Characterisation of selected neuropeptide systems in the marine model organism *Platynereis dumerilii*

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Abstract

Neuropeptides are an important class of signaling molecules produced by the nervous system to regulate diverse body functions such as physiology, development and behavior. Several neuropeptide families, which have originated early in animal evolution, are shared between protostomes and deuterostomes (including vertebrates) and may hence have relevance for fundamental processes. In my thesis, I investigate two of these ancient families of neuropeptides: the Oxytocin/Vasopressin (OT/AVP) family and the GnRH-like family. Both families have relevance for vertebrate reproduction, however their ancient function is difficult to conclude given their challenging evolutionary history (Gruber, 2014) (Roch et al., 2014a). The marine annelid Platynereis dumerilii, consistent with its slow evolutionary pace, has retained both OT/AVP-like and invGnRH-like peptide hormones (Tessmar-Raible et al., 2007; unpublished). Like many other marine species, it has a tightly regulated reproductive cycle, which is timed according to the lunar phase and is under the control of a molecular circa-lunar clock (Zantke et al., 2013). Moreover, the reproductive timing is dependent on other metabolic factors and hormonal cues, which still remain elusive. Through qPCR, I was able to confirm that these selected neuropeptide precursors are involved in its reproductive biology, since their transcripts are significantly upregulated during maturation.

The first part of my thesis will focus on the characterisation of the ancient OT/AVP signaling system, by trying to decipher the role of Vasotocin, the single peptide correlate in Platynereis. Initially, I was able to validate the binding of VTN to 2 ortholog receptors, named Pdu-VtnR1 and VtnR2, confirming VTN ligand belongs to this orthologous group of peptides. Transcripts levels of vtn and vtnr genes, which were found to be more abundant in heads than elsewhere in the body, were too low to be localized through whole mount in-situ hybridization. Nevertheless, with the help of anti-VTN antibodies, I succeeded in detecting the active VTN neuropeptide in the ventral brain area of adult worms. Importantly, as part of my project, I also contributed to the establishment of the first molecular gene knockout tool in Platynereis by creating functional TALENs constructs for
the OT/AVP-like gene vasotocin-neurophysin (vtn) (Bannister et al., 2014). The mutant strains I generated will now be relevant for uncovering the function of Vtn in Platyneireis

According to the most recent phylogenetic studies, GnRH-like peptides and their receptors are part of a wider AKH/CRZ/ACP/GnRH superfamily having a common evolutionary origin, which dates back to the split of Proto- and Deuterostomia (Hauser and Grimmelikhuijzen, 2014). In Protostomia these families have then diverged and subfunctionalised. As a second part of my study, I investigated this hypothesis in Platyneireis using different approaches. First, I showed that Platyneireis transcriptome encodes 4 peptide precursors that cluster into the AKH and the CRZ/invGnRH subgroups. However, evolutionary conclusions on such short sequences are difficult to determine. Phylogenetic analyses on candidate G-protein coupled receptors (GPCRs) for these ligands revealed that Platyneireis possesses 2 AKHR-type and 1 CRZ/invGnRHR-type receptor candidates. This phylogenetic assignment was independently confirmed by a cell-based validation assay, performed by another student and collaborators. More interestingly, the discovery that Platyneireis invGnRHR3 binds to both CRZ/invGnRH-type and AKH-type ligands suggests rather a crosstalk and adds complexity to the system. Through whole mount in situ hybridization I localized Platyneireis crz/gnrh1, akh1 and akh2 precursors to different domains of the worm’s brain, indicating peptides might have distinct roles, but still signal to the same receptive cells. Finally, to shed more light onto the enigmatic role of invGnRHs, I analysed a Platyneireis crz/gnrh1 mutant strain. My analyses revealed that homozygous females, when tested for their maturation timing, spawned significantly earlier compared to wild-type females and males, which showed no difference. These results are compatible with a role of invGnRHs in energy-switch processes and/or maturation, therefore affecting reproductive timing, though the phenotype might still be compensated by the other ligands acting on the same receptor and other functions could be unmasked by knocking out Platyneireis invGnRHR3 receptor gene.
Zusammenfassung


