Ancient peptidergic neurons regulate ciliary swimming and settlement in *Platynereis dumerilii*

Dissertation
Der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
Markus Conzelmann
aus Sigmaringen

Tübingen
2012

Tag der mündlichen Qualifikation:	19. 02. 2013
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Dr. Gáspár Jékely
2. Berichterstatter:	Prof. Dr. Nico Michiels
3. Berichterstatter:	Prof. Dr. Andreas Wanninger

ACKNOWLEDGEMENT

First, I would like to acknowledge all the scientific support that I received during the course of my dissertation. Foremost, I would like to thank my supervisor Gáspár Jékely for his continuous support and the exciting opportunity of initiating the neuropeptide-project. Gáspár's enthusiastic and prospective view of daily scientific work was one main reason for me to proceed my career in academia. It was fascinating for me to be part of his fast-growing and fast-developing lab.

I would also like to thank all of my current and former lab-mates, with a special thanks to those that collaborated with me on diverse projects including Sarah-Lena Offenburger for her effort on electrophysiology, Albina Asadulina for her magic image alignment skills, Nadine Randel and Reza Shahidi for their electron-microscopy work, and Elizabeth A. Williams for her enormous contribution in the settlement project and for proofreeding my thesis. I'm also thankful for the collaborators from other institutes that brought special flavors into my work such as Thomas Münch (ZIN, Tübingen), Sorin Tunaru (MPI, Bad Nauheim) and Stefan Offermanns (MPI, Bad Nauheim).

Zum Schluß möchte ich mich bei meiner Familie bedanken, die mich im Laufe meines Studiums stetig unterstützt hat. Danke an meine Eltern Elvira und Rolf und meinen Bruder Michael. Dziękuję bardzo Basiu, Krzysiu i Pauli.

Danke an meine kleine Eva. Ich liebe Dich. Danke an meinen Sohn Ludwig der mich jeden Tag stolz und glücklich macht. Emil, ich bin froh daß ich dich kennen lernen durfte.

Table of Contents

ZUSAMMENFASSUNG

Larven, die sich mittels Zilien fortbewegen, sind in marinen Invertebraten weit verbreitet. Diese Larven müssen ihre Schwimmtiefe regulieren, um ihr Überleben sicherzustellen und um geeignete Lebensräume für eine spätere Ansiedlung zu finden. Die vorliegenden Arbeit beschreibt, wie durch Neuropeptide ziliäres Schwimmen und Ansiedlungsverhalten in Larven des marinen Anneliden *Platynereis dumerilii* reguliert werden.

Eine Gruppe von *Platynereis* Neuropeptiden ist in sensorischen Neuronen exprimiert, welche direkt das larvale Zilienband innervieren. Diese Peptide beeinflussen die Zilienaktivität, wodurch die Schwimmtiefe der Larven reguliert wird. Die Ergebnisse zeigen, dass das larvale Gehirn von *Platynereis* als einfacher Tiefenzähler fungieren könnte, der die Schwimmtiefe der Larven in Abhängigkeit zahlreicher sensorischen Umwelteinflüssen justiert.

Einige dieser Neuropeptide sind in auch in anderen marinen Invertebraten weit verbreitet. Färbungen mit Antikörpern gegen kurze konservierte Neuropeptid Sequenzmotive zeigen peptiderge Innervierung von Zilienbändern in Anneliden, Mollusken, Bryozoen und Quallenlarven. Diese Beobachtungen deuten darauf hin, dass die Regulation ziliären Schwimmens durch Neuropeptide evolutionär konserviert sein könnte.

In *Platynereis* ist ein konserviertes MIP (Myoinhibitorisches Peptid) Rezeptor-Liganden Modul in chemosensorisch-neurosekretorischen Neuronen exprimiert. Dieses Modul reguliert das Ansiedlungsverhalten von *Platynereis* Larven. Orthologe Peptide (Wamide) sind auch an der Regulation des Ansiedlungsverhalten von Quallenlarven beteiligt. Dies deutet darauf hin, daß eine ursprünglichen Funktionen von Wamiden die Regulation von marinen Lebenszyklen gewesen sein könnte.

Die Ergebnisse meiner Arbeit zeigen, dass das larvale Gehirn von *Platynereis* zahlreiche ursprünglichen Neuronentypen besitzt, welche eine sensorisch-motorische oder sensorisch-neurosekretorische Doppelfunktion haben. Das larvale Gehirn von *Platynereis* könnte daher ein Relikt aus einer frühen Stufe der Nervensystem Evolution darstellen.

SUMMARY

Ciliated larval stages are widespread among marine invertebrates with a biphasic life cycle. Larvae have to regulate their swimming depth for optimal development and survival and to find suitable habitats for their settlement. In the present thesis I characterized the role of neuropeptides on ciliary swimming and settlement in larvae of the marine annelid *Platynereis dumerilii*.
A set of *Platynereis* neuropeptides is expressed in sensory neurons that directly innervate the larval ciliary band. These neuropeptides influence two parameters of ciliary activity: ciliary beat frequency and ciliary closures. Thereby, they also regulate the swimming depth of *Platynereis* larvae in vertical migration assays. The *Platynereis* larval brain, that is predominantly organized of direct neuropeptidergic sensory-motor neurons, could therefore function as a simple depth gauge, responsible for adjusting the swimming depth in accordance to a variety of sensory environmental cues.
Several of these *Platynereis* neuropeptides show a broad phyletic distribution. Specific antibodies for short conserved neuropeptide epitopes revealed a broad occurrence of peptidergic innervation of ciliary bands in mollusk, bryozoan and cnidarian larvae. These observations suggest a conserved role of neuropeptides in the regulation of ciliary swimming.
In *Platynereis,* a MIP (myoinhibitory peptide) receptor-ligand pair is expressed in larval apical organ neurons. MIP-expressing cells have characteristics for chemosensory-neurosecretory neurons, regulating *Platynereis* larval settlement. Since orthologous peptides (Wamides) are also involved in the regulation of metamorphosis and settlement in cnidarian larvae, these data suggest that one of the ancient functions of Wamides may have been the regulation of marine life cycle transitions.

Overall, the present work illustrates that the larval brain of *Platynereis* is predominantly composed of ancestral metazoan neuron types including cells with dual sensory-motor and chemosensory-neurosecretory function. Therefore, I propose that the *Platynereis* larval brain may represent a relic of an early stage of nervous system evolution.

PUBLICATIONS INCORPORATED INTO THE THESIS

M Conzelmann, SL Offenburger, A Asadulina, T Keller, TA Munch, G Jekely:
Neuropeptides regulate swimming depth of *Platynereis* larvae.
Proceedings of the National Academy of Sciences of the United States of America 2011, **108:**E1174-1183.
Author summary:
Proceedings of the National Academy of Sciences of the United States of America 2011, **108:**18593-18594.

M Conzelmann and G Jekely:
Antibodies against conserved amidated neuropeptide epitopes enrich the comparative neurobiology toolbox.
Evodevo 2012, **3**(1):23.

M Conzelmann *, EA Williams *, S Tunaru, N Randel, R Shahidi, A Asadulina, J Berger, S Offermanns, G Jekely:
Conserved MIP receptor–ligand pair regulates *Platynereis* larval settlement.
Proceedings of the National Academy of Sciences of the United States of America 2013 * Contributed equally.

INTRODUCTION

The pelagobenthic biphasic life cycle

Most marine invertebrates, from sponges and cnidarians to bilaterians and deuterostomes, have a biphasic pelagobenthic life cycle, usually consisting of a sessile or bottom-dwelling (benthic) adult stage that produces freely-swimming (pelagic) larvae [1]. These larvae can spend up to several months as part of the marine zooplankton [2, 3]. After the pelagic phase, larvae settle in suitable benthic habitats and undergo metamorphosis into the adult form [4, 5]. The widespread occurrence of the pelagobenthic strategy suggests that a pelagic phase in the life cycle may be advantageous. In essence, a planktonic larval stage facilitates dispersal, which reduces competition for local resources (food, space) between parents and offspring, and promotes the colonization of new habitats. However, pelagic larvae are particularly vulnerable to unfavorable conditions in the ocean (e.g., UV-damage, predators). A pelagic larval phase also increases the likelihood of dispersal away from favorable habitats, which could lead to settlement under suboptimal conditions and finally to death (reviewed in [6]). Therefore, it is essential for survival and reproductive success of the species that pelagic larvae detect unfavorable conditions or suitable settlement habitats and adjust their swimming appropriately.

Locomotion of ciliated zooplankton

Many planktonic larvae swim using motile cilia, organelles with conserved structure across eukaryotes [7, 8], arranged in circumferential ciliary bands. Ciliary locomotion occurs in larvae of sponges, cnidarians, lophotrochozoans (e.g., annelids and mollusks), and deuterostomes [9, 10].

The vertical distribution of larvae in the ocean is thought to be the outcome of the alternation of active swimming and passive sinking due to negative buoyancy [3], often following a specific dial vertical migration pattern [11]. In general, the vertical distribution of larvae in the ocean is regarded to be dependent on biological parameters including diet [12] and predation [13], hydrodynamic parameters including water turbulence and currents [3] and the

responses of larvae to external sensory cues. Marine larvae are capable of responding to various environmental cues such as light [2, 14], gravity [15], temperature [16, 17], pressure [18, 19], oxygen concentration [20], salinity [21], and ultraviolet light [22, 23]. Ciliated larvae could use these factors to regulate their swimming and sinking rates accordingly, ultimately maintaining a depth where the environment is most favorable for their development.

External cues induce settlement of ciliated larvae

Towards the end of the pelagic phase, larvae gain competence for settlement and subsequent metamorphosis on suitable benthic habitats [4, 5]. The competence for undergoing settlement is thought to be dependent on developmental status and food availability [24]. In ciliated larvae, settlement is initiated by a slowing of swimming, caused by an inhibition of ciliary activity, accompanied by larval substrate attachment and exploration behavior [25-27]. Settlement is often triggered by external chemical cues released from conspecifics, biofilms and prey species [24, 25], or induced by direct surface contact with physical cues from the substrate [28-30].

Neuropeptides are ancient and ubiquitous neuronal signaling molecules

Animal nervous systems contain four major groups of signaling molecules: classical synaptic transmitters (acetylcholine, glutamate, glycine), monoamine neurotransmitters (serotonin, dopamine), permeable mediators (nitric oxide), and neuropeptides. Neuropeptides, which represent the largest group of transmitters in the animal kingdom, are considered the oldest neuronal signaling molecules in metazoans [31, 32]. Core enzymes for neuropeptide processing, maturation and neurosecretion can even be found in organisms without a nervous system, such as sponge [33] and placozoans [34, 35], suggesting a deep evolutionary origin of peptidergic signaling.

From the neuropeptide precursor to bioactive peptides

In contrast to classical neurotransmitters that are enzymatically synthesized in the presynapse of a neuron, neuropeptides are translated as larger inactive protein precursors in the cell body of neurons. These large precursors are

further processed and modified to generate smaller biologically active and stable peptides. The main processing steps are illustrated in Figure 1.

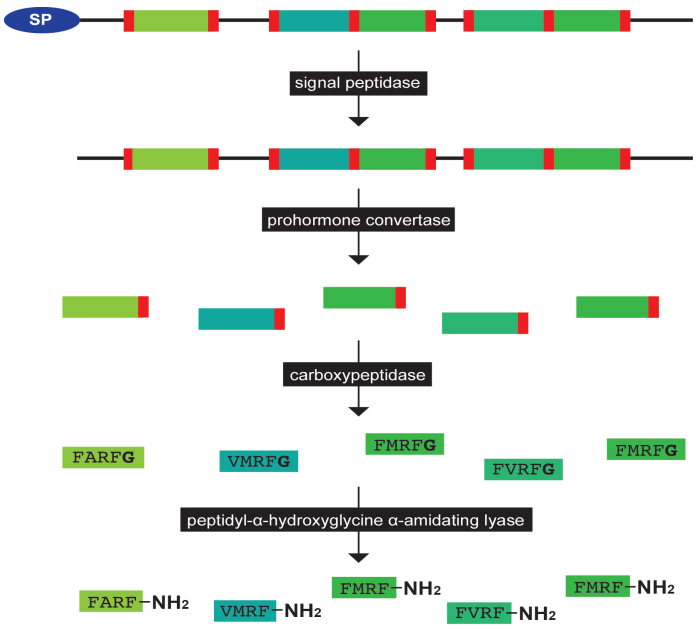


Figure 1: Processing of a neuropeptide precursor protein

The signal peptide (SP) is shown in blue, peptide blocks are shown in green, and basic cleavage sites are shown in red. The SP is removed by the enzyme signal peptidase. Prohormone convertase and carboxypeptidase yield smaller peptides that can be modified by converting a C-terminal glycine into an amide group through peptidyl-α-hydroxy-glycine α-amidating lyase.

A typical neuropeptide precursor protein consists of a N-terminal signal peptide that targets the precursor towards the secretory machinery, and several copies of smaller peptide blocks, flanked by basic cleavage sites [36]. Upon entry into the secretory machinery of the cell, the signal peptide is enzymatically removed and the precursor is packed into secretory vesicles in the Golgi apparatus. Here, proteolytic enzymes cleave the precursor C-terminally to the dibasic cleavage sites followed by a removal of the remaining C-terminal basic residues [37]. During the maturation process, peptides can be further modified to protect the N- and C-termini of these short peptides from unspecific proteolytic degradation and to confer biological activity (e.g. receptor binding [38]). These modifications can include N-terminal acetylations, glycosylations, and methylations. A common post translational modification is alpha-amidation, where a C-terminal glycine is enzymatically converted into an amide residue [39].

Neuropeptide transport, release and function

Mature neuropeptides are stored in large dense cored vesicles that derive from the trans-Golgi network and travel via fast microtubule-based neuronal transport to their release site [40]. Large dense cored vesicles often contain

cocktails of different peptides that are coexpressed in the cell [41, 42]. In contrast to vesicles of classical neurotransmitters that are located in proximity to synaptic zones, peptides are usually localized and released at extra-synaptic locations [43] including axon varicosities [44] and dendrites [45]. Neuropeptides are released from large dense cored vesicles upon an increase of the cellular calcium concentration, after intense and repetitive stimulation of the neuron [46, 47]. Release can happen via relatively slow exocytosis [48], or by a fast “kiss-and-run” mechanism via a transient fusion pore [49]. After release, peptides bind to their receptors, commonly G-protein coupled receptors (GPCR) [50]. Ligand binding activates a cascade of enzymatic events, which can cause various cellular responses. Neuropeptides can also trigger rapid cellular responses independent of GPCRs, via direct activation of fast-acting ion-channels [51].

Compared to classical neurotransmitters, peptides often have an approximately 1000 times higher binding affinity to their receptors, which is thought to be due to their larger size [52]. Moreover, since peptides are removed by unspecific proteolytic degradation rather than a fast and specific re-uptake mechanism, their half-lives in the extracellular space are remarkably long [53]. Both high receptor affinity and long half-lives enable peptides to have prolonged effects over relatively long distances [54].

Neuropeptides can regulate swimming and settlement

Limited studies revealed that neuropeptide signaling effect ciliary activity in marine invertebrate larvae [55-57]. Moreover, immunohistological approaches often describe a contribution of FMRFamide neuropeptides to the innervation of ciliary bands [58, 59]. In ciliated larvae, settlement cues are thought to be detected by a specialized anterior cluster of sensory neurons, the larval apical organ [60-62]. It has been shown that larval apical organs have neuroendocrine function and express neuropeptides [63, 64]. Several studies performed on cnidarians also reveal that neuropeptides, including Wamides, can influence settlement [65-67]. These Wamides belong to an ancient neuropeptide family that also includes arthropod myoinhibitory peptide (MIP) [68]. MIP is involved in adjusting the levels of key hormones that regulate life

cycle transitions between juvenile stages in arthropods [69, 70]. All these findings suggest that neuropeptides may play a general role in regulating ciliary swimming and settlement in marine invertebrates.

Platynereis dumerilii, an emerging lab-model

To investigate ciliary swimming and settlement of zooplankton, I studied the larva of the marine annelid *Platynereis dumerilii*, belonging to the bilaterian phylum lophotrochozoa. *Platynereis* has a pelagobenthic biphasic life cycle with a pelagic larval phase that uses cilia for swimming (2 days – 3 days after fertilization (dpf)). Ciliary movement is controlled by a simple organized brain, potentially regulated by external sensory cues (Figure 2A). Later in development (3 dpf – 5dpf), larvae show a mixed lifestyle with both pelagic cilia-based swimming and benthic muscle-based crawling (Figure 2B).

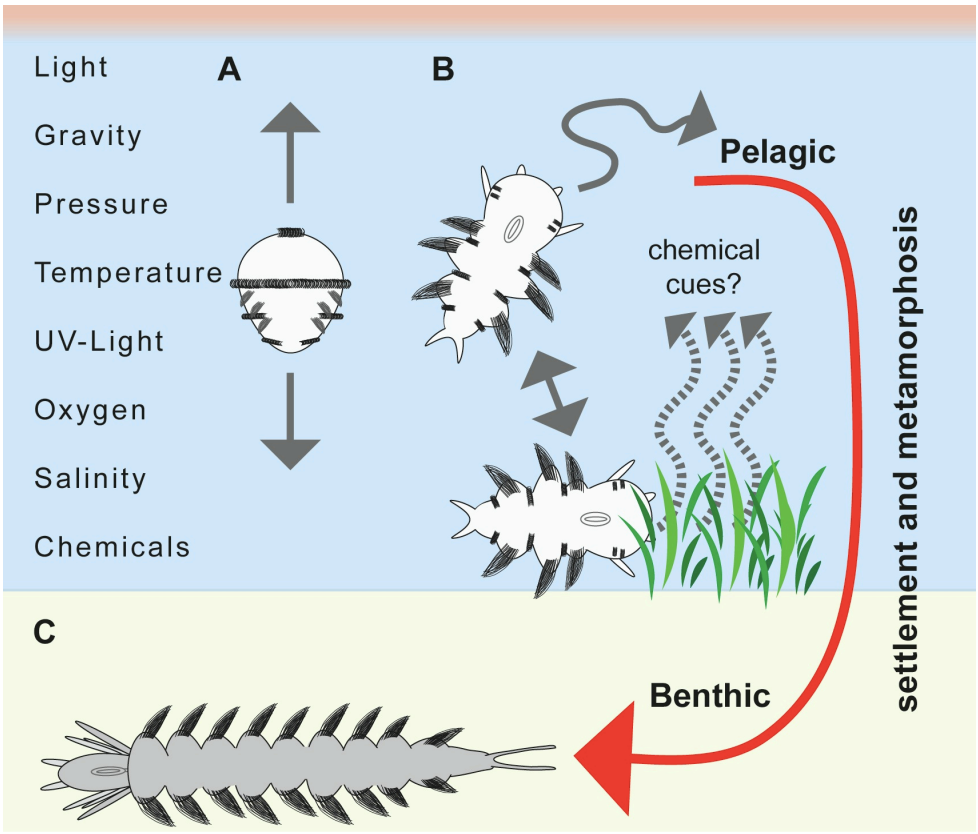


Figure 2: *Platynereis* has a pelagobenthic life cycle

(A) 2-3 dpf *Platynereis* larvae use cilia for swimming, oriented with their apical side upwards. Swimming can potentially be regulated by multiple environmental cues (listed on the left).

(B) Older *Platynereis* stages show a mixed lifestyle with periods of ciliary swimming and muscle based crawling.

(C) After settlement and metamorphosis, *Platynereis* shows benthic lifestyle.

Settlement, triggered by as yet unknown cues, occurs prior to the onset of feeding (5 dpf – 7 dpf). Then, larvae undergo metamorphosis into benthic juveniles and grow into small worms via the consecutive addition of segments (Figure 2C). Eventually they develop into sexually mature worms [71].

Platynereis has emerged as a useful model for studying the evolution of development and neurobiology. Compared to the traditional protostome model organisms (*Drosophila melanogaster* and *Caenorhabditis elegans),* the gene structure [72] an neuronal development of *Platynereis* is more characteristic of the ancestral bilaterian state, and shares more features with vertebrate neurodevelopment [73]. Moreover, the *Platynereis* larval brain (larval episphere) contains ancestral metazoan neuron types, such as simple larval eyespots with dual sensory-motor function, which regulate larval phototaxis by adjusting ciliary activity [14]. The larval brain has a few prominent features such as a sensory apical organ and a ciliary band nerve that regulates the activity of the larval ciliary band (Figure 3).

These larvae have recently also been established as a behavioral neurobiology model for studying the underlying sensory-motor neuronal circuits controlling ciliary swimming [14]. Therefore, *Platynereis* is a suitable model for studying ciliary locomotion and settlement in the pelagobenthic life cycle of marine zooplankton in the context of nervous system evolution.

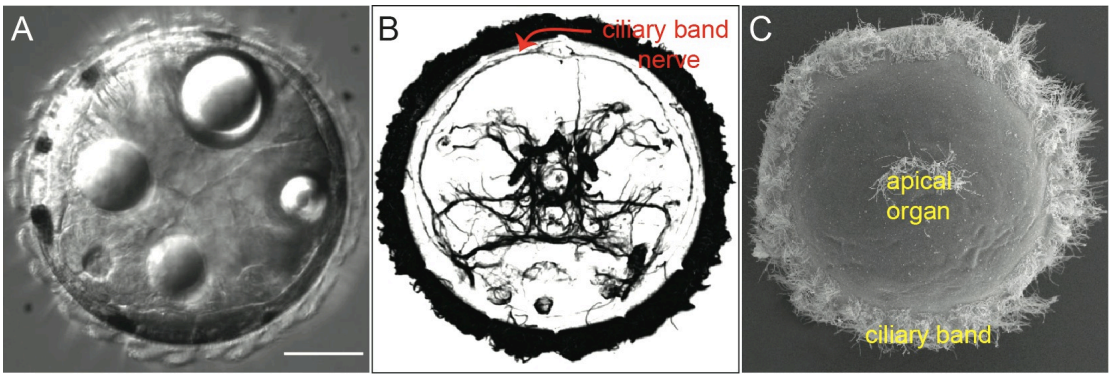


Figure 3: The ciliated larva of *Platynereis* has a simple brain

(A) Light micrograph of a *Platynereis* larva.

(B) Confocal scan of a larva stained for acetylated tubulin that labels axons, dendrites and cilia.

(C) Scanning electron microscopy image.

The *Platynereis* stage in (A-C) is 48 hours post fertilization; images are anterior views; scale bar: 50µm.

AIM OF THE THESIS

Remarkably little is known about the underlying neurobiology that enables ciliated zooplankton to regulate their swimming depth according to environmental factors. The regulation of swimming is crucial, since harmful conditions such as high UV-exposure or extreme temperatures could have a deleterious impact on larval survival. Therefore, it is essential for larvae to stay in an appropriate swimming depth. Moreover, the detection of suitable benthic habitats for settlement, which is often triggered by chemical cues, is imperative for the reproductive success and hence the survival of the species.

For my studies I primarily used the marine annelid *Platynereis dumerilii,* an emerging laboratory model organism. I will characterize neuropeptides in the larval nervous system of *Platynereis,* since these ancient signaling molecules have widespread roles in the regulation of animal behaviors, including neural circuits that are involved in the regulation of locomotion, ciliary activity, and life cycle transitions in marine invertebrates.

The major goal of my doctoral thesis is to characterize the morphology and function of neuropeptidergic circuits underlying cilia-based locomotion in *Platynereis* larvae. This includes aspects of larval behaviors such as cilia-based swimming and larval settlement.

RESULTS

Summary of Publication 1:

M Conzelmann, SL Offenburger, A Asadulina, T Keller, TA Munch, G Jekely:
Neuropeptides regulate swimming depth of *Platynereis* larvae.

Proceedings of the National Academy of Sciences of the United States of America 2011, 108:E1174-1183; Author summary: *PNAS* **108:**18593-18594

Most swimming planktonic larvae are driven by their ciliary bands, adjusting their swimming depth by changing the rate of active upward and passive downward sinking which is thought to define their vertical distribution in the ocean [3]. How the simple nervous system of planktonic swimmers translates environmental cues (e.g. gravity, pressure, temperature) into locomotory output to fine-tune their vertical distribution, and hence swimming depth, is largely unknown. To gain an insight into the neuronal mechanisms of depth regulation and swimming coordination in ciliated zooplankton, I set out to study neuropeptides in the ciliated larva of the marine annelid *Platynereis dumerilii*.

I identified 11 neuropeptide precursors in *Platynereis* transcriptome sources. Using RNA *in situ* hybridizations, I found precursor expression in different neuronal subsets. Through high-resolution confocal imaging of individual peptidergic neurons, I observed that these cells are likely sensory. These neurons are flask-shaped and have apical dendrites with distinctive sensory structures (bulbous endings, split endings) projecting to the surface of the larva. An *in silico* expression atlas, containing all 11 neuropeptide precursors defined a broad region of sensory-peptidergic neurons in the larval episphere. Overall, the finding that *Platynereis* neuropeptides are expressed in mostly non-overlapping neurons with various sensory-dendritic structures suggests that these peptidergic cells have a sensory function and can detect different environmental cues (e.g., salinity, temperature, pressure, chemicals).

To characterize the entire morphology of the peptidergic neurons, I also developed specific antibodies against single mature *Platynereis* neuropeptides from each precursor. Immunostainings indicated that neuropeptide-expressing sensory cells directly innervate the larval ciliary

band, shown by the presence of neuropeptide-positive varicose thickenings on the ciliary band nerve. This prominent nerve is responsible for the nervous regulation of ciliary activity, implying that neuropeptides are involved in the regulation of ciliary swimming in *Platynereis* larvae.

To test if neuropeptides regulate ciliary swimming, I analyzed two parameters of ciliary activity - ciliary beat frequency and the duration of ciliary arrests - on immobilized *Platynereis* larvae that were exposed to synthetic neuropeptides. I identified a group of activators that increased ciliary beat frequency, some of which additionally suppressed ciliary arrests (RYamide, FVMamide, DLamide, FMRFamide, FVamide, LYamide, and L11 peptide); and I found neuropeptides that inhibited ciliary beat frequency and also increased the duration of ciliary arrests (FLamide and WLD peptide).

In accordance to observations on mollusks [74, 75], sharp spikes in calcium concentrations that caused ciliary arrestswere observed in electro-physiological measurements on *Platynereis* larvae. I discovered that the L-type calcium channel blocker nifedipine efficiently abolished calcium-induced ciliary arrests, suggesting that neuropeptide signaling could initiate arrests via L-type calcium ion channels. Moreover, I noticed that the membrane permeable cAMP analog 8-Bromo-cAMP increased ciliary beat frequency, suggesting that cAMP levels regulate beating activity of cilia, which is in accordance with previous observations in motile cilia [7, 76]. The signaling could occur via peptide GPCRs, activating the GPCR-Gi/s-alpha-cAMP cascade to adjust cellular cAMP levels in ciliated cells and hence the beat frequency of cilia.

It has been proposed that the vertical position of ciliated pelagic larvae in the ocean can be adjusted by changes in ciliary activity [3]. To test this hypothesis, I studied the effect of *Platynereis* neuropeptides on freely swimming larvae. I developed a vertical migration setup, composed of an array of 25 cm high Plexiglas columns. In vertical swimming experiments, untreated control larvae were uniformly distributed along the vertical axis as a steady state where phases of active upward swimming and passive downwards sinking were balanced. Synthetic neuropeptides induced upward swimming when I applied activators of cilia activity (peptides that increased ciliary beat frequency and suppressed ciliary arrests), leading to an upward

shift in the steady state vertical distribution of larvae. On the other hand, inhibitors of ciliary activity (peptides that decreased ciliary beat frequency and increased the duration of ciliary arrests) led to a sinking of larvae and to a downward shift in the larval vertical distribution.

Overall, the results of this study revealed an unexpected richness of peptidergic regulation of ciliary activity in *Platynereis*. The atlas of *Platynereis* neuropeptides showed that these neuropeptides are expressed in largely non-overlapping subsets of sensory neurons. These neurons have a direct sensory-motor morphology as shown by immunostainings, suggesting a simple reflex circuit. This simple circuitry might allow larvae to respond to various environmental stimuli (e.g., pressure, salinity, temperature) and to adjust their swimming depth accordingly.

Summary of Publication 2:

M Conzelmann and G Jekely:
Antibodies against conserved amidated neuropeptide epitopes enrich the comparative neurobiology toolbox.
Evodevo 2012, **3**(1):23.

Antibodies that label neuron-populations and their projections (axons and dendrites) across diverse species and phyla are powerful tools in neurobiology, allowing comparative approaches for neurodevelopment and neuroanatomy. To date, studies on non-model invertebrates employ a limited set of antibodies, including sera against serotonin and the neuropeptide FMRFamide (e.g. [77-80]). RFamide neuropeptides are widely distributed among eumetazoans, showing the strongest sequence conservation in their RFamide C-terminus [81]. Like RFamides, other *Platynereis* neuropeptides such as DLamides, FVamides, FLamides, GWamides, and RYamides can be found in various invertebrate phyla with the strongest sequence conservation in the C-terminus. Since alpha-amidation is thought to confer a high immunogenic potential [82], I hypothesized that short amidated dipeptide epitopes could have sufficient immunogenicity to generate specific cross-

species reactive antibodies, as previously shown for a RFamide antibody [83]. Using a high stringency affinity purification protocol on amidated dipeptide epitopes DLamide, FVamide, FLamide, GWamide, and RYamide, I obtained antibodies that I further tested for immunoreactivity and specificity on larvae of *Platynereis.* For all antibodies, I found neuronal signals in sensory cells and their projections in the larval episphere (the larval brain). Absorbing the antibodies in synthetic full-length *Platynereis* peptides impeded these stainings. Moreover, cell body positions obtained in antibody-stainings closely correlated with the expression patterns of the respective precursors; and a recently described set of antibodies against the full length *Platynereis* peptides also showed highly similar neuronal signals (see publication 1 in this thesis [84]). These initial experiments show that this antibody set is indeed specific for their short amidated dipeptide epitopes.

Next, I determined the utility of these antibodies as cross-species neuronal markers in various marine ciliated larvae using another annelid, *Capitella teleta,* which is distantly related to *Platynereis* [85], two mollusk species, the nudibranch *Phestilla sibogae* and the bivalve *Pecten maximus*, a bryozoan *Cryptosula* species, and two cnidarian species *Aurelia aurita* and *Clava multicornis.* I also used nauplius crustacean larvae that were sampled from the plankton (Gullmar Fjord, Kristineberg Marine Station, Sweden).

I showed the usefulness of the DLamide antibody as a universal neuronal marker for annelids, where DLamide peptides have been described recently [84, 86]. I also observed neuronal staining for both the FLamide and FVamide antibodies in both annelids and mollusks, where these peptides have been identified [87]. GWamide neuropeptides have also been described in crustaceans and insects [88-90]. In accordance with this, the GWamide antibody stains neurons and their projections in ciliated larvae of annelids, mollusks and a crustacean nauplius larva. RYamide peptides show the broadest phyletic distribution and have been described in various marine phyla as well as in terrestrial invertebrates such as nematodes and insects [90-92]. I found RYamide immunoreactivity in ciliated larvae of annelids, a bryozoan, mollusks, cnidarians, and a crustacean larva.

The comparative survey across several marine phyla revealed a broad occurrence of peptidergic innervation of larval ciliary bands. For the anti-

DLamide, anti-FVamide, and anti-RYamide antibodies, I observed staining in the ciliary band nerve of both species of annelid larvae. In mollusk larvae, I found FVamide and RYamide immunoreactivity in a nerve running along the ciliated velum in *Pecten maximus* and the ciliated foot of *Phestilla sibogae*. Additionally, I discovered that RYamide-expressing neurons innervate locomotor cilia in bryozoan and cnidarian larvae.

In this work, I successfully developed five cross-species reactive antibodies that recognize conserved amidated dipeptide motifs. These antibodies can be applied as neuronal markers in various marine invertebrates, including annelids, mollusks, bryozoans, crustaceans, and cnidarians. I also consider that the ongoing sampling of neuropeptide diversity will allow the development of other similar antibodies in the near future to further enrich the comparative neurobiology toolbox. In immunostainings I furthermore discovered a broad occurrence of peptidergic cilia innervation in pelagic invertebrate larvae. These observations suggest that neuropeptides could regulate cilia activity in these larvae, as described for *Platynereis* [84], which points towards a general role of neuropeptides in the regulation of ciliary locomotion [93].

Summary of Manuscript 3:

M Conzelmann *, EA Williams *, S Tunaru, N Randel, R Shahidi, A Asadulina, J Berger, S Offermanns, G Jekely:

Conserved MIP receptor–ligand pair regulates *Platynereis* larval settlement.

Proceedings of the National Academy of Sciences of the United States of America 2013 *authors contributed equally

We identified myoinhibitory peptide (MIP) in transcriptome sources of *Platynereis dumerilii*, a neuropeptide with strong similarity to a phyletically conserved W(X_{5-8})Wamide motif in its mature peptide sequences.

In pharmacological assays using synthetic *Platynereis* MIP on 2 dpf pelagic larvae, I discovered that the peptide triggered long and frequent ciliary arrests in a concentration-dependent manner. Peptide treatment also caused downwards sinking of larvae in a vertical swimming setup. I observed that

larvae showed a sustained attachment behavior, characterized by a cilia-driven crawling on the substrate, mimicking a settlement behavior observed for other pelagic invertebrate larvae [26, 27].

Recently it has been shown that insect MIPs signal via the sex peptide receptor [94, 95]. We identified orthologs of the sex-peptide receptor in two annelids, *Platynereis,* and a distantly related annelid, *Capitella.* In ligand stimulation assays performed in cell culture (using a procedure similar to [96]) we showed that annelid MIP were highly specific activators of their respective sex-peptide receptor othologs in a nanomolar range (now referred to as MIP-receptors). To confirm that the observed settlement induction caused by synthetic MIP was due to signaling via the MIP-receptor, we knocked down the *Platynereis* MIP-receptor using morpholinos. In MIP-receptor knockdown larvae, I found that synthetic MIP could no longer trigger settlement behavior, confirming that MIP induced settlement via the MIP-receptor in *Platynereis.*

MIP is expressed in sensory neurons of the apical organ, a neuronal cluster that is thought to be involved in chemosensation and settlement in other pelagic larvae [60-62]. Using a specific antibody against *Platynereis* MIP, I observed that these neurons terminate in a nerve plexus with neurosecretory activity [64]. Additionally, immunostainings revealed similar MIP neurons in the apical organ and the neurosecretory nerve plexus in *Capitella*.

An *in silico* expression map (using an image registration method derived from [97]) showed that the *Platynereis* MIP receptor is also expressed in apical organ neurons, interspersed between, and largely non-overlapping with the MIP expressing cells. The MIP receptor-ligand module is expressed in a region with a neurosecretory molecular fingerprint, as shown by a broad coexpression of vertebrate and insect neuroendocrine determinant genes (*dimmed* [98] and *otp* [64] respectively).

In vivo laser ablation of *Platynereis* apical sensory neurons that were labeled with the fluorescent dye mitotracker, combined with subsequent MIP immunostaining, showed that the ablated apical cells were MIP neurons. Since dye-filling is a characteristic of chemosensory neurons in *Caenorhabditis elegans* [99], these experiments indicate that the MIP sensory neurons might have a chemosensory function in *Platynereis*.

In a dataset of ultrathin transmission electron microscopy sections, we identified two MIP neurons based on their characteristic dendritic anatomy and cell body position. We found that the dendritic ultrastructure of these cells is characteristic of chemosensory neurons, with a morphology as described by Purschke [100]. Moreover, we clearly identified these cells as being exclusively neurosecretory, with extensively branching axons that project to the larval apical neurosecretory plexus and are packed with large dense cored vesicles. Our ultrastructural characterizations corroborated that MIP neurons in the larval apical organ of *Platynereis* have a dual chemosensory-neurosecretory function.

We discovered that MIP is expressed in neurons with dual chemosensory-neurosecretory function in the annelid larval apical organ. MIP signals via the MIP-receptor, an orthologue of the *Drosophila* sex peptide receptor, to regulate *Platynereis* settlement. Interestingly, MIP is also present in mollusks [87, 101], suggesting a general function of MIP in the settlement process of lophotrochozoans. Protostome MIPs belong to an ancient metazoan Wamide peptide superfamily, including cnidarian GLWamides [68]. GLWamides are regulators of cnidarian metamorphosis and settlement [67, 102, 103], expressed in sensory cells at the most anterior larval pole [104]. The cnidarian anteriormost pole shares a regulatory signature with bilaterian apical organs [105] in which annelid and cnidarian Wamides are expressed. Both the shared expression domain of Wamide neurons in cnidarians and annelids and the similar function of Wamide peptides in the regulation of settlement and metamorphosis suggests that an ancient function of Wamides may have been the regulation of a life cycle transition in the last common metazoan ancestor.

