 006 to 8.1E – 006			1.0E – 005 to 0.0023	3.1E – 006 to 1.3E – 005

The most effective natural ligand for each receptor is shown in bold. 95% confidence intervals (*CI*) for the EC_{50} (M)/IC_{50} (M) values are given in every second line. The lowest EC_{50} value for each receptor was compared to the next lowest one using the extra sum-of-squares F test. *$P < 0.05$; ***$P < 0.0001$; *ns* not significant. Significance values are shown for the compared pairs

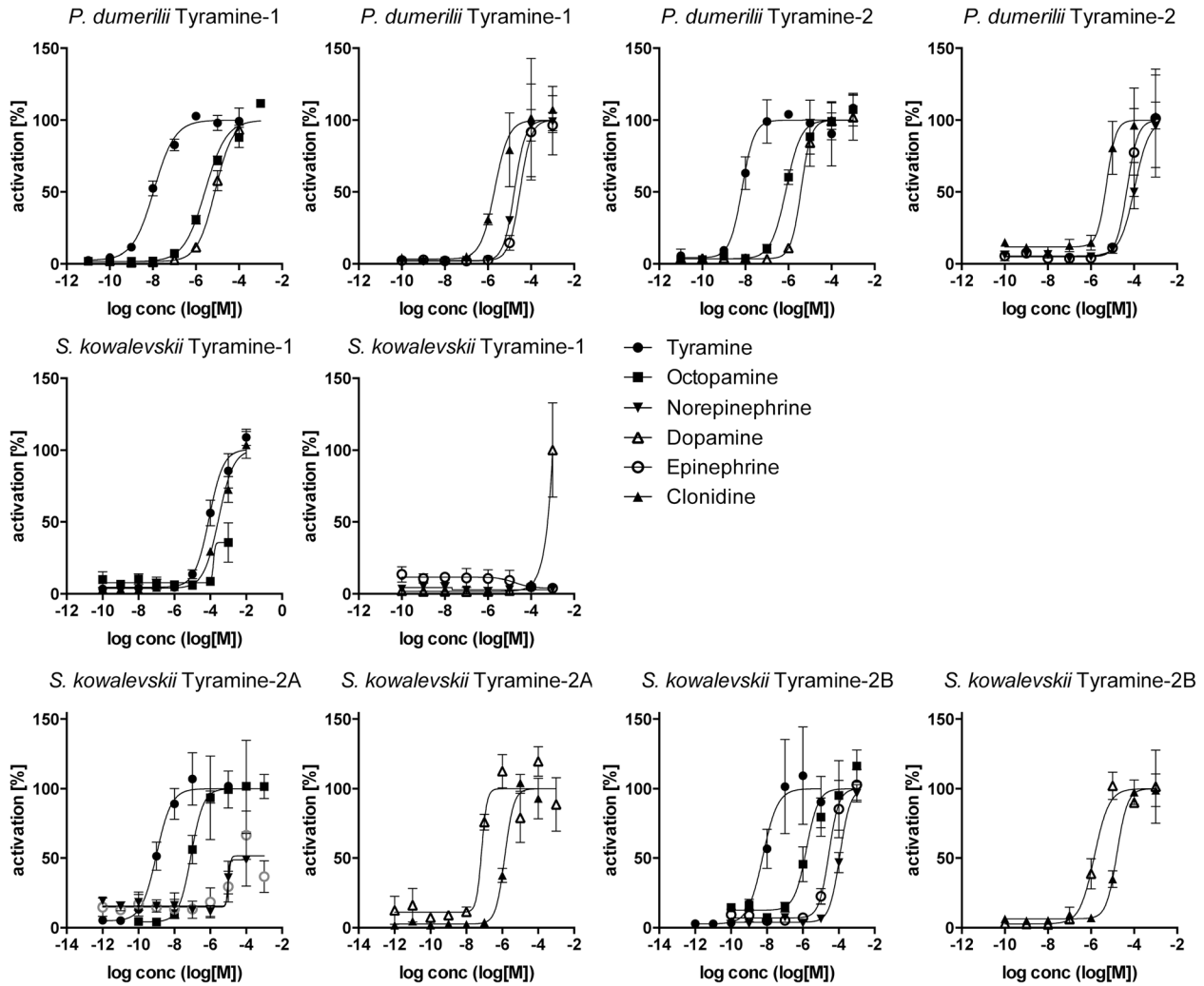

Fig. 3 Dose–response curves of tyramine G-protein-coupled receptors from *Platynereis dumerilii* and *Saccoglossus kowalevskii* treated with varying concentrations of ligand. Data, representing luminescence units relative to the maximum of the fitted dose–response curves, are shown as mean ± standard error of the mean (n = 3). EC_{50} values and significance values are listed in Table 1

not active in our assay. These results show that specific receptor systems for norepinephrine, octopamine, and tyramine coexist in *Platynereis dumerilii* and very likely also *Priapulus caudatus*.