beigetragen, indem TALEN-Konstrukte für das OT/AVP-ähnliche Gen Vasotocin-Neurophysin (Vtn) hergestellt wurden (Bannister et al., 2014). Diese Entdeckungen lassen schließen, dass Pdu-Vasotocin eine neuromodulatorische Funktion hat und möglicherweise eine Rolle im reproduktiven Verhalten spielt. Diese Hypothese kann nun mithilfe homozygoter Tiere aus der von mir hergestellten Mutantenlinie verifiziert werden.“

Introduction

**What are neuropeptides?**

Neuropeptides are used in all bilaterian animals to regulate diverse biological processes such as physiology, development and behavior. These molecular signals are produced within the central nervous system and diffuse either to other neurons (acting as neuromodulators) or to distant target tissues through bodily fluids (acting as hormones). Another classification has to do with the cell types that release and respond to these signaling molecules; neuropeptides are secreted from neuronal cells (primarily neurons but also glia) and signal to neighboring cells (primarily neurons). In contrast, peptide hormones are secreted from neuroendocrine cells and travel through the blood to distant tissues where they elicit a response. Both neuropeptides and peptide hormones are synthesized as ‘preprohormones’, larger proteins that are processed by the same set of enzymes, which selectively cleave the peptide precursor at specific processing sites to generate the active peptides.

**Biosynthesis of Neuropeptides and how they signal through their Receptors (GPCRs)**

Most peptide precursors contain a signal peptide on their N-terminus, which is cleaved off by signal peptidases once it reaches the processing site, usually cellular vesicles. Here, endoproteases, like prohormone convertases 1 and 2 (PC1 and PC2) recognize dibasic cleavage sites like KR, KK, RR and release a pre-mature peptide from the rest of the protein. The exopeptidase carboxypeptidase E (CPE) and the peptidylglycine α-amidating mono-oxygenase (PAM) serve as amidating enzymes of the C-terminus of the peptide. Other possible post-translational modifications are disulfide bond formation, the addition and subsequent modification of N-linked and O-linked oligosaccharides, phosphorylation and sulfation. These steps occur as the maturing neuropeptides travel down the axon in large dense core vesicles (LDCVs) toward the synapse, where they are released outside of the cells. Neuropeptides signal by interacting extracellularly to their specific transducing membrane receptors (Zupanc, 1996). These mostly belong to the G-protein coupled
receptor family (GPCRs) and are composed of 7 transmembrane α-helices domains, an extracellular N-terminus site and a C-terminus, which is into the cytoplasm. The extracellular domains bind specifically the ligand, while intracellularly the receptor interacts with a GTP-binding protein (G proteins), consisting of an α-,β-, and γ-subunit (Pin and Bockaert, 1999). When a GPCR is activated by its ligand, it leads to the dissociation of the α-subunit of the G protein from the βγ-dimer and both will be released in the cytoplasm. Subsequently, they will interact with specific effector proteins, depending on the type of α-subunit (Gq, Gs and Gi/o) triggering a cellular signalling response. One of the most common signalling pathways is the activation, by means of Gq subunit, of phospholipase Cβ (PLCβ) which results in the formation of diacylglycerol (DAG) and inositol triphosphate (IP3). DAG activates protein kinase C (PKC) and IP3 finally mobilizes the second messenger Ca2+ from intracellular stores like the endoplasmic reticulum.

How ancient is the neuropeptide signalling system?