DISCUSSION AND CONCLUSION

The *Platynereis* larval brain is a sensory-motor depth gauge

Larval stages that use cilia for locomotion are present in most marine metazoan clades [9, 10]. These larvae are often pelagic, connected to a benthic adult stage via settlement and metamorphosis in a pelagobenthic life cycle [1]. Despite the significance of ciliated pelagic larvae for the survival of the species [6], or their importance as a food source in marine ecosystems [106], we have little knowledge about the nervous control of ciliary movement.

In the present doctoral thesis I described various *Platynereis* neuropeptides that influenced ciliary activity in *Platynereis* larvae and hence rates of active upwards swimming and passive downwards sinking, which ultimately determined the vertical distribution of larvae. Since upward swimming and passive sinking is the default behavior, many of the neuropeptides that activate cilia may mediate avoidance reactions to noxious conditions that larvae could encounter in deeper water layers (e.g. low temperature, hypoxia). The identified peptide-expressing neurons control the ciliary band in a direct sensory-motor fashion. The simplicity of cilia-regulating neurons I discovered for *Platynereis* is in stark contrast to muscle-regulating systems that involve sensory, motor, and inter-neurons even in their simplest forms [107]. Intriguingly, the brain of the pelagic larva is also different from the trunk nervous system of *Platynereis*, which is devoid of sensory-motor neurons, instead possessing distinct interneurons and motor neurons to control muscle-based crawling in the benthic phase [71, 73].

For pelagic *Platynereis* larvae, I propose a scenario in which simple peptide-expressing neurons with dual sensory-motor function can respond to sensory cues to trigger changes in ciliary activity. One type of neuropeptidergic neuron could respond to one particular sensory modality in a one-neuron-one-behavior-like fashion. The *Platynereis* larval brain could work as a simple depth gauge: The combination of various sensory modalities that larvae encounter in the ocean (e.g. light, pressure, salinity, temperature, gravity) could elicit the release of a neuropeptide-cocktail at the larval ciliary band, hence fine-tuning the actual swimming depth in the ocean.

Is the neuropeptidergic control of ciliary locomotion ancient?

The cilia-regulating neuropeptides that I found in *Platynereis* are also present in various other marine invertebrates. The phyletic conservation is often restricted to a short amidated dipeptide C-terminus in the respective neuropeptide. Specific antibodies against these short conserved epitopes revealed a broad occurrence of peptidergic innervation in a sensory-motor fashion for DLamide, FVamide and RYamide neuropeptides in ciliated larvae of annelids and mollusks. For RYamide, I even identified innervation of ciliary bands in a bryozoan larva and ciliated planula larvae of two cnidarian species. The presence of related peptidergic neurons in ciliated cnidarian larvae suggests that sensory-motor neurons regulating cilia could trace back to the last common ancestor of eumetazoans.

MIP signaling regulates settlement in annelids

Besides the neuropeptides that regulate swimming depth of *Platynereis*, I also identified the neuropeptide MIP that evoked a distinctive settlement-like behavior in pelagic larvae. We identified the MIP-receptor for *Platynereis* and *Capitella* as orthologous to the *Drosophila* sex-peptide receptor [94, 95]. In pelagic *Platynereis* larvae, the MIP receptor-ligand module regulates settlement through an inhibition of ciliary activity via the induction of ciliary closures, combined with a substrate attachment behavior, and a cilia driven exploratory crawling behavior on the substrate. Similar behaviors have been described for other marine invertebrate larvae that encounter a natural settlement cue [26, 27]. My settlement assay results also confirmed that *Platynereis* MIP induces a characteristic and unique behavior in the pelagic larval stage that is different from peptides of the depth-gauge system.

The morphology of the MIP sensory neurons is also different to the depth-gauge peptidergic cells: MIP neurons do not directly innervate the ciliary band. MIP is expressed in chemosensory-neurosecretory neurons, suggesting that these cells detect inductive chemical settlement cues and release MIPs upon activation to trigger downstream events via the MIP-receptor.

The MIP receptor-ligand pair is expressed in the larval apical organ in a region with a neurosecretory molecular identity. It has previously been hypothesized that the larval apical organ is involved in settlement in marine

invertebrate larvae [61, 62]. The results extend this hypothesis, designating the annelid apical organ as a neuroendocrine signaling center that regulates settlement. MIPs have also been described in mollusks [87, 101]. As for annelids and insects, the MIP-receptor has been identified in *Aplysia californica* as an ortholog of the sex-peptide receptor [94]. The pelagobenthic life cycle is a common strategy in mollusks; therefore it is likely that the MIP receptor-ligand module is also involved in mollusk settlement.

Platynereis larvae have a protobrain with ancestral metazoan neurons

The present work illustrates that the brain of ciliated *Platynereis* larvae is largely composed of simple peptidergic neurons with dual sensory-motor or chemosensory-neurosecretory function. The observed abundance of these simple neuron types in the larval episphere of *Platynereis* is not due to a simple nervous system organization in the whole larva, because in the pelagic larval stage the trunk nervous system already harbors distinct interneurons and motor neurons for muscle regulation [71, 73].

It is plausible that the first metazoan neurons combined a sensory with a motor or neurosecretory function [93, 108, 109]. It has also been proposed that neuroendocrine centers in animals evolved by the internalization of chemosensory-neurosecretory cells, which in the course of evolution lost their chemosensory function and instead receive neuronal inputs from other neurons [110]. From these ancestral metazoan neurons or protoneurons, complex circuits may have evolved in a process of cell duplication and functional segregation, during which one neuron specialized in a sensory and the other in a motor or neurosecretory neuron [93, 108, 109].

Conclusion

The study of neuropeptides and their function in pelagic ciliated larvae of *Platynereis* revealed an unexpected abundance of ancestral metazoan neuron types with dual function that are involved in the depth-gauge-system of the larva as well as in the transition from a pelagic larva into a benthic adult. As a result, I propose that the larval brain of *Platynereis* represents a relic of an early stage of nervous system evolution.

BIBLIOGRAPHY

1. Rieger RM: **The Biphasic Life-Cycle - a Central Theme of Metazoan Evolution.** *Am Zool* 1994, **34:**484-491.

2. Thorson G: **Light as an ecological factor in the dispersal and settlement of larvae of marine bottom invertebrates.** *Ophelia* 1964, **1:**167–208.

3. Chia FS, Bucklandnicks J, Young CM: **Locomotion of Marine Invertebrate Larvae - a Review.** *Can J Zool* 1984, **62:**1205-1222.

4. Hadfield MG, Carpizo-Ituarte EJ, del Carmen K, Nedved BT: **Metamorphic competence, a major adaptive convergence in marine invertebrate larvae.** *Am Zool* 2001, **41:**1123-1131.

5. Bishop CD, Huggett MJ, Heyland A, Hodin J, Brandhorst BP: **Interspecific variation in metamorphic competence in marine invertebrates: the significance for comparative investigations into the timing of metamorphosis.** *Integrative and comparative biology* 2006, **46:**662-682.

6. Pechenik JA: **On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles.** *Mar Ecol-Prog Ser* 1999, **177:**269-297.

7. Salathe M: **Regulation of mammalian ciliary beating.** *Annu Rev Physiol* 2007, **69:**401-422.

8. Satir P: **The cilium as a biological nanomachine.** *Faseb J* 1999, **13:**S235-S237.

9. Nielsen C: **Trochophora larvae: cell-lineages, ciliary bands and body regions. 2. Other groups and general discussion.** *Journal of experimental zoology Part B, Molecular and developmental evolution* 2005, **304:**401-447.

10. Nielsen C: **Trochophora larvae: cell-lineages, ciliary bands, and body regions. 1. Annelida and Mollusca.** *Journal of experimental zoology Part B, Molecular and developmental evolution* 2004, **302:**35-68.

11. Enright JT, Hamner WM: **Vertical Diurnal Migration and Endogenous Rhythmicity.** *Science* 1967, **157:**937-&.

12. Strathmann RR: **Feeding and Nonfeeding Larval Development and Life-History Evolution in Marine-Invertebrates.** *Annu Rev Ecol Syst* 1985, **16:**339-361.

13. Gliwicz MZ: **Predation and the Evolution of Vertical Migration in Zooplankton.** *Nature* 1986, **320:**746-748.

14. Jekely G, Colombelli J, Hausen H, Guy K, Stelzer E, Nedelec F, Arendt D: **Mechanism of phototaxis in marine zooplankton.** *Nature* 2008, **456:**395-399.

15. Harris JE: **Physical Factors Involved in the Vertical Migration of Plankton.** *Q J Microsc Sci* 1953, **94:**537-550.

16. Hidu H, Haskin HH: **Swimming Speeds of Oyster Larvae Crassostrea-Virginica in Different Salinities and Temperatures.** *Estuaries* 1978, **1:**252-255.

17. Forward RB: **Behavioral-Responses of Crustacean Larvae to Rates of Temperature-Change.** *Biol Bull-Us* 1990, **178:**195-204.

18. Knightjones EW, Qasim SZ: **Responses of Some Marine Plankton Animals to Changes in Hydrostatic Pressure.** *Nature* 1955, **175:**941-942.

19. Bayne BL: **Responses of Mytilus Edulis Larvae to Increases in Hydrostatic Pressure.** *Nature* 1963, **198:**406-&.

20. Kuang S, Doran SA, Wilson RJ, Goss GG, Goldberg JI: **Serotonergic sensory-motor neurons mediate a behavioral response to hypoxia in pond snail embryos.** *Journal of neurobiology* 2002, **52:**73-83.

21. Lougee LA, Bollens SM, Avent SR: **The effects of haloclines on the vertical distribution and migration of zooplankton.** *J Exp Mar Biol Ecol* 2002, **278:**111-134.

22. Cooke SL, Williamson CE, Leech DM, Boeing WJ, Torres L: **Effects of temperature and ultraviolet radiation on diel vertical migration of freshwater crustacean zooplankton.** *Can J Fish Aquat Sci* 2008, **65:**1144-1152.

23. Leech DM, Williamson CE, Moeller RE, Hargreaves BR: **Effects of ultraviolet radiation on the seasonal vertical distribution of zooplankton: a database analysis.** *Arch Hydrobiol* 2005, **162:**445-464.

24. Rodriguez SR, Ojeda FP, Inestrosa NC: **Settlement of Benthic Marine-Invertebrates.** *Mar Ecol-Prog Ser* 1993, **97:**193-207.

25. Pawlik JR: **Chemical Ecology of the Settlement of Benthic Marine-Invertebrates.** *Oceanogr Mar Biol* 1992, **30:**273-335.

26. Qian PY: **Larval settlement of polychaetes.** *Hydrobiologia* 1999, **402:**239-253.

27. Hadfield MG, Koehl MA: **Rapid behavioral responses of an invertebrate larva to dissolved settlement cue.** *The Biological bulletin* 2004, **207:**28-43.

28. Johnson CR, Sutton DC, Olson RR, Giddins R: **Settlement of Crown-of-Thorns Starfish - Role of Bacteria on Surfaces of Coralline Algae and a Hypothesis for Deep-Water Recruitment.** *Mar Ecol-Prog Ser* 1991, **71:**143-162.

29. Lam C, Harder T, Qian PY: **Induction of larval settlement in the polychaete Hydroides elegans by extracellular polymers of benthic diatoms.** *Mar Ecol-Prog Ser* 2005, **286:**145-154.

30. Morse ANC, Morse DE: **Recruitment and Metamorphosis of Haliotis Larvae Induced by Molecules Uniquely Available at the Surfaces of Crustose Red Algae.** *J Exp Mar Biol Ecol* 1984, **75:**191-215.

31. Wegener C, Gorbashov A: **Molecular evolution of neuropeptides in the genus Drosophila.** *Genome biology* 2008, **9**.

32. Watanabe H, Fujisawa T, Holstein TW: **Cnidarians and the evolutionary origin of the nervous system.** *Development, growth & differentiation* 2009, **51:**167-183.

33. Srivastava M, Simakov O, Chapman J, Fahey B, Gauthier ME, Mitros T, Richards GS, Conaco C, Dacre M, Hellsten U, et al: **The Amphimedon queenslandica genome and the evolution of animal complexity.** *Nature* 2010, **466:**720-726.

34. Srivastava M, Begovic E, Chapman J, Putnam NH, Hellsten U, Kawashima T, Kuo A, Mitros T, Salamov A, Carpenter ML, et al: **The Trichoplax genome and the nature of placozoans.** *Nature* 2008, **454:**955-960.

35. Attenborough RM, Hayward DC, Kitahara MV, Miller DJ, Ball EE: **A "Neural" Enzyme in Nonbilaterian Animals and Algae: Preneural Origins for Peptidylglycine alpha-Amidating Monooxygenase.** *Molecular biology and evolution* 2012.

36. Veenstra JA: **Mono- and dibasic proteolytic cleavage sites in insect neuroendocrine peptide precursors.** *Archives of insect biochemistry and physiology* 2000, **43:**49-63.

37. Hook V, Funkelstein L, Lu D, Bark S, Wegrzyn J, Hwang SR: **Proteases for processing proneuropeptides into peptide neurotransmitters and hormones.** *Annual review of pharmacology and toxicology* 2008, **48:**393-423.

38. Edison AS, Espinoza E, Zachariah C: **Conformational ensembles: the role of neuropeptide structures in receptor binding.** *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1999, **19:**6318-6326.

39. Eipper BA, Stoffers DA, Mains RE: **The biosynthesis of neuropeptides: peptide alpha-amidation.** *Annual review of neuroscience* 1992, **15:**57-85.

40. Goldstein LS, Yang Z: **Microtubule-based transport systems in neurons: the roles of kinesins and dyneins.** *Annual review of neuroscience* 2000, **23:**39-71.

41. Merighi A, Polak JM, Gibson SJ, Gulbenkian S, Valentino KL, Peirone SM: **Ultrastructural Studies on Calcitonin Gene-Related Peptide-Immunoreactive, Tachykinin-Immunoreactive and Somatostatin-Immunoreactive Neurons in Rat Dorsal-Root Ganglia - Evidence for the Colocalization of Different Peptides in Single Secretory Granules.** *Cell and tissue research* 1988, **254:**101-109.

42. Gulbenkian S, Merighi A, Wharton J, Varndell IM, Polak JM: **Ultrastructural Evidence for the Coexistence of Calcitonin Gene-Related Peptide and Substance-P in Secretory Vesicles of Peripheral-Nerves in the Guinea-Pig.** *J Neurocytol* 1986, **15:**535-542.

43. de Wit J, Toonen RF, Verhage M: **Matrix-dependent local retention of secretory vesicle cargo in cortical neurons.** *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2009, **29:**23-37.

44. Wong MY, Zhou C, Shakiryanova D, Lloyd TE, Deitcher DL, Levitan ES: **Neuropeptide delivery to synapses by long-range vesicle circulation and sporadic capture.** *Cell* 2012, **148:**1029-1038.

45. Ludwig M, Leng G: **Dendritic peptide release and peptide-dependent behaviours.** *Nature Reviews Neuroscience* 2006, **7:**126-136.

46. Verhage M, Mcmahon HT, Ghijsen WEJM, Boomsma F, Scholten G, Wiegant VM, Nicholls DG: **Differential Release of Amino-Acids, Neuropeptides, and Catecholamines from Isolated Nerve-Terminals.** *Neuron* 1991, **6:**517-524.

47. Berwin B, Floor E, Martin TFJ: **CAPS (mammalian UNC-31) protein localizes to membranes involved in dense-core vesicle exocytosis.** *Neuron* 1998, **21:**137-145.

48. Barg S, Olofsson CS, Schriever-Abeln J, Wendt A, Gebre-Medhin S, Renstrom E, Rorsman P: **Delay between fusion pore opening and peptide release from large dense-core vesicles in neuroendocrine cells.** *Neuron* 2002, **33:**287-299.

49. Artalejo CR, Elhamdani A, Palfrey HC: **Secretion: Dense-core vesicles can kiss-and-run too.** *Current Biology* 1997, **8:**R62-R65.

50. Hewes RS, Taghert PH: **Neuropeptides and neuropeptide receptors in the Drosophila melanogaster genome.** *Genome Res* 2001, **11:**1126-1142.

51. Lingueglia E, Deval E, Lazdunski M: **FMRFamide-gated sodium channel and ASIC channels: A new class of ionotropic receptors for FMRFamide and related peptides.** *Peptides* 2006, **27:**1138-1152.

52. Salio C, Lossi L, Ferrini F, Merighi A: **Neuropeptides as synaptic transmitters.** *Cell and tissue research* 2006, **326:**583-598.

53. Mens WBJ, Witter A, Greidanus TBV: **Penetration of Neurohypophyseal Hormones from Plasma into Cerebrospinal-Fluid (Csf) - Half-Times of Disappearance of These Neuropeptides from Csf.** *Brain research* 1983, **262:**143-149.

54. Merighi A, Salio C, Ferrini F, Lossi L: **Neuromodulatory function of neuropeptides in the normal CNS.** *J Chem Neuroanat* 2011, **42:**276-287.

55. Willows AOD, Pavlova GA, Phillips NE: **Modulation of ciliary beat frequency by neuropeptides from identified molluscan neurons.** *J Exp Biol* 1997, **200:**1433-1439.

56. Katsukura Y, Ando H, David CN, Grimmelikhuijzen CJP, Sugiyama T: **Control of planula migration by LWamide and RFamide neuropeptides in Hydractinia echinata.** *J Exp Biol* 2004, **207:**1803-1810.

57. Braubach OR, Dickinson AJG, Evans CCE, Croll RP: **Neural control of the velum in larvae of the gastropod, Ilyanassa obsoleta.** *J Exp Biol* 2006, **209:**4676-4689.

58. Dickinson AJG, Nason J, Croll RP: **Histochemical localization of FMRFamide, serotonin and catecholamines in embryonic Crepidula fornicata (Gastropoda, Prosobranchia).** *Zoomorphology* 1999, **119:**49-62.

59. Gruhl A: **Serotonergic and FMRFamidergic nervous systems in gymnolaemate bryozoan larvae.** *Zoomorphology* 2009, **128:**135-156.

60. Hadfield MG, Meleshkevitch EA, Boudko DY: **The apical sensory organ of a gastropod veliger is a receptor for settlement cues.** *The Biological bulletin* 2000, **198:**67-76.

61. Nielsen C: **Larval and adult brains.** *Evolution & development* 2005, **7:**483-489.

62. Rentzsch F, Fritzenwanker JH, Scholz CB, Technau U: **FGF signalling controls formation of the apical sensory organ in the cnidarian Nematostella vectensis.** *Development* 2008, **135:**1761-1769.

63. Tessmar-Raible K: **The evolution of neurosecretory centers in bilaterian forebrains: insights from protostomes.** *Seminars in cell & developmental biology* 2007, **18:**492-501.

64. Tessmar-Raible K, Raible F, Christodoulou F, Guy K, Rembold M, Hausen H, Arendt D: **Conserved sensory-neurosecretory cell types in annelid and fish forebrain: Insights into hypothalamus evolution.** *Cell* 2007, **129:**1389-1400.

65. Katsukura Y, David CN, Grimmelikhuijzen CJP, Sugiyama T: **Inhibition of metamorphosis by RFamide neuropeptides in planula larvae of Hydractinia echinata.** *Dev Genes Evol* 2003, **213:**579-586.

66. Leitz T, Lay M: **Metamorphosin-a Is a Neuropeptide.** *Roux Arch Dev Biol* 1995, **204:**276-279.

67. Erwin PM, Szmant AM: **Settlement induction of Acropora palmata planulae by a GLW-amide neuropeptide.** *Coral Reefs* 2010, **29:**929-939.

68. Liu F, Baggerman G, Schoofs L, Wets G: **The construction of a bioactive peptide database in Metazoa.** *Journal of proteome research* 2008, **7:**4119-4131.

69. Truman JW, Riddiford LM: **The origins of insect metamorphosis.** *Nature* 1999, **401:**447-452.

70. De Loof A: **Ecdysteroids, juvenile hormone and insect neuropeptides: Recent successes and remaining major challenges.** *General and comparative endocrinology* 2008, **155:**3-13.

71. Fischer AH, Henrich T, Arendt D: **The normal development of Platynereis dumerilii (Nereididae, Annelida).** *Frontiers in zoology* 2010, **7:**31.

72. Raible F, Tessmar-Raible K, Osoegawa K, Wincker P, Jubin C, Balavoine G, Ferrier D, Benes V, de Jong P, Weissenbach J, et al: **Vertebrate-type intron-rich genes in the marine annelid Platynereis dumerilii.** *Science* 2005, **310:**1325-1326.

73. Denes AS, Jekely G, Steinmetz PR, Raible F, Snyman H, Prud'homme B, Ferrier DE, Balavoine G, Arendt D: **Molecular architecture of annelid nerve cord supports common origin of nervous system centralization in bilateria.** *Cell* 2007, **129:**277-288.

74. Mackie GO, Singla CL, Thiriotquievreux C: **Nervous Control of Ciliary Activity in Gastropod Larvae.** *Biol Bull-Us* 1976, **151:**182-199.

75. Arkett SA, Mackie GO: **Neuronal Control of Ciliary Locomotion in a Gastropod Veliger (Calliostoma).** *Am Zool* 1986, **26:**A126-A126.

76. Hamasaki T, Barkalow K, Richmond J, Satir P: **Camp-Stimulated Phosphorylation of an Axonemal Polypeptide That Copurifies with the 22s Dynein Arm Regulates Microtubule Translocation Velocity and Swimming Speed in Paramecium.** *Proceedings of the National Academy of Sciences of the United States of America* 1991, **88:**7918-7922.

77. Hay-Schmidt A: **The evolution of the serotonergic nervous system.** *Proceedings Biological sciences / The Royal Society* 2000, **267:**1071-1079.

78. Brinkmann N, Wanninger A: **Neurogenesis suggests independent evolution of opercula in serpulid polychaetes.** *BMC evolutionary biology* 2009, **9:**270.

79. Raikova OI, Reuter M, Jondelius U, Gustafsson MK: **The brain of the Nemertodermatida (Platyhelminthes) as revealed by anti-5HT and anti-FMRFamide immunostainings.** *Tissue & cell* 2000, **32:**358-365.

80. Stegner ME, Richter S: **Morphology of the brain in Hutchinsoniella macracantha (Cephalocarida, Crustacea).** *Arthropod structure & development* 2011, **40:**221-243.

81. Walker RJ, Papaioannou S, Holden-Dye L: **A review of FMRFamide- and RFamide-like peptides in metazoa.** *Invertebrate neuroscience : IN* 2009, **9:**111-153.

82. Maillere B, Herve M: **The specificity of antibodies raised against a T cell peptide is influenced by peptide amidation.** *Molecular immunology* 1997, **34:**1003-1009.

83. Grimmelikhuijzen CJP: **Antisera to the Sequence Arg-Phe-Amide Visualize Neuronal Centralization in Hydroid Polyps.** *Cell and tissue research* 1985, **241:**171-182.

84. Conzelmann M, Offenburger SL, Asadulina A, Keller T, Munch TA, Jekely G: **Neuropeptides regulate swimming depth of Platynereis larvae.** *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108:**E1174-1183.

85. Struck TH, Paul C, Hill N, Hartmann S, Hosel C, Kube M, Lieb B, Meyer A, Tiedemann R, Purschke G, Bleidorn C: **Phylogenomic analyses unravel annelid evolution.** *Nature* 2011, **471:**95-98.

86. Veenstra JA: **Neuropeptide evolution: neurohormones and neuropeptides predicted from the genomes of Capitella teleta and Helobdella robusta.** *General and comparative endocrinology* 2011, **171:**160-175.

87. Veenstra JA: **Neurohormones and neuropeptides encoded by the genome of Lottia gigantea, with reference to other mollusks and insects.** *General and comparative endocrinology* 2010, **167:**86-103.

88. Gade G, Hoffmann KH, Spring JH: **Hormonal regulation in insects: facts, gaps, and future directions.** *Physiological reviews* 1997, **77:**963-1032.

89. Christie AE, Cashman CR, Brennan HR, Ma M, Sousa GL, Li L, Stemmler EA, Dickinson PS: **Identification of putative crustacean neuropeptides using in silico analyses of publicly accessible expressed sequence tags.** *General and comparative endocrinology* 2008, **156:**246-264.

90. Christie AE, Stemmler EA, Dickinson PS: **Crustacean neuropeptides.** *Cellular and molecular life sciences : CMLS* 2010, **67:**4135-4169.

91. McVeigh P, Mair GR, Atkinson L, Ladurner P, Zamanian M, Novozhilova E, Marks NJ, Day TA, Maule AG: **Discovery of multiple neuropeptide families in the phylum Platyhelminthes.** *International journal for parasitology* 2009, **39:**1243-1252.

92. Hauser F, Neupert S, Williamson M, Predel R, Tanaka Y, Grimmelikhuijzen CJ: **Genomics and peptidomics of neuropeptides and protein hormones present in the parasitic wasp Nasonia vitripennis.** *Journal of proteome research* 2010, **9:**5296-5310.

93. Jekely G: **Origin and early evolution of neural circuits for the control of ciliary locomotion.** *Proceedings Biological sciences / The Royal Society* 2011, **278:**914-922.

94. Kim YJ, Bartalska K, Audsley N, Yamanaka N, Yapici N, Lee JY, Kim YC, Markovic M, Isaac E, Tanaka Y, Dickson BJ: **MIPs are ancestral ligands for the sex peptide receptor.** *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107:**6520-6525.

95. Yamanaka N, Hua YJ, Roller L, Spalovska-Valachova I, Mizoguchi A, Kataoka H, Tanaka Y: **Bombyx prothoracicostatic peptides activate the sex peptide receptor to regulate ecdysteroid biosynthesis.** *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107:**2060-2065.

96. Tunaru S, Lattig 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:**1271-1280.

97. Tomer R, Denes AS, Tessmar-Raible K, Arendt D: **Profiling by image registration reveals common origin of annelid mushroom bodies and vertebrate pallium.** *Cell* 2010, **142:**800-809.

98. Park D, Taghert PH: **Peptidergic neurosecretory cells in insects: organization and control by the bHLH protein DIMMED.** *General and comparative endocrinology* 2009, **162:**2-7.

99. Perkins LA, Hedgecock EM, Thomson JN, Culotti JG: **Mutant Sensory Cilia in the Nematode Caenorhabditis-Elegans.** *Dev Biol* 1986, **117:**456-487.

100. Purschke G: **Sense organs in polychaetes (Annelida).** *Hydrobiologia* 2005, **535:**53-78.

101. Moroz LL, Edwards JR, Puthanveettil SV, Kohn AB, Ha T, Heyland A, Knudsen B, Sahni A, Yu F, Liu L, et al: **Neuronal transcriptome of Aplysia: neuronal compartments and circuitry.** *Cell* 2006, **127:**1453-1467.

102. Leitz T, Morand K, Mann M: **Metamorphosin-a - a Novel Peptide Controlling Development of the Lower Metazoan Hydractinia-Echinata (Coelenterata, Hydrozoa).** *Dev Biol* 1994, **163:**440-446.

103. Gajewski M, Leitz T, Schlossherr J, Plickert G: **LWamides from Cnidaria constitute a novel family of neuropeptides with morphogenetic activity.** *Roux Arch Dev Biol* 1996, **205:**232-242.

104. Piraino S, Zega G, Di Benedetto C, Leone A, Dell'Anna A, Pennati R, Carnevali DC, Schmid V, Reichert H: **Complex Neural Architecture in the Diploblastic Larva of Clava multicornis (Hydrozoa, Cnidaria).** *J Comp Neurol* 2011, **519:**1931-1951.

105. Sinigaglia C, Busengdal H, Leclere L, Technau U, Rentzsch F: **The Bilaterian Head Patterning Gene six3/6 Controls Aboral Domain Development in a Cnidarian.** *Plos Biol* 2013, **11**.

106. Frederiksen M, Edwards M, Richardson AJ, Halliday NC, Wanless S: **From plankton to top predators: bottom-up control of a marine food web across four trophic levels.** *Journal of Animal Ecology* 2006, **75:**1259-1268.

107. Li W, Feng Z, Sternberg PW, Xu XZ: **A C. elegans stretch receptor neuron revealed by a mechanosensitive TRP channel homologue.** *Nature* 2006, **440:**684-687.

108. Arendt D, Hausen H, Purschke G: **The 'division of labour' model of eye evolution.** *Philos T R Soc B* 2009, **364:**2809-2817.

109. Arendt D: **The evolution of cell types in animals: emerging principles from molecular studies.** *Nature Reviews Genetics* 2008, **9:**868-882.

110. Hartenstein V: **The neuroendocrine system of invertebrates: a developmental and evolutionary perspective.** *J Endocrinol* 2006, **190:**555-570.

OWN CONTRIBUTION TO THE PUBLICATIONS

M Conzelmann, SL Offenburger, A Asadulina, T Keller, TA Munch, G Jekely:
Neuropeptides regulate swimming depth of *Platynereis* larvae:
Figures 1 and S1: Myself did the analysis of precursor sequences.
Figure 2; Movie S1: I characterized neuropeptide precursor expression. High-resolution scans of single neurons was shared with GJ. Raw data images (at least 5 confocal scans per gene) for the *in silico* expression atlas were taken by myself, GJ and TK.
Figure 3; Movies S2, S3: I developed neuropeptide antibodies and did the immunostainings.
Figures 4-6; Movies S4 and S5: I performed the pharmacological experiments on ciliary activity and on freely swimming larvae in the vertical migration setup.
Figure 7: I characterized the behavioral effects of Br-cAMP and nifedipin.
Author summary figure: preparation was shared with GJ.
Manuscript writing and figure preparation and design of the study was shared equally with GJ.

M Conzelmann and G Jekely:
Antibodies against two amino-acid amidated neuropeptide epitopes enrich the comparative neurobiology toolbox:
I performed all experiments and prepared all the figures; design of the study was shared with GJ; myself prepared manuscript writing with support from GJ.

M Conzelmann *, EA Williams *, S Tunaru, N Randel, R Shahidi, A Asadulina, J Berger, S Offermanns, G Jekely: * Contributed equally
Conserved MIP receptor-ligand pair regulates *Platynereis* larval settlement.
Figures 1 and S2; Movies S1 – S5: I performed all MIP assays on larval settlement.
Figures 2 and S3: I characterized MIP expression. I developed neuropeptide antibodies and did the immunostainings.
Figure 3: I did the immunostainings and performed the confocal scans.
Figure 4: Myself did the MIP-receptor clonings. I also measured ciliary closure on MIP-receptor knockdown larvae.
The figure preparation, drafting of the manuscript, and study design was shared equally with GJ and EAW.

APPENDIX: PUBLICATIONS

Publication 1: **Neuropeptides regulate swimming depth of *Platynereis* larvae;** *PNAS* (2011)

Conzelmann *et al*.

Neuropeptides regulate swimming depth of *Platynereis* larvae

Markus Conzelmann[a], Sarah-Lena Offenburger[a,b], Albina Asadulina[a], Timea Keller[a], Thomas A. Münch[b], and Gáspár Jékely[a,1]

[a]Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany; and [b]Werner Reichardt Centre for Integrative Neuroscience, 72076 Tübingen, Germany

Edited by Cornelia Bargmann, The Rockefeller University, New York, NY, and approved September 22, 2011 (received for review June 7, 2011)

Cilia-based locomotion is the major form of locomotion for microscopic planktonic organisms in the ocean. Given their negative buoyancy, these organisms must control ciliary activity to maintain an appropriate depth. The neuronal bases of depth regulation in ciliary swimmers are unknown. To gain insights into depth regulation we studied ciliary locomotor control in the planktonic larva of the marine annelid, *Platynereis*. We found several neuropeptides expressed in distinct sensory neurons that innervate locomotor cilia. Neuropeptides altered ciliary beat frequency and the rate of calcium-evoked ciliary arrests. These changes influenced larval orientation, vertical swimming, and sinking, resulting in upward or downward shifts in the steady-state vertical distribution of larvae. Our findings indicate that *Platynereis* larvae have depth-regulating peptidergic neurons that directly translate sensory inputs into locomotor output on effector cilia. We propose that the simple circuitry found in these ciliated larvae represents an ancestral state in nervous system evolution.

neural circuit | zooplankton | sensory-motor neuron | FMRFamide-related peptides

Two different types of locomotor systems are present in animals, one muscle based and the other cilia based. The neuronal control of muscle-based motor systems is well understood from studies on terrestrial model organisms. In contrast, our knowledge of the neuronal control of ciliary locomotion is limited, even though cilia-driven locomotion is prominent in the majority of animal phyla (1).

Ciliary swimming in open water is widespread among the larval stages of marine invertebrates, including sponges, cnidarians, and many protostomes and deuterostomes (2–5). Freely swimming ciliated larvae often spend days to months as part of the zooplankton (1, 6). The primary axis for ciliated plankton is vertical, and body orientation is maintained either by passive (buoyancy) or active (gravitaxis, phototaxis) mechanisms. When cilia beat, larvae swim upward, and when cilia cease beating, the negatively buoyant larvae sink. During swimming, the thrust exerted on the body is proportional to the beating frequency of cilia (7–9). The alternation of active upward swimming and passive sinking, together with swimming speed and sinking rate, is thought to determine vertical distribution in the water (8). Because several environmental parameters, including water temperature, light intensity, and phytoplankton abundance, change with depth, swimming depth will influence the speed of larval development, the magnitude of UV damage, and the success of larval feeding and settlement. To stay at an appropriate depth, planktonic swimmers must therefore sense environmental cues and regulate ciliary beating.

The ciliated larvae of the marine annelid *Platynereis dumerilii* provide an accessible model for the study of ciliary swimming in marine plankton (10). *Platynereis* can be cultured in the laboratory, and thousands of synchronously developing larvae can be obtained daily year-round (11). *Platynereis* has emerged as a model for the study of the evolution of development (evo-devo) and neurobiology. Its neuronal development is more representative of the ancestral bilaterian condition than that of conventional protostome models (flies, nematodes) and shares many features with vertebrate neurodevelopment (12). *Platynereis* has also retained ancestral neuron types, including ciliary photoreceptors and vasotocin–neurophysin-producing sensory–neurosecretory cells, shared with vertebrates but absent from flies and nematodes (13, 14). Such conservation makes *Platynereis* an interesting model for the reconstruction of the ancestral state of the bilaterian nervous system. The larval nervous system of *Platynereis* also shows surprising simplicity in its circuitry. The photoreceptor cell of the larval eyespot was shown to directly synapse on the ciliated cells and regulate phototactic turning (10). Such a sensory-motor system, directly regulating cilia, may be a relic from the earliest stages of the evolution of eyes and neural circuits (10, 15).

Planktonic ciliated larvae also adjust their ciliary activity in response to several environmental cues other than light (16–20). It is unclear, however, how other cues affect cilia and whether the innervation of ciliary bands by other neurons is as simple as that of the larval eyespots. Anatomical studies have revealed that larval ciliary bands receive extensive innervation from the nervous system, both in *Platynereis* and in other species (21). In protostome larvae, neurons expressing the neuropeptide FMRF-amide often contribute to this innervation (22–24). Neurons with related F-amide neuropeptides also innervate ciliary bands in sea urchin larvae (25), suggesting that neuropeptides may have a general role in the regulation of larval locomotion in both protostomes and deuterostomes. However, these limited studies have not revealed the general neural circuit architecture of ciliated larvae and the role of neuropeptides in regulating ciliary swimming.

Neuropeptides are considered the oldest neuronal signaling molecules in animals (26). They are produced from inactive precursor proteins by proteolytic cleavage and further processing (e.g., amidation) (27–29) and are released into the hemolymph to act as hormones or at synapses to regulate target cells. Neuropeptides have a wide range of functions in the control of neural circuits and physiology, including the modulation of locomotion and rhythmic pattern generators (30–33), presynaptic facilitation and remodeling of sensory networks (34, 35), and the regulation of reproduction (36, 37). We have only limited information about the role of neuropeptides in the regulation of ciliary beating (38, 39).

Author contributions: T.A.M. and G.J. designed research; M.C., S.-L.O., A.A., and T.K. performed research; M.C., S.-L.O., A.A., T.A.M., and G.J. analyzed data; and M.C. and G.J. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. JF811323–JF811333).

[1]To whom correspondence should be addressed. E-mail: gaspar.jekely@tuebingen.mpg.de.

See Author Summary on page 18593.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1109085108/-/DCSupplemental.

To gain further insights into ciliary locomotor control we characterized neuropeptide functions and the associated neural circuits in the larvae of *Platynereis*. We found diverse neuropeptides expressed in larval sensory neurons that directly innervate the ciliary band. Application of synthetic neuropeptides altered both ciliary beat frequency and the rate and duration of ciliary arrests, indicating that neuropeptides act as neurotransmitters on the ciliated cells. The neuropeptide-induced changes in ciliary activity altered the swimming trajectories and shifted the vertical distribution of the larvae. Our results suggest that in planktonic swimmers, neuropeptides released upon sensory stimulation at neurociliary synapses modulate ciliary activity, ultimately resulting in changes in swimming depth. The control of ciliary swimming, the ancestral form of animal locomotion, by a simple sensory-motor nervous system suggests that these locomotor circuits may represent an ancestral stage of neural circuit evolution.