When did tyramine and octopamine signaling originate? To answer this, we surveyed available genome sequences for tyramine and octopamine receptors. As expected, we identified several receptors across the protostomes, including ecdysozoans and lophotrochozoans (Additional files 5, 6, 7, and 8). We also identified receptors for tyramine, but not octopamine, in xenacoelomorphs. However, chordate genomes lacked orthologs of these receptors. Strikingly, we identified tyramine type 1 and 2 and octopamine α and β receptor orthologs in the genome of the hemichordate *S. kowalevskii* (Fig. 1b, c, Additional files 5, 6, 7, and 8). In phylogenetic analyses, we recovered at least one *S. kowalevskii* sequence in each of the four receptor clades (one octopamine α, one octopamine β, two tyramine type 1, and two tyramine type 2 receptors), establishing these sequences as deuterostome orthologs of these predominantly protostome GPCR families (Additional files 5, 6, 7, and 8).

We cloned the candidate *S. kowalevskii* tyramine and octopamine receptors and performed ligand activation experiments. The *S. kowalevskii* type 2 receptors were preferentially activated by tyramine in the nanomolar range. The type 1 receptor was only activated at higher ligand concentrations. The octopamine α and β receptors were preferentially activated by octopamine in the nanomolar range (Figs 3 and 4, Table 1). These data show that octopamine and tyramine signaling also coexist with adrenergic signaling in this deuterostome, as in *Platynereis dumerilii* and *Priapulus caudatus*. The presence of tyramine signaling in *S. kowalevskii* is also

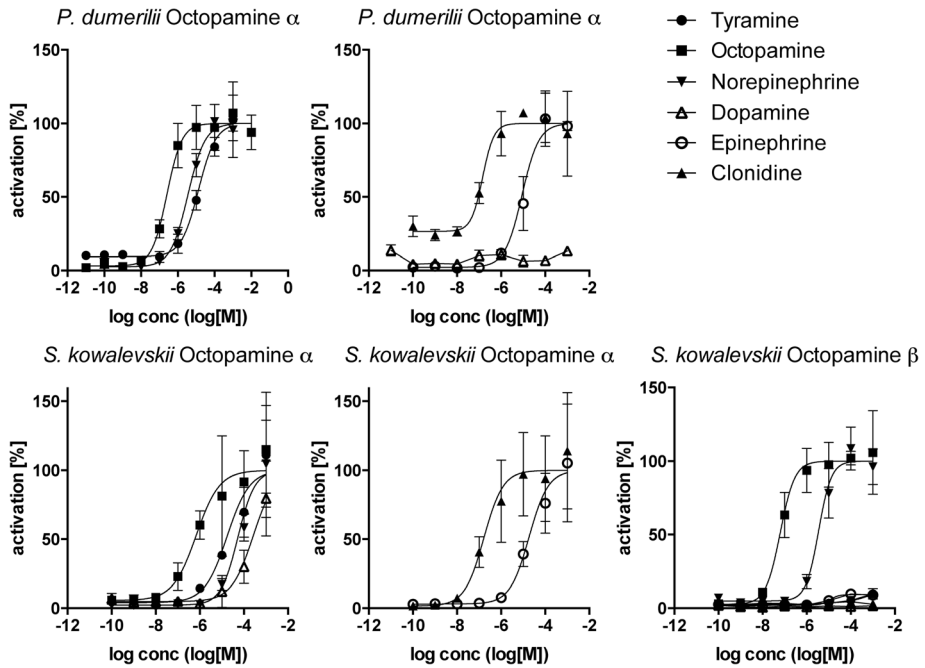

Fig. 4 Dose–response curves of octopamine G-protein-coupled receptors from *Platynereis dumerilii* and *Saccoglossus kowalevskii* treated with varying concentrations of ligand. Data, representing luminescence units relative to the maximum of the fitted dose–response curves, are shown as mean ± standard error of the mean (n = 3). Half maximal effective concentration (EC_{50}) values and significance values are listed in Table 1

supported by the phylogenetic distribution of tyrosine decarboxylase, a specific enzyme for tyramine synthesis [32]. Tyrosine decarboxylase is present in protostomes and *S. kowalevskii* but is absent from other deuterostomes (Additional file 9). In mammals, aromatic amino acid decarboxylases are involved in synthesizing low amounts of tyramine [33].