It is a long-standing belief that the neuropeptide GPCR subfamilies, just like the other families that signal through a variety of different ligands, originated before the divergence of Protostomes and Deuterostomes. This led to a great diversification but, remarkably across evolution, they all share a well-conserved structure (Caers et al., 2012). Neuropeptides were first discovered more than 40 years ago by psychopharmacology researchers, and since then neuroendocrine control mechanisms have been observed in all animals that possess a nervous system. Today a lot is known in humans about the different families of neuropeptides and studies focus on elucidating the many fascinating functions they have, for example on social behavior. In evolutionary terms, neuropeptides were established very early as molecules mediating intercellular communication. In cnidarians, one of the lowest animal groups with a nervous system, the nerve net is strongly peptidergic. In fact, in Hydra, there are many peptides used in neurotransmission, but many of the “conventional” neurotransmitter systems, such as acetylcholine (ACh), catecholamines and serotonin, are not found (Grimmelikhuijzen et al., 1996). Moreover recent analyses of neuroendocrine functions in invertebrate model systems reveal a great degree of similarity between phyla as far apart as nematodes, arthropods, and chordates. Studies that emphasize the comparison between different animal groups will help to shed
light on questions regarding the common evolutionary origin and possible homologies between neuroendocrine systems.

In my thesis I will focus on the characterisation and evolutionary relationship of neuropeptides belonging to two major signaling systems, the Oxytocin/Vasopressin and the GnRH/AKH/ACP/CRZ superfamily in an invertebrate emerging model organism, the marine annelid *Platynereis dumerilii*.

**The Nonapeptide family: Oxytocin/Vasopressin-like hormones**

The Nonapeptide hormonal system includes of Oxytocin (OT) and Arg-Vasopressin (AVP) in mammals. As the name suggests, mature peptides comprise 9 amino acids. They are synthesized in the soma of neuronal secretory cells as longer pre-prohormones. These are then cleaved by processing enzymes, like prohormone convertases PC1 and PC2 which cut at dibasic sites leaving the short peptide and a carrier protein, named neurophysin. These enzymes are found in the LDCVs, where oxytocin and vasopressin are further processed by alpha-amidation of the last Glutamine and disulfide bond formation from the two Cysteine residues. The active cyclic peptides are then packed into these vesicles and transported along the axons to the release sites.

In vertebrates, Vasopressin and Oxytocin and their homologues across species are secreted by the hypothalamus and project to the neurohypophysis (pituitary) from where they are released into the blood stream to act on distant target organs. In addition they are diffused into the liquor cerebrospinalis to act as neuromodulators on behavior (Donaldson and Young, 2008; Heinrichs and Domes, 2008). In mammals Oxytocin was first discovered as a paracrine factor stimulating the uterus contraction during delivery (Gimpl, 2001), while currently there is a great interest on its neuroregulatory function in social cognition and behavior, mother-newborn bonding (Broad et al., 2006) as well as pair bonding (Young and Wang, 2004). Arg-Vasopressin, on the other side, is also called the anti-diuretic hormone in mammals for its role in regulating blood pressure and water homeostasis, by acting on the V2 receptors expressed in the loop of Hendel region of the kidneys (Burgess et al., 1994). This fluid homeostasis function is also shared among non-mammals (McCormick and Bradshaw, 2006). In fishes a direct connection between plasma osmolarity and their ortholog peptide Arg-Vasotocin levels has been found (Hyodo et al., 2004; Warne and
As a neuromodulator acting locally within the brain, Arg-Vasotocin has been linked by many studies in teleosts to male social behavior and aggression (Goodson and Bass, 2001) and is believed to be implicated in courtship behavior (Semsar and Godwin, 2004).