Results

Identification of *Platynereis* Neuropeptides. Given the widespread role of neuropeptides in regulating animal locomotion (30–32), we set out to characterize neuropeptides of the *Platynereis* larval nervous system. Here we describe 11 neuropeptide precursors identified in a *Platynereis* larval transcriptome resource using a combination of BLAST and pattern searches. On the basis of the 11 precursor sequences we predicted 120 *Platynereis* neuropeptides forming 11 distinct groups of similar peptides (Fig. 1). Full-length precursor sequences have an N-terminal signal peptide and contain repetitions of similar short neuropeptide sequences flanked by dibasic cleavage sites (KR, RK, or KK) for prohormone convertases (27, 28). We deduced the structure of mature *Platynereis* neuropeptides using NeuroPred (40) and manual curation (Fig. 1). In 8 precursors most peptides contain a Gly residue before the dibasic cleavage site. These peptides are expected to be further processed by α-amidating enzymes (29) and to terminate in an α-amide (RYa, FVMa, DLa, FMRFa, FVa, LYa, YFa, and FLa; "a", "amide"). Other precursors give rise to peptides with a carboxyl terminus (L11, SPY, and WLD).

The *Platynereis* neuropeptide precursors are related to the widely distributed family of RFamide–like neuropeptide precursors. Members of this family are present in all eumetazoans, and their mature peptides can have diverse functions in the regulation of neuronal circuits (31, 37, 41–44). Many *Platynereis* neuropeptides have close relatives in other lophotrochozoan species (mollusks and annelids) and some also outside the lophotrochozoans (RYa, RFa, and L11). Conservation of amidated neuropeptides is restricted to a few residues N-terminal to the cleavage site and amidation signature (Fig. S1). Amidated neuropeptides within the same precursor protein also show higher conservation close to the amidated terminus (Fig. 1), indicating that the functionally important residues are located there. In contrast, nonamidated neuropeptides often show stronger N-terminal conservation both between species and within the same precursor (Fig. 1 and Fig. S1).

Neuropeptides Are Expressed in Distinct Sensory Neurons in the *Platynereis* Larval Nervous System. To map neuropeptide expression in the *Platynereis* larval nervous system we performed in situ hybridizations on whole larvae with precursor-specific RNA probes. We combined in situ hybridization with fluorescent antibody staining using an anti-acetylated tubulin antibody that stains stabilized microtubules in cilia, axons, and dendrites. The samples were scanned in a confocal microscope combining fluorescence

Precursor name	Predicted precursor and mature neuropeptides	Neuropeptide logos
RYa	VFRYa(3x), GTLMRYa(2x), GSLMRYa(2x), GTLLRYa, LFRWa, IFRYa	
FVMa	NDGDYSKFVMa(6x), NADDYSKFVMa(2x), ASPNYQDFVLa(2x), NVDDYSKFVMa, DDNDYSNFVMa, NDKDYSNFVMa, NDGDYSNFVMa, NNGDYSKFVMa, NGEDYSKFVMa, NGEDYSHFVMa, NANEYSKFVMa, NPKDLDSFYMa, SIPEYDAFVMa, KNYQDFVMa, AAGNYQDFVMa, GAPQYQDFVMa, GAPHYQDFVLa, SPNYQDFVLa, AEPNYQDFVLa, ASANYQDFVLa, NSQNYQDFVLa, GSADYQDFVFa, SHGPQNYENFVME, EQKPDYQSFLLa	
DLa	YYGFNNDLa(2x), YSSFRADLa(2x), YAFNADLa, FAAFNTDLa, MGFNADLa, YMGFNADLa, FMFRQDLa, AMFRGDLa, SYGFRSDLa, FSSFRADLa, YSGFRADLa	
FMRFa	FMRFa(21x), GLKFa, GGGYIRFa, AGGHYMRFa, GEKGFMRFa, DGENGFMRFa, SFDPSLYLQMRQa	
FVa	AHRFVa, AHMFVa(2x), RLFVa(2x), NRMFVa(2x), AAHRFVa, PHNFVa, GHMFVa, RMFIa, ARMFIa, RMFIa, NRMFIa, RYFIa, RQLYIa, DGISRDIRRLWIa	
LYa/ SFD	QLDSLGGAEIPLYa(2x), GQSARQLDSLGGAEIPLYa, QLDSLGGAQIPLYa, SIVDDDDENSIYAa, QLDTLGGGQIPAED, SFDSIGHSSNFAGLD(10x), PFDSIGHSSSFAGL(2x), PFDSIGQSSNFAGLD(2x), AFDSIGHHSAFGGL(2x), SFNSIGHASNFAGL, PFDSIGHSSNFAGLD, AFDSIGHSSNFAGLD, AFDSIGHHSAFSGL	
YFa	KMVYFa(3x), RMVYFa, YFa, YPNTVLFa, APLFKFa, QFMFa, KFYFa, FFLa	
L11	PDCTRFVFHPSCRGVAA, SESEEFVLDP, SSINQEGGADHYISNGADEDFIEFSEDDFAEN, QADQASSYDLGLAAKLWPHI, ALLGRTN, GSQDVEAFAPQ	
SPY	SPYAGFMTGD, SPYAGFLASNSED, SPYAKFLNED, SPYAKFMTGDEE, SPYANFMAGD, SPYAKFMGSSED, SPYAKFMGSSED, SPYAKFMGSNEE, SPYAQFMGSNEE, SPYAKYMYNEND, SPYGAYMGMMSDD, SPGFRSM, SPGFRHM, SPGFRFM, GAGFRSM	
FLa	AKYFLa(6x), QKYFLa, SPSPFLa, SPFLa, PRNNFLa	
WLD	WLDNSQFRDE(4x), WLDNSHFNED(4x), WLDHSQFRED(3x), WLDNSQFKDED(2x), WLDNSQFKDED, WLDNSQFIED, WLDNSQFKDDD, WIDASQFADD, WVDMMHFNDE, WLDSMQFNED, WVDPLNYGLD	

Fig. 1. Neuropeptide precursors and their predicted neuropeptides in *Platynereis*. Schematic drawings of *Platynereis* neuropeptide precursors are shown with the location of the signal peptide (blue), the amidated (yellow) and nonamidated (green) neuropeptides, and cleavage sites (red). Sequences and the number of neuropeptides predicted from each precursor are shown. Sequence logos were generated on the basis of alignments of all neuropeptides from one precursor. The amidation signature C-terminal Gly is included in the logos. For the L11 precursor no logo is shown because the peptides are not similar. Neuropeptides used for the pharmacological experiments and immunizations (with an extra N-terminal Cys) are shown in red. For SPY and WLD we did not obtain a working antibody.

and reflection microscopy, a technique that visualizes the in situ hybridization signal [alkaline phosphatase/nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) staining] by reflection of the laser beam (45). Using this technique we obtained high-resolution three-dimensional information on the expression patterns and also on the morphology of the labeled cells.

We found that all precursors were expressed 48 h post-fertilization (hpf) in the larval episphere (the anterior part of the larva) in a cell-type–specific manner (Fig. 2) in patterns that were largely invariant for the same gene from larva to larva. For all precursors, expression was detected in 2–12 neurons in the larval episphere.

To obtain a molecular map of all peptidergic neurons at cellular resolution we calculated an average expression pattern for each neuropeptide on the basis of three to five confocal scans and then registered these averages to a reference axonal scaffold.

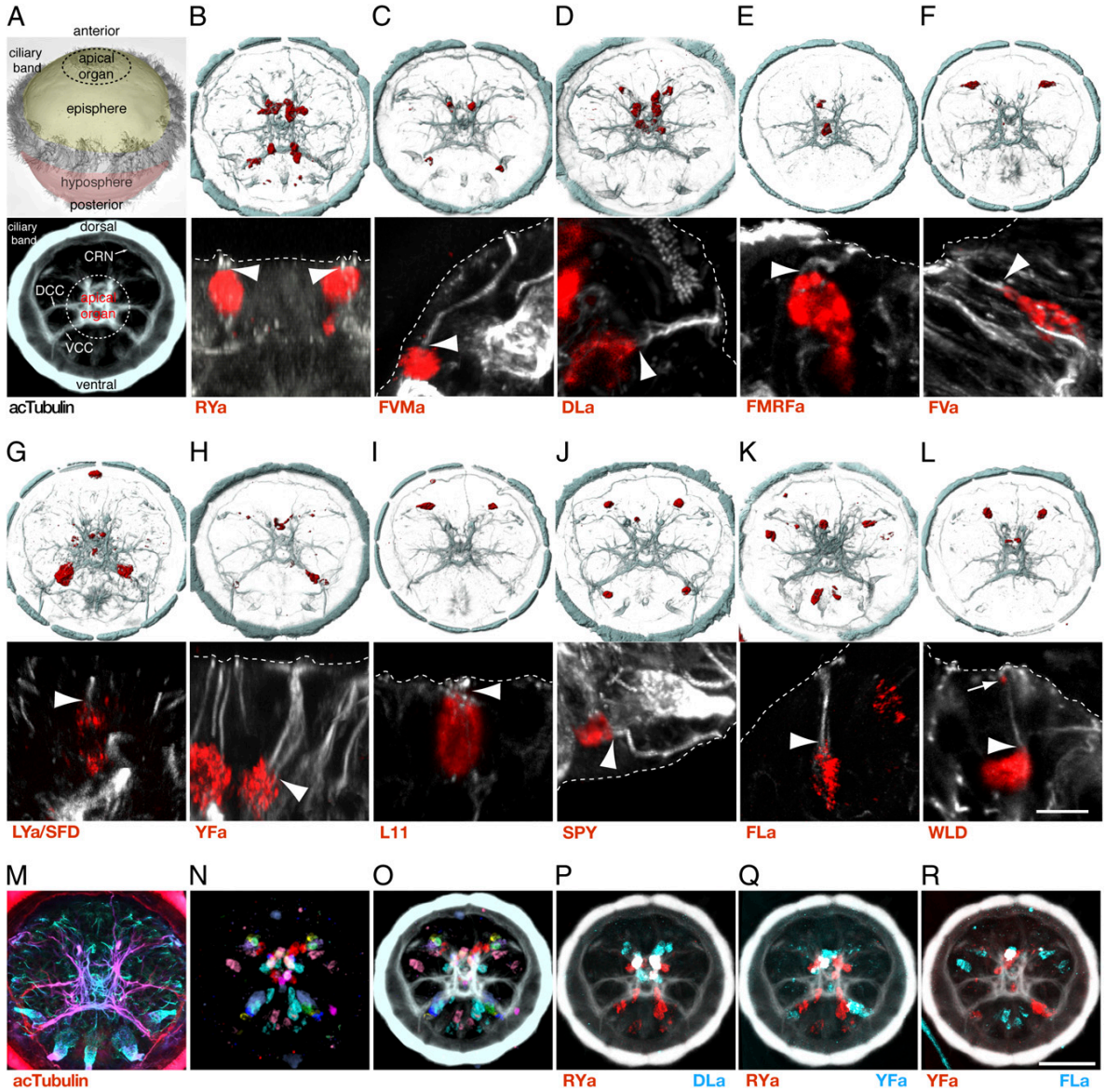


Fig. 2. Expression of neuropeptide precursors in *Platynereis* larval sensory cells. (*A*) SEM image of a *Platynereis* larva with the main body regions indicated (*Upper*) and anterior view of the larval axonal scaffold with the main nerves indicated (*Lower*). (*B–L*) Whole-mount in situ hybridization for *RYa* (*B*), *FVMa* (*C*), *DLa* (*D*), *FMRFa* (*E*), *FVa* (*F*), *LYa/SFD* (*G*), *YFa* (*H*), *L11* (*I*), *SPY* (*J*) *FLa* (*K*), and *WLD* (*L*) neuropeptide precursor mRNAs (red) counterstained with anti-acTubulin antibody (cyan, *Upper*; white, *Lower*). (*Lower*) close-up images of neuropeptide-expressing sensory cells. (*M*) Apical sensory dendrites (in cyan) in a depth-encoded acetylated tubulin confocal stack of a 48-hpf larva. (*N* and *O*) Expression of all neuropeptides (*N*) in the larval episphere, projected on a common reference scaffold (*O*) by image registration. (*P–R*) Overlap of *RYa* and *DLa* (*P*), *RYa* and *YFa* (*Q*) and *YFa* and *FLa* (*R*) expression, determined by image registration. All images are of 48-hpf larvae. *A* is a dorsal view, and *B–R* are anterior views. The in situ signal (red) labels the cell bodies. (*Lower*) Arrowheads point to the base of the apical sensory dendrites. Arrow in *L* points to WLD signal localized to the tip of the dendrite. Dashed lines indicate the apical margin of the neuroepithelium. [Scale bars: *B–L* (*Lower*), 5 μm; and *B–L* (*Upper*) and *M–R*, 50 μm.] CRN, ciliary ring nerve; DCC, dorsal branch of the circumesophageal connectives; VCC, ventral branch of the circumesophageal connectives.

This procedure is possible because *Platynereis* larvae, like many spiralians, have a strict cell lineage (46) and there is very little cellular-level variation among larvae of the same developmental stage. For image registration we used a procedure similar to the one recently described for *Platynereis* (47), taking advantage of the highly stereotypic acetylated-tubulin reference channel.

Placing these average expression maps into the common acetylated-tubulin reference (Fig. 2*A*) revealed that neuropeptides are expressed broadly in the larval nervous system (Fig. 2 *N* and *O* and Movie S1). Many peptidergic neurons concentrated in the central region of the episphere, the apical organ (Fig. 2 *B*–*E*, *N*, and *O*). We further defined a bilaterally arranged dorso-lateral cluster of peptidergic neurons, in the region of the developing cerebral eyes, and a ventro-lateral cluster, above the ventral branch of the circumesophageal connectives (Fig. 2 *F*–*L*). The SD of cell center positions among different registered scans for the same gene ranged between 0.6 and 3.34 μm (median = 2.63; along the *x* axis), whereas the average cell diameter was 11.1 μm (SD = 4.11; along the *x* axis). This cellular resolution allowed us to show that most neuropeptides were not coexpressed and therefore define distinct peptidergic neurons in the *Platynereis* larva. We found only three pairs of neuropeptides with partially overlapping expression. DLa and RYa were coexpressed in three apical organ cells, whereas YFa overlapped with both FLa and RYa in two different cells of the apical organ (Fig. 2 *P*–*R*).

To characterize the morphology of the neuropeptidergic cells we performed high-resolution confocal scans of the NBT/BCIP signal together with the acetylated tubulin signal. The acetylated tubulin signal revealed that the larval episphere forms a polarized neural tissue with apical dendrites and a basal axonal scaffold (Fig. 2*M*). The neuropeptide precursors were all expressed in neurons with differentiated morphology, with cell bodies continuing in basally projecting axons that joined the axonal scaffold of the larval nervous system. Most of the peptidergic neurons

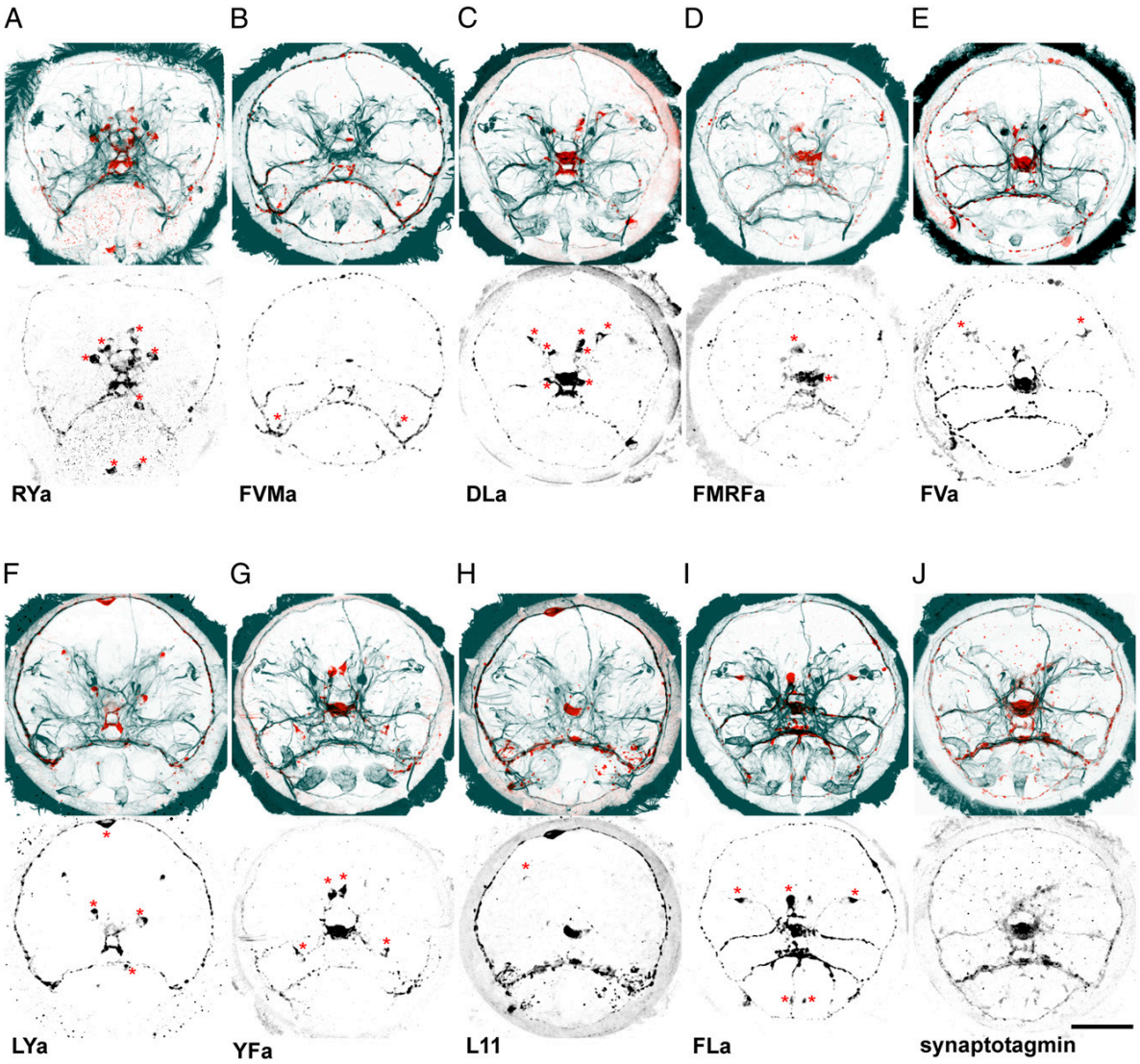


Fig. 3. Neuropeptidergic sensory neurons directly innervate larval ciliary bands. Immunostainings with RYa (*A*), FVMa (*B*), DLa (*C*), FMRFa (*D*), FVa (*E*), LYa (*F*), YFa (*G*), L11 (*H*), FLa (*I*), and synaptotagmin (*J*) antibodies are shown in red (*Upper*) and in black (*Lower*), counterstained with anti-acTubulin (cyan). Labeled cell bodies that correspond to the cells labeled with in situ hybridization are marked with an asterisk. All images are anterior views of 48-hpf larvae. (Scale bar: 50 μm.)

were flask shaped and also had a dendrite extending apically (Fig. 2 *B–L*). Such flask-shaped sensory morphology has already been described with immunolabeling for the FMRFa-expressing cells in the *Platynereis* larva that we localize here with the FMRFa precursor (14). The ultrastructural reconstruction of these cells revealed that the apical dendrite has two sensory cilia. These cilia extend into the subcuticular space that has access to the external environment (14). Similar sensory cell morphologies have also been described ultrastructurally in various other annelids (48). The peptidergic sensory cells we found also had acetylated-tubulin positive projections that reach the apical-most side of the neuroepithelium and therefore are in direct contact with the external environment. The dendrites of different cells had terminals with various apical morphological modifications, including split (Fig. 2 *B*, *D*, and *H*) and bulbous endings (Fig. 2*C*), indicating that the neuropeptidergic neurons have various sensory modalities. We also observed that the mRNA of the WLD precursor localized to the tip of the sensory dendrite (Fig. 2*L*). We did not find apical dendrites on the large ventral LYa-expressing cells (Fig. 2*G*); these cells therefore likely do not have a sensory function.

Overall, our neuropeptide expression data revealed a cellular resolution molecular map of sensory neurons in the 48-hpf *Platynereis* larva.

Peptidergic Sensory Neurons Directly Innervate the Larval Ciliary Band. Maturing neuropeptides can be transported to synaptic release sites via axonal transport. To visualize axonal projections of *Platynereis* sensory neurons, we developed antibodies against one mature peptide from each precursor (Fig. 1). We affinity purified the antibodies using the respective peptides immobilized to a resin and then performed immunostainings on 48-hpf larvae. The immunostainings revealed cell body and axonal signals for all neuropeptides. We always found a strong correlation in the number and position of the cell bodies between our in situ hybridization and immunostaining data, showing that the antibodies do not cross-react and label the same cells that express the respective precursors. (Fig. 3; compare with Fig. 2 and Movies S2 and S3). Although the cell body labeling was weak for FVMa and L11 (Fig. 3 *B* and *H*), upon inspection of 3D renderings of the data (Movies S2 and S3) we could nevertheless see them in positions corresponding to precursor expression. Using the FMRFa antibody, in addition to the two cells that also showed precursor expression (Fig. 2*E*) we observed three faintly labeled cells. Similarly, for LYa we observed two faint cells located more dorsally. These signals could be due either to the higher sensitivity of antibody stainings or to weak unspecific labeling.

We also observed staining of the axonal projections of the neuropeptidergic sensory neurons. Axons for all neuron types run along the ventral branch of the circumesophageal connectives (Fig. 3 *A–I*) and in some cases also along the dorsal branch (FVMa, DLa, FVa, YFa, and FLa; Fig. 3 *B*, *C*, *E*, *G*, and *I*). Axons joined the ciliary ring nerve (CRN) innervating the main ciliary band of the larva (Fig. 3 *A–I*; compare with Fig. 2*A*) and formed several varicose thickenings along its entire length (Fig. 3 *A–I*), indicating *en passant* synaptic contacts to the ciliated cells. Direct synaptic contact between ciliated cells and the ciliary ring nerve has also been described by electron microscopy for the larval eye photoreceptor axon (10).

To confirm that the ciliary ring nerve has synaptic zones all along its length and not only where the eyespot photoreceptor axon forms synapses (10) we generated an antibody against *Platynereis* synaptotagmin (12), a transmembrane protein involved in the fusion of synaptic vesicles and large dense-cored vesicles (49, 50). Anti-synaptotagmin antibody staining revealed strong signal in the median nervous system, along the circumesophageal connectives, and along the entire length of the ciliary ring nerve (Fig. 3*J*).

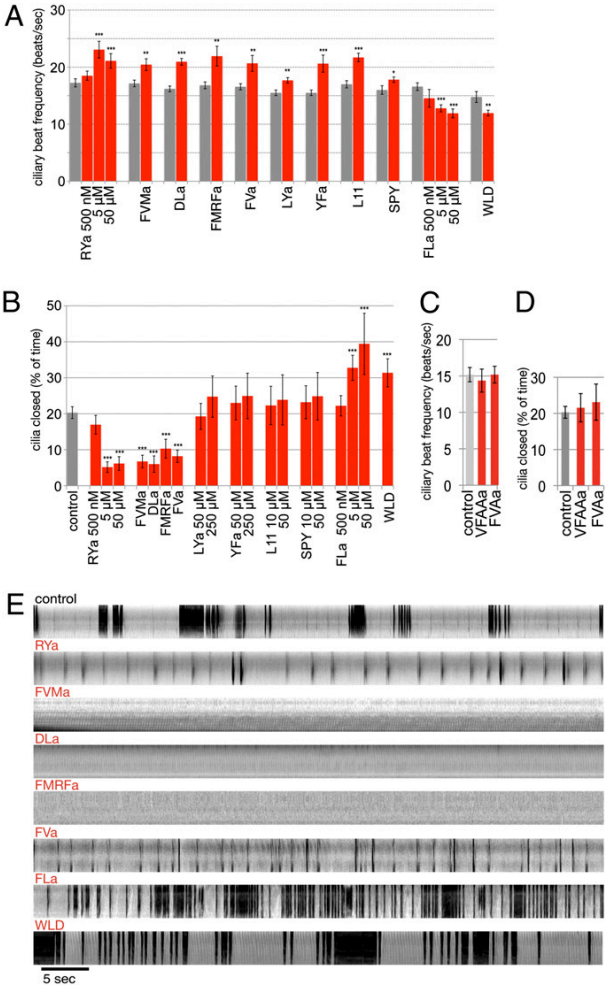


Fig. 4. Neuropeptides regulate ciliary beating and arrests. (*A*) Ciliary beat frequency in the presence of neuropeptides. Gray bars represent untreated controls from the same batch of larvae. (*B*) Quantification of ciliary arrests in the presence of neuropeptides. (*C*) Ciliary beat frequency in control larvae (pooled from several batches) and in the presence of the Ala substituted neuropeptides. (*D*) Quantification of ciliary arrests in the presence of the Ala substituted neuropeptides. For *B* and *D* the same pooled control is shown. (*E*) Representative kymographs of ciliary beating (light gray sections) and ciliary arrests (dark gray sections) in the presence of neuropeptides, generated from 1-min videos on single immobilized larvae. The kymographs were generated from line selections perpendicular to the beating cilia (compare Movie S4). In *A–D* data are shown as mean ± SEM. *P* values of an unpaired *t* test are indicated: $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$. $n > 10$ larvae for *A–D*. In *A* the final concentrations are indicated for RYa and FLa; for the other peptides we used 5 µM (DLa), 10 µM (L11 and SPY), 20 µM (FVMa and WLD), and 50 µM (FMRFa, FVa, LYa, and YFa). In *B* we used concentrations as indicated or 5 µM (DLa), 20 µM (FVMa and WLD), and 50 µM (FMRFa and FVa), and in *C* and *D* we used 5 µM (VFAAa) and 20 µM (FVAa). For the full peptide sequences used see Fig. 1.

Many neuropeptides (DLa, FMRFa, FVa, YFa, L11, and FLa) also showed intense labeling in the apical neurosecretory plexus of the larva, indicating that they may also be released here (Fig. 3 *C–E* and *G–I*). A neurosecretory function has already been suggested for FMRFa (14). The innervation of the ciliary band by the diverse neuropeptidergic sensory neurons suggests that these cells, in addition to having a possible neurosecretory

function, are involved in the direct motor control of the ciliary band and that there is extensive peptidergic regulation during ciliary swimming.

Neuropeptides Influence Ciliary Beating and Arrests. The innervation of the ciliary band by neuropeptidergic cells suggested that neuropeptides could directly influence ciliary activity. To uncover neuropeptide effects we characterized ciliary activity using video microscopy on immobilized *Platynereis* larvae. We scored ciliary beat frequency and the rate and duration of ciliary arrests. Control larvae (50–60 hpf) beat with their cilia with a frequency of 16.2 beats/s (SEM = 0.26, n = 146 larvae). Complete arrests of all cilia occurred at regular intervals and lasted for several seconds (Movie S4 and Fig. 4*E*). The ratio of the total duration of arrested to beating periods was 0.22 (SEM = 0.02, n = 28 larvae).

We next tested the effect of synthetic neuropeptides on ciliary beat frequency and ciliary arrests (500 nM to 250 μM concentration range, see Fig. 4 legend). Nine neuropeptides increased ciliary beat frequency (RYa, FVMa, DLa, FMRFa, FVa, LYa, YFa, L11, and SPY), and two decreased ciliary beat frequency (FLa and WLD, Fig. 4*A*).

The neuropeptides also affected ciliary arrests as quantified by the total duration of arrests during 1-min recordings (Fig. 4*B*) and visualized by kymographs (Fig. 4*E* and Movie S4). In the presence of RYa, FVMa, DLa, FMRFa, and FVa, ciliary arrests were strongly reduced (Fig. 4 *B* and *E* and Movie S4). In contrast, FLa and WLD led to very frequent and sustained arrests (Fig. 4 *B* and *E* and Movie S4). LYa, YFa, L11, and SPY had no effect on ciliary arrests even at concentrations five times higher than the concentrations significantly increasing ciliary beat frequency (Fig. 4*B*). These effects were dose dependent as determined for RYa and FLa (Fig. 4 *A* and *B*).

Next we tested the importance of conserved C-terminal residues in mature neuropeptides. Whereas the naturally occurring RYa (full sequence: VFRYa) increased ciliary beat frequency and abolished sustained ciliary arrests (Fig. 4 *A*, *B*, and *E*), changing the RY residues to Ala (VFAAa) resulted in a loss of these effects (Fig. 4 *C* and *D*). FVMa (full sequence: NDGDYSKFVMa) also increased ciliary beat frequency and inhibited arrests (Fig. 4 *A*, *B*, and *E*), but its effects were lost when the C-terminal Met residue was changed to Ala (Fig. 4 *C* and *D*).

These experiments show that *Platynereis* neuropeptides have strong and sequence-specific effects on two parameters of larval ciliary activity, ciliary beat frequency and ciliary arrests.

Neuropeptides Regulate Depth in the Water Column. It has been proposed that changes in ciliary beat frequency and ciliary arrests regulate the depth of ciliary planktonic swimmers (8). To quantitatively test this hypothesis and to analyze the effects of neuropeptides on larval swimming behavior, we developed a vertical migration setup consisting of 25-cm–high tubes. This setup allowed us to record the swimming activity and the steady-state vertical distribution of large populations of larvae under different conditions.

First, we recorded swimming larvae at high resolution and found that they swam with a right-handed spiral, with their anterior end pointing upward. Occasionally, larvae sank with their anterior end pointing up, indicating that the center of gravity is closer to the posterior end (Movie S5). The analysis of swimming tracks in populations of untreated control larvae revealed that active upward and lateral swimming and passive sinking were balanced (no average displacement in the vertical direction). The angular plots of the displacement vectors of larval tracks showed a broad bimodal distribution, with the majority of tracks pointing upward or downward (Fig. 5*A*). Such nonbiased upward and downward displacement in the vertical tubes maintained a uniform vertical distribution as a steady state (Fig. 6*A*).

Next, we tested how changes in ciliary beat frequency and ciliary arrests induced by the neuropeptides alter the directionality of larval movement and the steady-state larval distribution. Neuropeptides that inhibited ciliary arrest and/or increased ciliary beat frequency led to biased upward swimming (RYa, FVMa, DLa, FMRFa, FVa, LYa, YFa, L11, and SPY; Fig. 5 *B*–*J*). This upward swimming was not observed when we used RYa and FVMa peptides with Ala substitutions (Fig. 5 *B* and *C*). In contrast, application of FLa and WLD, two peptides that caused lower ciliary beat frequency and frequent ciliary arrests, resulted in downward displacement of larvae (Fig. 5 *K* and *L*).

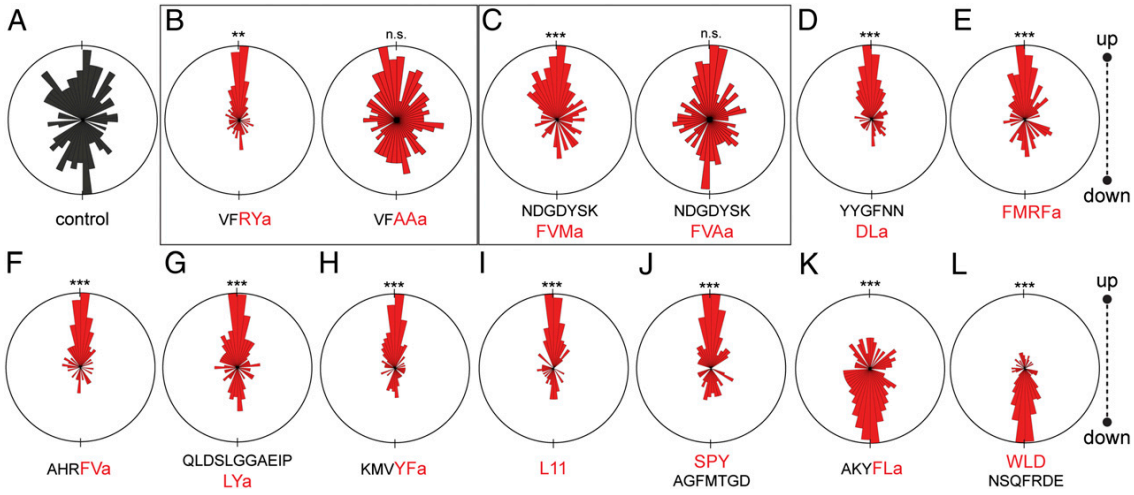


Fig. 5. Neuropeptides regulate larval swimming directions. Angular plots are shown of the displacement vectors of larval swimming tracks for (*A*) control larvae and (*B*–*L*) larvae in the presence of the indicated neuropeptides. *P* values of a χ^2-test comparing the number of upward and downward swimming larvae are indicated: $^{**}P < 0.01$; $^{***}P < 0.001$. For each peptide the χ^2-test was performed with a measurement on control larvae from the same batch. In *A*, a representative control is shown. $n > 121$ larvae for all measurements. The final concentrations were 5 μM (RYa, AAa, DLa, and FLa), 10 μM (L11 and SPY), 20 μM (FVMa, FVAa, and WLD), and 50 μM (FMRFa, FVa, LYa, and YFa).

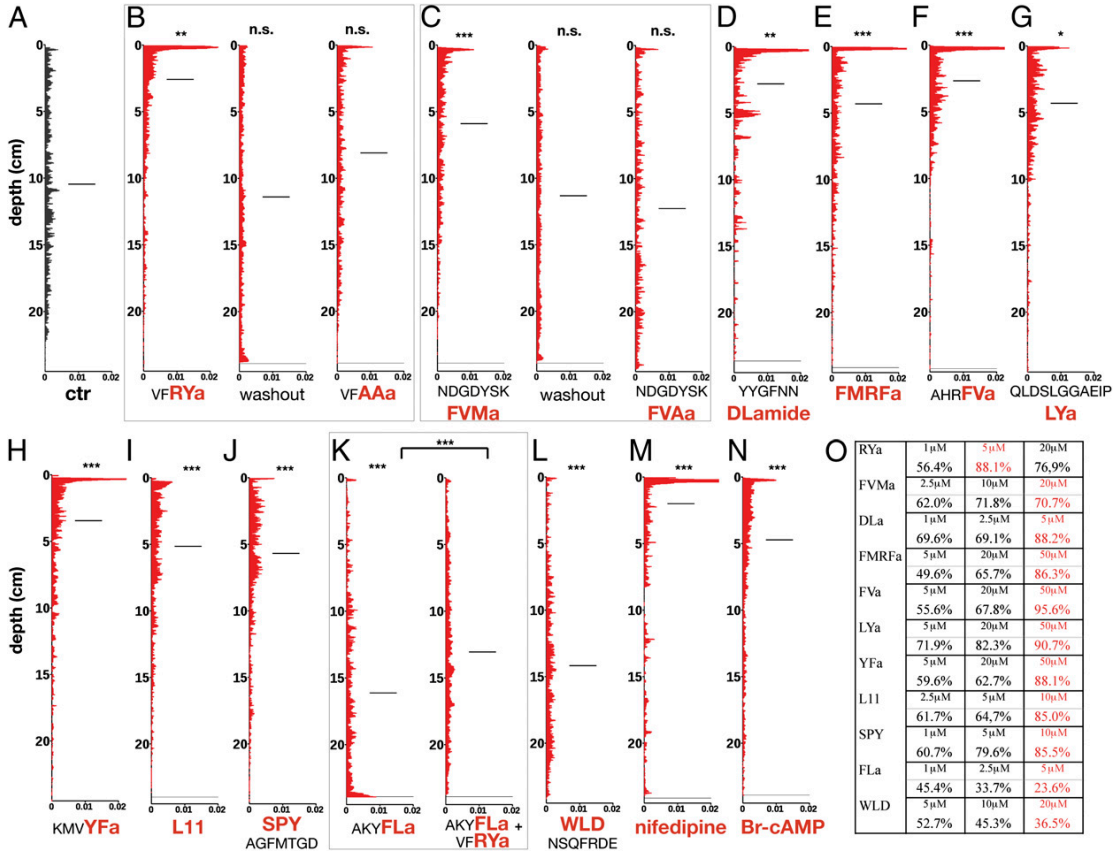


Fig. 6. Neuropeptides regulate larval vertical distribution. Vertical distribution of control larvae (*A*) and larvae in the presence of VFRYa, washout, and VFAAa (*B*); NDGDYSKFVMa, washout, and NDGDYSKFVAa (*C*); YYGFNNDLa (*D*); FMRFa (*E*); AHRFVa (*F*); QLDSLGGAEIPLYa (*G*); KMVYFa (*H*); PDCTRFVFHPSCRGVAA or L11 (*I*); SPYAGFMTGD (*J*); AKYFLa and AKYFLa+VFRYa (*K*); WLDNSQFRDE (*L*); nifedipine (10 μM) (*M*); and Br-cAMP (5 μM) (*N*). (*O*) Percentage of larvae in the upper half of the tubes in the presence of different peptide concentrations. The concentrations shown in red were used in the other experiments. The horizontal lines in *A*–*N* indicate the mean. *P* values of a χ^2-test are indicated: $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$. $n > 69$ larvae. The final concentrations of neuropeptides in *B*–*L* were 5 μM (RYa, AAa, DLa, and FLa), 10 μM (L11 and SPY), 20 μM (FVMa, FVAa, and WLD), and 50 μM (FMRFa, FVa, LYa, and YFa).