We also tested the α adrenergic agonist clonidine and the GPCR antagonists mianserin and yohimbine on several receptors from all three species. These chemicals did not show specificity for any of the receptor types, suggesting these chemicals may not be useful for studying individual biogenic amine receptors in vivo (Table 1 and Additional file 10).

Discussion

The discovery of adrenergic signaling in some protostomes and xenacoelomorphs and octopamine and tyramine signaling in a deuterostome changes our view on the evolution of monoamine signaling in bilaterians (Fig. 5). It is clear from the phylogenetic distribution of orthologous receptor systems that at least six families of octopamine,

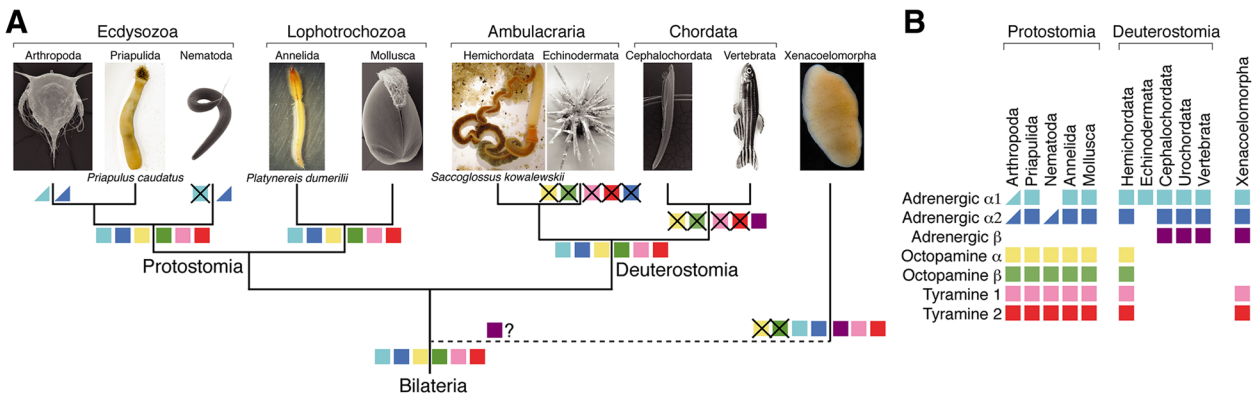

Fig. 5 Evolution of adrenergic, octopamine, and tyramine signaling in bilaterians. **a** Phylogenetic tree of major clades of bilaterian animals with the presence/loss of specific G-protein-coupled receptor (GPCR) families indicated. **b** Phyletic distribution of adrenergic, octopamine, and tyramine GPCR families across major bilaterian clades. Half squares mean losses in a large number of species in a phylum

tyramine, and adrenergic receptors were present in the bilaterian last common ancestor (Additional file 11). These include the adrenergic α1 and α2 receptors, the tyramine type 1 and type 2 receptors, and the octopamine α and β receptors. From the six ancestral families, the octopamine and tyramine receptors have been lost from most deuterostomes, and the adrenergic receptors from most ecdysozoans. Interestingly, the xenacoelomorph *M. stichopi* also has an adrenergic β receptor, representing the only ortholog outside chordates. Octopamine α receptors have likely been lost from xenacoelomorphs, given that the split of the six receptor families (four with well-resolved xenacoelomorph sequences) pre-dated the divergence of the main lineages of bilaterians (Fig. 1c).

Although we performed the receptor activation assays in a heterologous system that might not mimic the in vivo situation very well, we found clear evidence of ligand preferences for each receptor. In general, there was two orders of magnitude difference in the EC_{50} values between the best ligand and other related ligands for the same receptor measured under the same conditions. We consider these in vitro ligand preferences as indicative of the physiological ligands for these receptors. Furthermore, there was high congruence between the in vitro ligand specificities and the phylogenetic placement of the different classes of receptors, further strengthening our receptor-type assignments. The most potent ligand of all six orthologous receptor families we analyzed was the same across protostomes and deuterostomes, indicating the evolutionary stability of ligand–receptor pairs, similar to the long-term stability of neuropeptide GPCR ligand–receptor pairs [34, 35].

Understanding the ancestral role of these signaling systems and why they may have been lost differentially in different animal groups will require functional studies in organisms where all three neurotransmitter systems coexist.