The enigmatic function of the Nonapeptide system in invertebrates

Current literature states that the Oxytocin/Vasopressin system has evolved more than 600 million years ago from a single ancestral gene duplication within the vertebrate lineage. In fact, basal vertebrates like lampreys and hagfishes have retained one single ortholog (Gwee et al., 2009). Importantly, the nonapeptide family is conserved across several classes of Lophotrochozoa such as mollusks (Octopus, Lymnea, Aplysia) and annelids (leech and earthworm) and invertebrate Deuterostomia, such as sea urchin and star fish (Elphick and Rowe, 2009) (Semmens et al., 2016). On the other hand, this family is only present in basal Ecdysozoa like Tribolium, while it has been lost in higher insects like Drosophila. Recently two more studies have brought to light the presence of an OT/AVT homologue in another class of Ecdysozoa, the nematode C. elegans. They found that nematocin has implications in the mating behavior of the animal and gustatory associative-learning (Beets et al., 2012) (Garrison et al., 2012). Currently, the physiological role of OT/AVP molecules across protostomes are diverse, but mostly classifiable between reproductive behavior, neuromodulation or water homeostasis regulation. Cephalotocin and octopressin are the two nonapeptides identified in Octopus. Their receptors have been localized in the nervous system and peripheral tissues such as the ovary, though there is no functional evidence of a role in reproduction or neurotransmission (Kanda et al., 2003) (Kanda et al., 2005). The same peptides when administered in vivo to Sepia had effects on long-term memory formation (Bardou et al., 2010). In Aplysia, neurophysiological studies associate conopressin G to the modulation of gill behavior (MARTÍNEZ-PADRÓN et al., 1992), while in annelids like the leech Hirudo medicinalis, injections of the same conopressin G induces spontaneous courtship behavior (Wagenaar et al., 2010). In another leech species, Whitmania pigra, nonapeptides had a role both in egg-laying behavior and osmoregulation with reduction of water content (Fujino et al., 1999) Oumi et al. (1996). In vivo studies on the flour beetle Tribolium demonstrated how the insect vasopressin-like peptide has a diuretic activity and has an indirect activity on Malpighian tubes (Aikins et al., 2008).
Therefore, given such diverse functions across invertebrates, the ancient role of OT/AVP system remains still an open question, which I have tried to address in my thesis using *Platynereis* as a suitable model organism.

**GnRH and GnRH-like family across vertebrates and invertebrates**

Vertebrates possess multiple Gonadotropin-Releasing Hormone (GnRH) isoforms that are classified into three main groups of paralogues, namely GnRH1, GnRH2, GnRH3. GnRH1 has a well-studied function, across the hypothalamic-pituitary-gonad (HPG) axis, where it regulates the production of gonadotropins such as LH and FSH and therefore has a key role in reproduction. GnRH2 acts locally as a neuropeptide, The respective neurons are located in the midbrain, project to the whole brain and are thought to be involved in sexual behaviour and food intake, while GnRH3 is present only in teleosts. Presence of homologues of the GnRH family of neuropeptides has also been shown across invertebrates. A GnRH-like peptide was confirmed in the cephalochordate amphioxus *Branchiostoma floridae* (Roch et al., 2014b); but also across Protostomes such as mollusks and annelids (Capitella, Helobdella). Of note, these inv-GnRHs show a higher molecular diversity than chordate orthologs. Studies on marine lophotrochozoans showed the existence of a GnRH-like molecule in Octopus, called oct-GnRH which was able to induce gonadal maturation and egg-laying behaviour by regulating steroidogenesis (Kanda et al., 2006) as well as stimulating reproductive responses when tested on quails (Minakata et al., 2009). Evidence from another close lophotrochozoan species, however, shows that the GnRH-like peptide found in *Aplysia Califoronica* (apl-GnRH) modulates a series of behavioural responses, but none related to reproduction like gonadal activity, oocyte growth or penile eversion (Tsai et al., 2010). In this model, the inv-GnRHs would not act as a vertebrate "gonadotropin-releasing hormone", but rather as a direct regulatory factor for the central nervous system and various peripheral tissues. To support this notion, phylogenetic studies on both GnRH-like peptides and receptors suggest that inv-GnRHs do not directly cluster with ver-GnRHs. Rather, structurally related families of invertebrate peptides including corazonin (CRZ) and adipokinetic hormone (AKH) form a superfamily of neuropeptides with the GnRH family (Roch et al., 2011). Moreover, a study on *C. elegans* found that a GnRHR-like receptor was activated by a AKH/GnRH-like cognate ligand, supporting a signaling crosstalk that arose very early in metazoan evolution (Lindemans et
Therefore, increasing evidence suggests that inv-GnRHs, AKHs, RPCHs, Corazonins all share the same ancestry to form a neuropeptide superfamily.