The changes triggered by the neuropeptides in the directionality of larval movement also shifted the mean vertical distribution at steady state in a dose-dependent manner. Activating peptides led to strong upward shifts, whereas inhibitory peptides caused downward shifts (Fig. 6). The peptide effects were lost following washout, as shown for RYa and FVMa (Fig. 6 *B* and *C*). When we combined RYa and FLa, two peptides with opposing effects, the effects canceled out, indicating that neuropeptide effects are additive (Fig. 6*K*). All neuropeptides affected vertical shifts in a concentration-dependent manner (Fig. 6*O*).

By influencing ciliary beat frequency and ciliary arrests, neuropeptides can change the net directionality of movement, leading to large shifts in the vertical distribution of populations of ciliated larvae.

Ciliary Arrests and Beat Frequency Have Distinct Contributions to Larval Swimming. All neuropeptides that affected ciliary closures also affected ciliary beat frequency (Fig. 4 *A* and *B*). To uncouple the contribution of ciliary arrests and beat frequency on larval swimming, we tried to influence these parameters separately. To interfere with ciliary arrests without changing beat frequency we first characterized the mechanism of arrests.

When performing extracellular current measurements with a recording pipette penetrated into the center of *Platynereis* larvae, we observed regular spiking activity. Combined extracellular recordings and video microscopy of cilia showed that the spikes correlated with ciliary arrests, with the first spike in a spike train preceding the arrest (Fig. 7*A*). Sustained arrests of cilia were accompanied by repeated spiking events of a frequency between 5 and 10 Hz, during the entire duration of the arrest episode. The long duration of the spiking events (full width at half max = 1.45 ms, SD = 0.17; Fig. 7*B*) indicated that they were likely evoked by calcium, rather than sodium. Calcium spikes were also shown to trigger ciliary arrests in mollusk larvae (19, 51). To test whether voltage-dependent calcium channels were responsible for the recorded spike events, we applied the L-type calcium-channel blocker nifedipine. In the presence of nifedipine, beat frequency was unaffected (Fig. 7*C*), but spikes and ciliary arrests were completely abolished (Fig. 7 *A* and *D*). These observations indicate that calcium spikes generated by L-type calcium channels induce and sustain ciliary arrests and also provided a tool to specifically block arrests without affecting ciliary beat frequency.

To test the contribution of ciliary arrests to the overall directionality of larval movement, we then tested the effect of nifedipine in vertical swimming assays. Application of nifedipine led to consistent upward swimming (Fig. 7 *E* and *F*; average upward displacement = 0.92 mm/s; control = −0.03 mm/s). Larval trajectories were straighter than in control larvae (Fig. 7*F*), also shown by the narrower distribution in the angular his-

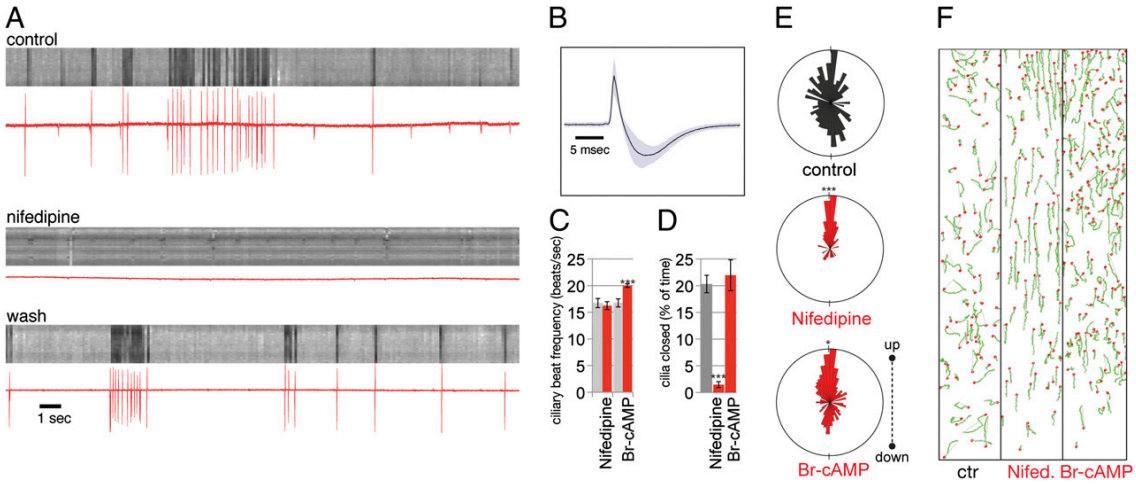


Fig. 7. Calcium-regulated arrests and ciliary beat frequency both affect larval swimming. (*A*) Kymographs of ciliary beating (light gray sections) and ciliary arrest (dark gray sections) with parallel extracellular current recordings (red traces) for control, nifedipine addition, and washout. (*B*) Average of 179 spikes from recordings with a 0.1-ms temporal resolution. (*C*) Ciliary beat frequency in the presence of nifedipine and Br-cAMP. (*D*) Quantification of ciliary arrests in the presence of nifedipine and Br-cAMP. (*E*) Angular plots of the displacement vectors of larval swimming tracks for control larvae and larvae in the presence of nifedipine and Br-cAMP. (*F*) Swimming trajectories of control larvae and larvae in the presence of nifedipine and Br-cAMP. Red dots indicate the end of the tracks. The beginning and end of each track is connected by a gray line. Tracks were generated from a 10-s section of 30-fps video recordings. In *C* and *D* data are shown as mean ± SEM. *P* values of an unpaired *t* test are indicated: $*P < 0.05$; and $***P < 0.001$. $n > 10$ larvae for *C* and *D*. Final concentration was 10 μM for nifedipine and 5 μM for Br-cAMP.

tograms (Fig. 7*E*). The consistent upward swimming led to an upward-shifted steady-state distribution (Fig. 6*M*).

The up-regulation of ciliary beat frequency also led to upward swimming, as shown by the neuropeptides that increased beat frequency without affecting arrests (LYa, YFa, L11, and SPY; Fig. 4 *A* and *B* and Fig. 5 *G*–*J*). To influence beat frequency independent of neuropeptides, we used the cell-permeable cAMP analog, Br-cAMP. cAMP is known to increase ciliary beat frequency in a wide range of eukaryotes (52) and it also increased beat frequency in *Platynereis* larvae, without affecting arrests (Fig. 7 *C* and *D*). As with neuropeptides, the Br-cAMP–induced increase in beat frequency led to upward swimming and higher steady-state vertical distribution (Fig. 7 *E* and *F* and Fig. 6*N*), but the swimming tracks remained irregular, similar to those in control larvae (Fig. 7*F*).

These results show that ciliary arrests and beat frequency have distinct contributions to larval swimming. Arrests allow larvae to sink and contribute to the maintenance of an unbiased net vertical displacement. Arrests also result in irregular swimming tracks (i.e., frequent turning events; compare Movie S5). Ciliary beating promotes upward swimming. Neuropeptides can influence both parameters, thereby modulating swimming directionality, the frequency of sinking events, and swimming pattern.

Discussion

Neuropeptides Regulate Ciliary Swimming. Animals moving with cilia can either crawl on a surface (e.g., flatworms) or swim freely in water. Ciliary swimming is characteristic of the larval stage of many marine invertebrates with a benthic adult. Such ciliated larvae are widespread in the animal kingdom (1). Among the bilaterians, the lophotrochozoans (e.g., annelids and molluscs) and the deuterostomes (e.g., echinoderms and hemichordates) often have ciliated larvae (2, 3), whereas the ecdysozoans (e.g., insects and nematodes) lack them. Outside the bilaterians, cilia-based locomotion is present in ctenophores and in the larval stages of many cnidarians. Additionally, sponges, the basal-most animal group relative to the eumetazoans, often have ciliated larvae (4, 5). This trait, together with the lack of muscles in sponges, indicates that ciliary swimming is likely the ancestral form of locomotion in animals. Despite its importance for marine life and our understanding of the evolution of locomotion, we know very little about the control of ciliary swimming.

Here we described an unexpected diversity of neuropeptides influencing ciliary activity in larvae of the annelid *Platynereis*. The peptides can either activate ciliary swimming (increased ciliary beat frequency and reduced ciliary arrests) or inhibit swimming (reduced ciliary beat frequency and more arrests), leading to upward and downward shifts in larval vertical distribution. The regulation of ciliary beat frequency and ciliary arrests both contribute to changes in larval distribution, with arrests also contributing to the irregularity of swimming tracks.

Our vertical larval swimming experiments support the model that upward swimming and sinking determine the vertical distribution of marine plankton (8). It is remarkable that actively swimming *Platynereis* larvae maintain a uniform vertical distribution at steady state. Such a distribution can be maintained only if the upward and downward net movement of the larvae is balanced. To achieve this, swimming and sinking parameters (ciliary beat frequency, ciliary arrests, directionality, and buoyancy) must be fine-tuned. We showed how neuropeptide signaling can alter some of these parameters, shifting the overall larval vertical distribution.

Our results provide a general framework for how neuropeptides and the neurons that express them can regulate the depth of ciliated zooplankton.

Sensory Neurons Directly Control Cilia. The morphology of the neuropeptidergic neurons suggests that these cells are dual-function sensory-motor cells directly translating sensory inputs into motor output on effector ciliated cells. The diversity of sensory neurons with cilia-regulating neuropeptides in the *Platynereis* larva is consistent with the multitude of environmental cues annelid and other ciliated larvae can respond to. These cues include light, pressure, salinity, and temperature, as well as settlement-inducing chemicals (6, 17, 18, 53). Some of these cues were shown to change the distribution of larvae, either by pro-

moting upward swimming (e.g., increased pressure) (53) or by inhibiting cilia (e.g., settlement cues) (17). The sensory-motor neurons described here are potentially involved in similar responses in *Platynereis* larvae. Our results therefore suggest a model where certain aversive sensory inputs (e.g., high pressure or low temperature) can elicit neuropeptide release at the ciliary band, thereby promoting upward swimming. Such simple depth-regulating escape circuits could contribute to the accumulation of many planktonic organisms at water interfaces (54).

An analogous serotonergic sensory-motor neuron, mediating a response to an aversive cue, has also been described in the embryo of the pond snail, *Helisoma trivolvis*. Here the sensory neuron directly innervates embryonic cilia and increases ciliary beat frequency upon hypoxic stimulation in the egg capsule (55).

As with these previous studies, our results suggest that ciliated larvae harbor simple sensory-motor reflex circuits of various sensory modalities, able to directly influence ciliary activity and thus vertical position in the water.

Differences Between Muscle- and Ciliary-Control Circuits. A direct sensory-motor regulation of locomotion, implicated by our findings, has not been described in muscle-based motor circuits in bilaterians. In such systems, distinct sensory, inter-, and motoneurons are always present, even in the simplest examples, such as the gill-withdrawal reflex of *Aplysia* (56) or stretch-receptor–mediated proprioception in *Caenorhabditis* (57). Muscle-regulating circuits always have interneurons because motor control can entail the contraction of several muscles in a strict temporal order (e.g., locomotor central pattern generators) (58), the integration of conflicting sensory inputs into different motor outputs (e.g., anterior and posterior stimulation during the nematode tap withdrawal reflex) (59), or the coordinated contraction and relaxation of antagonistic muscles (e.g., tendon jerk reflex in tetrapods) (60).

In contrast, the nervous system of the *Platynereis* larval episphere is organized predominantly as a direct sensory-motor system. This system of simple organization is not due to the simplicity or young age of these larvae, because at the corresponding stage the trunk nervous system already harbors distinct types of interneurons, likely involved in regulating the trunk musculature (12). The structural difference between the trunk and episphere nervous system therefore seems to reflect the different functionality of the two systems, in that the trunk system regulates muscles whereas the episphere system regulates cilia. Because ciliary locomotion does not require a complex coordination of various motor structures, simple sensory-motor neurons may be adequate to perform all activatory and inhibitory functions on a single ciliary band. If the innervation runs to all ciliated cells, as found for the neurons described here, regulation can occur uniformly along the entire ciliary band. Alternatively, regulation can be differential, due to local innervation of a segment of the ciliary band, allowing tactic turning behavior (10).

Are Ciliary Locomotor Circuits Ancestral? Our results show that ciliated larvae use a very simple functional circuitry, regulating swimming by direct sensory-motor innervation. Such sensory-motor neurons are common in cnidarians (61–63); however, in bilaterians they have to date been described only in ciliated larvae (10, 55). This observation raises the interesting possibility that ciliary locomotor circuits in bilaterian larvae have retained an ancestral state of nervous system organization. If this scenario is true, ciliated larval circuitry may give insights into the evolutionary origin of the first nervous systems (15).

The cilia regulatory neuropeptides described here are widely conserved among marine invertebrates, including other annelids, mollusks, and cnidarians. Because all of these groups have ciliated larvae, our results have broad implications for the understanding of ciliary locomotor control in a wide range of invertebrate larvae.

Methods

Bioinformatics. For the identification of neuropeptides we used BLAST searches and pattern searches using repetitions of the motive K[K/R]-x(3-10). The GenBank accession numbers are JF811323–JF811333.

Antibodies and Staining. For antibody production, rabbits were immunized with neuropeptides coupled to a carrier via an N-terminal Cys. Sera were affinity purified on a SulfoLink resin (Pierce) coupled to the Cys-containing peptides. The bound antibodies were washed extensively with PBS and with 0.5 M NaCl to remove weakly bound antibodies. Fractions were collected upon elution with 100 mM glycine, pH 2.7 and 2.3. Inmunostainings and whole-mount in situ hybridization were performed on precisely staged larvae raised at 18 °C. Imaging of in situ hybridization samples was performed as previously described (45).

Microscopy and Image Processing. Confocal images were taken on an Olympus Fluoview-1000 confocal microscope with a 60× water-immersion objective using 488- and 635-nm laser lines and a pinhole of airy unit 1. For volume scans 512 × 512-pixel image stacks were recorded at a *Z*-distance of 1 μm. Recordings of ciliary beating and arrest were performed with a Zeiss Axioimager microscope and a DMK 21BF04 camera (The Imaging Source) at 60 frames/s or 15 frames/s to quantify arrests. Larvae were immobilized between a slide and a coverslip spaced with adhesive tape. Freely swimming larvae in vertical tubes were recorded with a DMK 21BF03 camera (The Imaging Source) at 30 frames/s. The tubes were illuminated laterally with red light-emitting diode (LED) lights that larvae are unable to detect at the stages studied. Stacks of confocal images or videos were processed using ImageJ 1.42 and Imaris 5.5.1. For the whole-mount in situ and immunostaining samples we generated projections using Imaris. Some images were filtered with a median filter (3 × 3 × 1 filter size), using Imaris. Contrast was always adjusted equally across the entire image. Larval swimming videos were analyzed using custom ImageJ macros and Perl scripts.

Image Registration. The expression patterns of the neuropeptides were registered to a reference axonal scaffold to obtain a combined molecular map of the peptidergic neurons. We established an image registration procedure similar to that in ref. 47, using the ITK toolkit (64). We first generated a reference scaffold by aligning 20 individual larvae to a representative scaffold, using affine and deformable transformations, and averaging them. Three to five gene expression patterns per gene were registered to the reference, using the acetylated tubulin channel. First affine (rotation, scaling, translation, shearing; itkAffineTransform class) and then deformable (nonuniform warp; itkBSplineDeformableTransform class) transformations were applied. For both steps the Mattes mutual information metric (itkMattesMutualInformationImageToImageMetric class) and the gradient step optimizer (itkRegularStepGradientDescentOptimizer class) were used. The multiresolution registration method was applied for both steps to speed up the process and make it more robust (itkMultiResolutionImageRegistrationMethod class).

Behavior. *Platynereis* larvae were obtained from a breeding culture, following ref. 11. Behavioral experiments were performed in seawater, using 48- to 60-hpf larvae (raised at 18 °C). Neuropeptides were added to the seawater and larvae were incubated for 2–5 min before recordings. For each peptide first we tested a range of different concentrations in the vertical migration assay and scored the effects by quantifying the larvae in the upper versus the lower half of the tubes (Fig. 6*O*). The lowest concentration with maximum effect was used in the single-larva assays.

Electrophysiology. Extracellular recordings were performed at room temperature with 29- to 33-hpf larvae immobilized with a holding pipette (opening ~30 μm). For recordings we used borosilicate capillaries (Science Products GB150F-8P) pulled with a Sutter P-1000 Flaming/Brown micropipette puller (Heka Elektronik) to 2–4 MΩ resistance. To facilitate penetration of the cuticle 0.002% trifluoromethanesulfonic acid was added to the larvae 10 min before the experiments. The recording solution contained 70 mM CsCl, 10 mM Hepes, 11 mM glucose, 10 mM glutathione, 5 mM EGTA, 500 mM aspartic acid, 5 mM ATP, and 0.1 mM GTP, and the pH was set to 7.3 with CsOH. The sample was illuminated with a Sharp DLP projector (PG-F212X-L) through a 740/10 band-pass filter, and ciliary beating was recorded at 6.5 frames/s. Recordings were done with the pipette in close proximity to the ciliated cells.

ACKNOWLEDGMENTS. We thank Elizabeth Williams, Andrew Renault, and Fulvia Bono for comments on the manuscript and Heinz Schwarz for advice on immunostainings. This work was supported by a Sequencing Grant to G.J.

(M.IF.A.ENTW8050) from the Max Planck Society. Work of T.A.M. and S.-L.O. is supported by the Centre for Integrative Neuroscience (Deutsche Forschungsgemeinschaft, EXC 307). The research leading to these results received funding from the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013)/European Research Council Grant Agreement 260821.

1. Young CM, ed (2002) *Atlas of Marine Invertebrate Larvae* (Academic, San Francisco), 1st Ed.
2. Nielsen C (2004) Trochophora larvae: Cell-lineages, ciliary bands, and body regions. 1. Annelida and Mollusca. *J Exp Zoolog B Mol Dev Evol* 302:35–68.
3. Nielsen C (2005) Trochophora larvae: Cell-lineages, ciliary bands and body regions. 2. Other groups and general discussion. *J Exp Zoolog B Mol Dev Evol* 304:401–447.
4. Maldonado M (2006) The ecology of the sponge larva. *Can J Zool* 84:175–194.
5. Nielsen C (2008) Six major steps in animal evolution: Are we derived sponge larvae? *Evol Dev* 10:241–257.
6. Thorson G (1964) Light as an ecological factor in the dispersal and settlement of larvae of marine bottom invertebrates. *Ophelia* 1:167–208.
7. Hamasaki T, Barkalow K, Richmond J, Satir P (1991) cAMP-stimulated phosphorylation of an axonemal polypeptide that copurifies with the 22S dynein arm regulates microtubule translocation velocity and swimming speed in Paramecium. *Proc Natl Acad Sci USA* 88:7918–7922.
8. Chia F, Buckland-Nicks J, Young C (1984) Locomotion of marine invertebrate larvae: A review. *Can J Zool* 62:1205–1222.
9. Satir P (1999) The cilium as a biological nanomachine. *FASEB J* 13(Suppl 2):S235–S237.
10. Jékely G, et al. (2008) Mechanism of phototaxis in marine zooplankton. *Nature* 456: 395–399.
11. Hauenschild C, Fischer A (1969) *Platynereis dumerilii. Mikroskopische Anatomie, Fortpflanzung, Entwicklung* (Gustav Fischer, Stuttgart).
12. Denes AS, et al. (2007) Molecular architecture of annelid nerve cord supports common origin of nervous system centralization in bilateria. *Cell* 129:277–288.
13. Arendt D, Tessmar-Raible K, Snyman H, Dorresteijn AW, Wittbrodt J (2004) Ciliary photoreceptors with a vertebrate-type opsin in an invertebrate brain. *Science* 306:869–871.
14. Tessmar-Raible K, et al. (2007) Conserved sensory-neurosecretory cell types in annelid and fish forebrain: Insights into hypothalamus evolution. *Cell* 129:1389–1400.
15. Jékely G (2011) Origin and early evolution of neural circuits for the control of ciliary locomotion. *Proc Biol Sci* 278:914–922.
16. Bayne B (1963) Responses of Mytilus edulis larvae to increases in hydrostatic pressure. *Nature* 198:406–407.
17. Hadfield MG, Koehl MA (2004) Rapid behavioral responses of an invertebrate larva to dissolved settlement cue. *Biol Bull* 207:28–43.
18. Hidu H, Haskin HH (1978) Swimming speeds of oyster larvae Crassostrea virginica in different salinities and temperatures. *Estuaries* 1:252.
19. Mackie GO, Singla CL, Thiriot-Quievreux C (1976) Nervous control of ciliary activity in gastropod larvae. *Biol Bull* 151:182–199.
20. Lacalli TC, Gilmour T (1990) Ciliary reversal and locomotory control in the Pluteus larva of Lytechinus pictus. *Philos Trans R Soc B* 330:391–396.
21. Lacalli TC (1984) Structure and organization of the nervous system in the Trochophore larva of Spirobranchus. *Philos Trans R Soc B* 306:79–135.
22. Dickinson A, Nason J, Croll R (1999) Histochemical localization of FMRFamide, serotonin and catecholamines in embryonic Crepidula fornicata (Gastropoda, Prosobranchia). *Zoomorphology* 119:49–62.
23. Voronezhskaya EE, Tsitrin EB, Nezlin LP (2003) Neuronal development in larval polychaete Phyllodoce maculata (Phyllodocidae). *J Comp Neurol* 455:299–309.
24. Gruhl A (2009) Serotonergic and FMRFamidergic nervous systems in gymnolaemate bryozoan larvae. *Zoomorphology* 128:135–156.
25. Beer A-J, Moss C, Thorndyke M (2001) Development of serotonin-like and SALMFamide-like immunoreactivity in the nervous system of the sea urchin Psammechinus miliaris. *Biol Bull* 200:268–280.
26. Watanabe H, Fujisawa T, Holstein TW (2009) Cnidarians and the evolutionary origin of the nervous system. *Dev Growth Differ* 51:167–183.
27. Chun JY, Korner J, Kreiner T, Scheller RH, Axel R (1994) The function and differential sorting of a family of aplysia prohormone processing enzymes. *Neuron* 12:831–844.
28. Hook V, et al. (2008) Proteases for processing proneuropeptides into peptide neurotransmitters and hormones. *Annu Rev Pharmacol Toxicol* 48:393–423.
29. Eipper BA, Stoffers DA, Mains RE (1992) The biosynthesis of neuropeptides: Peptide alpha-amidation. *Annu Rev Neurosci* 15:57–85.
30. McVeigh P, Kimber MJ, Novozhilova E, Day TA (2005) Neuropeptide signalling systems in flatworms. *Parasitology* 131(Suppl):S41–S55.
31. Nelson LS, Rosoff ML, Li C (1998) Disruption of a neuropeptide gene, flp-1, causes multiple behavioral defects in Caenorhabditis elegans. *Science* 281:1686–1690.
32. Marder E, Bucher D (2001) Central pattern generators and the control of rhythmic movements. *Curr Biol* 11:R986–R996.
33. Marder E, Richards KS (1999) Development of the peptidergic modulation of a rhythmic pattern generating network. *Brain Res* 848:35–44.
34. Root CM, Ko KI, Jafari A, Wang JW (2011) Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search. *Cell* 145:133–144.
35. Chalasani SH, et al. (2010) Neuropeptide feedback modifies odor-evoked dynamics in Caenorhabditis elegans olfactory neurons. *Nat Neurosci* 13:615–621.
36. Ringstad N, Horvitz H (2008) FMRFamide neuropeptides and acetylcholine synergistically inhibit egg-laying by C. elegans. *Nat Neurosci* 11:1168–1176.
37. Collins, JJ, 3rd, et al. (2010) Genome-wide analyses reveal a role for peptide hormones in planarian germline development. *PLoS Biol* 8:e1000509.
38. Willows AO, Pavlova GA, Phillips NE (1997) Modulation of ciliary beat frequency by neuropeptides from identified molluscan neurons. *J Exp Biol* 200:1433–1439.
39. Katsukura Y, Ando H, David CN, Grimmelikhuijzen CJP, Sugiyama T (2004) Control of planula migration by LWamide and RFamide neuropeptides in Hydractinia echinata. *J Exp Biol* 207:1803–1810.
40. Southey BR, Amare A, Zimmerman TA, Rodriguez-Zas SL, Sweedler JV (2006) NeuroPred: A tool to predict cleavage sites in neuropeptide precursors and provide the masses of the resulting peptides. *Nucleic Acids Res* 34(Web Server issue):W267–W272.
41. Gustafsson MKS, et al. (2002) Neuropeptides in flatworms. *Peptides* 23:2053–2061.
42. Hewes RS, Taghert PH (2001) Neuropeptides and neuropeptide receptors in the Drosophila melanogaster genome. *Genome Res* 11:1126–1142.
43. Plickert G, et al. (2003) The role of alpha-amidated neuropeptides in hydroid development—LWamides and metamorphosis in Hydractinia echinata. *Int J Dev Biol* 47:439–450.
44. Nathoo AN, Moeller RA, Westlund BA, Hart AC (2001) Identification of neuropeptide-like protein gene families in Caenorhabditis elegans and other species. *Proc Natl Acad Sci USA* 98:14000–14005.
45. Jékely G, Arendt D (2007) Cellular resolution expression profiling using confocal detection of NBT/BCIP precipitate by reflection microscopy. *Biotechniques* 42: 751–755.
46. Dorresteijn AWC (1990) Quantitative analysis of cellular differentiation during early embryogenesis of Platynereis dumerilii. *Rouxs Arch Dev Biol* 199:14–30.
47. Tomer R, Denes AS, Tessmar-Raible K, Arendt D (2010) Profiling by image registration reveals common origin of annelid mushroom bodies and vertebrate pallium. *Cell* 142: 800–809.
48. Purschke G (2005) Sense organs in polychaetes (Annelida). *Hydrobiologia* 535:53–78.
49. Walch-Solimena C, et al. (1993) Synaptotagmin: A membrane constituent of neuropeptide-containing large dense-core vesicles. *J Neurosci* 13:3895–3903.
50. Voets T, et al. (2001) Intracellular calcium dependence of large dense-core vesicle exocytosis in the absence of synaptotagmin I. *Proc Natl Acad Sci USA* 98:11680–11685.
51. Arkett S, Mackie G, Singla C (1987) Neuronal control of ciliary locomotion in a gastropod veliger (Calliostoma). *Biol Bull* 173:513–526.
52. Salathe M (2007) Regulation of mammalian ciliary beating. *Annu Rev Physiol* 69: 401–422.
53. Knight-Jones EW, Qasim SZ (1955) Responses of some marine plankton animals to changes in hydrostatic pressure. *Nature* 175:941–942.
54. Harder W (1968) Reactions of plankton organisms to water stratification. *Limnol Oceanogr* 13:156–168.
55. Kuang S, Doran SA, Wilson RJA, Goss GG, Goldberg JI (2002) Serotonergic sensory-motor neurons mediate a behavioral response to hypoxia in pond snail embryos. *J Neurobiol* 52:73–83.
56. Castellucci V, Pinsker H, Kupfermann I, Kandel ER (1970) Neuronal mechanisms of habituation and dishabituation of the gill-withdrawal reflex in Aplysia. *Science* 167: 1745–1748.
57. Li W, Feng Z, Sternberg PW, Xu XZS (2006) A C. elegans stretch receptor neuron revealed by a mechanosensitive TRP channel homologue. *Nature* 440:684–687.
58. Grillner S (2003) The motor infrastructure: From ion channels to neuronal networks. *Nat Rev Neurosci* 4:573–586.
59. Wicks SR, Rankin CH (1995) Integration of mechanosensory stimuli in Caenorhabditis elegans. *J Neurosci* 15:2434–2444.
60. Liddell E, Sherrington C (1924) Reflexes in response to stretch (myotatic reflexes). *Proc R Soc Lond B* 96:212–242.
61. Westfall JA (1973) Ultrastructural evidence for a granule-containing sensory-motor-interneuron in Hydra littoralis. *J Ultrastruct Res* 42:268–282.
62. Westfall JA, Kinnamon JC (1978) A second sensory–motor–interneuron with neurosecretory granules in Hydra. *J Neurocytol* 7:365–379.
63. Westfall JA, Wilson JD, Rogers RA, Kinnamon JC (1991) Multifunctional features of a gastrodermal sensory cell in Hydra: Three-dimensional study. *J Neurocytol* 20: 251–261.
64. Yoo TS, et al. (2002) Engineering and algorithm design for an image processing Api: A technical report on ITK—the Insight Toolkit. *Stud Health Technol Inform* 85:586–592.

Supporting Information

Conzelmann et al. 10.1073/pnas.1109085108

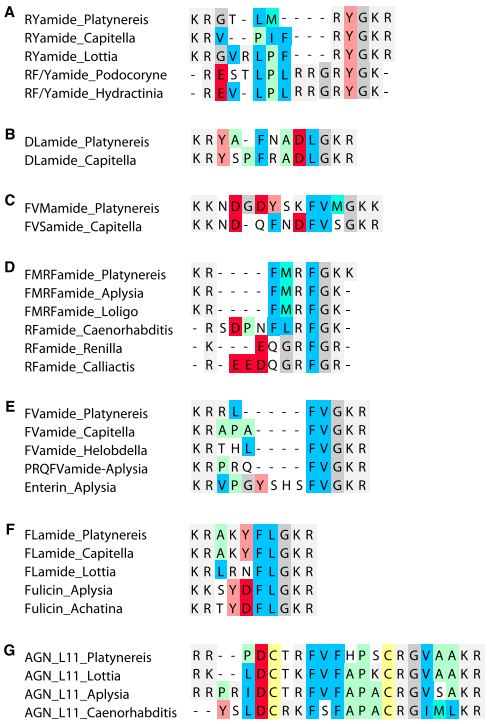
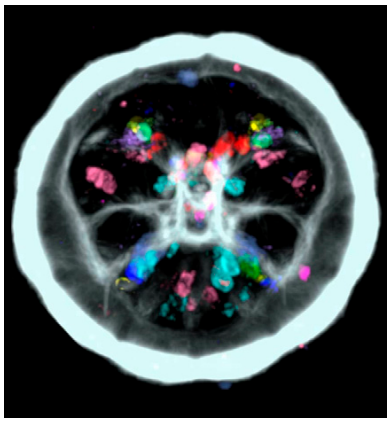
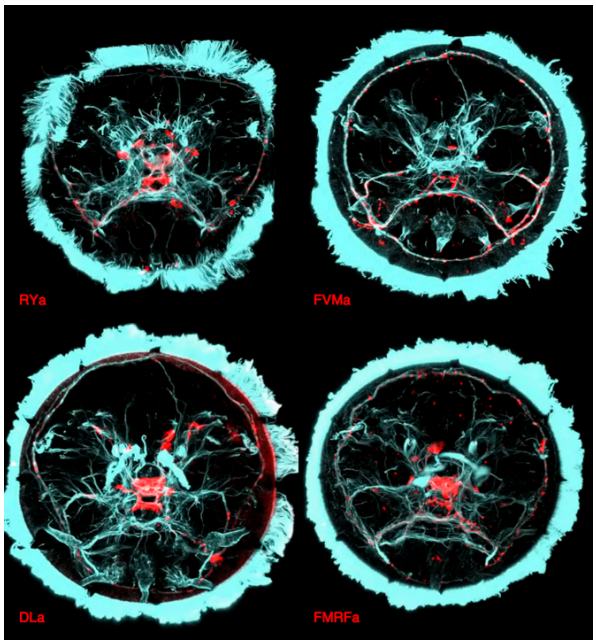


Fig. S1. (*A–G*) Multiple alignment of conserved neuropeptides. Multiple alignments of *Platynereis* RYa, DLa, FVMa, FMRFa, FVa, FLa, and L11 neuropeptides are shown with related neuropeptides from other metazoans including cnidarians (*Podocoryne*, *Hydractinia*, *Renilla*, and *Calliactis*), Lophotrochozoans (*Capitella*, *Lottia*, *Helobdella*, *Aplysia*, *Loligo*, and *Achatina*), and Ecdysozoan (*Caenorhabditis*).



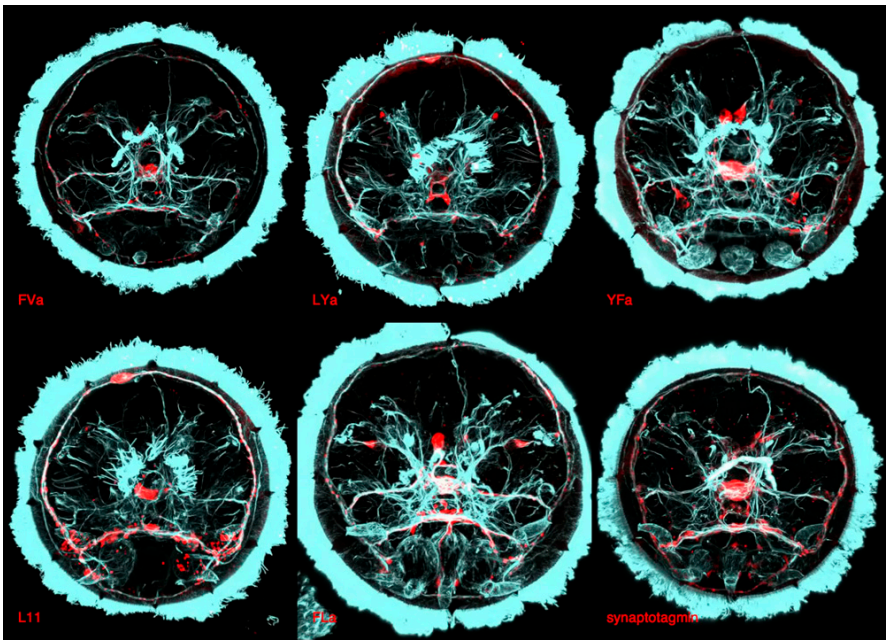
Movie S1. Neuropeptide expressions in the *Platynereis* larva. Average expression pattern of each neuropeptide was registered to the same acetylated tubulin reference. Anterior view of a 48-hpf larva is shown.

Movie S1



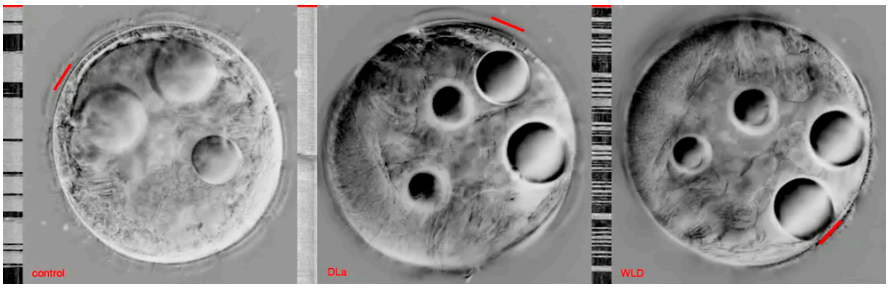
Movie S2. Neuropeptide antibody labelings in the *Platynereis* larva. Immunostainings with RYa, FVMa, DLa, and FMRFa antibodies (red) and the acetylated tubulin antibody (white) are shown. All images are anterior views of 48-hpf larvae.