Conclusions

We have established the coexistence of adrenergic, octopaminergic, and tyraminergic signaling in the deuterostome *S. kowalevskii* and the protostomes *Platynereis dumerilii* and *Priapulus caudatus.* Signaling by norepinephrine in vertebrates has often been considered as equivalent to signaling by octopamine in invertebrates. Our results change this view and show that these signaling systems coexisted ancestrally and still coexist in some bilaterians. The extent of functional redundancy in species where all six receptor systems coexist will require experimental studies. It may be that some of these monoamines ancestrally had partially overlapping roles. In that case, following the loss of a receptor, functions associated with that ligand–receptor pair may have been taken over by another pair. However, regardless of such potential shifts in function, it is clear that octopamine signaling in invertebrates and adrenergic signaling in vertebrates is not equivalent or homologous from an evolutionary point of view. This has important implications for our interpretation of comparative studies of the function of these neurotransmitter systems and their neural circuits. Our study also contributes to the understanding of nervous system evolution in bilaterians by revealing extensive losses during the history of one of the major classes of neurotransmitter systems.

Methods

Gene identification and receptor cloning

Platynereis protein sequences were collected from a *Platynereis* mixed-stage transcriptome assembly [36]. GPCR sequences from other species were downloaded from NCBI. GPCRs were cloned into pcDNA3.1(+) (Thermo Fisher Scientific, Waltham, MA, USA) as described before [31]. Forward primers consisted of a spacer (ACAATA) followed by a BamHI or EcoRI restriction site, the Kozak consensus sequence (CGCCACC), a start codon (ATG), and a sequence corresponding to the target sequence. Reverse primers consisted of a spacer (ACAATA), a NotI restriction site, a STOP codon, and a reverse complementary sequence to the target sequence. Primers were designed to end with a C or G with a 72 °C melting temperature. Polymerase chain reaction was performed using Phusion polymerase (New England Biolabs GmbH, Frankfurt, Germany). The sequences of all *Platynereis* GPCRs tested here were deposited in GenBank (accession numbers: α1-adrenergic receptor [GenBank: KX372342]; α2-adrenergic receptor [GenBank: KX372343], Tyramine-1 receptor [GenBank: KP293998]; Tyramine-2 receptor [GenBank: KU715093]; Octopamine α receptor [GenBank: KU530199]; Octopamine β receptor [GenBank: KU886229]). Tyramine receptor 1 has previously been published [31] as Pdu orphan GPCR 48. The GenBank accession numbers of the *S. kowalevskii* and *Priapulus caudatus* sequences tested are: *S. kowalevskii* α1-adrenergic [GenBank: ALR88680]; *S. kowalevskii* α2-adrenergic [GenBank: XP_002734932]; *Priapulus caudatus* α1-adrenergic [GenBank: XP_014662992]; *Priapulus caudatus* α2-adrenergic [GenBank: XP_014681069]; *S. kowalevskii* Tyramine-1 [GenBank: XP_002742354]; *S. kowalevskii* Tyramine-2A [GenBank: XP_002734062]; *S. kowalevskii* Tyramine-2B [GenBank: XP_006812999]; *S. kowalevskii* Octopamine α, [GenBank: XP_006823182]; and *S. kowalevskii* Octopamine β [GenBank: XP_002733926].

Cell culture and receptor deorphanization

Cell culture assays were done as described before [31]. Briefly, CHO-K1 cells were kept in Ham's F12 Nut Mix

medium (Thermo Fisher Scientific) with 10% fetal bovine serum and penicillin-streptomycin (PenStrep, Thermo Fisher Scientific). Cells were seeded in 96-well plates (Thermo Fisher Scientific) at approximately 10,000 cells/well. After 1 day, cells were transfected with plasmids encoding a GPCR, the promiscuous Gα-16 protein [37], and a reporter construct GFP-apoaequorin [38] (60 ng each) using 0.375 μL of the transfection reagent TurboFect (Thermo Fisher Scientific). After 2 days of expression, the medium was removed and replaced with Hank's Balanced Salt Solution (HBSS) supplemented with 1.8 mM Ca^{2+}, 10 mM glucose, and 1 mM coelenterazine h (Promega, Madison, WI, USA). After incubation at 37 °C for 2 h, cells were tested by adding synthetic monoamines (Sigma, St. Louis, MO, USA) in HBSS supplemented with 1.8 mM Ca^{2+} and 10 mM glucose. Solutions containing norepinephrine, epinephrine, or dopamine were supplemented with 100 μM ascorbic acid to prevent oxidation. Luminescence was recorded for 45 s in a plate reader (BioTek Synergy Mx or Synergy H4; BioTek, Winooski, VT, USA). For inhibitor testing, the cells were incubated with yohimbine or mianserin (Sigma) for 1 h. Then, synthetic monoamines were added to yield in each case the smallest final concentration expected to elicit the maximal response in the absence of inhibitor, and luminescence was recorded for 45 s. Data were integrated over the 45-s measurement period. Data for dose–response curves were recorded as technical triplicates for each concentration. Measurements were performed from adjacent wells on the same plate to minimize variation introduced by cell seeding and transfection. Dose–response curves were fitted with a four-parameter curve using Prism 6 (GraphPad, La Jolla, CA, USA). The curves were normalized to the calculated upper plateau values (100% activation). The different EC_{50} values for each receptor were compared with the extra sum-of-squares F test in a pairwise manner using Prism 6.