**AKH, Corazonin and ACP neuropeptide families**

The Adipokinetic Hormones (AKHs) were first discovered in the corpora cardiaca of Ecdysozoa and are known to regulate carbohydrate homeostasis and lipid mobilization for several metabolic functions. They are octa-nona-decapeptides with a fixed N-terminal pyro-glutamate and a C-terminal amide group. AKHs also occur in crustaceans, where they are called Red-Pigment-Concentrating-Hormones (RPCHs), involved in color adaptation and camouflage. Given structural similarities they belong to a larger group called the AKH/RPCH family (Gade, 1996). Recently, an AKH-type ligand has also been confirmed in the marine lophotrochozoan *A. Californica*, where it significantly inhibited feeding, reduced body and gonadal mass and oocyte diameter. These effects are substantially different from the ones elicited by the related peptide apl-GnRH, suggesting the two systems have diverged functionally in invertebrates (Johnson et al., 2014).

Another group of peptides structurally related to AKHs, are Corazonins (CRZs), which were also first discovered in the insect corpora cardiaca and later in crustaceans. They are normally 1-3 amino acids longer than AKHs and maintain an invariable structure across arthropods, with the most common sequence being pQTFQYSRGWTNa. Their functions though are very diverse and it’s difficult to find a generalised role. They have a cardiostimulatory effect in cockroaches, pigment-increase role in locusts, a role in the initiation of moulting behavior in moths, and regulate insulin-producing cells in *Drosophila*, to list some (Veenstra, 1989) (Tawfik et al., 1999) (Kim et al., 2004) (Kapan et al., 2012). Moreover, a recent finding of a novel AKH/corazonin-related neuropeptide (ACP) in the insect *A. gambiae* also suggests the close relationship between these two peptide groups (Hansen et al., 2010). So far no clear functions have been assigned to the ACP signaling system, but the high expression shortly before and after hatching of *T. castaneum* suggests a role in early larval development (Hansen et al., 2010). These invertebrate peptides and their receptors have been therefore comprised into a larger AKH/CRZ/ACP neuropeptide superfamily.
Phylogenetic studies state AKH/ACP/CRZ/GnRH systems belong to a superfamily of signaling molecules

According to the most recent phylogenetic studies by Hauser and Grimmelikhuijzen, these AKH/CRZ/ACP/GnRH signaling systems are closely related and have a common evolutionary origin, which dates back to the split of Proto- and Deuterostomia, about 700 million years ago (Hauser and Grimmelikhuijzen, 2014): in Protostomia the ancestral receptor gene duplicated together with its ligand, leading to GnRH/Corazonin(i) and AKH(ii) families in Mollusca/Annelida. Then the AKH duplicated once more into what describes now the arthropods hormonal system, made of 3 independent systems: AKH/CRZ/ACP through a receptor-ligand coevolution. The different neuropeptide subgroups have hence diverged and subfunctionalised. To complement, another study suggests that vertebrates have lost the inv-type GnRH and its receptor, while the ancestral deuterostomes had both: an AKHR/GnRHR and Corazonin/invGnRHR families. This study supports evidence for a common ancestor for the ver-type GnRH, inv-type GnRH, CRZ and AKH signaling molecules (Roch et al., 2014a).