Movie S2



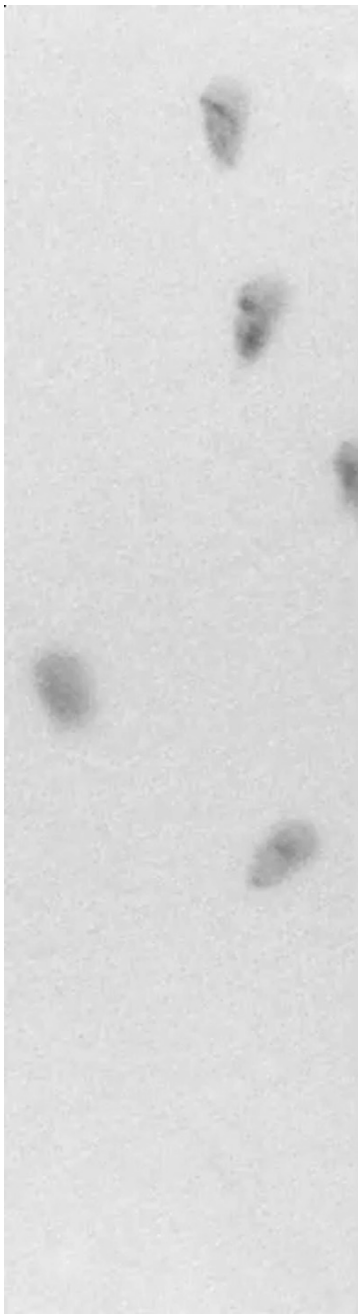
Movie S3. Neuropeptide antibody labelings in the *Platynereis* larva. Immunostainings with FVa, LYa, YFa, L11, FLa, and synaptotagmin antibodies (red) and the acetylated tubulin antibody (white) are shown. All images are anterior views of 48-hpf larvae.

Movie S3



Movie S4. Ciliary beating and arrests in *Platynereis* larvae. Ciliary beating and arrests in an immobilized, control 50-hpf *Platynereis* larva and larvae in the presence of 20 μM YYGFNNDLa or WLDNSQFRDE neuropeptide are shown. The recordings were done at 15 fps.

Movie S4



Movie S5. Vertical swimming of *Platynereis* larvae. *Platynereis* larvae (50 hpf) swimming in a vertical column are shown (recorded at 30 fps).

Movie S5

Author summary: **Neuropeptides regulate swimming depth of *Platynereis* larvae;** *PNAS* (2011)

Conzelmann *et al*.

Neuropeptides regulate swimming depth of *Platynereis* larvae

Markus Conzelmann[a], Sarah-Lena Offenburger[a,b], Albina Asadulina[a], Timea Keller[a], Thomas A. Münch[b], and Gáspár Jékely[a,1]

[a]*Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany; and* [b]*Werner Reichardt Centre for Integrative Neuroscience, 72076 Tübingen, Germany*

AUTHOR SUMMARY

Cilia-based locomotion is the major form of locomotion for microscopic planktonic organisms in the ocean. It is prominent during the larval stages of many marine invertebrates, providing an efficient means of dispersal. Ciliated larvae are negatively buoyant, meaning that the gravitational pull is greater than the buoyant force, and must therefore control ciliary activity to maintain an appropriate depth. However, the neuronal basis of depth regulation in ciliary swimmers remains unknown. To gain an insight into depth regulation by these organisms, we studied ciliary locomotor control in the planktonic larva of the marine annelid *Platynereis*. We found several neuropeptides, which were expressed in distinct sensory neurons that innervate locomotor cilia. The neuropeptides altered ciliary beat frequency and the rate of ciliary arrest. These changes influenced larval orientation, vertical swimming, and sinking, which resulted in upward or downward shifts in the steady-state vertical distribution of groups of larvae (Fig. P1). Our findings indicate that *Platynereis* larvae have depth-regulating peptidergic neurons that directly translate sensory inputs into locomotor output by modulating the activity of cilia. We propose that the simple circuitry found in these ciliated larvae represents an ancestral state in nervous system evolution.

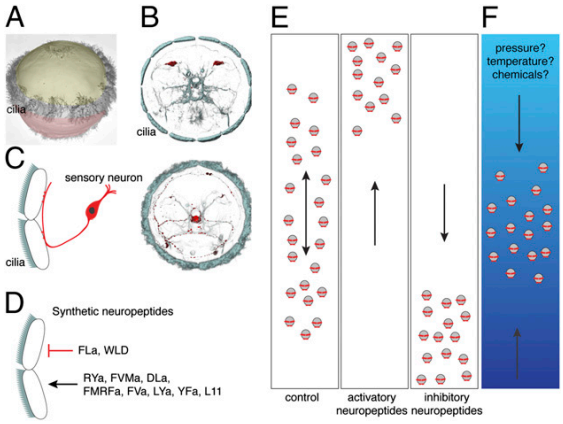


Fig. P1. Sensory-motor peptidergic neurons regulate swimming depth in ciliated larvae. (*A*) Scanning electron microscope image of a 2-d-old *Platynereis* larva with an equatorial band of cilia. (*B*) Expression of 1 of the 11 studied neuropeptide precursors in larval sensory cells. (*C*) Immunostaining (i.e., an antibody-based method to detect a specific protein in a sample) with FV-amide neuropeptide-specific antibodies revealed the innervation of the ciliary band by the sensory cell axons. (*D*) Synthetic neuropeptides either activate (black arrow) or inhibit (red bar) ciliary activity in immobilized larvae. (*E*) Neuropeptides trigger upward swimming or sinking, thus shifting the vertical distribution of larval populations. (*F*) A model for sensory-motor depth regulation in ciliated marine zooplankton. Neuropeptides induce shifts in the vertical distribution of larval populations; however, it is not yet known how external environmental factors influence the release of specific neuropeptides.

Two different types of locomotor systems are present in animals: One is muscle based, and the other is cilia based. The neuronal control of muscle-based motor systems is well understood from studies on terrestrial model organisms. In contrast, knowledge about neuronal control in ciliary locomotion is limited, even though cilia-driven locomotion is prominent in the majority of animal phyla (1).

A large proportion of marine plankton is composed of the ciliated larval stages of marine bottom invertebrates that have a biphasic life cycle (i.e., initial development as swimming larvae followed by metamorphosis to the sessile adult form). Ciliated larvae may spend from days to months in the planktonic form, freely swimming in the open water. During this planktonic phase, the larvae are passively drifted by sea currents, yet they also actively regulate their cilia, thereby maintaining their vertical position in the water column. Regulated migration in the vertical direction ensures that the larvae eventually settle at an appropriate depth for survival as adults. Ciliated larvae have a simple nervous system that is involved in locomotor control, including larval phototaxis, which is a type of directional locomotion in response to light stimulus (2). However, other than phototaxis, the neuronal basis of locomotor control and depth regulation remains poorly understood.

To gain an insight into the neuronal mechanisms of depth regulation in ciliated zooplankton, we studied the ciliated larvae of the marine annelid *Platynereis dumerilii*. Although there is detailed information about the gross anatomy of the nerves in the *Platynereis* larva, in addition to the anatomy and function of the simple larval eyes (2), little is known about its neuronal circuit architecture and how larval swimming is regulated. To characterize larval circuitry and neuronal regulation in *Platynereis*, we focused on neuropeptides, due to their widespread roles in the regulation of neural circuits and locomotion. Using molecular biology and bioinformatics techniques we identified 11 *Platynereis* neuropeptide precursors belonging to the RF-amide family of neuropeptides, an ancient and widespread family of animal neuropeptides. We first characterized the expression of the neuropeptide precursors by using a standard technique called RNA in situ hybridization. We found that the precursors were expressed in distinct sensory cells of the nervous system at the anterior half of the larva (the prospective

Author contributions: T.A.M. and G.J. designed research; M.C., S.-L.O., A.A., and T.K. performed research; M.C., S.-L.O., A.A., T.A.M., and G.J. analyzed data; and M.C. and G.J. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. JF811323–JF811333).

[1]To whom correspondence should be addressed. E-mail: gaspar.jekely@tuebingen.mpg.de.

See full research article on page E1174 of www.pnas.org.

Cite this Author Summary as: PNAS 10.1073/pnas.1109085108.

adult brain), revealing the presence of a broad region of sensory peptide-secreting (peptidergic) neurons. We then generated 9 neuropeptide-specific antibodies and used them to obtain information about the axonal projections of the sensory peptidergic neurons. Remarkably, the results showed that the sensory cells directly innervated the larval ciliary band, implying that neuropeptides regulate cilia in direct sensory-motor control.

To determine how neuropeptides affect cilia, we analyzed ciliary activity and larval swimming in the presence of synthetic neuropeptides. Video recordings of immobilized larvae showed that neuropeptides altered ciliary beat frequency, in addition to the rate and duration of ciliary arrests. We identified activatory and inhibitory neuropeptides for both ciliary beat frequency and arrests. In addition, we used electrophysiology experiments on the larvae to show that ciliary arrests were triggered by sharp spikes in calcium concentrations, implying that neuropeptide signaling could affect arrests via voltage-dependent calcium channels, which are protein molecules that allow diffusion of calcium ions in and out of the cells. We also developed a vertical migration assay, which served as a model for plankton migration, to study larval swimming behavior in populations of a few hundred larvae. In these vertical-swimming experiments, control larvae were distributed uniformly along the vertical axis. This uniform distribution was maintained as a steady state, in which upward swimming and sinking were balanced. Neuropeptides induced upward swimming or sinking, causing large upward or downward shifts in the vertical distribution of larvae. Upward swimming was also triggered by blocking ciliary arrests with a blocker of calcium channels or the pharmacological increase of ciliary beat frequency. These results allowed us to evaluate the contribution of ciliary arrests and ciliary beat frequency to swimming behavior and to better interpret the effects of neuropeptides on larval swimming behavior.

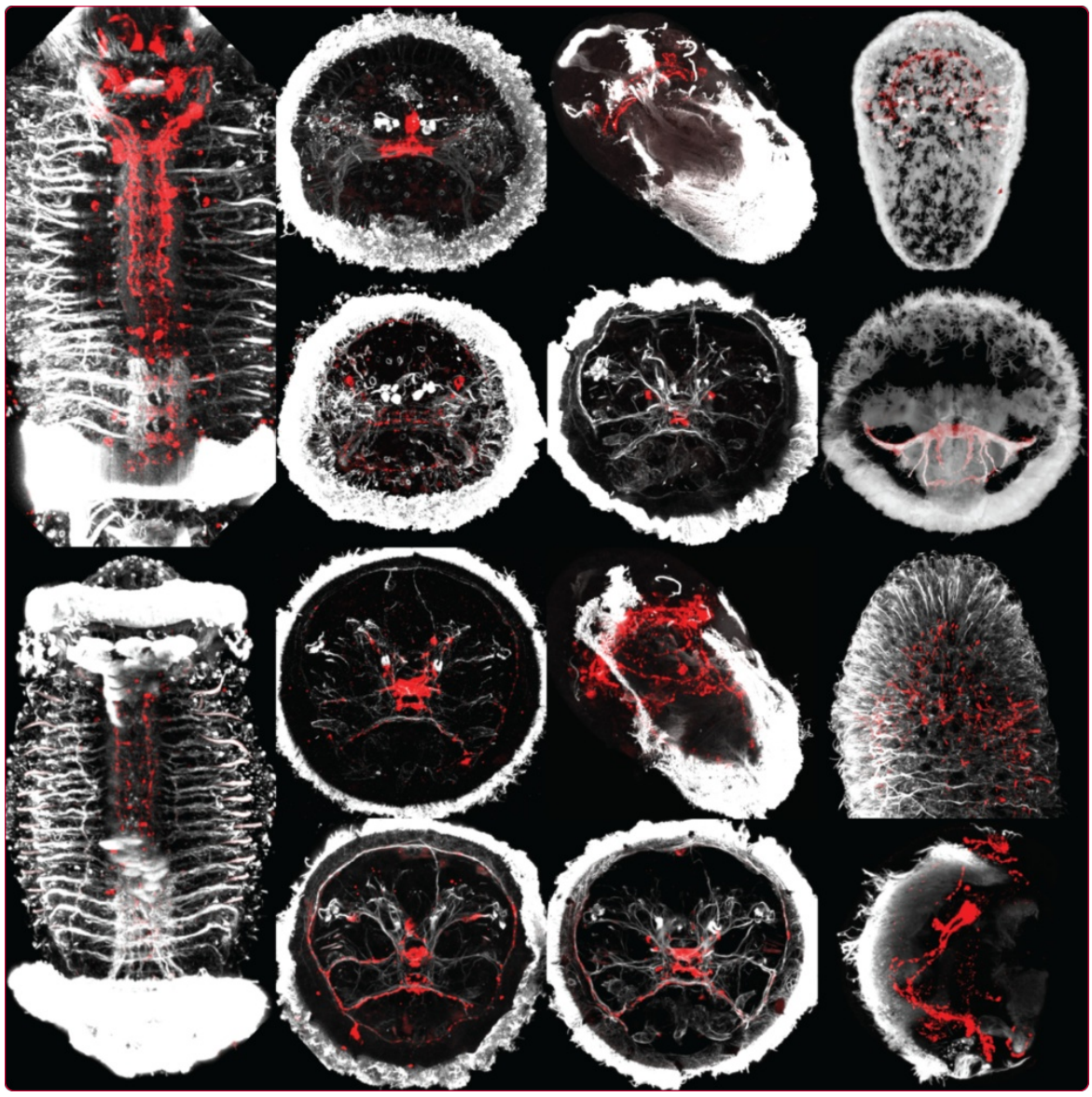
Overall, our results revealed an unexpected complexity of peptidergic regulation of ciliary activity and established a laboratory model for depth regulation in marine zooplankton. The sensory-motor morphology of the neuropeptidergic cells indicated that neuropeptides could be released at synapses formed on the ciliated cells. Upon sensory stimulation, neuropeptides could change the ciliary activity of the larvae, promoting either upward swimming or sinking. Such simple reflex circuits might allow larvae to respond to various stimuli during their vertical migration and adjust their swimming depth accordingly. In our experiments, we incubated larvae in synthetic neuropeptides, bypassing the need for sensory stimulation. An interesting challenge for the future would be to link the natural sensory stimuli (i.e., pressure, temperature, chemicals) that are known to influence zooplankton migration (3) to the sensory-motor peptidergic neurons described here. The cilia-regulatory neuropeptides characterized in *Platynereis* are widely conserved among marine invertebrates, including other annelids, mollusks, and cnidarians. Because all of these groups have ciliated larvae, our results also have broad implications for the understanding of neural circuits that control ciliary locomotion in a wide range of invertebrate larvae.

1. Young CM, ed (2002) *Atlas of Marine Invertebrate Larvae* (Academic, San Francisco), 1st Ed.
2. Jékely G, et al. (2008) Mechanism of phototaxis in marine zooplankton. *Nature* 456: 395–399.
3. Chia F, Buckland-Nicks J, Young C (1984) Locomotion of marine invertebrate larvae: A review. *Can J Zool* 62:1205–1222.

Publication 2: **Antibodies against conserved amidated neuropeptide epitopes enrich the comparative neurobiology toolbox;** *EvoDevo* (2012)

Conzelmann & Jekely

EvoDevo



Antibodies against conserved amidated neuropeptide epitopes enrich the comparative neurobiology toolbox

Conzelmann and Jékely

BioMed Central

Conzelmann and Jékely *EvoDevo* 2012, **3**:23
http://www.evodevojournal.com/content/3/1/23

Conzelmann and Jékely *EvoDevo* 2012, **3**:23
http://www.evodevojournal.com/content/3/1/23

EvoDevo

RESEARCH Open Access

Antibodies against conserved amidated neuropeptide epitopes enrich the comparative neurobiology toolbox

Markus Conzelmann and Gáspár Jékely*

Abstract

Background: Neuronal antibodies that show immunoreactivity across a broad range of species are important tools for comparative neuroanatomy. Nonetheless, the current antibody repertoire for non-model invertebrates is limited. Currently, only antibodies against the neuropeptide RFamide and the monoamine transmitter serotonin are extensively used. These antibodies label respective neuron-populations and their axons and dendrites in a large number of species across various animal phyla.

Results: Several other neuropeptides also have a broad phyletic distribution among invertebrates, including DLamides, FVamides, FLamides, GWamides and RYamides. These neuropeptides show strong conservation of the two carboxy-terminal amino acids and are α-amidated at their C-termini. We generated and affinity-purified specific polyclonal antibodies against each of these conserved amidated dipeptide motifs. We thoroughly tested antibody reactivity and specificity both by peptide pre-incubation experiments and by showing a close correlation between the immunostaining signals and mRNA expression patterns of the respective precursor genes in the annelid *Platynereis*. We also demonstrated the usefulness of these antibodies by performing immunostainings on a broad range of invertebrate species, including cnidarians, annelids, molluscs, a bryozoan, and a crustacean. In all species, the antibodies label distinct neuronal populations and their axonal projections. In the ciliated larvae of cnidarians, annelids, molluscs and bryozoans, a subset of antibodies reveal peptidergic innervation of locomotor cilia.

Conclusions: We developed five specific cross-species-reactive antibodies recognizing conserved two-amino-acid amidated neuropeptide epitopes. These antibodies allow specific labelling of peptidergic neurons and their projections in a broad range of invertebrates. Our comparative survey across several marine phyla demonstrates a broad occurrence of peptidergic innervation of larval ciliary bands, suggesting a general role of these neuropeptides in the regulation of ciliary swimming.

Background

Antibodies that show specific immunoreactivity across a broad range of species are valuable tools for comparative neuroanatomy in non-model organisms. For example, antibodies against serotonin commonly label cell bodies and their projections, allowing comparative studies of neurodevelopment and neuroanatomy across diverse species and phyla [1]. Another commonly used antibody is that against FMRFamide, a neuropeptide first discovered in molluscs [2,3]. Similar RFamide neuropeptides were later found to be widespread among eumetazoans [4-6]. A pioneering work reported the development of antibodies against the conserved amidated dipeptide motif RFamide [7]. This RFamide and other FMRFamide antibodies have been extensively used in invertebrate neuroanatomy, owing to the broad distribution of RFamide-like peptides [8]. The RFamide antibody labels distinct neuronal subsets and their projections, and can be applied as a neuronal marker to increase morphological resolution in complex adult tissues [9], or to reveal aspects of nervous system development and organization, allowing the clarification of phylogenetic relationships within phyla [10-12] or the study of nervous system evolution between related groups [13].

* Correspondence: gaspar.jekely@tuebingen.mpg.de
Max Planck Institute for Developmental Biology, Spemannstrasse 35, Tübingen 72076, Germany

Neuropeptides are signalling molecules that are translated as precursor molecules, typically consisting of an N-terminal signal peptide and multiple copies of similar peptide motifs, flanked by dibasic cleavage sites (Lys and Arg residues). The precursor is cleaved and often further modified to yield shorter active neuropeptides [14,15]. α-amidation is the most common post-translational modification, where a C-terminal glycine is enzymatically converted into an amide group. This modification protects the small peptides from degradation and is critical for receptor binding [16-18]. Amidation is also thought to confer high immunogenic potential to short neuropeptides [19-21] and antibodies raised against amidated peptides are highly specific for the amidated peptide moiety [21]. Changes in hydrogen bonding capability caused by the amide group may lead to the improved receptor binding and increased immunogenicity of C-terminally amidated peptides [22].

The C-terminal residues in amidated neuropeptides are often highly conserved across different species and even phyla [23]. We reasoned that, like the RFamide antibodies, other dipeptide antibodies could also potentially be used as neuronal markers across a wide range of species. Here we report the development of specific neuronal antibodies against the amidated dipeptide motifs of five conserved neuropeptides, DLamide, FVamide, FLamide, GWamide and RYamide. We show that these antibodies recognize specific subsets of neurons and their projections in cnidarian, annelid, mollusc, bryozoan and crustacean larvae. Furthermore, our antibody stainings reveal that the neuropeptidergic innervation of locomotor cilia is a general feature of ciliated larvae.

Methods

Generation of polyclonal neuropeptide antibodies

The amidated peptides, coupled to an adjuvant (lipoadjuvant Pam3) via an N-terminal cysteine (CRYamide, CGWamide, CFVamide, CFLamide, CDLamide), were used to immunize rabbits. Sera were affinity-purified on the respective peptide epitopes using a SulfoLink resin (Thermo Scientific, Rockford, USA) that allows the coupling of cysteine containing peptides via a disulphide bond. After coupling of 1 mg peptide epitope to 2 ml resin in Coupling Buffer (CB; 50 mM TRIS pH 8.5, 5 mM EDTA), the resin was washed three times with 10 ml CB. Excess reactive sites were blocked by incubating the resin in 2 ml 50 mM cysteine for 45 min, followed by three washes with 1 M NaCl and three washes with 25 ml phosphate buffered saline (PBS). Next, 25 ml serum was applied to the resin and this was incubated overnight to allow antibody binding. After flow-through of the serum, the resin was washed five times with 25 ml PBS followed by a wash with 15 ml 0.5 M NaCl/PBS and again twice with 10 ml PBS. The antibodies were eluted and fractionated with eight times 1 ml of 100 mM glycine pH 2.7, eight times 1 ml of 100 mM glycine pH 2.3 and eight times 1 ml of 100 mM glycine pH 2.0. The fractions were neutralized by directly collecting them in an adequate volume (about 40, 75 and 95 μl for the different pH solutions) of 1 M TRIS–HCl pH 9.5. The protein concentration of each fraction was determined, and the first two fractions of the pH 2.7 peak (usually fractions 2 and 3) were discarded, since these contained the lowest affinity antibodies. The peak fractions and the end-of-peak fractions were pooled, and concentrated, if necessary, using Vivaspin centrifugation tubes with a molecular weight cut-off of 10 kDa (Sartorius, Göttingen, Germany). Antibodies were stored in 50% glycerol at −20°C for mid-term (up to 1 year), and −80°C for long-term storage. A detailed protocol is available [24].

Immunohistochemistry

For immunostainings, larvae were fixed in 4% formaldehyde in PTW (PBS + 0.1% Tween-20) for 2 h and stored in 100% methanol at −20°C until use. After stepwise rehydration to PTW, samples were permeabilized with proteinase-K treatment (100 μg/ml in PTW, for 1 to 3 min). To stop proteinase-K activity, larvae were rinsed with glycine buffer (5 μg/ml in PTW) and post-fixed in 4% formaldehyde in PTW for 20 min followed by two 5 minwashes in PTW and two 5 minwashes in THT (0.1 M TRIS–HCl pH 8.5 + 0.1% Tween-20). Larvae and antibodies were blocked in 5% sheep serum in THT for 1 h. Primary antibodies were used at a final concentration of 1 μg/ml for rabbit neuropeptide antibodies and 0.5 μg/ml for mouse anti-acetylated tubulin antibody (Sigma, Saint Louis, USA) and incubated overnight at 6°C. Weakly bound primary antibodies were removed by two 10 min washes in 1 M NaCl in THT, followed by five 30 min washes in THT. Larvae were incubated overnight at 6°C in the dark in 1 μg/ml anti-rabbit Alexa Fluor® 647 antibody (Invitrogen, Carlsbad, CA, USA) and in 0.5 μg/ml anti-mouse FITC antibody (Jackson Immuno Research, West Grove, PA, USA) and then washed six times for 30 min with THT-buffer, and mounted in 87% glycerol including 2.5 mg/ml of the anti-photobleaching reagent 1,4-diazabicyclo[2.2.2]octane (Sigma, St. Louis, MO, USA). *Pecten* larvae were additionally treated with 4% paraformaldehyde in PBS with 50 μM EDTA pH 8.0 for 1 h to decalcify their shells before the immunostaining procedure (performed as described previously). For cnidarian larvae, we also used a mouse anti-tyrosylated tubulin antibody (Sigma, Saint Louis, USA) at 1 μg/ml. For immunostaining with multiple rabbit primary antibodies in the same sample, antibodies were directly labelled with a fluorophore using the Zenon® Tricolour Rabbit IgG Labelling Kit (Invitrogen, Carlsbad, CA, USA) and used in combination with mouse anti-acetylated tubulin antibody.

For blocking experiments, we pre-incubated the antibodies in 5 mM of the respective full-length *Platynereis* peptides (YYGFNNDLamide, AHRFVamide, AKYFLamide, VFRYamide, RGWamide) for 2 h before immunostainings.

Microscopy and image processing

Images were taken on an Olympus Fluoview-1000 confocal microscope (Olympus Deutschland GmbH, Hamburg, Germany) using a 60× water-immersion objective and the appropriate laser lines to capture fluorescent signals. Signals from RNA *in situ* hybridizations (nitro blue tetrazolium chloride/5-Bromo-4-cloro-3-indolyl phosphate precipitate) were imaged with reflection confocal microscopy as described [25]. Images were processed with Imaris 6.4 (BitPlane Inc., Saint Paul, USA) and ImageJ 1.45 software [26]. All image stacks are available [24].

Bioinformatic tools

Neuropeptide prediction was performed using NeuroPred [27], N-terminal signal peptides were predicted using SignalP 4.0 Server [28]. For multiple sequence alignments, we used ClustalW [29]. The GenBank accession number for the Platynereis RGWamide neuropeptide precursor: JX412226.

Results

Generation of specific antibodies against amidated dipeptide epitopes of neuropeptides

We set out to develop antibodies against the conserved C-amidated dipeptides DLa, FVa, FLa, GWa and RYa ('a' = 'amide') neuropeptides that are conserved across phyla (Figure 1) [23,25-36]. We have recently shown in the marine annelid model *Platynereisdumerilii* that the precursor mRNAs for these neuropeptides are expressed in largely non-overlapping subsets of neurons in the larval episphere. None of these neuropeptides co-expresses with FMRFamide in *Platynereis* [23], suggesting that antibodies against their conserved amidated dipeptides could also substantially increase the number of neurons that can be labelled in other species.

Rabbits were immunized with the short amidated peptides extended with an N-terminal cysteine to allow coupling to a carrier during the immunization procedure. We also used the cysteine residue to couple the peptides to a resin and to affinity purify the antibodies from the respective sera. We employed a high stringency affinity purification protocol including high salt washes and low pH elution to obtain high-affinity antibody fractions.

Next, we tested the reactivity of the affinity purified neuropeptide antibodies in whole mount immunostainings on *Platynereis* larvae. We found labelling for all antibodies in a subset of neurons and their axons in the larval episphere (Figure 2A-D). To test the specificity of our antibodies, we pre-incubated them in the synthetic amidated full-length *Platynereis* peptides. This treatment led to a complete block of the signal for the anti-DLa, anti-FVa and anti-RYa antibodies (Figure 3A,C,E) and a strong reduction in signal intensity for the anti-FLa and anti-GWa antibodies (Figure 3B,D). These results indicate that the antibodies bind to the respective peptides and this prevents further binding to epitopes in the tissue. The specificity of the antibodies is further supported by the close correlation between the cell body positions revealed by immunostaining and the expression patterns of the respective precursors (Figure 3 A-E, bottom panels, asterisks) as shown by whole-mount RNA

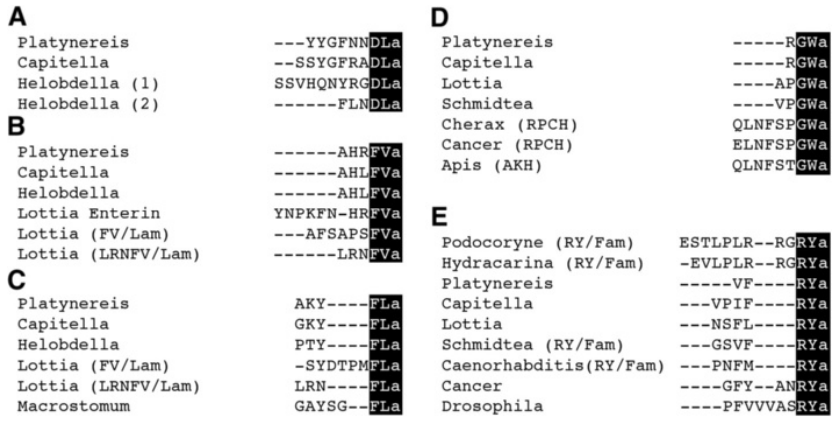


Figure 1 Two amino-acid amidated motifs are conserved in neuropeptides across phyla. Multiple sequence alignment of mature neuropeptides with a conserved C-terminus for DLamides (**A**), FVamides (**B**), FLamides (**D**), GWamides (**D**) and RYamides (**E**) from annelids *(Platynereis, Capitella, Helobdella)*, molluscs *(Lottia)*, platyhelminthes *(Schmidtea, Macrostomum)*, nematodes *(Caenorhabditis)*, arthropods *(Cherax, Cancer, Drosophila, Apis)* and cnidarians (*Podocoryne, Hydractinia).* The conserved C-termini that were used for antibody production are highlighted in black. RPCH, red pigment concentrating hormone; AKH, adipokinetic hormone.

Conzelmann and Jékely *EvoDevo* 2012, **3**:23
http://www.evodevojournal.com/content/3/1/23

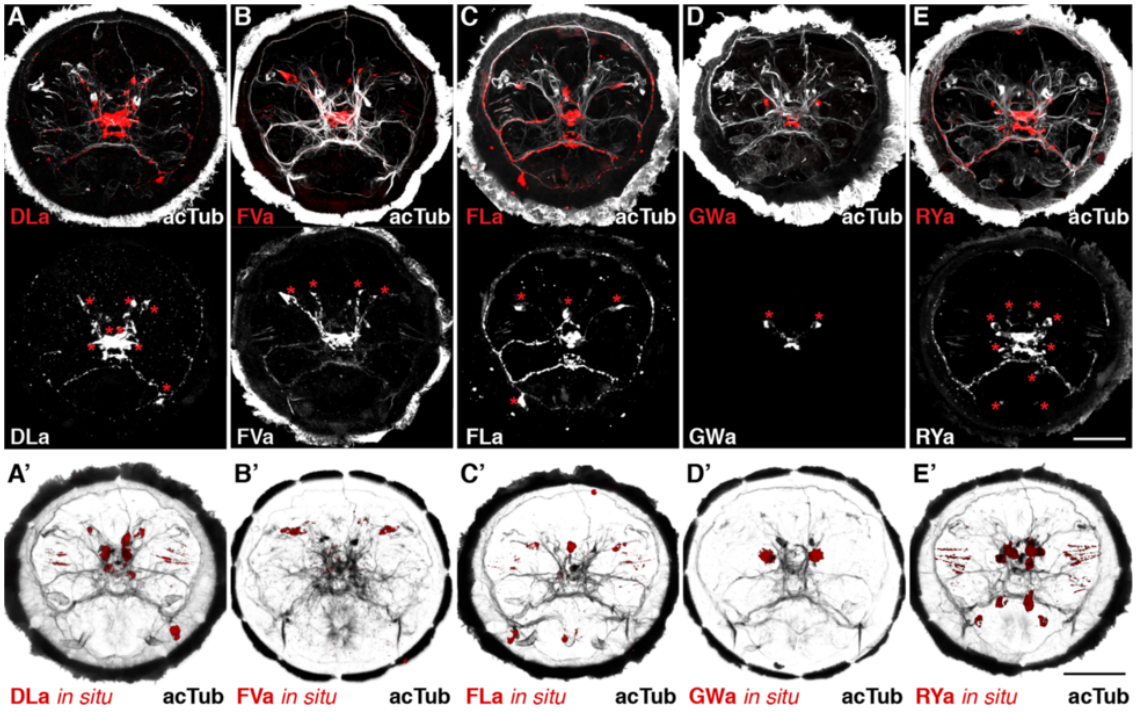


Figure 2 Correspondence of antibody signals with the respective precursor mRNA expression in *Platynereis*. (**A**-**E**) Immunostaining (red) with the DLamide (**A**), FVamide (**B**), FLamide (**C**), GWamide (**D**) and RYamide (**E**) antibodies counterstained for acetylated tubulin (acTub, white) in the upper panels. Bottom panels show immunostainings for the respective antibodies in white. (**A′**-**E′**) mRNA *in situ* hybridization (red) counterstained for acetylated tubulin (acTub, black) for the DLamide (**A′**), FVamide (**B′**), FLamide (**C′**), GWamide (**D′**) and RYamide (**E′**) neuropeptide precursors. With the exception of GWamide, all precursors*in situ* were described in [23] and are shown here for comparison only. All images are anterior views of *Platynereis* larvae 48 h post fertilization (hpf). Asterisks indicate cells that show a spatial correspondence with the mRNA *in situ* hybridization signals in **A′**-**E′**. Scale bars: 50 μm.

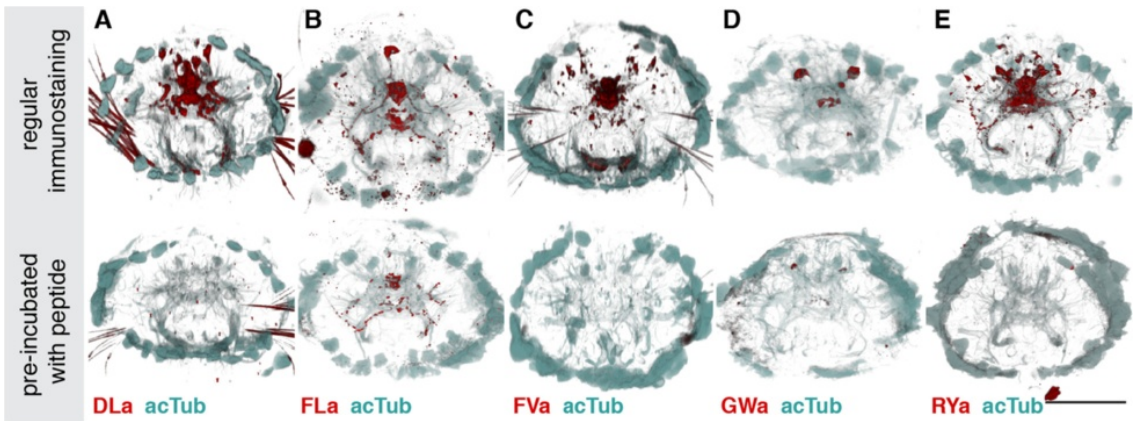


Figure 3 Blocking of immunostaining signals with peptide pre-incubation. (**A**-**E**) Regular immunostaining (upper panels), and stainings with antibodies that were pre-incubated with the corresponding synthetic *Platynereis* full length neuropeptide (bottom panels) for DLamide (**A**), FVamide (**B**), FLamide (**C**), GWamide (**D**) and RYamide (**E**), all shown in red. Samples were counterstained for acetylated tubulin (acTub, cyan). All images are anterior views of *Platynereis* larvae 72 h post fertilization (hpf). Scale bar: 50 μm.

in situ hybridization (Figure 3 A′-E′). The recently described antibodies raised against full length *Platynereis* DLa, FVa, FLa and RYa peptides also show very similar neuronal signals [23].

Overall, our specificity tests in *Platynereis* demonstrate that the antibodies raised against amidated dipeptide motifs are remarkably specific and can be used to obtain high-quality tissue stainings. To test the utility of our antibody collection as cross-species-reactive neuronal markers, we performed immunostainings on a variety of marine larvae from different species and phyla.

DLamide immunoreactivity in annelids

DLa neuropeptides have been described from the errant annelid (Errantia) *Platynereis* and the sedentary annelids (Sedentaria) *Capitella* and *Helobdella* [23,36] (Figure 1A). Since errant and sedentary annelids encompass most of annelid diversity [37], DLa neuropeptides are potentially widely distributed among annelids. To test whether our DLa antibody could be used as a pan-annelid nervous system marker, we also tested its reactivity in *Capitella*. In *Capitella* larvae, we found staining in neurons of the apical organ. These neurons have a flask-shaped morphology typical of sensory cells and project to the larval ciliary band (Figure 4A, arrow) in a similar fashion to that observed for *Platynereis* larvae (compare with Figure 2A). We also observed strong staining in the ventral nerve cord in older *Capitella* larvae (Figure 4B). The specific reactivity of the DLa antibody in both errant and sedentary annelid species demonstrates its usefulness as a pan-annelid neuronal marker.

FVamide and FLamide immunoreactivity in annelids and molluscs

FVa and FLa neuropeptides have been described in annelids, molluscs and platyhelminths. In the annelids *Platynereis* and *Capitella,* there is one FVa neuropeptide precursor, whereas there are three different precursors in the mollusc *Lottia gigantea* (Figure 1B,C) [35]. FLa peptides are either encoded by a separate precursor gene and expressed in distinct subsets of cells, as in annelids, or co-occur on the same precursor together with FVa peptides, as in molluscs. Regardless of the number of precursor genes, the conserved FVa and FLa epitopes could allow the labelling of all FVa and FLa expressing neurons in annelids and molluscs. We tested the reactivity of both antibodies on *Capitella* larvae and on larvae of the bivalve mollusc *Pecten maximus* and the nudibranch mollusc *Phestilla sibogae* (for morphological details see [38]). In *Capitella,* we found FVa immunoreactivity in apical organ neurons with projections to the ciliary band (Figure 5A, arrow, compare with Figure 2B), and also in the ventral nerve cord (Figure 5B). The FLa antibody labels neurons in the brain and in the ventral nerve cord in older stages of *Capitella* (Figure 5F). In *Pecten* veliger larvae, the FVa antibody labels a small number of neurons in the cerebral and visceral ganglia, some of which project to the ciliated velum (Figure 5C). In *Phestilla,* both antibodies show strong staining in the cerebropleural ganglion between the eyes (Figure 5D,G). The FVa antibody also labels two nerve fibres in the ciliated foot (Figure 5E, arrows).