Bioinformatics

Protein sequences were downloaded from the NCBI. Redundant sequences were removed from the collection using CD-HIT [39] with an identity cutoff of 70%. Sequence cluster maps were created with CLANS2 [40] using the BLOSUM62 matrix and a *P*-value cutoff of 1e−70. For phylogenetic trees, protein sequences were aligned with MUSCLE [41]. Alignments were trimmed with TrimAI [42] in "Automated 1" mode. The best amino acid substitution model was selected using ProtTest 3 [43]. Maximum likelihood trees were calculated with RAxML [44] using the CIPRES Science Gateway [45] or with IQ-TREE and automatic model selection (http://www.iqtree.org/). Bootstrap analysis in RAxML was done and automatically stopped [46] when the Majority Rule Criterion (autoMRE) was met. The resulting trees were visualized with FigTree (http://tree.bio.ed.ac.uk/software/figtree/). The identifiers of deorphanized adrenergic, octopamine, and tyramine receptors [12, 29, 47–59] were tagged with _AA1, AA2, _Oa, _Ob, _T1, or _T2. The trees were rooted on 5HT receptors. The full phylogenetic tree is available in nexus format (Additional file 11).

Additional files

Additional file 1: Maximum likelihood tree of adrenergic, octopamine, and tyramine receptors. Bootstrap support values are shown. This tree contains all investigated GPCRs. The tree was rooted on 5HT receptor sequences. Sub-trees are shown in Additional files 2, 3, 4, 5, 6, 7, and 8. (PDF 118 kb)

Additional file 2: Maximum likelihood tree of α1-adrenergic receptors. Bootstrap support values are shown for selected nodes. This tree is part of a larger tree containing all investigated GPCRs. (PDF 16992 kb)

Additional file 3: Maximum likelihood tree of α2-adrenergic receptors. Bootstrap support values are shown for selected nodes. This tree is part of a larger tree containing all investigated GPCRs. (PDF 17168 kb)

Additional file 4: Maximum likelihood tree of β-adrenergic receptors. Bootstrap support values are shown for some nodes of interest. This tree is part of a larger tree containing all investigated GPCRs. (PDF 759 kb)

Additional file 5: Maximum likelihood tree of tyramine type 1 receptors. Bootstrap support values are shown for selected nodes. This tree is part of a larger tree containing all investigated GPCRs. The identifiers of deorphanized tyramine receptors were tagged with _T1. (PDF 17028 kb)

Additional file 6: Maximum likelihood tree of tyramine type 2 receptors. Bootstrap support values are shown for selected nodes. This tree is part of a larger tree containing all investigated GPCRs. The identifiers of deorphanized tyramine receptors were tagged with _T2. (PDF 17007 kb)

Additional file 7: Maximum likelihood tree of octopamine-α receptors. Bootstrap support values are shown for selected nodes. This tree is part of a larger tree containing all investigated GPCRs. The identifiers of deorphanized octopamine receptors were tagged with _Oa. (PDF 16730 kb)

Additional file 8: Maximum likelihood tree of octopamine-β receptors. Bootstrap support values are shown for selected nodes. This tree is part of a larger tree containing all investigated GPCRs. The identifiers of deorphanized octopamine receptors were tagged with _Ob. (PDF 16730 kb)

Additional file 9: Maximum likelihood tree of tyrosine decarboxylase and aromatic amino acid decarboxylase enzymes. Bootstrap support values are shown for selected nodes. *P. dumerilii*, *P. caudatus*, and *S. kowalevskii* sequences are highlighted in color. The *Caenorhabditis elegans* tyrosine decarboxylase was experimentally shown to be required for tyramine biosynthesis [32]. (PDF 566 kb)