Therefore, the inv-type GnRH peptides belong to a wider CRZ/GnRH group, which shows a variety of functions, but has only been studied so far in 2-3 animal models (like Octopus, and Aplysia). While in arthropods there is abundant literature on the characterization of AKH, ACP and CRZ neuropeptides and receptor systems, not much is known about the other protostomian CRZ/GnRH and AKH peptides and their roles and more suitable animal models are required. In my thesis, I therefore investigated the invertebrate GnRH system, taking advantage of the annelid model Platynereis dumerilii and its genetic/molecular tools.

Platynereis dumerilii: a model organism for evolutionary studies

Platynereis dumerilii is a marine polychaete worm belonging to the Annelida Phylum. Annelida, together with mollusks, belong to one main branch of protostomes, the Lophotrochozoa. The other branch, called Ecdysozoa, includes well-represented model organisms such as the nematode C. elegans and the arthropod D. melanogaster. Protostomes along with the deuterostomes, where vertebrates are found, are the two main branches of the Bilateria and they diverged about 600 million years ago (Figure 1). Lophotrochozoa are evolutionary distant to arthropods, nematodes and vertebrates and
are also largely under-represented in today's molecular research, therefore more studies could provide new insights into the evolution of bilaterians (Raible et al., 2005).

**Figure 1: Simplified tree of Bilateria.** Protostomes are divided mainly in Lophotrochozoa (red), to which *P. dumerilii* belongs, and Ecdysozoa (yellow). In green are represented Deuterostomes, which include echinoderms, ascidians and chordates, among which vertebrates.

What makes *Platynereis* interesting in this context is that it is considered a 'living fossil'. In fact it was shown to be a molecularly slow-evolving species, as its protein sequences, the intron number and positions in the genome show more similarity to vertebrates than to other protostomes like nematodes and insects (Raible et al., 2005). On the cellular level, *Platynereis* possesses more ancestral cell types than other vertebrate and invertebrate model organisms based on molecular fingerprints and cellular morphologies, especially in studies on photosensory and neurosecretory systems (Arendt et al., 2002) (Arendt et al., 2008) (Jekely et al., 2008) (Tessmar-Raible et al., 2007) (Tomer et al., 2010). It has a central nervous system and a development that also dates back to the morphology of the Urbilateria, the last common ancestor of all Bilateria (Denes et al., 2007) (Kerner et al.,
Moreover, gene collections from annelids and mollusks suggest that Lophotrochozoa have preserved a larger fraction of vertebrate-type protein coding genes. Notably, *Platynereis* possesses not only the orthologs of OT/AVP and GnRH-like families, which will be discussed in my thesis, but also glycoprotein-beta genes which constitute gonadotropins in mammals, insulin-like peptides, the peptide processing enzymes prohormone convertases and estrogen-like receptors (Keay and Thornton, 2009). This is in line with recent gene-inventory comparisons suggesting that the annelid lineage has evolved at a slower evolutionary pace (Raible et al., 2005) and indicates that a large set of vertebrate-type neuropeptides already existed in Urbilateria. The combination of these features makes *Platynereis* a very suitable model system to study the evolution of the neuropeptide systems.