GWamide immunoreactivity in annelids, molluscs and crustaceans

GWa neuropeptides are present in annelids, molluscs (APGWa), platyhelminths, crustaceans (as red pigment concentrating hormone, RPCH) and insects (as adipokinetic hormone, AKH, Figure 1D). Although the sequence similarity is limited, the annelid and mollusc GWa precursors are the likely lophotrochozoan orthologues of arthropod RPCH and AKH neuropeptide precursors [39]. We tested our GWa antibody in the annelid *Capitella,* the molluscs *Pecten* and *Phestilla* and a nauplius larva from a cirripede crustacean (for morphological details, see [40]) collected from a plankton sample (Figure 6). Like *Platynereis, Capitella* larvae show staining in a small number of neurons in the apical organ (Figure 6A, compare with Figure 2D) and in the ventral nerve cord of older larvae (Figure 6B), with no ciliary innervation. In *Pecten*

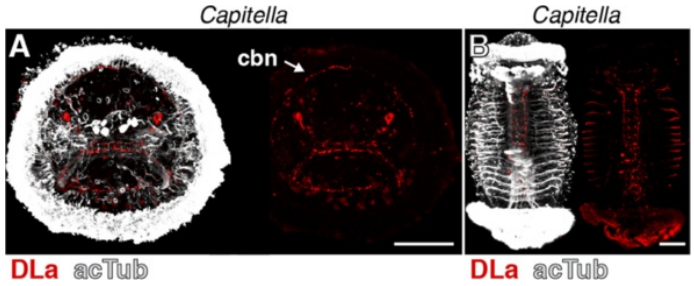


Figure 4 DLamide immunoreactivity in *Capitella* larvae. (**A**) Anterior view of an early *Capitella* larva and (**B**) ventral view of a late *Capitella* larva stained with the DLamide antibody (red) counterstained for acetylated tubulin (acTub, white). Scale bars: 50 μm. cbn, ciliary band nerve.

Conzelmann and Jékely *EvoDevo* 2012, **3**:23
http://www.evodevojournal.com/content/3/1/23

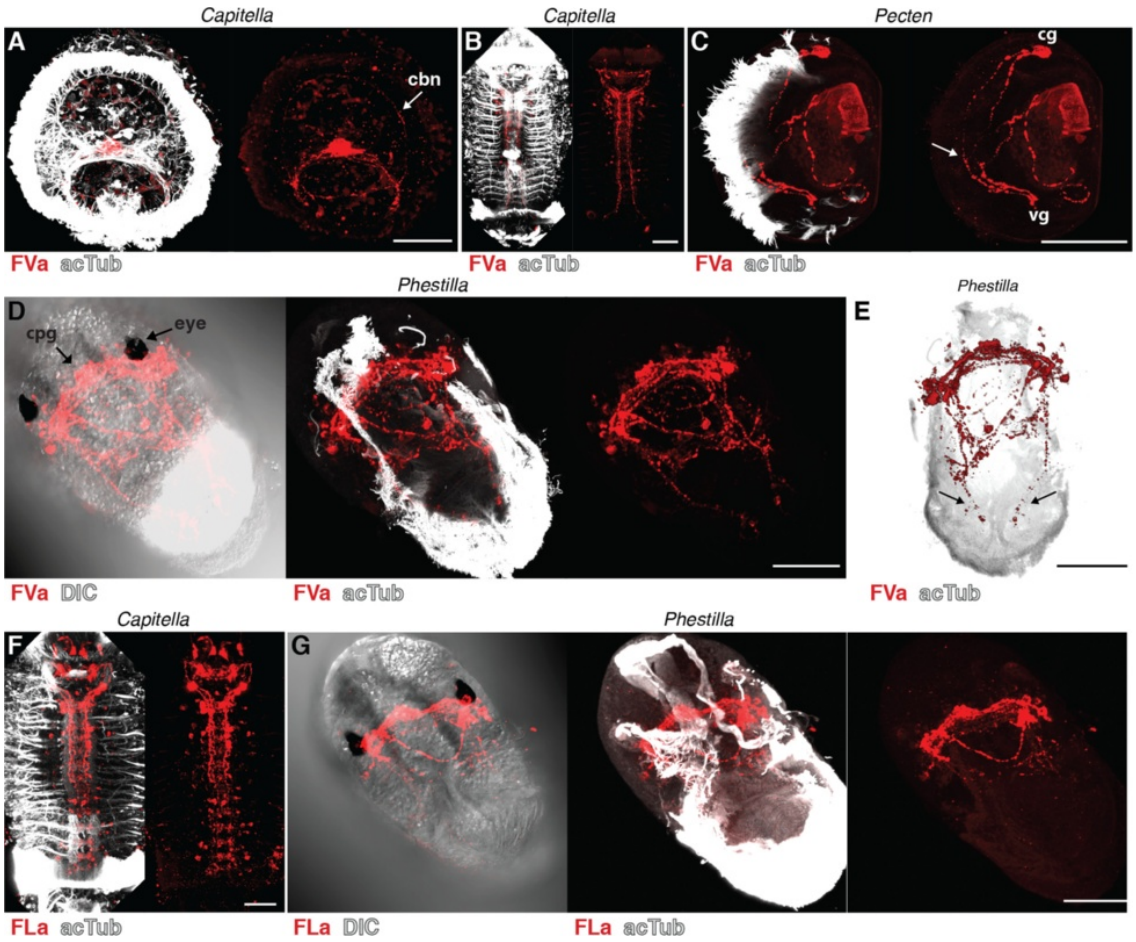


Figure 5 FVamide and FLamide immunoreactivity in annelid and mollusc larvae. Immunostainings with the FVamide antibody (red) counterstained for acetylated tubulin (acTub, white) in (**A**) an early *Capitella* larva, anterior view, (**B**) a late *Capitella* larva, ventral view, (**C**) a *Pecten* veliger larva, lateral view, and (**D**, **E**) a *Phestilla* larva, ventral (**D**) and dorsal (**E**) views. Immunostainings with the FLamide antibody (red) counterstained for acetylated tubulin (acTub, white) in (**F**) a late *Capitella* larva, ventral view, and (**G**) a *Phestilla* larva, ventral view. Arrows in (**C**) and (**E**) point at projections that run along the ciliated velum of *Pecten* and the ciliated foot of *Phestilla*. Scale bars: 50 μm. cbn, ciliary band nerve; cg, cerebral ganglion; cpg, cerebropleural ganglion; DIC, differential interference contrast; vg, visceral ganglion.

veliger larvae, the GWa antibody labels a small number of neurons and their projections (Figure 6C). In *Phestilla*, we found staining in the cerebropleural ganglion between the eyes (Figure 6D). In the crustacean larvae, the antibody labels two cerebral neurons that project to the protocerebral neuropil and a pair of neurons on either side of the labrum (Figure 6E).

RYamide immunoreactivity in cnidarian, annelid, bryozoan, mollusc and crustacean larvae

RYa neuropeptides have been described in a number of marine phyla, including cnidarians, annelids, molluscs, platyhelminthes and crustaceans. They are also present in terrestrial invertebrates, such as nematodes and insects (Figure 1E). In cnidarians, platyhelminthes and nematodes, RYa peptides co-occur with RFa peptides on the same precursor [33,34,41], whereas in most other phyla they originate from a distinct precursor. Given the broad phyletic distribution of RYa peptides and the observation that they often derive from distinct precursors expressed in different cells than RFa [23], the RYa antibody could have a great value for comparative neuroanatomical studies. To explore the potential of the RYa antibody, we tested its reactivity in cnidarians, annelids, molluscs, bryozoans and crustaceans.

In *Capitella* larvae, we found RYa staining in individual sensory neurons in the apical organ. As with DLa and FVa, these neurons project to the ciliary band nerve

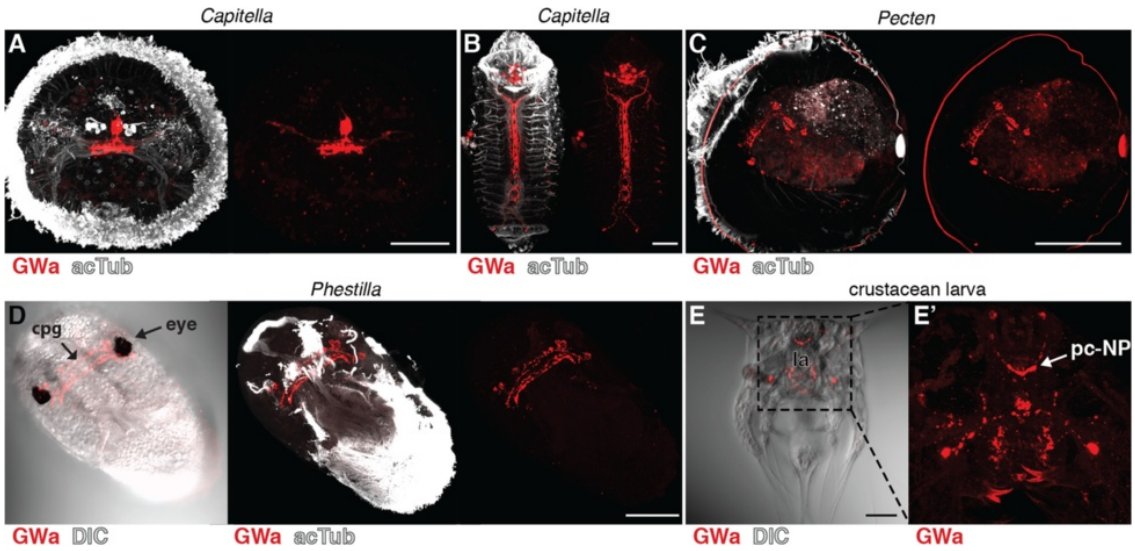


Figure 6 GWamide immunoreactivity in annelid, mollusc and crustacean larvae. Immunostainings with the GWamide antibody (red) counterstained for acetylated tubulin (acTub, white) in (**A**) an early *Capitella* larva, anterior view, (**B**) a late *Capitella* larva, ventral view, (**C**) a *Pecten* veliger larva, lateral view, (**D**) a *Phestilla* larva, ventral view, (**E**) and a crustacean larva, ventral view and (**E'**) a close-up confocal scan. Scale bars: 50 μm. cpg, cerebropleural ganglion; DIC, differential interference contrast; la, labrum; pc-Np, protocerebral neuropil.

(Figure 7A, arrow, compare with Figure 2E), suggesting a role for RYa neuropeptides in regulating ciliary activity in *Capitella*. We also observed a strong staining in the ventral nerve cord in older *Capitella* larvae (Figure 7B). In larvae of the bryozoan *Cryptosula* species (for morphological details see [42]), we detected strong RYa immunoreactivity in the nerve nodule and in the lateral nerves projecting to the coronal ciliary band (Figure 7C, arrows) and several axons that embrace the pyriform organ. In *Pecten* larvae, we detected two pairs of neurons and their projections along the ciliated velum (Figure 7D, arrow). Using primary antibodies directly pre-labelled with different fluorophores, we also co-stained *Pecten* larvae for RYa and FVa. We observed co-labelling only in a subset of neurons, suggesting that these cells co-express RYa and FVa neuropeptides (Figure 7E, asterisks). This result demonstrates that these antibodies can also be used in combination in a single specimen. In *Phestilla* larvae, we detected a strong RYa signal in the apical organ, the cerebropleural ganglion between the eyes and in the pedal ganglion, as well as in nerves connecting these ganglia (Figure 7F), and also in projections running to the ciliated foot (Figure 7G, arrows). In the nauplius larvae, we found RYa in a group of cerebral neurons that have a flask-shaped morphology and in various neurons surrounding the labrum (Figure 7H). In the cnidarians *Aurelia* and *Clava*, we detected RYa staining in sensory neurons of the ciliated planula larvae, mainly located at the aboral pole (Figure 7I-K). These neurons have sensory morphology with apical sensory dendrites projecting to the surface of the ciliated neuroectoderm (Figure 7K). The basal neuronal projections run along the basal side of the ciliated neuroectoderm and terminate in the anterior plexus. These results show that the RYa antibody is a neuronal marker widely applicable across several invertebrate phyla. It should be noted that the RYa antibody may cross-react with invertebrate neuropeptides belonging to the NPF/NPY (short neuropeptide F, NPF; short neuropeptide Y, NPY) family, that sometimes have a C-terminal RYa, such as in *Apis mellifera* and *Bombyx mori* NPFs [43].

Discussion

Amidated dipeptide epitopes allow the generation of specific antibodies

We have shown, using a variety of species, that our antibodies against amidated dipeptides can be used to label distinct subsets of peptidergic neurons (Figure 8, confocal stacks are available [24]). Our finding that several different amidated dipeptides could be used to generate specific antibodies broadens our understanding of the immunogenic potential of peptide sequences. It is interesting to note that the company we contacted for antibody production initially warned us not to carry out the project, arguing that 'It is considered that up to 5aa peptides are not immunogenic at all.' The strong immunogenicity of these peptides must be due to their

Conzelmann and Jékely *EvoDevo* 2012, **3**:23
http://www.evodevojournal.com/content/3/1/23

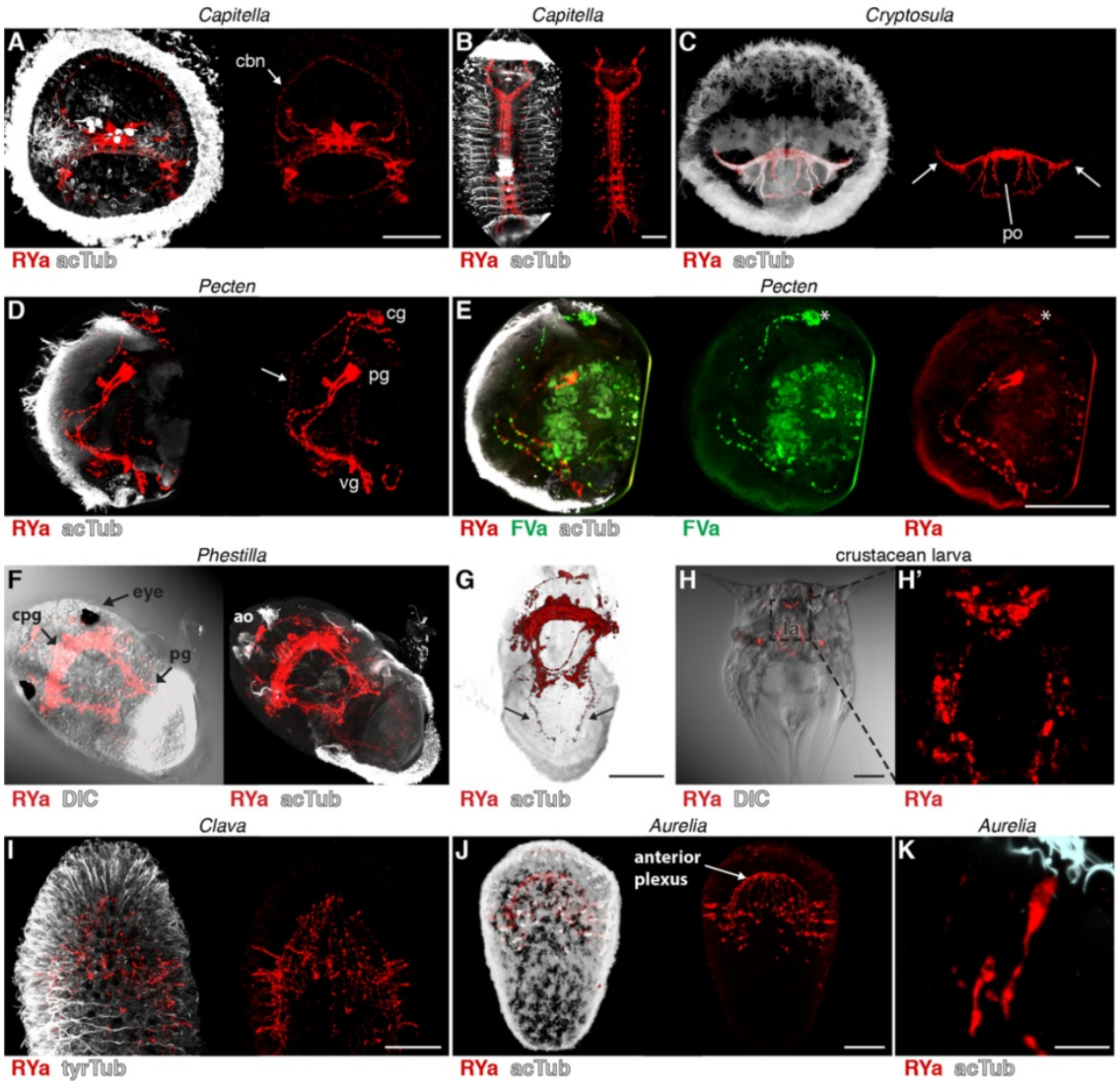


Figure 7 RYamide immunoreactivity in annelid, bryozoan, mollusc, crustacean and cnidarian larvae. Immunostainings with the RYamide antibody (red) counterstained for acetylated tubulin (acTub, white) in (**A**) an early *Capitella* larva, anterior view, (**B**) a late *Capitella* larva, ventral view, (**C**) a *Cryptosula* larva, anterior view, (**D**) a *Pecten* veliger larva, anterior view, (**E**) a *Pecten* veliger larva counterstained with the anti-FVamide antibody (green), anterior view, (**F**) a *Phestilla* larva, ventral and (**G**) dorsal view, (**H**) a crustacean larva, ventral view and (**H'**) a close-up confocal scan. Immunostainings with the RYamide antibody (red) counterstained for acetylated or tyrosylated tubulin (acTub, white) in (**I**) a *Clava* planula larva, lateral view, (**J**) an *Aurelia* planula larva, lateral view, and (**K**) close-up of a sensory neuron of *Aurelia*. Arrows in (**C**) point at axons that project to the ciliary band of *Cryptosula*. Arrows in (**D**) and (**G**) point at projections to the ciliated velum of *Pecten* and the ciliated foot of *Phestilla*. Asterisks in (**E**) indicate neurons that are co-labelled for RYa and FVa. Scale bars: (A-J) 50 μm, (K) 10 μm. ao, apical organ; cbn, ciliary band nerve; cg, cerebral ganglion; cpg, cerebropleural ganglion; DIC, differential interference contrast; la, labrum; pg, pedal ganglion; po, pyriform organ; vg, visceral ganglion.

C-terminal amidation, in the context of the two residues. It is not the amide group that is recognized alone, since all peptides have it, yet we see no cross-reactivity. The importance of amidation, and not the two amino acids alone, is supported by the observation that such dipeptide motifs can be found in thousands of other proteins (for example, 61,717 DL, 32,582 FV, 9,459 GW and 18,792 RY in the *Capitella* predicted proteome), yet we do not see strong background staining in our immunostainings on many different invertebrate species.

Conzelmann and Jékely *EvoDevo* 2012, **3**:23
http://www.evodevojournal.com/content/3/1/23

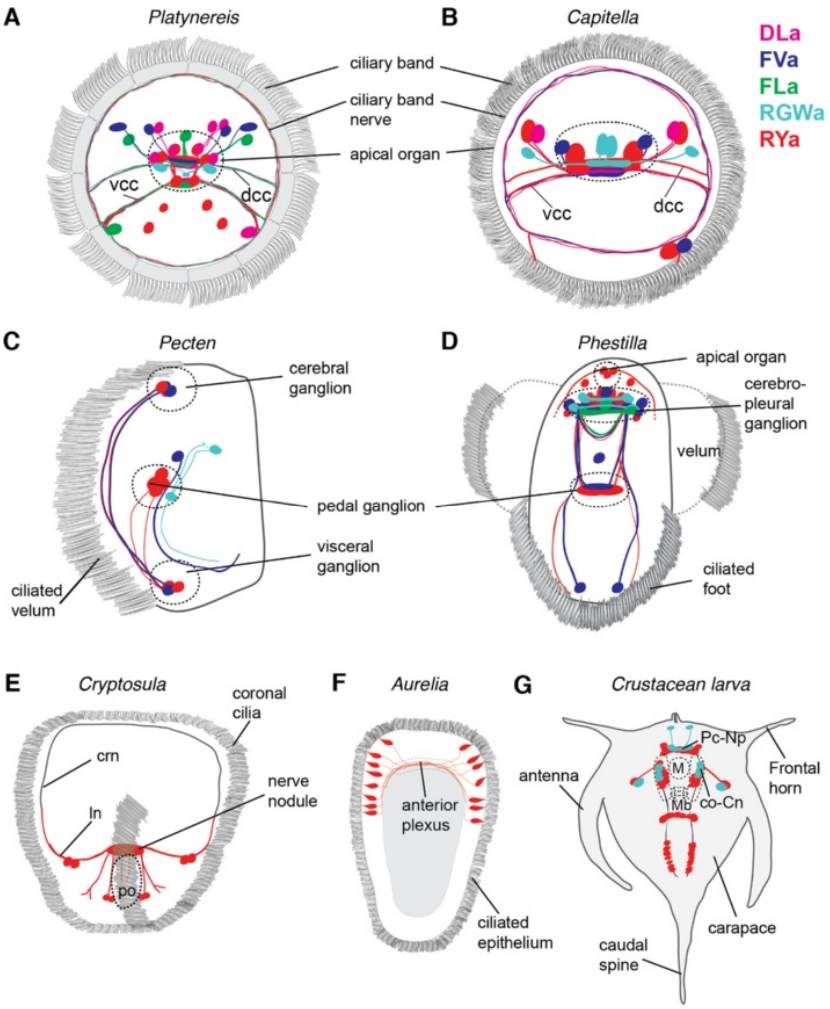


Figure 8 Summary diagrams of peptidergic cells in various species. Summary diagrams showing the position of peptidergic cells labelled with our cross-species reactive neuropeptide antibodies for *Platynereis*, anterior view (**A**), *Capitella*, anterior view (**B**), *Pecten*, lateral view (**C**), *Phestilla*, ventral view (**D**), *Cryptosula*, anterior view (**E**), *Aurelia*, lateral view (**F**) and the crustacean larva, ventral view (**G**). co-Cn, circumoral connective; crn, coronal ring nerve; dcc, dorsal branch of the circum-oesophageal connectives; ln, lateral nerve; m, mouth; mb, mandible; Pc-Np - protocerebral neuropil; po, pyriform organ; vcc, ventral branch of the circum-oesophageal connectives.

Overall, our data argue that the antibodies strongly and specifically bind the amidated peptides we used for immunization. First, the stringent affinity purification protocol we employed together with the peptide-blocking experiments indicates that the antibodies strongly bind to the short amidated peptides. Second, the specific neuronal stainings in tissues corresponding to the expression patterns of the precursor genes in *Platynereis* show that the antibodies specifically bind to the respective peptides.

The strategy we employed to generate specific cross-species antibodies could also be applied to other conserved neuropeptides present in diverse taxa. With the increasing sampling of metazoan genomes and transcriptomes, and the accumulation of data from understudied groups (for example, hemichordates, platyhelminths, priapulids), we will have the chance to identify further conserved peptide motifs. Further sampling will also allow the identification of other taxonomic groups in which the antibodies described here could be used as neuronal markers. Given the brevity of the sequences, reactivity to multiple neuropeptide families with the same amidated termini cannot be excluded. For the proper interpretation of staining patterns, it is therefore also important to study mRNA expression and to scrutinize available transcriptomic and genomic resources. Importantly, our results

show that these antibodies are not widely cross-reactive and do not recognize other amidated peptides. A single amino acid change seems to be sufficient to prevent antibody binding, since the DLa, FLa and FVa antibodies all recognize different cells.

Finally, C-terminal amidation is commonly used for immunization for peptides that derive from an internal part of the protein, to keep the peptide closer to its natural state. Our results caution that such an unnatural terminal amide in internal peptide sequences may trigger an undesired immune response, and potentially cause cross-reactivity to naturally occurring amidated peptides.

Cross-species antibodies suggest that the neuropeptidergic control of cilia is widespread in marine larvae

With the DLa, FVa and RYa antibodies, we commonly observe projections to larval ciliary bands. We have recently shown that the neurons expressing these neuropeptides also innervate the ciliary band in *Platynereis* larvae, and that these peptides regulate the activity of cilia. All three peptides increase the beating frequency of cilia and inhibit ciliary arrests, thereby influencing the swimming depth of planktonic *Platynereis* larvae [23]. In *Capitella* larvae, all three neuropeptides are present in the ciliary band nerve. In *Pecten,* we found FVa and RYa immunoreactivity in nerves running along the ciliated velum, and in *Phestilla* in projections in the ciliated foot. RYa neurons also seem to innervate locomotor cilia in bryozoan and cnidarian larvae. This suggests that these peptides may also regulate ciliary activity in these larvae, indicating a general role for neuropeptides in the regulation of ciliary locomotion in marine invertebrate larvae [44].

Conclusions

We developed specific cross-species reactive antibodies that recognize the conserved neuropeptide motifs DLamide, FVamide, FLamide, GWamide and RYamide. These antibodies can be used in a wide range of marine invertebrates, including annelids, molluscs, bryozoans and cnidarians. Further genomic and transcriptomic sampling could identify other animal groups where these peptide motifs are conserved and where our antibodies could also be employed. Our work also highlights the antigenic potential of very short amidated peptide motifs. The ongoing sampling of neuropeptide diversity will allow the development of other similar antibodies, to enrich further the comparative neurobiology toolbox. Our sampling across diverse marine larvae demonstrates the broad utility of these antibodies, and also indicates that the neuropeptidergic regulation of ciliary locomotion may be a general feature of marine ciliated larvae.

Abbreviations

acTub: Acetylated tubulin; AKH: Adipokinetic hormone; ao: Apical organ; cbn: Ciliaryband nerve; cg: Cerebral ganglion; co-Cn: Circumoral connective; cpg: Cerebropleural ganglion; crn: Coronal ring nerve; Dcc: Dorsal branch of the circum-oesophageal connectives; DIC: Differential interference contrast; Hpf: Hours post fertilization; la: Labrum; ln: Lateral nerve; m: Mouth; mb: Mandible NPF; F: Short neuropeptide; NPY: Short neuropeptide; Pc-Np: Protocerebral neuropil; pg: Pedal ganglion; po: Pyriform organ; RPCH: Red pigment concentrating hormone; tyrTub: Tyrosylated tubulin; vcc: Ventral branch of the circum-oesophageal connectives; vg: Visceral ganglion.

Competing interests

A patent application has been submitted.

Authors' contribution

MC carried out antibody purification, tissue stainings and imaging and wrote the paper. GJ conceived the study, participated in its design and wrote the paper. Both authors read and approved the final manuscript.

Acknowledgement

We thank Harald Hausen for our *Capitella* stock and Andreas Bick for live *Aurelia.* We thank Elizabeth A. Williams for collecting *Clava* larvae and for comments on the manuscript, Nadine Randel and Michael G. Hadfield for *Phestilla* larvae, and Mechtild Seyboldt for collecting *Pecten* larvae. The *Clava* work was supported by an ASSEMBLE grant 227799. The research leading to these results received funding from the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013)/ European Research Council Grant Agreement 260821.

Received: 26 April 2012 Accepted: 23 July 2012
Published: 1 October 2012

References

1. Hay-Schmidt A: **The evolution of the serotonergic nervous system.** *Proc Biol Sci* 2000, **267:**1071–1079.
2. Frontali N, Williams L, Welsh JH: **Heart excitatory and inhibitory substances in molluscan ganglia.** *Comp Biochem Physiol* 1967, **22:**833–841.
3. Price DA, Greenberg MJ: **Structure of a molluscan cardioexcitatory neuropeptide.** *Science* 1977, **197:**670–671.
4. Dockray GJ, Reeve JR, Shively J, Gayton RJ, Barnard CS: **A novel active pentapeptide from chicken brain identified by antibodies to FMRFamide.** *Nature* 1983, **305:**328–330.
5. Dockray GJ, Vaillant C, Williams RG: **New vertebrate brain-gut peptide related to a molluscan neuropeptide and an opioid peptide.** *Nature* 1981, **293:**656–657.
6. Walker RJ, Papaioannou S, Holden-Dye L: **A review of FMRFamide- and RFamide-like peptides in metazoa.** *Invertebr Neurosci* 2009, **9:**111–153.
7. Grimmelikhuijzen CJ: **Antisera to the sequence Arg-Phe-amide visualize neuronal centralization in hydroid polyps.** *Cell Tissue Res* 1985, **241:**171–182.
8. Walker RJ: **Neuroactive peptides with an RFamide or Famide carboxyl terminal.** *Comp Biochem Physiol C, Comp Pharmacol Toxicol* 1992, **102:**213–222.
9. Heuer CM, Loesel R: **Immunofluorescence analysis of the internal brain anatomy of *Nereis diversicolor* (Polychaeta, Annelida).** *Cell Tissue Res* 2008, **331:**713–724.
10. Brinkmann N, Wanninger A: **Neurogenesis suggests independent evolution of opercula in serpulid polychaetes.** *BMC Evol Biol* 2009, **9:**270.
11. Raikova OI, Reuter M, Jondelius U, Gustafsson MK: **The brain of the *Nemertodermatida* (Platyhelminthes) as revealed by anti-5HT and anti-FMRFamide immunostainings.** *Tissue Cell* 2000, **32:**358–365.
12. Stegner MEJ, Richter S: **Morphology of the brain in *Hutchinsoniella macracantha* (Cephalocarida, Crustacea).** *Arthropod Struct Dev* 2011, **40:**221–243.
13. Kristof A, Wollesen T, Wanninger A: **Segmental mode of neural patterning in *Sipuncula*.** *CurrBiol* 2008, **18:**1129–1132.
14. Chun JY, Korner J, Kreiner T, Scheller RH, Axel R: **The function and differential sorting of a family of aplysia prohormone processing enzymes.** *Neuron* 1994, **12:**831–844.
15. Hook V, Funkelstein L, Lu D, Bark S, Wegrzyn J, Hwang S-R: **Proteases for processing proneuropeptides into peptide neurotransmitters and hormones.** *Annu Rev Pharmacol Toxicol* 2008, **48:**393–423.

16. Edison AS, Espinoza E, Zachariah C: **Conformational ensembles: the role of neuropeptide structures in receptor binding.** *J Neurosci* 1999, **19:**6318–6326.
17. Eipper BA, Stoffers DA, Mains RE: **The biosynthesis of neuropeptides: peptide α-amidation.** *Annu Rev Neurosci* 1992, **15:**57–85.
18. Han S-K, Dong X, Hwang J-I, Zylka MJ, Anderson DJ, Simon MI: **Orphan G protein-coupled receptors MrgA1 and MrgC11 are distinctively activated by RF-amide-related peptides through the $G\alpha_{q/11}$ pathway.** *P Natl Acad Sci USA* 2002, **99:**14740–14745.
19. Minth CD, Qiu H, Akil H, Watson SJ, Dixon JE: **Two precursors of melanin-concentrating hormone: DNA sequence analysis and *in situ* immunochemical localization.** *P Natl Acad Sci USA* 1989, **86:**4292–4296.
20. Nahon JL, Presse F, Bittencourt JC, Sawchenko PE, Vale W: **The rat melanin-concentrating hormone messenger ribonucleic acid encodes multiple putative neuropeptides coexpressed in the dorsolateral hypothalamus.** *Endocrinology* 1989, **125:**2056–2065.
21. Maillère B, Hervé M: **The specificity of antibodies raised against a T cell peptide is influenced by peptide amidation.** *Mol Immunol* 1997, **34:**1003–1009.
22. In Y, Ono H, Ishida T: **Structural studies on C-amidated amino acids and peptides: function of amide group in molecular association in crystal structures of Val-Gly-NH2, Ser-Phe-NH2, Gly-Tyr-NH2 and Pro-Tyr-NH2 hydrochloride salts.** *Chem Pharm Bull* 2002, **50:**571–577.
23. Conzelmann M, Offenburger S-L, Asadulina A, Keller T, Münch TA, Jékely G: **Neuropeptides regulate swimming depth of *Platynereis* larvae.** *Proc Natl Acad Sci U S A* 2011, **108:**E1174–E1183.
24. Jékely G: **Max Planck Institute for Developmental Biology; Jékely Lab.** http://jekely-lab.tuebingen.mpg.de/.
25. Jékely G, Arendt D: **Cellular resolution expression profiling using confocal detection of NBT/BCIP precipitate by reflection microscopy.** *Biotechniques* 2007, **42:**751–755.
26. Schneider CA, Rasband WS, Eliceiri KW: **NIH Image to ImageJ: 25 years of image analysis.** *Nat Methods* 2012, **9:**671–675.
27. Southey BR, Amare A, Zimmerman TA, Rodriguez-Zas SL, Sweedler JV: **NeuroPred: a tool to predict cleavage sites in neuropeptide precursors and provide the masses of the resulting peptides.** *Nucleic Acids Res* 2006, **34:**W267–72.
28. Petersen TN, Brunak S, von Heijne G, Nielsen H: **SignalP 4.0: discriminating signal peptides from transmembrane regions.** *Nat Methods* 2011, **8:**785–786.
29. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD: **Multiple sequence alignment with the Clustal series of programs.** *Nucleic Acids Res* 2003, **31:**3497–3500.
30. Christie AE, Cashman CR, Brennan HR, Ma M, Sousa GL, Li L, Stemmler EA, Dickinson PS: **Identification of putative crustacean neuropeptides using *in silico* analyses of publicly accessible expressed sequence tags.** *Gen Comp Endocrinol* 2008, **156:**246–264.
31. Christie AE, Stemmler EA, Dickinson PS: **Crustacean neuropeptides.** *Cell Mol Life Sci* 2010, **67:**4135–4169.
32. Gäde G, Hoffmann KH, Spring JH: **Hormonal regulation in insects: facts, gaps, and future directions.** *Physiol Rev* 1997, **77:**963–1032.
33. Gajewski M, Schmutzler C, Plickert G: **Structure of neuropeptide precursors in *Cnidaria*.** *Ann N Y Acad Sci* 1998, **839:**311–315.
34. Mcveigh P, Mair GR, Atkinson L, Ladurner P, Zamanian M, Novozhilova E, Marks NJ, Day TA, Maule AG: **Discovery of multiple neuropeptide families in the phylum Platyhelminthes.** *Int J Parasitol* 2009, **39:**1243–1252.
35. Veenstra JA: **Neurohormones and neuropeptides encoded by the genome of *Lottia gigantea*, with reference to other molluscs and insects.** *Gen Comp Endocrinol* 2010, **167:**86–103.
36. Veenstra JA: **Neuropeptide evolution: neurohormones and neuropeptides predicted from the genomes of *Capitella teleta* and *Helobdella robusta*.** *Gen Comp Endocrinol* 2011, **171:**160–175.
37. Struck TH, Paul C, Hill N, Hartmann S, Hösel C, Kube M, Lieb B, Meyer A, Tiedemann R, Purschke G, Bleidorn C: **Phylogenomic analyses unravel annelid evolution.** *Nature* 2011, **471:**95.
38. Croll RP: **Development of embryonic and larval cells containing serotonin, catecholamines, and FMRFamide-related peptides in the gastropod mollusc *Phestilla sibogae*.** *Biol Bull* 2006, **211:**232–247.
39. Martínez-Pérez F, Becerra A, Valdés J, Zinker S, Aréchiga H: **A possible molecular ancestor for mollusc APGWamide, insect adipokinetic hormone, and crustacean red pigment concentrating hormone.** *J MolEvol* 2002, **54:**703–714.
40. Semmler H, Wanninger A, Høeg JT, Scholtz G: **Immunocytochemical studies on the naupliar nervous system of *Balanus improvisus* (Crustacea, Cirripedia, Thecostraca).** *Arthropod Struct Dev* 2008, **37:**383–395.
41. Li C, Kim K, Nelson LS: **FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans*.** *Brain Res* 1999, **848:**26–34.
42. Santagata S: **Evolutionary and structural diversification of the larval nervous system among marine bryozoans.** *Biol Bull* 2008, **215:**3.
43. Nässel DR, Wegener C: **A comparative review of short and long neuropeptide F signaling in invertebrates: any similarities to vertebrate neuropeptide Y signaling?** *Peptides* 2011, **32:**1335–1355.
44. Jékely G: **Origin and early evolution of neural circuits for the control of ciliary locomotion.** *P R Soc B* 2011, **278:**914–922.

doi:10.1186/2041-9139-3-23
Cite this article as: Conzelmann and Jékely: **Antibodies against conserved amidated neuropeptide epitopes enrich the comparative neurobiology toolbox.** *EvoDevo* 2012 **3**:23.

Publication 3: **Conserved MIP receptor-ligand pair regulates *Platynereis* larval settlement;** *PNAS* (2013)

Conzelmann, Williams *et al*.