Additional file 10: Dose–response curves of adrenergic, tyramine, and octopamine receptors from *P. dumerilii*, *P. caudatus*, and *S. kowalevskii* treated with varying concentrations of inhibitors. Data, representing luminescence units relative to the maximum of the fitted dose–response curves, are shown as mean ± SEM (n = 3). IC_{50} values are listed in Table 1. (TIF 956 kb)

Additional file 11: Maximum likelihood tree of octopamine, tyramine, and adrenergic α receptors, in nexus format. (NEXUS 37 kb)

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The funding bodies had no role in the design of the study; collection, analysis, and interpretation of data; or in writing the manuscript.

Availability of data and materials
GenBank accession numbers are listed in the Methods. All data generated or analyzed during this study are included in this published article and its supplementary information files. All data on which our conclusions depend are available on reasonable request.

Authors' contributions
PG and GJ performed phylogenetic analysis. PG performed gene cloning and receptor pharmacology. PB and GJ designed the study and wrote the paper. Both authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

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References

1. Singh C, Oikonomou G, Prober DA. Norepinephrine is required to promote wakefulness and for hypocretin-induced arousal in zebrafish. elife. 2015;4, e07000.
2. Marino MD, Bourdélat-Parks BN, Cameron Liles L, Weinshenker D. Genetic reduction of noradrenergic function alters social memory and reduces aggression in mice. Behav Brain Res. 2005;161:197–203.
3. Kim CH, Zabetian CP, Cubells JF, Cho S, Biaggioni I, Cohen BM, Robertson D, Kim KS. Mutations in the dopamine beta-hydroxylase gene are associated with human norepinephrine deficiency. Am J Med Genet. 2002;108:140–7.
4. Roeder T. Tyramine and octopamine: ruling behavior and metabolism. Annu Rev Entomol. 2005;50:447–77.
5. Zhou C, Rao Y, Rao Y. A subset of octopaminergic neurons are important for Drosophila aggression. Nat Neurosci. 2008;11:1059–67.
6. Crocker A, Sehgal A. Octopamine regulates sleep in drosophila through protein kinase A-dependent mechanisms. J Neurosci. 2008;28:9377–85.
7. Crisp KM, Grupe RE, Lobsang TT, Yang X. Biogenic amines modulate pulse rate in the dorsal blood vessel of *Lumbriculus variegatus*. Comp Biochem Physiol C Toxicol Pharmacol. 2010;151:467–72.
8. Florey E, Rathmayer M. The effects of octopamine and other amines on the heart and on neuromuscular transmission in decapod crustaceans: further evidence for a role as neurohormone. Comp Biochem Physiol Part C. 1978; 61:229–37.
9. Jin X, Pokala N, Bargmann CI. Distinct circuits for the formation and retrieval of an imprinted olfactory memory. Cell. 2016;164:632–43.
10. Nagaya Y, Kutsukake M, Chigusa SI, Komatsu A. A trace amine, tyramine, functions as a neuromodulator in *Drosophila melanogaster*. Neurosci Lett. 2002;329:324–8.
11. Saudou F, Amlaiky N, Plassat JL, Borrelli E, Hen R. Cloning and characterization of a Drosophila tyramine receptor. EMBO J. 1990;9:3611–7.
12. Rex E, Komuniecki RW. Characterization of a tyramine receptor from *Caenorhabditis elegans*. J Neurochem. 2002;82:1352–9.
13. Kutsukake M, Komatsu A, Yamamoto D, Ishiwa-Chigusa S. A tyramine receptor gene mutation causes a defective olfactory behavior in *Drosophila melanogaster*. Gene. 2000;245:31–42.
14. Selcho M, Pauls D, El Jundi B, Stocker RF, Thum AS. The role of octopamine and tyramine in Drosophila larval locomotion. J Comp Neurol. 2012;520:3764–85.
15. Huang J, Liu W, Qi YX, Luo J, Montell C. Neuromodulation of courtship drive through tyramine-responsive neurons in the Drosophila brain. Curr Biol. 2016;26:2246–56.
16. Wallace BG. The biosynthesis of octopamine–characterization of lobster tyramine beta-hydroxylase. J Neurochem. 1976;26:761–70.
17. Monastirioti M, Linn CE, White K. Characterization of Drosophila tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. J Neurosci. 1996;16:3900–11.