Conserved MIP receptor–ligand pair regulates *Platynereis* larval settlement

Markus Conzelmann[a,1], Elizabeth A. Williams[a,1], Sorin Tunaru[b], Nadine Randel[a], Réza Shahidi[a], Albina Asadulina[a], Jürgen Berger[a], Stefan Offermanns[b], and Gáspár Jékely[a,2]

[a]Max Planck Institute for Developmental Biology, 72076 Tuebingen, Germany; and [b]Max Planck Institute for Heart and Lung Research, 61231 Bad Nauheim, Germany

Edited by Cornelia Bargmann, The Rockefeller University, New York, NY, and approved March 14, 2013 (received for review November 21, 2012)

Life-cycle transitions connecting larval and juvenile stages in metazoans are orchestrated by neuroendocrine signals including neuropeptides and hormones. In marine invertebrate life cycles, which often consist of planktonic larval and benthic adult stages, settlement of the free-swimming larva to the sea floor in response to environmental cues is a key life cycle transition. Settlement is regulated by a specialized sensory–neurosecretory system, the larval apical organ. The neuroendocrine mechanisms through which the apical organ transduces environmental cues into behavioral responses during settlement are not yet understood. Here we show that myoinhibitory peptide (MIP)/allatostatin-B, a pleiotropic neuropeptide widespread among protostomes, regulates larval settlement in the marine annelid *Platynereis dumerilii*. MIP is expressed in chemosensory–neurosecretory cells in the annelid larval apical organ and signals to its receptor, an orthologue of the *Drosophila* sex peptide receptor, expressed in neighboring apical organ cells. We demonstrate by morpholino-mediated knockdown that MIP signals via this receptor to trigger settlement. These results reveal a role for a conserved MIP receptor–ligand pair in regulating marine annelid settlement.

Metazoan life cycles show great diversity in larval, juvenile, and adult forms, as well as in the timing and ecological context of the transitions between these forms. In many animal species, neuroendocrine signals involving hormones and neuropeptides regulate life cycle transitions (1–3). Environmental cues are often important instructors of the timing of life cycle transitions (4), and can affect behavioral, physiological, or morphological change via neuroendocrine signaling (5).

Marine invertebrate larval settlement is a prime example of the strong link between environmental cues and the timing of life-cycle transitions. Marine invertebrate life cycles often consist of a free-swimming (i.e., pelagic) larval stage that settles to the ocean floor and metamorphoses into a bottom-dwelling (i.e., benthic) juvenile (6–8). In many invertebrate larvae, a pelagic–benthic transition is induced by chemical cues from the environment (9, 10). Larval settlement commonly includes the cessation of swimming and the appearance of substrate exploratory behavior, including crawling on or attachment to the substrate (11–14). In diverse ciliated marine larvae (15), the apical organ, an anterior cluster of larval sensory neurons (16) with a strong neurosecretory character (17–20), has been implicated in the detection of cues for the initiation of larval settlement (21). Although molecular markers of the apical organ have been described (22–24), our knowledge of the neuroendocrine mechanisms with which apical organ cells transmit signals to initiate larval settlement behavior is incomplete.

Here, we identify a conserved myoinhibitory peptide (MIP)/allatostatin-B receptor–ligand pair as a regulator of larval settlement behavior in the marine polychaete annelid *Platynereis dumerilii*. MIPs are pleiotropic neuropeptides (25) first described in insects as inhibitors of muscle contractions (26, 27). In some insect species, MIPs modulate juvenile hormone (28) or ecdysone synthesis (29), and are also referred to as allatostatin-B or prothoracicostatic peptide (30, 31). These peptides are known to signal via a G protein-coupled receptor, the sex peptide receptor (SPR) (31, 32). MIPs show sequence similarity to cnidarian GLWamides and belong to an ancestral eumetazoan Wamide family that also includes mollusk APGWamides and other peptides (33). We found that *Platynereis* MIP is expressed in chemosensory–neurosecretory cells in the apical organ and triggers larval settlement behavior by signaling via an SPR orthologue expressed in adjacent apical organ cells. Our results identify a conserved neuropeptide receptor–ligand pair in the apical organ, which may transduce environmental signals to initiate settlement in a pelagic–benthic life cycle.

Results

MIP Triggers Larval Settlement in *Platynereis*. *Platynereis* has a pelagic–benthic life cycle with a freely swimming ciliated larval stage that spends as long as several days in the plankton before transitioning to a benthic lifestyle (34, 35). Searching for regulators of annelid settlement, we identified a neuropeptide, an orthologue of arthropod MIP (Fig. S1 *A* and *B*), that efficiently triggers settlement of *Platynereis* larvae. The peptide is derived from a precursor protein that shows high sequence similarity to the MIP preprotein from the distantly related polychaete *Capitella teleta* (36, 37) and to MIP precursors from arthropods (also called allatostatin-B or prothoracicostatic peptide) (30). The predicted mature protostome MIP peptides share a conserved W($X_{5–8}$)W sequence motif and are amidated (Fig. S1*B*). We did not identify any MIP orthologue in deuterostomes.

Treatment of free-swimming *Platynereis* larvae in a vertical swimming assay (19) with synthetic *Platynereis* MIP peptide rapidly induced downward vertical movement in trochophore and nectochaete larval stages (Fig. 1*A*, Fig. S2, and Movies S1, S2, and S3), an effect that could be reversed by washout (Fig. 1*A*). Different versions of MIPs derived from the same *Platynereis* precursor protein, including a nonamidated MIP, also rapidly triggered larval sinking (Fig. S2 *A–E*). We focused on MIP7 in subsequent experiments because this peptide closely matches the consensus sequence derived from all *Platynereis* MIPs (Fig. S1 *C* and *D*). Substitution of the last (W10A), but not the first (W2A), tryptophan residue to alanine rendered MIP7 inactive in the vertical swimming assay

Author contributions: M.C., E.A.W., S.O., and G.J. designed research; M.C., E.A.W., S.T., N.R., R.S., J.B., and G.J. performed research; M.C. and E.A.W. contributed new reagents/analytic tools; M.C., E.A.W., S.T., N.R., A.A., S.O., and G.J. analyzed data; and M.C., E.A.W., and G.J. wrote the paper.

Conflict of interest statement: A patent application on the potential use of MIP/allatostatin-B has been submitted.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. JX513876, JX513877, and JX513878).

[1]M.C. and E.A.W. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: gaspar.jekely@tuebingen.mpg.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1220285110/-/DCSupplemental.

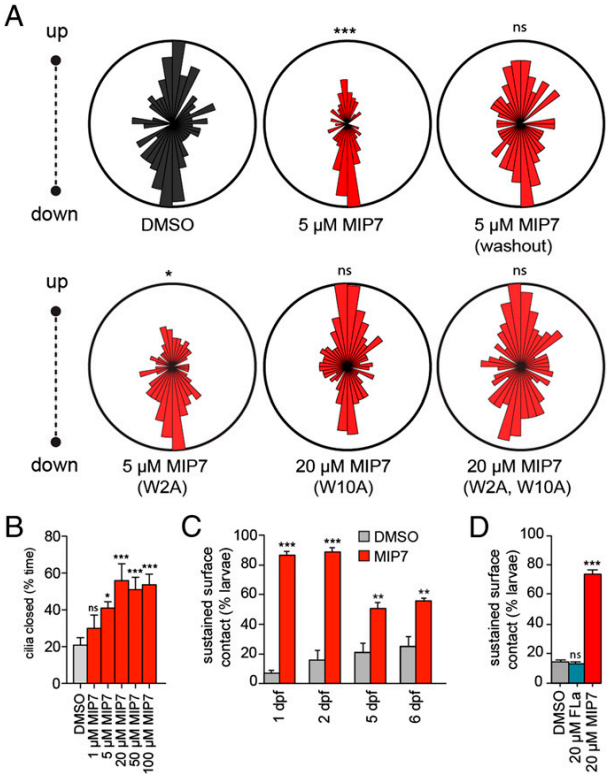


Fig. 1. MIP triggers larval settlement behavior in *Platynereis*. (*A*) Angular histograms of the displacement vectors of swimming tracks for larvae treated with DMSO (control) and larvae treated with the indicated peptide [$n >$ 100 larvae (55–60 hpf) each]. (*B*) Percentage of time in which cilia are closed in DMSO-treated larvae (control) vs. larvae exposed to MIP7 [$n > 10$ larvae (48–50 hpf) each]. (*C*) Percentage of 1-, 2-, 5-, and 6-dpf larvae that showed sustained substrate contact after 2 min exposure to DMSO (control), 20 µM MIP7 (for 1 dpf and 2 dpf), or 50 µM MIP7 (for 5 dpf and 6 dpf; $n = 4$ repetitions with >30 larvae). (*D*) Percentage of larvae that showed sustained substrate contact after 90 min exposure to DMSO (control), AKYFLamide (FLa), and MIP7 [$n = 10$ repetitions with >30 larvae (50 hpf) each]. In *A*, the *P* values of an χ^2 test comparing the number of upward and downward swimming larvae are indicated as $*P < 0.05$ and $***P < 0.001$. Data in *B–D* are shown as mean ± SEM. *P* values of an unpaired *t* test are indicated as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, with "ns" indicating $P > 0.05$.

(Fig. 1*A*). To understand the mechanism of downward movement, we analyzed the effects of MIP7 treatment on ciliary activity. MIP7 did not significantly alter the beat frequency of cilia (Fig. S2*G*), but triggered long and frequent ciliary arrests in a concentration-dependent manner (Fig. 1*B*). Close-up videos of 2-d-old larvae treated with MIP7 in the vertical column showed that the larvae were sinking with their anterior pointed upward (Movie S2). Larvae at age 3 d showed downward movement with their anterior pointed downward, implying more complex behavioral effects in older larvae (Movie S3).

After MIP7-treated larvae reached the bottom of the culture dish, they showed sustained exploratory crawling behavior, with frequent touching of the apical side to the substrate (Fig. 1 *C* and *D* and Movies S4 and S5). Such substrate exploratory behavior could also be observed at low frequency for control larvae, with late nectochaete stages [5–6 d postfertilization (dpf)] showing a greater tendency to contact the substrate. MIP7 treatment strongly induced sustained substrate contact between 1 and 6 d of development (Fig. 1*C*). Another *Platynereis* neuropeptide, AKYFLamide, which has previously been shown to trigger larval sinking (19), was inactive in the crawling assay even after 90 min of incubation (Fig. 1*D*). Overall, MIP treatment can rapidly induce two distinct behaviors in *Platynereis* larvae: (*i*) inhibition of cilia and cessation of swimming and (*ii*) crawling on the substrate, both considered hallmarks of marine invertebrate larval settlement (11–14). These results identify *Platynereis* MIP as a settlement-inducing neuropeptide.

MIP Is Expressed in Neurosecretory Annelid Apical Organ. Whole-mount in situ hybridization on *Platynereis* larvae revealed *MIP* mRNA expression in sensory cells of the apical organ from 20 h postfertilization (hpf) on, as well as two pairs of cells in the trunk from 48 hpf on. The apical organ expression was observed in an increasing number of cells with age (Fig. 2 *A–D* and Fig. S3 *B–H*). Immunostaining with a specific MIP antibody (Fig. S3*A*) showed that the axonal projections of these cells terminate in the apical nerve plexus (Fig. 2 *E* and *F*), a region of strong neurosecretory activity (18, 19). By using the *Platynereis* MIP antibody or an antibody against the conserved C-amidated dipeptide VWamide that is strongly conserved in mollusks and annelids (Figs. S1*B* and S3*A*), we observed similar immunolabeling in sensory cells in the larval apical organ and in the nerve plexus of *Capitella* (Fig. 1 *G* and *H* and Fig. S3*I*).

To confirm the neurosecretory nature of the *MIP*-expressing cells, we analyzed *MIP* coexpression with known neuroendocrine markers by using image registration of in situ hybridization scans to an average anatomical reference template (19, 38, 39). We scanned, registered, and averaged at least five individual larvae per gene from whole-mount in situ hybridization samples. We analyzed the neurosecretory marker prohormone convertase *prohormone convertase 2* (*phc2*) and the neuroendocrine transcription factors *orthopedia* (*otp*) (18) and *dimmed* (*dimm*) (Fig. S4 *A–D*). In *Drosophila*, *dimm* directs the differentiation of neuroendocrine neurons and is coexpressed with *MIP* in the median brain (40, 41). In vertebrates, *otp* is required for the terminal differentiation of hypothalamic neuropeptidergic neurons (42). Image registration revealed that the average *MIP* signal colocalized with *phc2*, and partially overlapped with *otp* and *dimm* average gene expression patterns. Additionally, *otp* and *dimm* colocalized broadly in the *Platynereis* apical organ neurons (Fig. S4 *E–J*). These results indicate that the *MIP* neurons are part of the neurosecretory apical organ.

***Platynereis* MIP Neurons Have Dual Chemosensory–Neurosecretory Function.** To investigate the sensory modality of the MIP neurons, we performed dye-filling experiments on live 30-h-old *Platynereis* larvae. In nematodes, dye-filling is the exclusive property of chemosensory neurons (43). Upon incubation of the larvae with fluorescent MitoTracker dye, we observed labeling of several flask-shaped neurons in the larval episphere, with long apical microvilli at the tip of their dendrites, including two prominent apical organ cells (Fig. 3 *A* and *B*). Laser ablation of these two apical organ cells, followed by subsequent fixation and MIP immunostaining, identified the cells as the two median MIP sensory neurons (Fig. 3 *A–D*, arrowheads). The dye-filling results together with the characteristic microvillar morphology of the MIP neurons suggest that these cells likely have a chemosensory function.

To further characterize the morphology of the MIP neurons in *Platynereis* at an ultrastructural level, we traced the entire volume of two MIP cells (Figs. 2 *D* and *E* and 3*C*, arrows) by using serial-sectioning transmission EM (TEM). In a dataset of 664 ultrathin sections (50 nm), we identified these two ventral MIP neurons based on the position of their cell bodies relative to large adjacent secretory gland cells and the characteristic shape and position of their dendrites and axons (Fig. S5 and Movies S6 and S7). Both identified neurons have a sensory dendrite with a cilium and long apical microvillar extensions surrounding the central cilium, characteristic of annelid chemosensory neurons (44) (Fig. 3*E*). These extensions run at the basal side of the cuticle in a subcuticular

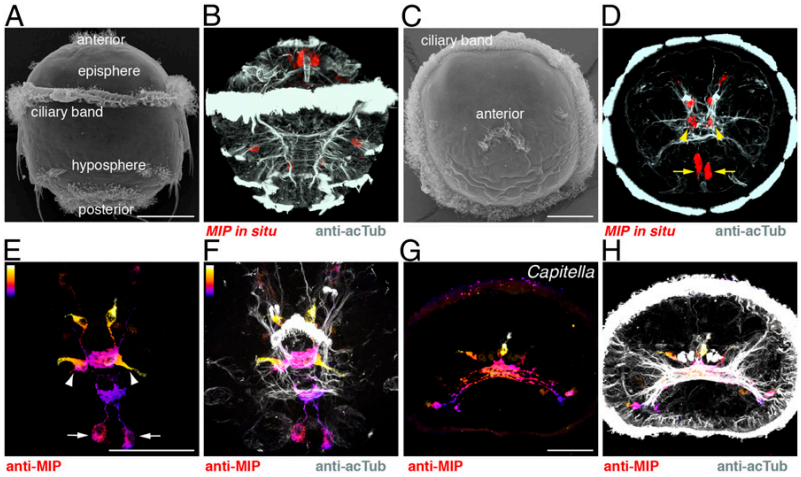


Fig. 2. MIP is expressed in apical organ neurons in *Platynereis* and *Capitella*. (*A*) SEM image of a 48-hpf *Platynereis* larva in ventral view. (*B*) Whole-mount mRNA in situ hybridization for *Platynereis MIP* (red) in a 48-hpf larva in ventral view. (*C*) SEM image of a 48-hpf *Platynereis* larva. (*D*) Whole-mount mRNA in situ hybridization for *MIP* (red) in a 48-hpf *Platynereis* larva. (*E* and *F*) MIP immunostaining on a 48-hpf *Platynereis* larva, color-coded for depth. (*G* and *H*) MIP immunostaining on a *Capitella* larva, color-coded for depth. In *B*, *D*, *F*, and *H*, larvae are counterstained for acetylated α-tubulin (white, anti-acTub). (*C*–*H*) Anterior views. (*D* and *E*) Arrowheads indicate two prominent apical organ cells, and arrows indicate ventral neurons that have been reconstructed by TEM (compare with Fig. 3). (Scale bars: 50 μm.)

NEUROSCIENCE

space that is permeable to seawater. The axons project to the dense apical nerve plexus of the larva, where they branch extensively (Fig. 3 *F* and *G* and Movie S7). We observed that the branched axons are full of large dense-cored vesicles (Fig. 3*H*), but are completely devoid of synapses that are typical for cells containing classical neurotransmitters (45), indicating that these MIP chemosensory neurons signal exclusively via the release of neuropeptides.

Annelid MIP Peptides Signal via Conserved G Protein-Coupled Receptor. In insects, MIP peptides signal via a G protein-coupled receptor, the SPR (31, 32). We identified the orthologues of insect SPRs in *Platynereis* and *Capitella* (Fig. S6) and tested whether these receptors could be activated by annelid MIP peptides. Ligand stimulation in CHO cells expressing the respective *Platynereis* or *Capitella* receptor, a bioluminescent Ca^{2+} reporter, and a promiscuous G protein (46, 47) showed that annelid MIP peptides are potent agonists for these receptors. Activation assays with increasing concentrations of ligand showed that *Platynereis* MIP activated the *Platynereis* receptor in the nanomolar range (EC_{50} of 10 nM; Fig. 4*A*). The activation was specific, as 12 other

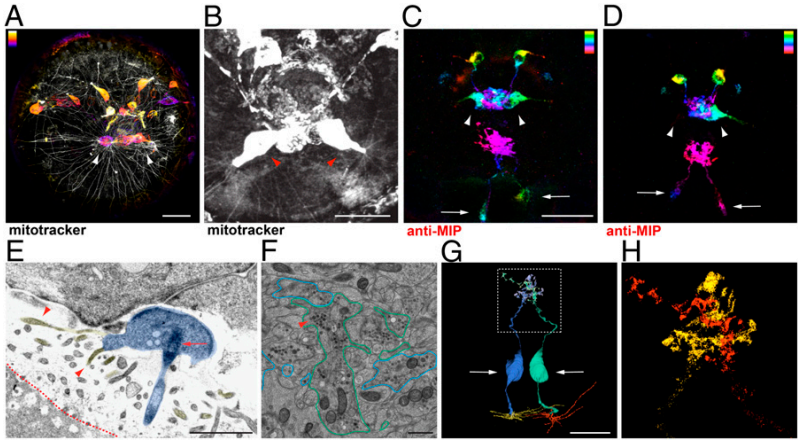


Fig. 3. *Platynereis* MIP neurons are chemosensory and neurosecretory. (*A* and *B*) Chemosensory neurons with long microvilli in the apical organ filled with MitoTracker dye in a 37-hpf *Platynereis* larva. Arrowheads indicate two prominently labeled apical organ cells, one of which was laser-ablated. (*C*) MIP immunostaining on a 52-hpf nonablated larva, color-coded for depth. (*D*) MIP immunostaining in a 52-hpf larva in which the MitoTracker-filled apical organ cell on the right side of the body (left side of image) was ablated. (*E*) Apical sensory ending of a ventral MIP neuron (blue) in a TEM image. Arrow points at the basal body of the sensory cilium, and arrowheads indicate apical microvilli. The red dashed line indicates the border of the cuticle. (*F*) TEM image of the neurosecretory projections of the two ventral MIP neurons filled with dense-cored vesicles (arrowhead). (*G*) Volume reconstruction from serial TEM sections in a 72-hpf larva of the two ventral MIP neurons indicated with arrows in *C* and *D*. The apical microvilli are shown in yellow and red. (*H*) Close-up of the area indicated by the dashed box in *G* shows a reconstruction from serial TEM images of all dense-cored vesicles in the two ventral MIP neurons. (*C* and *D*) Arrowheads indicate two MIP neurons, one of which has been ablated in *D*; arrows indicate ventral neurons that have been reconstructed by TEM. (*A*–*D*) Anterior views. (Scale bars: *A* and *B*, 20 μm; *C* and *D*, 25 μm; *E* and *F*, 1 μm; *G*, 10 μm.)

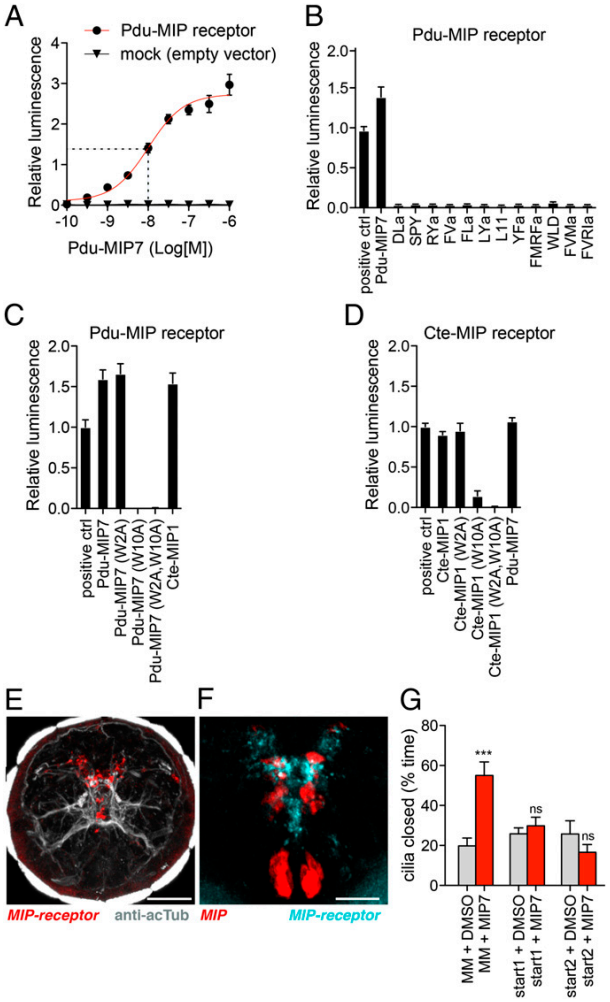


Fig. 4. Annelid MIP peptides signal via a G protein-coupled receptor. (*A*) Dose–response curve of the *Platynereis* (Pdu) MIP receptor treated with varying concentrations of *Platynereis* MIP7. (*B*) Activation of the *Platynereis* MIP receptor by *Platynereis* MIP7 exclusive of other *Platynereis* neuropeptides. All peptides were used at 1 μM concentration. (*C*) Activation of the *Platynereis* MIP receptor by Ala-substituted *Platynereis* MIP7 and *Capitella* (Cte) MIP1. All peptides were used at 1 μM concentration. (*D*) Activation of the *Capitella* MIP receptor by Ala-substituted *Capitella* MIP1 and *Platynereis* MIP7. All peptides were used at 1 μM concentration. (*E*) Whole-mount in situ hybridization for *Platynereis MIP-receptor* (red) in a 48-hpf *Platynereis* larva, counterstained for acetylated α-tubulin (white, anti-acTub), in anterior view. (*F*) Average expression patterns of *MIP* (red) and *dimm* (cyan) obtained by image registration. (*G*) Percentage of time in which cilia are closed in uninjected *Platynereis* larvae, and in MIP-receptor-mismatch (MM), MIP-receptor-start1 (start1), and MIP-receptor-start2 (start2) MO-injected larvae, exposed to 5 μM MIP7. In *B–D*, the positive control represents activation of a human G-protein coupled receptor 109a with 100 μM nicotinic acid. The values are shown as mean ± SEM ($n = 3$ measurements). In *G*, *P* values of an unpaired *t* test are indicated: ***$P < 0.001$ and "ns" represents $P > 0.05$; $n > 10$ larvae (48 hpf). (Scale bars: *E*, 50 μm; *F*, 20 μm.)

Platynereis neuropeptides (19, 45) did not activate the MIP receptor (Fig. 4*B*). Consistently, *Capitella* MIP activated the *Capitella* SPR orthologue (Fig. 4*D*). Substitution of the last, but not the first, tryptophan residue with alanine in the *Platynereis* and the *Capitella* synthetic peptides resulted in a loss of activity (Fig. 4 *C* and *D*), indicating that the conserved C-terminal tryptophan residue is crucial for receptor activation. This result is consistent with our observations in the vertical swimming assays (compare with Fig. 1*A*). We also found that MIP peptides from the two annelid species cross-activated the receptor from the other species (Fig. 4 *C* and *D*).

By using mRNA in situ hybridization, we found that the *MIP-receptor* is expressed in cells of the *Platynereis* apical organ, interspersed between the *MIP*-expressing neurons with little overlap (Fig. 4 *E* and *F*), as revealed by image registration. Average *MIP-receptor* expression also colocalizes with *phc2*, *otp*, and *dimm* average gene expression, indicating that these cells are also part of a neurosecretory apical organ system (Fig. S7).

We next asked if the observed effects of synthetic MIP peptides on *Platynereis* larval settlement were caused by signaling via the MIP receptor. To test this, we knocked down the *Platynereis* receptor by microinjecting two different translation-blocking morpholinos (MOs) and a control mismatch MO into fertilized *Platynereis* eggs. In MIP-receptor–knockdown larvae, we no longer observed an effect of MIP peptide on ciliary closures in 2-d-old larvae, showing that MIP triggers settlement behavior by signaling via the MIP receptor (Fig. 4*G*).

Discussion

MIP Orchestrates *Platynereis* Larval Settlement Behavior via Neuroendocrine Signaling in Apical Organ. Understanding the molecular machinery that operates within larval sensory structures is essential to our understanding of how the environment shapes larval behavior and development. The behavior evoked by synthetic MIP in *Platynereis* larvae mimics the settlement behavior described for other marine larvae upon encountering a natural inductive settlement cue (11–14), suggesting that MIP activates a behavioral program for settlement. In laboratory culture, in the apparent absence of a cue, *Platynereis* larvae gradually transition from a pelagic to a benthic lifestyle over a period of approximately 6 d (35). Our results suggest that, were favorable chemical cues encountered, larvae would be competent to transition to a benthic lifestyle at an earlier stage. This is supported by the early expression of the *MIP* precursor in sensory cells of the apical organ and the strong settlement-inducing effects of MIP from early larval stages on. In early larvae, MIP is exclusively expressed in the apical organ (Fig. S3*B*); therefore, by adding exogenous peptide, we likely mimic the endogenous release of MIP from these apical sensory cells following chemosensory stimulation. A direct demonstration of this model will require the identification of the currently unknown settlement-inducing chemicals for *Platynereis* and their chemoreceptors in the MIP cells, in combination with genetic manipulations to show a link among environmental chemical cues, chemoreceptors, and settlement.

The integrative nature of the settlement behaviors induced by MIP (sinking followed by substrate exploration) is characteristic of neuroendocrine signaling, which can act simultaneously on a number of neurons and genes within an organism (48, 49). In *Platynereis*, several other neuropeptides are expressed in sensory neurons in the apical organ and regulate larval swimming depth during the pelagic phase of the life cycle (19). MIP is unique among these neuropeptides in that it can trigger rapid sinking and substrate exploratory behavior. The expression of the *MIP* precursor broadens and MIP effects become more complex with age, implying an elaboration of MIP targets during development.

The chemosensory MIP cells in the apical organ are also neurosecretory, as they have high concentrations of dense core vesicles and express the neurosecretory cell markers *phc2*, *otp*, and *dimm*. These cells could directly release MIP upon sensory stimulation. The expression of the *MIP-receptor* in apical organ cells adjacent to the MIP cells indicates that there is peptidergic paracrine signaling between the MIP sensory neurons and the receptor-expressing cells. The settlement-inducing effects of MIP were blocked by the MO-mediated knockdown of the receptor, indicating that the MIP receptor is required for the orchestration

of peptide-induced settlement behavior. The molecular fingerprint of the *MIP-receptor*–expressing neurons shows that these cells are also neurosecretory and may release downstream signaling molecules in a neuroendocrine cascade.

Wamide Signaling: Ancestral Component of Eumetazoan Anterior Nervous System? The conservation of MIP and its receptor in *Platynereis* and *Capitella* indicates that MIP signaling is widespread among annelids. MIP is also present in other Lophotrochozoans (Fig. S1) (50, 51). As for insects and annelids, the MIP receptor in the sea hare *Aplysia californica* is also an SPR orthologue (32).

In annelids and insects, a prominent domain of MIP expression is in the most anterior neurosecretory region of the developing brain (30), an area demarcated by the expression of the homeobox gene *six3* (20). The MIP neurons in annelids and insect also express *dimm* and *phc2*. These similarities identify the MIP neurons as a conserved neurosecretory cell type in the anterior protostome brain. Although MIP seems to have been lost along the deuterostome lineage, the coexpression of *dimm* and *otp* in the *Platynereis* apical organ also supports a link among insect, annelid, and vertebrate neuroendocrine centers (18, 52).

Protostome MIPs belong to a diverse and ancient Wamide neuropeptide family that also includes cnidarian GLWamides (33), inducers of cnidarian larval metamorphosis (53–55). In cnidarians, GLWamides are expressed in sensory cells at the anterior pole of the larva (8, 56). This anterior territory shares a conserved regulatory signature with apical organs in many bilaterian ciliated larvae (24, 57). MIP and LWamide may thus represent a conserved effector gene in the neurosecretory anterior brain of cnidarians and protostomes. The role of MIP in annelid settlement and LWamide in cnidarian metamorphosis suggests that one of the ancestral functions of Wamides may have been the regulation of a life cycle transition.

Methods

Gene Identification. *Platynereis* genes were identified from expressed sequence tag (EST) sequences generated from a full-length normalized cDNA library from mixed larval stages. *Capitella* genes were identified at the Joint Genome Institute Genome Portal (58).

Behavioral Assays. *Platynereis* larvae were obtained from an in-house breeding culture, following a previous publication (59). Vertical larval swimming and ciliary beating assays were performed and analyzed as previously described (19).

The substrate–contact assay was carried out in Nunclon 24-well tissue culture dishes or in customized quartz cuvettes. We increased the peptide concentration for 5-dpf and 6-dpf larvae from 20 μM to 50 μM because, in later stages, penetration of peptides could be impaired as a result of a thickening of the outer larval cuticle. After peptide application, sustained contact of the larva with the bottom of the tissue-culture dish was scored. A larva was scored to show sustained substrate contact behavior if it was in contact with the bottom of the dish for at least 5 s (75 frames in a 15 frames-per-second movie).

Antibodies and Tissue Staining. Rabbit antisera against *Platynereis* MIP7 and against the conserved C-terminal VWamide motif were affinity-purified by using the respective peptides coupled to a SulfoLink resin (Thermo Scientific) via an N-terminal Cys (CAWNKNSMRVWamide and CVWamide) as previously described (60). In situ hybridization was performed as previously described (39).

Dye Filling and Laser Ablation. MitoTracker red FM (50 μg; special packaging; Invitrogen) was freshly dissolved in 100 μL DMSO. The solution was added to 30-hpf larvae at 1:500 dilution and incubated for 1 h for optimal dye filling. Single larvae were mounted on a glass slide with two layers of adhesive tape on both sides in 20 μL natural seawater and covered with a coverslip to immobilize them. Dye-filled neurons were ablated on an Olympus FV1000 confocal microscope equipped with a 355 nm-pulsed laser (Teem Photonics) coupled via air and controlled by the SIM Scanner (Olympus). The ablated larvae were recovered, fixed at 48 or 52 hpf, and processed for anti-MIP immunostaining.

Light Microscopy and Image Registration. Confocal imaging was performed as previously described (19). Image registration to a 48-hpf whole-body nuclear reference template and colocalization analysis were performed as previously described (39).

TEM. *Platynereis* larvae (72 hpf) were frozen using a high-pressure freezer (BAL-TEC HPM 010; Balzers) and quickly transferred to liquid nitrogen. Frozen samples were treated with a substitution medium containing 2% (wt/vol) osmium tetroxide in acetone and 0.5% uranyl acetate in a cryosubstitution unit (EM AFS-2; Leica). The samples were cryosubstituted through gradually rising temperatures and embedded in Epon. Serial sections were cut at 50 nm on a Reichert Jung Ultracut E microtome and collected on single-slotted copper grids (NOTSCH-NUM 2 × 1 mm; Science Service) with Formvar support film, contrasted with uranyl acetate and Reynolds lead citrate, and carbon-coated to stabilize the film. Imaging of one specimen (*Platynereis*-72-HT-9-3) was performed at a pixel size of 3.87 nm on a TECNAI Spirit transmission electron microscope (FEI) equipped with an UltraScan 4000 4 × 4 k camera by using Digital Micrograph (Gatan) Stitching and alignment were done by using TrakEM2 (61). All structures were segmented manually as area lists, which were exported into 3Dviewer and Imaris.

Receptor Deorphanization. *Platynereis* and *Capitella* SPR orthologues were cloned into a pcDNA3.1+ vector (Invitrogen) with HindIII and NotI. The *Platynereis* receptor was PCR-amplified from larval cDNA by using the primers 5′-ACAATAAAGCTTGCCACCATGATGGAAGTAAGCTATTCAAATGGAAATG (including HindIII site and Kozak consensus) and 5′-ACAATAGCGGCCGCTTAAATATTTGTAGTTTTAGTCGTGTGATCG (including NotI site), and the *Capitella* receptor clone used was a synthetic construct (GenScript). CHO-K1 cells stably expressing a calcium-sensitive bioluminescent fusion protein were transfected, and receptor activation was measured. Measurements were performed by using a fluorescent plate reader. The area under each calcium transient (measured for 1 min) was calculated by using Ascent software (Thermo Electron) and expressed as integrated luminescence units (relative units).

MO Injection. For microinjections, fertilized *Platynereis* eggs developing at 16 °C were rinsed approximately 1 h after fertilization with sterile filtered seawater in a 100-μm sieve to remove the egg jelly, followed by treatment with 70 μg/mL proteinase K for 1 min to soften the vitellin envelope. Injections were carried out by using Femtotip II needles with a FemtoJet microinjector (Eppendorf) on a Zeiss Axiovert 40 CL inverted microscope equipped with a Luigs and Neumann micromanipulator. The temperature of the developing zygotes was maintained at 16 °C throughout injection by using a Luigs and Neumann Badcontroller V cooling system and a Cyclo 2 water pump (Roth).

Two translation-blocking MOs and one corresponding 5-bp mismatch control MO were designed to target the *Platynereis MIP-receptor* gene. MOs with the following sequences were purchased from Gene Tools: MIP-receptor-start1, TCCATCATTTTGAATGTTGAATGCA; MIP-receptor-mismatch, TCCAT***G***ATTTT***C***AAT***C***TT***C***AAT***C***CA; and MIP-receptor-start2, GTCAATGAGGTCACAAACATCCAAC. Nucleotides complementary to the start codon (ATG) are underlined; nucleotides altered in mismatch control MOs are denoted by boldface italics.

MOs were diluted in water with 12 μg/μL fluorescein dextran (M_r of 10,000 dalton; Invitrogen) as a fluorescent tracer. MOs (0.6 mM) were injected with an injection pressure of 600 hPa for 0.1 s and a compensation pressure of 35 hPa. Injected zygotes were kept in Nunclon six-well plates in 10 mL filtered seawater, and their development was monitored daily. Injected larvae at 48 hpf were used for ciliary resting measurements in the presence of 5 μM synthetic MIP7 peptide or DMSO as control.

ACKNOWLEDGMENTS. We thank Harald Hausen for *Capitella* larvae; Detlev Arendt, Raju Tomer, Heidi Roebert, and Nicola Kegel for advice on *Platynereis* microinjection; and Aurora Panzera for help with microscopy. This work was supported by Max Planck Society Sequencing Grant M.IF.A.ENTW8050 (to G.J.). The research leading to these results was supported by the European Research Council under European Union Seventh Framework Programme FP7/2007–2013 and European Research Council Grant Agreement 260821.

1. Truman JW, Riddiford LM (1999) The origins of insect metamorphosis. *Nature* 401(6752):447–452.

2. Heyland A, Moroz LL (2006) Signaling mechanisms underlying metamorphic transitions in animals. *Integr Comp Biol* 46(6):743–759.