18. Evans PD, Maqueira B. Insect octopamine receptors: a new classification scheme based on studies of cloned Drosophila G-protein coupled receptors. Invert Neurosci. 2005;5:111–8.
19. Cazzamali G, Klaerke DA, Grimmelikhuijzen CJ. A new family of insect tyramine receptors. Biochem Biophys Res Commun. 2005;338:1189–96.
20. Borowsky B, Adham N, Jones KA, Raddatz R, Artymyshyn R, Ogozalek KL, Durkin MM, Lakhlani PP, Bonini JA, Pathirana S, Boyle N, Pu X, Kouranova E, Lichtblau H, Ochoa FY, Branchek TA, Gerald C. Trace amines: identification of a family of mammalian G protein-coupled receptors. Proc Natl Acad Sci U S A. 2001;98:8966–71.
21. Eyun SI, Moriyama H, Hoffmann FG, Moriyama EN. Molecular evolution and functional divergence of trace amine-associated receptors. PLoS One. 2016; 11, e0151023.
22. Gallo VP, Accordi F, Chimenti C, Civinini A, Crivellato E. Catecholaminergic system of invertebrates: comparative and evolutionary aspects in comparison with the octopaminergic system. Int Rev Cell Mol Biol. 2016; 322:363–94.
23. Arakawa S, Gocayne JD, McCombie WR, Urquhart DA, Hall LM, Fraser CM, Venter JC. Cloning, localization, and permanent expression of a Drosophila octopamine receptor. Neuron. 1990;4:343–54.
24. Dunn C, Giribet G, Edgecombe G, Hejnol A. Animal phylogeny and its evolutionary implications. Annu Rev Ecol Evol Syst. 2014;45:371–95.
25. Bourlat SJ, Juliusdottir T, Lowe CJ, Freeman R, Aronowicz J, Kirschner M, Lander ES, Thorndyke M, Nakano H, Kohn AB, Heyland A, Moroz LL, Copley RR, Telford MJ. Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida. Nature. 2006;444:85–8.
26. Cannon JT, Vellutini BC, Smith J, Ronquist F, Jondelius U, Hejnol A. Xenacoelomorpha is the sister group to Nephrozoa. Nature. 2016;530:89–93.
27. Philippe H, Brinkmann H, Copley RR, Moroz LL, Nakano H, Poustka AJ, Wallberg A, Peterson KJ, Telford MJ. Acoelomorph flatworms are deuterostomes related to *Xenoturbella*. Nature. 2011;470:255–8.
28. Krishnan A, Almén MS, Fredriksson R, Schiöth HB. Remarkable similarities between the hemichordate (*Saccoglossus kowalevskii*) and vertebrate GPCR repertoire. Gene. 2013;526:122–33.
29. Wu SF, Xu G, Qi YX, Xia RY, Huang J, Ye GY. Two splicing variants of a novel family of octopamine receptors with different signaling properties. J Neurochem. 2014;129:37–47.
30. Tunaru S, Lättig J, Kero J, Krause G, Offermanns S. Characterization of determinants of ligand binding to the nicotinic acid receptor GPR109A (HM74A/PUMA-G). Mol Pharmacol. 2005;68:1271–80.
31. Bauknecht P, Jékely G. Large-scale combinatorial deorphanization of *Platynereis* neuropeptide GPCRs. Cell Rep. 2015;12:684–93.
32. Alkema MJ, Hunter-Ensor M, Ringstad N, Horvitz HR. Tyramine functions independently of octopamine in the *Caenorhabditis elegans* nervous system. Neuron. 2005;46:247–60.
33. Lovenberg W, Weissbach H, Udenfriend S. Aromatic L-amino acid decarboxylase. J Biol Chem. 1962;237:89–93.
34. Jékely G. Global view of the evolution and diversity of metazoan neuropeptide signaling. Proc Natl Acad Sci U S A. 2013;110:8702–7.
35. Mirabeau O, Joly JS. Molecular evolution of peptidergic signaling systems in bilaterians. Proc Natl Acad Sci U S A. 2013;110:E2028–37.
36. Conzelmann M, Williams EA, Krug K, Franz-Wachtel M, Macek B, Jékely G. The neuropeptide complement of the marine annelid *Platynereis dumerilii*. BMC Genomics. 2013;14:906.
37. Offermanns S, Simon MI. G alpha 15 and G alpha 16 couple a wide variety of receptors to phospholipase C. J Biol Chem. 1995;270:15175–80.
38. Baubet V, Le Mouellic H, Campbell AK, Lucas-Meunier E, Fossier P, Brúlet P. Chimeric green fluorescent protein-aequorin as bioluminescent Ca2+ reporters at the single-cell level. Proc Natl Acad Sci U S A. 2000;97:7260–5.
39. Li W, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics. 2006;22:1658–9.
40. Frickey T, Lupas A. CLANS: a Java application for visualizing protein families based on pairwise similarity. Bioinformatics. 2004;20:3702–4.

41. Edgar RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics. 2004;5:113.
42. Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics. 2009;25:1972–3.
43. Darriba D, Taboada GL, Doallo R, Posada D. ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics. 2011;27:1164–5.
44. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30:1312–3.
45. Miller MA, Pfeiffer W, Schwartz T. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: 2010 Gateway Computing Environments Workshop (GCE). New Orleans: IEEE; 2010:1–8
46. Pattengale ND, Alipour M, Bininda-Emonds OR, Moret BM, Stamatakis A. How many bootstrap replicates are necessary? J Comput Biol. 2010;17:337–54.
47. Balfanz S, Jordan N, Langenstück T, Breuer J, Bergmeier V, Baumann A. Molecular, pharmacological, and signaling properties of octopamine receptors from honeybee (*Apis mellifera*) brain. J Neurochem. 2014;129:284–96.
48. Verlinden H, Vleugels R, Marchal E, Badisco L, Pflüger HJ, Blenau W, Broeck JV. The role of octopamine in locusts and other arthropods. J Insect Physiol. 2010;56:854–67.
49. Gross AD, Temeyer KB, Day TA, de Pérez León AA, Kimber MJ, Coats JR. Pharmacological characterization of a tyramine receptor from the southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*. Insect Biochem Mol Biol. 2015;63:47–53.
50. Kastner KW, Shoue DA, Estiu GL, Wolford J, Fuerst MF, Markley LD, Izaguirre JA, McDowell MA. Characterization of the *Anopheles gambiae* octopamine receptor and discovery of potential agonists and antagonists using a combined computational-experimental approach. Malar J. 2014;13:434.
51. Wu SF, Yao Y, Huang J, Ye GY. Characterization of a β-adrenergic-like octopamine receptor from the rice stem borer (*Chilo suppressalis*). J Exp Biol. 2012;215(Pt 15):2646–52.
52. Huang J, Wu SF, Li XH, Adamo SA, Ye GY. The characterization of a concentration-sensitive α-adrenergic-like octopamine receptor found on insect immune cells and its possible role in mediating stress hormone effects on immune function. Brain Behav Immun. 2012;26:942–50.
53. Lind U, Alm Rosenblad M, Hasselberg Frank L, Falkbring S, Brive L, Laurila JM, Pohjanoksa K, Vuorenpää A, Kukkonen JP, Gunnarsson L, Scheinin M, Mårtensson Lindblad LG, Blomberg A. Octopamine receptors from the barnacle *Balanus improvisus* are activated by the alpha2-adrenoceptor agonist medetomidine. Mol Pharmacol. 2010;78:237–48.
54. Chen X, Ohta H, Ozoe F, Miyazawa K, Huang J, Ozoe Y. Functional and pharmacological characterization of a beta-adrenergic-like octopamine receptor from the silkworm *Bombyx mori*. Insect Biochem Mol Biol. 2010;40:476–86.
55. Blais V, Bounif N, Dubé F. Characterization of a novel octopamine receptor expressed in the surf clam *Spisula solidissima*. Gen Comp Endocrinol. 2010;167:215–27.
56. Chang DJ, Li XC, Lee YS, Kim HK, Kim US, Cho NJ, Lo X, Weiss KR, Kandel ER, Kaang BK. Activation of a heterologously expressed octopamine receptor coupled only to adenylyl cyclase produces all the features of presynaptic facilitation in aplysia sensory neurons. Proc Natl Acad Sci U S A. 2000;97:1829–34.
57. Gerhardt CC, Bakker RA, Piek GJ, Planta RJ, Vreugdenhil E, Leysen JE, Van Heerikhuizen H. Molecular cloning and pharmacological characterization of a molluscan octopamine receptor. Mol Pharmacol. 1997;51:293–300.
58. Wu SF, Xu G, Ye GY. Characterization of a tyramine receptor type 2 from hemocytes of rice stem borer, *Chilo suppressalis*. J Insect Physiol. 2015;75:39–46.
59. Jezzini SH, Reyes-Colón D, Sosa MA. Characterization of a prawn OA/TA receptor in *Xenopus* oocytes suggests functional selectivity between octopamine and tyramine. PLoS One. 2014;9, e111314.