3. Laudet V (2011) The origins and evolution of vertebrate metamorphosis. *Curr Biol* 21(18):R726–R737.
4. Gilbert SF (2012) Ecological developmental biology: Environmental signals for normal animal development. *Evol Dev* 14(1):20–28.
5. Denver RJ (2009) Stress hormones mediate environment-genotype interactions during amphibian development. *Gen Comp Endocrinol* 164(1):20–31.
6. Rieger RM (1994) The biphasic life cycle—a central theme of metazoan evolution. *Am Zool* 34:484–491.
7. Hadfield MG (2000) Why and how marine-invertebrate larvae metamorphose so fast. *Semin Cell Dev Biol* 11(6):437–443.
8. Hodin J (2006) Expanding networks: Signaling components in and a hypothesis for the evolution of metamorphosis. *Integr Comp Biol* 46(6):719–742.
9. Pawlik J (1992) Chemical ecology of the settlement of benthic marine invertebrates. *Oceanogr Mar Biol* 30:273–335.
10. Zimmer RK, Butman CA (2000) Chemical signaling processes in the marine environment. *Biol Bull* 198(2):168–187.
11. Qian P (1999) Larval settlement of polychaetes. *Hydrobiologia* 402:239–253.
12. Hadfield MG, Koehl MAR (2004) Rapid behavioral responses of an invertebrate larva to dissolved settlement cue. *Biol Bull* 207(1):28–43.
13. Walters LJ, Miron G, Bourget E (1999) Endoscopic observations of invertebrate larval substratum exploration and settlement. *Mar Ecol Prog Ser* 182:95–108.
14. Maldonado M, Young CM (1996) Effects of physical factors on larval behavior, settlement and recruitment of four tropical demosponges. *Mar Ecol Prog Ser* 138: 169–180.
15. Young CM, ed. (2002) *Atlas of Marine Invertebrate Larvae* (Academic, San Francisco). 1st Ed.
16. Nielsen C (2005) Larval and adult brains. *Evol Dev* 7(5):483–489.
17. Page LR, Parries SC (2000) Comparative study of the apical ganglion in planktotrophic caenogastropod larvae: Ultrastructure and immunoreactivity to serotonin. *J Comp Neurol* 418(4):383–401.
18. Tessmar-Raible K, et al. (2007) Conserved sensory-neurosecretory cell types in annelid and fish forebrain: Insights into hypothalamus evolution. *Cell* 129(7):1389–1400.
19. Conzelmann M, et al. (2011) Neuropeptides regulate swimming depth of *Platynereis* larvae. *Proc Natl Acad Sci USA* 108(46):E1174–E1183.
20. Steinmetz PR, et al. (2010) Six3 demarcates the anterior-most developing brain region in bilaterian animals. *Evodevo* 1(1):14.
21. Hadfield MG, Meleshkevitch EA, Boudko DY (2000) The apical sensory organ of a gastropod veliger is a receptor for settlement cues. *Biol Bull* 198(1):67–76.
22. Rentzsch F, Fritzenwanker JH, Scholz CB, Technau U (2008) FGF signalling controls formation of the apical sensory organ in the cnidarian *Nematostella vectensis*. *Development* 135(10):1761–1769.
23. Jackson DJ, et al. (2010) Developmental expression of COE across the Metazoa supports a conserved role in neuronal cell-type specification and mesodermal development. *Dev Genes Evol* 220(7-8):221–234.
24. Santagata S, Resh C, Hejnol A, Martindale MQ, Passamaneck YJ (2012) Development of the larval anterior neurogenic domains of *Terebratalia transversa* (Brachiopoda) provides insights into the diversification of larval apical organs and the spiralian nervous system. *Evodevo* 3:3.
25. Nässel DR, Winther AME (2010) *Drosophila* neuropeptides in regulation of physiology and behavior. *Prog Neurobiol* 92(1):42–104.
26. Schoofs L, Holman GM, Hayes TK, Nachman RJ, De Loof A (1991) Isolation, identification and synthesis of locustamyoinhibiting peptide (LOM-MIP), a novel biologically active neuropeptide from *Locusta migratoria*. *Regul Pept* 36(1):111–119.
27. Schoofs L, Veelaert D, Broeck JV, De Loof A (1996) Immunocytochemical distribution of locustamyoinhibiting peptide (Lom-MIP) in the nervous system of *Locusta migratoria*. *Regul Pept* 63(2-3):171–179.
28. Lorenz MW, Kellner R, Hoffmann KH (1995) A family of neuropeptides that inhibit juvenile hormone biosynthesis in the cricket, *Gryllus bimaculatus*. *J Biol Chem* 270(36): 21103–21108.
29. Hua YJ, et al. (1999) Identification of a prothoracicostatic peptide in the larval brain of the silkworm, *Bombyx mori*. *J Biol Chem* 274(44):31169–31173.
30. Williamson M, Lenz C, Winther AM, Nässel DR, Grimmelikhuijzen CJ (2001) Molecular cloning, genomic organization, and expression of a B-type (cricket-type) allatostatin preprohormone from *Drosophila melanogaster*. *Biochem Biophys Res Commun* 281(2):544–550.
31. Yamanaka N, et al. (2010) *Bombyx* prothoracicostatic peptides activate the sex peptide receptor to regulate ecdysteroid biosynthesis. *Proc Natl Acad Sci USA* 107(5): 2060–2065.
32. Kim Y-J, et al. (2010) MIPs are ancestral ligands for the sex peptide receptor. *Proc Natl Acad Sci USA* 107(14):6520–6525.
33. Liu F, Baggerman G, Schoofs L, Wets G (2008) The construction of a bioactive peptide database in Metazoa. *J Proteome Res* 7(9):4119–4131.
34. Fischer A, Dorresteijn A (2004) The polychaete *Platynereis dumerilii* (Annelida): A laboratory animal with spiralian cleavage, lifelong segment proliferation and a mixed benthic/pelagic life cycle. *Bioessays* 26(3):314–325.
35. Fischer AH, Henrich T, Arendt D (2010) The normal development of *Platynereis dumerilii* (Nereididae, Annelida). *Front Zool* 7:31.
36. Struck TH, et al. (2011) Phylogenomic analyses unravel annelid evolution. *Nature* 471(7336):95–98.
37. Veenstra JA (2011) Neuropeptide evolution: Neurohormones and neuropeptides predicted from the genomes of *Capitella teleta* and *Helobdella robusta*. *Gen Comp Endocrinol* 171(2):160–175.
38. Tomer R, Denes AS, Tessmar-Raible K, Arendt D (2010) Profiling by image registration reveals common origin of annelid mushroom bodies and vertebrate pallium. *Cell* 142(5):800–809.
39. Asadulina A, Panzera A, Veraszto C, Liebig C, Jékely G (2012) Whole-body gene expression pattern registration in *Platynereis* larvae. *EvoDevo* 3:27.
40. Park D, et al. (2008) The Drosophila basic helix-loop-helix protein DIMMED directly activates PHM, a gene encoding a neuropeptide-amidating enzyme. *Mol Cell Biol* 28(1):410–421.
41. Hamanaka Y, et al. (2010) Transcriptional orchestration of the regulated secretory pathway in neurons by the bHLH protein DIMM. *Curr Biol* 20(1):9–18.
42. Acampora D, et al. (1999) Progressive impairment of developing neuroendocrine cell lineages in the hypothalamus of mice lacking the Orthopedia gene. *Genes Dev* 13(21): 2787–2800.
43. Perkins LA, Hedgecock EM, Thomson JN, Culotti JG (1986) Mutant sensory cilia in the nematode *Caenorhabditis elegans*. *Dev Biol* 117(2):456–487.
44. Purschke G (2005) Sense organs in polychaetes (Annelida). *Hydrobiologia* 535:53–78.
45. Jékely G, et al. (2008) Mechanism of phototaxis in marine zooplankton. *Nature* 456(7220):395–399.
46. Baubet V, et al. (2000) Chimeric green fluorescent protein-aequorin as bioluminescent Ca2+ reporters at the single-cell level. *Proc Natl Acad Sci USA* 97(13):7260–7265.
47. Tunaru S, Lättig J, Kero J, Krause G, Offermanns S (2005) Characterization of determinants of ligand binding to the nicotinic acid receptor GPR109A (HM74A/PUMA-G). *Mol Pharmacol* 68(5):1271–1280.
48. Gilbert SF, Epel D (2009) *Ecological Developmental Biology* (Sinauer, Sunderland, MA).
49. Kim Y-J, Zitňan D, Galizia CG, Cho K-H, Adams ME (2006) A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. *Curr Biol* 16(14):1395–1407.
50. Moroz LL, et al. (2006) Neuronal transcriptome of *Aplysia*: Neuronal compartments and circuitry. *Cell* 127(7):1453–1467.
51. Veenstra JA (2010) Neurohormones and neuropeptides encoded by the genome of *Lottia gigantea*, with reference to other mollusks and insects. *Gen Comp Endocrinol* 167(1):86–103.
52. Wirmer A, Bradler S, Heinrich R (2012) Homology of insect corpora allata and vertebrate adenohypophysis? *Arthropod Struct Dev* 41(5):409–417.
53. Leitz T, Morand K, Mann M (1994) Metamorphosin A: A novel peptide controlling development of the lower metazoan *Hydractinia echinata* (Coelenterata, Hydrozoa). *Dev Biol* 163(2):440–446.
54. Gajewski M, Leitz T, Schloßherr J, Plickert G (1996) LWamides from Cnidaria constitute a novel family of neuropeptides with morphogenetic activity. *Rouxs Arch Dev Biol* 205:232–242.
55. Erwin PM, Szmant AM (2010) Settlement induction of *Acropora palmata* planulae by a GLW-amide neuropeptide. *Coral Reefs* 29:929–939.
56. Piraino S, et al. (2011) Complex neural architecture in the diploblastic larva of *Clava multicornis* (Hydrozoa, Cnidaria). *J Comp Neurol* 519(10):1931–1951.
57. Sinigaglia C, Busengdal H, Leclère L, Technau U, Rentzsch F (2013) The bilaterian head patterning gene *six3/6* controls aboral domain development in a cnidarian. *PLoS Biol* 11(2):e1001488.
58. Grigoriev IV, et al. (2012) The genome portal of the Department of Energy Joint Genome Institute. *Nucleic Acids Res* 40(database issue):D26–D32.
59. Hauenschild C, Fischer A (1969) *Platynereis Dumerilii. Mikroskopische Anatomie, Fortpflanzung, Entwicklung* (Gustav Fischer, Stuttgart).
60. Conzelmann M, Jékely G (2012) Antibodies against conserved amidated neuropeptide epitopes enrich the comparative neurobiology toolbox. *Evodevo* 3(1):23.
61. Cardona A, et al. (2010) An integrated micro- and macroarchitectural analysis of the Drosophila brain by computer-assisted serial section electron microscopy. *PLoS Biol* 8(10):8.

Supporting Information

Conzelmann et al. 10.1073/pnas.1220285110

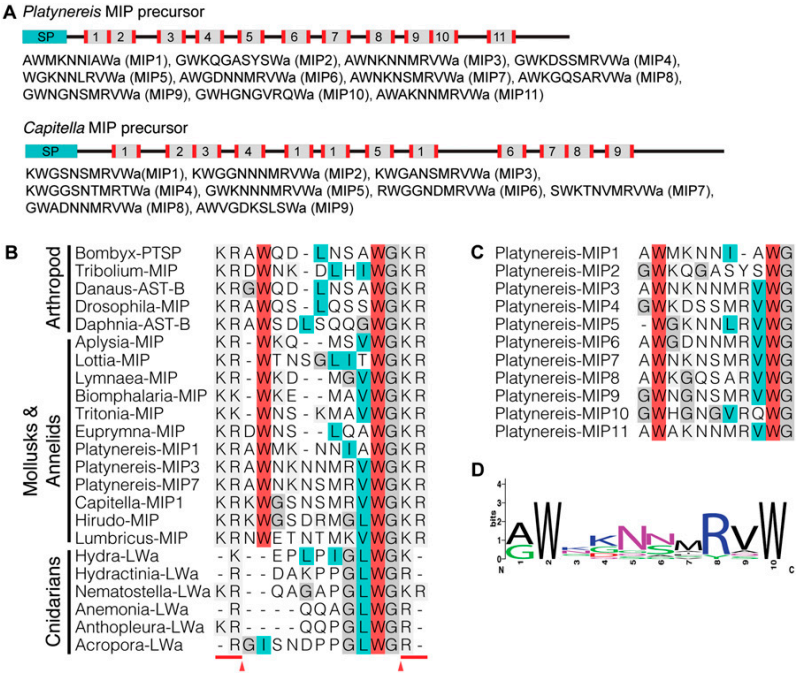


Fig. S1. Annelid myoinhibitory peptide (MIP) neuropeptide precursors. (*A*) Schematics of MIP precursor proteins for *Platynereis dumerilii* and *Capitella teleta*. N-terminal signal peptides (teal) and the predicted peptides (gray) flanked by basic cleavage sites (red) are shown. The predicted mature MIP sequences and their numbering as used in the text are listed. (*B*) Multiple alignment of arthropod, mollusk, and annelid MIP and cnidarian GLWamide sequences show the mono- or dibasic cleavage sites (underlined in red), the amidation signature glycine, the conserved tryptophan (red), and aliphatic residues (teal). The peptides liberated following protease cleavages are indicated between the arrowheads. (*C*) Multiple alignment of predicted mature *Platynereis* MIPs. The C-terminal Gly is expected to be converted into an amide group. (*D*) MIP sequence logo created from an alignment of all *Platynereis* MIPs. MIP7 is the peptide with the highest sequence similarity to the consensus and was therefore used for most behavioral assays, receptor deorphanization, and antibody design.

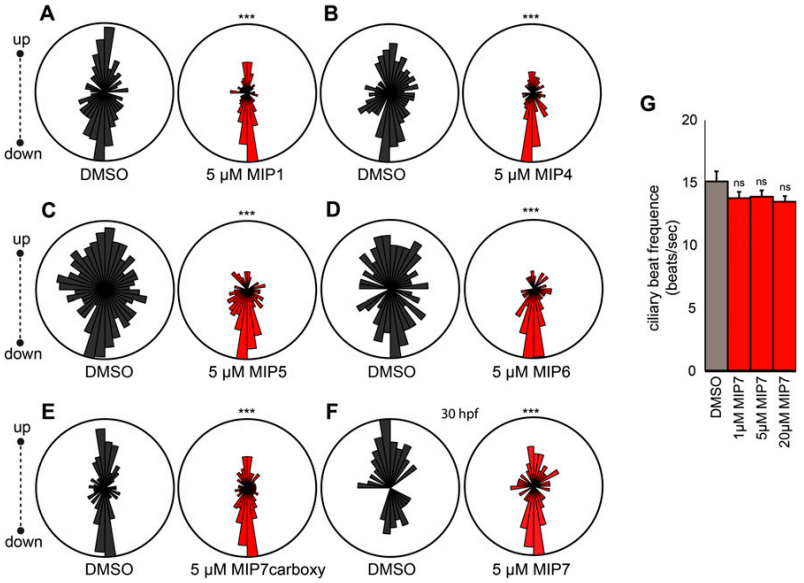


Fig. S2. Different synthetic MIPs trigger larval settlement in *Platynereis* without significantly affecting ciliary beat frequency. (*A*–*E*) Angular histograms of the displacement vectors of swimming tracks for 55 to 60 h postfertilization (hpf) control larvae (gray) and larvae in the presence of the indicated peptides (red). (*F*) Angular histograms of the displacement vectors of swimming tracks for 30-hpf control larvae (gray) and larvae in the presence of 5 µM MIP7. (*A*–*F*) *P* values of an χ^2 test comparing the number of larvae swimming upward and downward are indicated: ***$P < 0.001$; $n > 100$ larvae. (*G*) Ciliary beat frequency in control larvae vs. larvae exposed to increasing concentrations of MIP7. Data are shown as mean ± SEM. *P* values of an unpaired *t* test are indicated: "ns" indicates $P > 0.05$; $n > 15$ larvae (50 hpf) each.

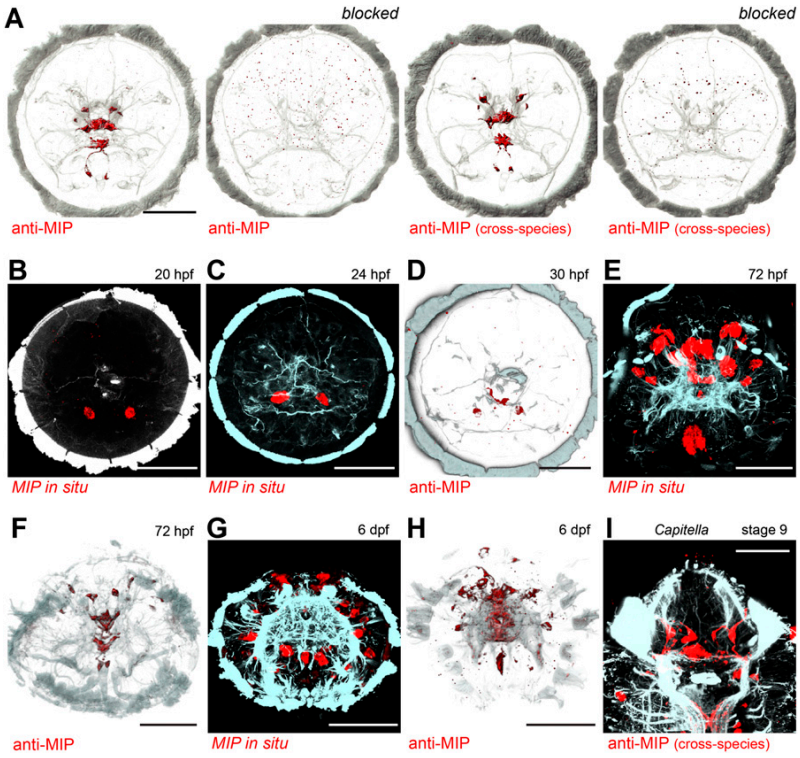


Fig. S3. MIP immunostaining and RNA in situ hybridization in *Platynereis* and *Capitella* larvae. (*A*) *Platynereis* MIP antibody and cross-species MIP antibody have specific affinity for their epitopes. MIP immunostaining on 48-hpf larvae with an antibody raised against CAWNKNSMRVWamide; MIP immunostaining (red) following preincubation of the antibody with 5 mM AWNKNSMRVWamide for 2 h (blocked); immunostaining with a cross-species–reactive MIP antibody raised against CVWamide (red); and immunostaining with a cross-species–reactive MIP antibody (red) following preincubation with 5 mM VWamide for 2 h (blocked). Images were acquired by using the same confocal microscopy and image processing parameters. (*B–I*) Spatial expression of MIP throughout annelid development; developmental stages are indicated. (*B*, *C*, *E*, and *G*) Whole-mount in situ hybridization for the *MIP* precursor mRNA on different *Platynereis* larval stages. (*D*, *F*, and *H*) Immunostaining with an MIP antibody on different *Platynereis* stages. (*I*) Immunostaining with a cross-species MIP antibody against the conserved dipeptide VWamide on a *Capitella* larva (ventral view). All larvae are counterstained for acetylated α-tubulin (white, gray, or cyan). (*A–H*) Anterior views. (Scale bars: 50 μm.)

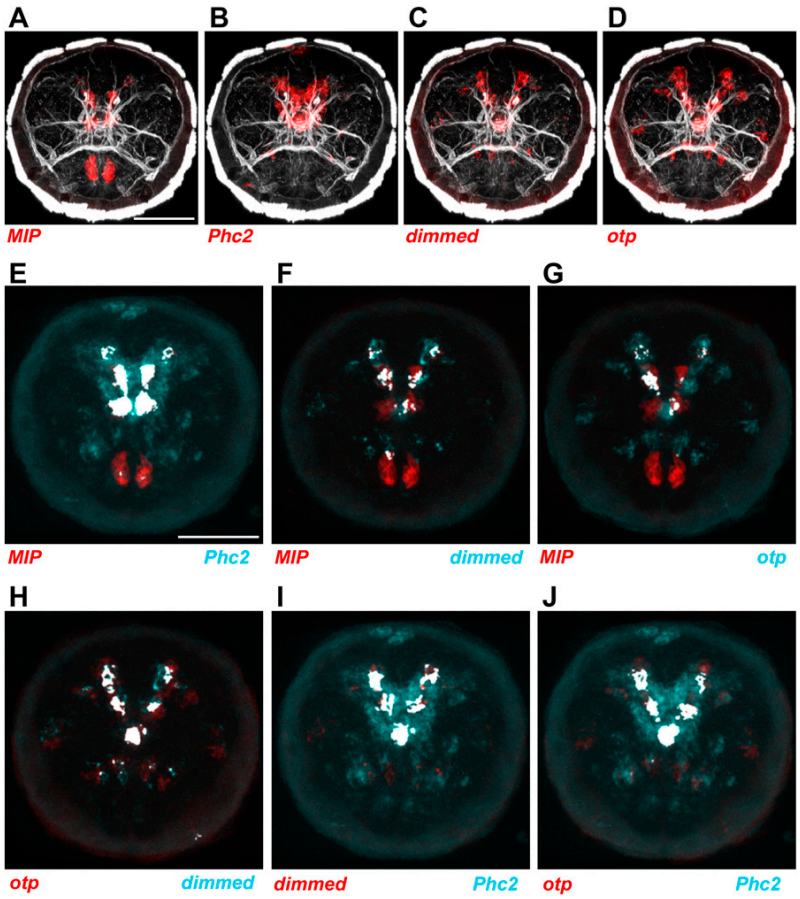


Fig. S4. Gene expression profiling by image registration of MIP-expressing neurons in 48-hpf *Platynereis* larvae. (*A–D*) Average expression patterns of *MIP* precursor RNA and the neuroendocrine genes *prohormone convertase 2* (*phc2*), *dimmed* (*dimm*), and *orthopedia* (*otp*) projected onto a common whole-body reference template. Only the larval episphere is shown. Acetylated tubulin signal was also projected onto the reference. (*E–J*) Pairwise colocalization of the average expression patterns of the indicated genes in the reference template. The overlap in the average gene expression 3D image stacks is shown in white. All images are anterior views. (Scale bars: 50 μm.)

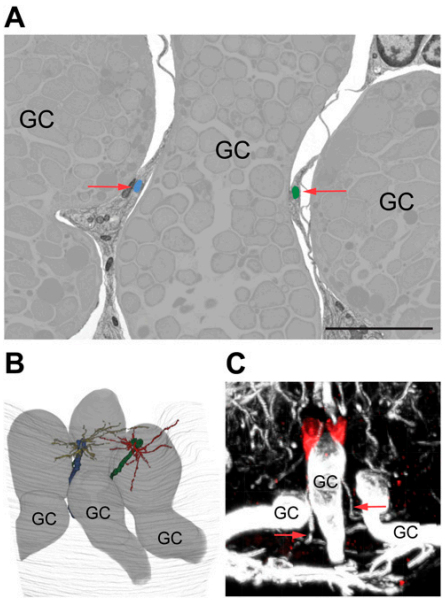


Fig. S5. Identification of two ventral MIP neurons in a serial transmission EM (TEM) dataset. (*A*) Dendrite cross-section of the two ventral MIP neurons at the level of the secretory gland cells. Arrows point at the dendrites (compare with Movie S6). (*B*) Reconstruction of the two ventral MIP neurons (blue and green) in relation to the three gland cells. The apical dendritic microvilli are shown in yellow and red (compare with Movie S7). (*C*) Close-up view of the two ventral MIP neurons labeled by MIP immunostaining (red) on a 72-hpf *Platynereis* larva, counterstained for acetylated α-tubulin (white). Arrows point at the long dendrites that project apically between the gland cells (GC). (Scale bar: 5 μm.)

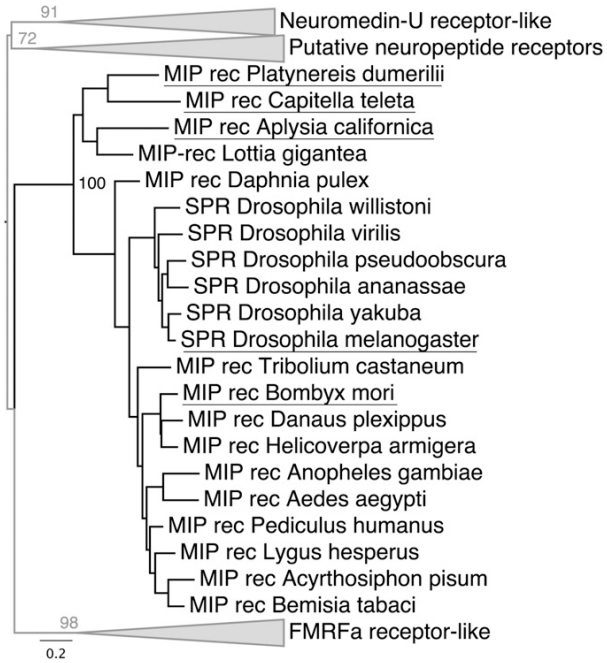


Fig. S6. Phylogenetic tree of MIP/sex peptide receptors. Neighbor-joining phylogenetic tree of MIP/sex peptide receptors. The receptors that have been deorphaned previously (1, 2) and in the present study are underlined. The bootstrap values at selected nodes are indicated.

1. Yamanaka N, et al. (2010) *Bombyx* prothoracicostatic peptides activate the sex peptide receptor to regulate ecdysteroid biosynthesis. *Proc Natl Acad Sci USA* 107(5):2060–2065.
2. Kim Y-J, et al. (2010) MIPs are ancestral ligands for the sex peptide receptor. *Proc Natl Acad Sci USA* 107(14):6520–6525.

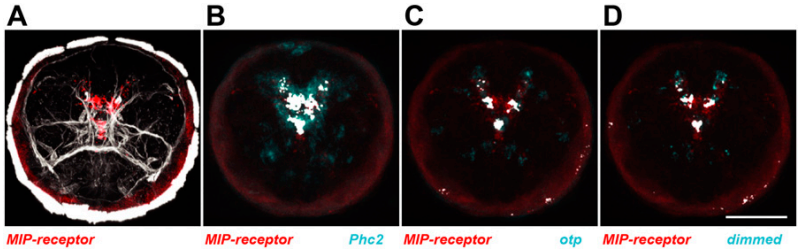
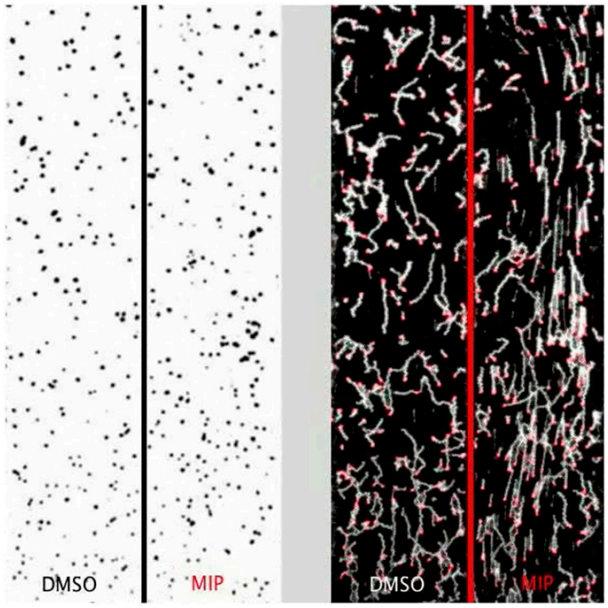
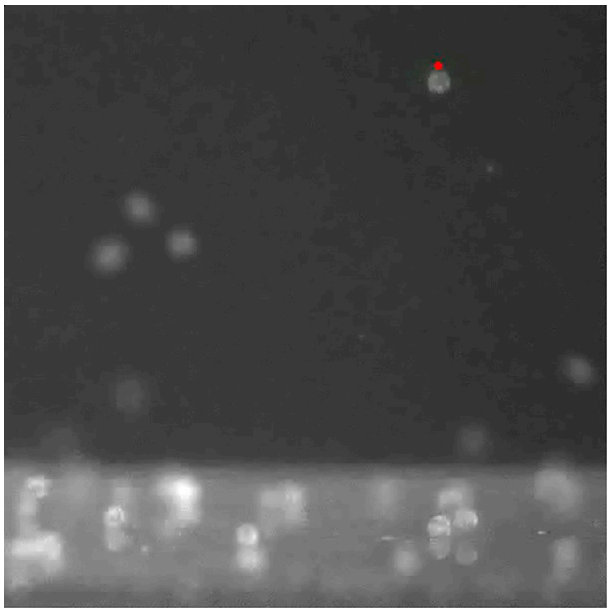


Fig. S7. Gene expression profiling by image registration of *MIP-receptor*–expressing neurons in 48-hpf *Platynereis* larvae. (*A*) Average expression pattern of *Platynereis MIP receptor* projected onto a common whole-body reference template. Only the larval episphere is shown. Acetylated tubulin signal was also projected onto the reference. (*B*–*D*) Colocalization of the *Platynereis MIP receptor* with the neuroendocrine markers *phc2*, *otp*, and *dimm*. The overlap in the average gene expression 3D image stacks is shown in white. All images are anterior views. (Scale bar: 50 μm.)



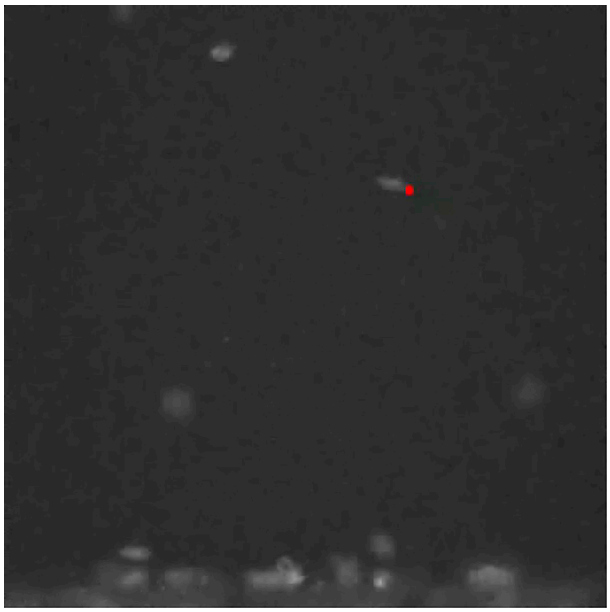
Movie S1. MIP treatment triggers sinking of *Platynereis* larvae. Time-lapse recording of 57-hpf untreated larvae (*Left*) and larvae in the presence of 5 μM MIP7 (*Right*) in vertical columns (up in the column is at the top of the movie). (*Left*) Background-subtracted raw videos. (*Right*) Larval tracks are shown in white, and red dots mark the position of the larvae. Three hundred frames of a 15-frames-per-second movie are shown (sped up 2×).

Movie S1



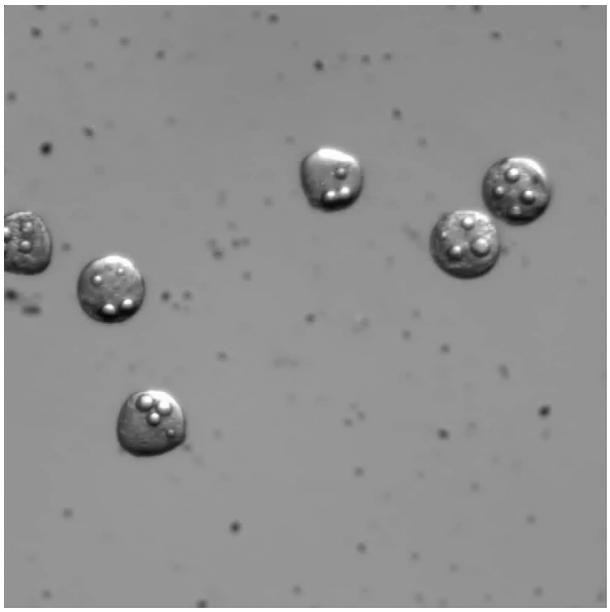
Movie S2. MIP-treated 2-d postfertilization (dpf) *Platynereis* larvae sink with their anterior pointing upward. Close-up recording of freely swimming *Platynereis* larvae after 2 min exposure to 5 μM MIP7 in a vertical column (up in the column is at the top of the movie). The apical pole of a larva is indicated by a red dot, indicating that 2-dpf larvae sink with their anterior pole pointing upward. Larvae that show exploratory behavior are visible at the bottom of the vertical columns.

Movie S2



Movie S3. MIP-treated 3-dpf *Platynereis* larvae sink with their anterior pointing downward. Close-up recording of freely swimming *Platynereis* larvae after 2 min exposure to 5 μM MIP7 in a vertical column (up in the column is at the top of the movie). The apical pole of a larva is indicated by a red dot, indicating that 3-dpf larvae sink with their anterior pole pointing downward.

Movie S3

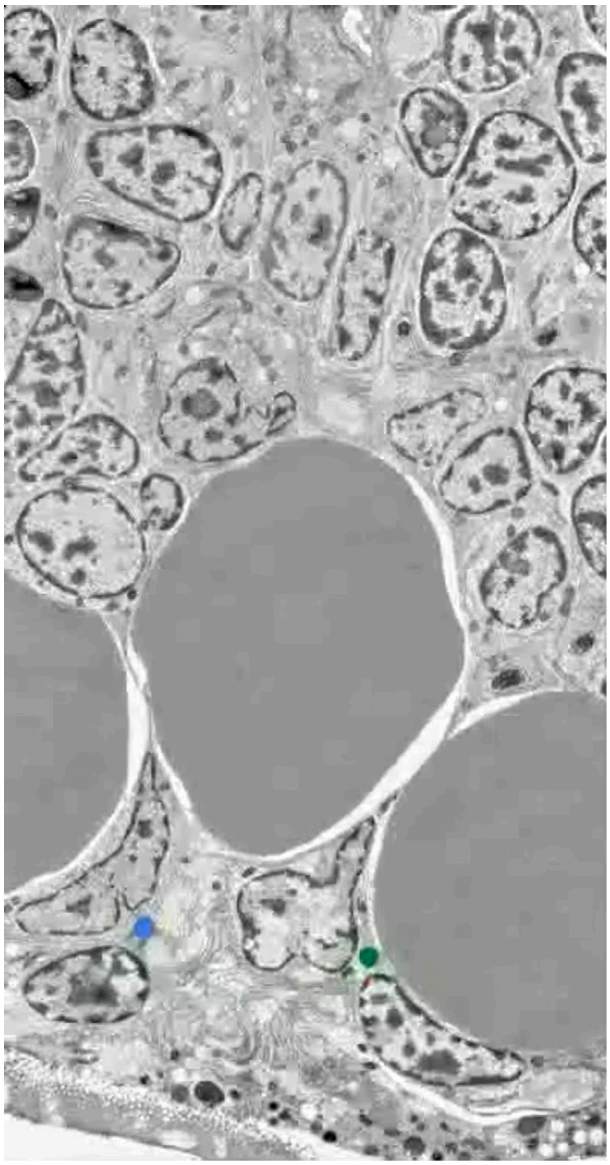


Movie S4. MIP treatment triggers sustained substrate contact of 2-dpf *Platynereis* larvae. Close-up recording of *Platynereis* larvae exposed to 5 μM MIP7 in a dish. Larvae show exploratory crawling behavior. The imaging focus is at the level of the bottom of the dish.

Movie S4

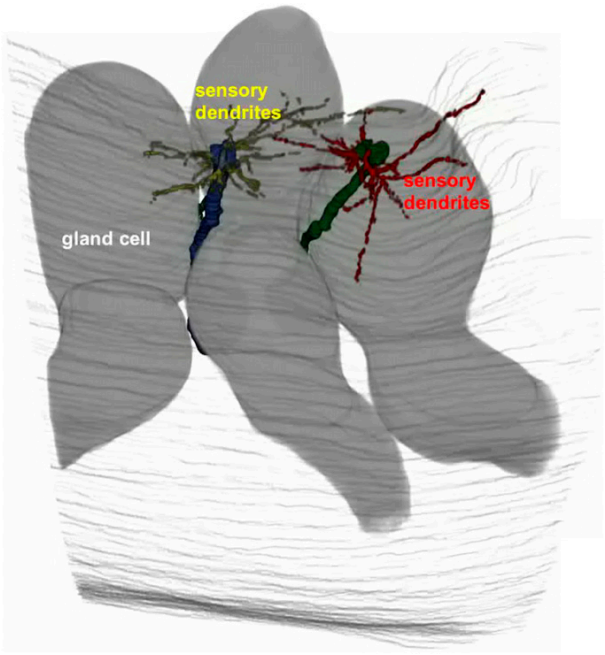
Movie S5. MIP treatment triggers sustained substrate contact and crawling of 5-dpf *Platynereis* larvae. Close-up recording of *Platynereis* larvae exposed to DMSO vs. 50 μM MIP7 in a cuvette. MIP peptide-treated larvae show an increase in crawling behavior compared with control larvae (i.e., DMSO). The imaging focus is at the level of the bottom of the cuvette. Larvae that crawl throughout the whole video were marked with a colored dot by using the Manual Tracking plugin in ImageJ.

Movie S5



Movie S6. TEM image series with the segmented ventral MIP neurons in a 72-hpf larva. The scale of the image is 30 μm.

Movie S6



Movie S7. Reconstruction of the two ventral MIP neurons from the TEM sections from a 72-hpf larva.

Movie S7