**Platynereis life cycle and its lunar reproductive rhythm**

Adding to these evolutionary considerations, another reason that makes the study of putative reproductive hormones interesting in *Platynereis* is the remarkable reproductive biology of this species. *Platynereis* populations live in shallow waters from 3-10m of depth. Throughout almost their entire life, from young juvenile worms till they are fully mature individuals, they are atokous animals dwelling in a self-constructed tube attached to the bottom of the sea (Fischer and Dorresteijn, 2004). They have a semelparous life cycle (i.e. they reproduce only once in the life time) that lasts from 3 months up to 2 years, depending on temperature, food availability and other factors, which are still not known. Sexually mature individuals, who become epitokes swimming up to the surface of the sea, undergo a dramatic morphogeneis of their tissues to become ‘sacks’ full of germ cells ready to be released by means of external fertilization. This happens through a ‘nuptial dance’ at night timed according to the lunar phase, where the male swims rapidly around the female, releasing a sperm cloud. This triggers the female to spawn synchronously by spinning fast and spreading the egg cells around. Pheromones play an important role in assuring the release of germ cells into the water. Uric acid is released together with the eggs from the coelomic fluid of the female (Zeeck et al., 1998), while L-ovothiol A is the male-specific pheromone that initiates egg release (Röhl et al., 1999). For animals undergoing external
fertilization, in order for it to be successful, it needs to be highly synchronized. Like many other marine species, Platynereis' mass spawning is synchronized to the moon phase, specifically to the new moon phase during night hours. At the end of the mating performance, the adult worms die soon after. Fertilized eggs develop into trochophora larvae after one day and live a planktonic and positively phototactic life for a few days. 3-4 days after fertilization the three-segmented juveniles settle down and start building living tubes, where they grow into atokous adults (Fischer and Dorresteijn, 2004) (see Figure 2 for a comprehensive overview).

Figure 2. Platynereis life cycle. (Drawing by Demilly et al, 2013)
**Platynereis** lunar reproductive periodicity is under the control of a circalunar molecular clock

Their nocturnal and lunar synchronized emergence has been first documented at the beginning of the 1900 and studied for over a century. Importantly, the zoologist Carl Hauenschild was the first to show across 1955-1960 that cultures of *Platynereis* under artificial illumination reproducing both diurnal light/dark cycles and dim nocturnal illumination mimicking full moon, also displayed a lunar reproductive behavior. Most interestingly, once the culture is entrained, their reproductive periodicity can be maintained for over 3 months even by omitting the nocturnal light stimulus (a condition which in chronobiology is called ‘free-running’) (Hauenschild, 1955)(Hauenschild, 1960). This showed the existence of an endogenous circalunar clock that can be entrained by dim nocturnal illumination. Recently, our lab confirmed this finding and, more importantly, identified the core circadian genes in *Platynereis* and showed how these can be influenced by the circalunar clock at a molecular level. In fact, the transcript levels of three circadian clock genes – pdp1, clock and period – are significantly elevated in free-running full moon (FR-FM) light regimes, compared to new moon conditions (Zantke et al., 2013).

The hypothesis is that the two clocks (circadian and circalunar) orchestrate a series of physiological and behavioral outputs in *Platynereis*, though the molecular mechanism by which the circalunar clock signal is transmitted to other tissues and regulates reproductive processes still remain elusive. What is known to also play a critical role in nereids’ regulation of maturation are hormones. Classical transplantation studies in the 70s by Hofmann postulated the presence of a ‘brain hormone’ that promotes regeneration in juvenile worms and blocks transition into the maturing state (Hofmann, 1976) (Hauenschild, 1956). This is why hormones are an attractive category of signaling molecules to study in our model organism, because they could mediate the signal between the endogenous clocks and the actual reproductive timing.

**Establishing *Platynereis dumerilii* as a new model system**

Since Hauenschild first dedicated his studies on the *Platynereis* population present in the gulf of Naples (1953) this organism has been studied mainly from its zoological and
developmental point of view. But he did more. He was also able to set up the first laboratory culture, by reproducing natural conditions and the animals have been bred in laboratories ever since without interruption. Platynereis therefore became an advantageous model species, given also its small average size of 2-3 cm when adult and the fact that one pair of worms can produce several thousands of offspring in a single spawning event. Molecular studies performed on larvae are also advantageous because of their transparency and abundance. It was only thanks to the recent creation, by different groups, of datasets of expressed sequence tags (EST), individual genomic BAC clones and a high-coverage reference genome (http://4dx.embl.de/platy/) that Platynereis is now useful also at a genetic and genomic level. Clearly, for all the characteristics mentioned above and in the paragraphs before, the development of genetic and genomic tools will serve to allow researchers to address important questions spanning evolution, development, gene regulation, chronobiology, and behavior on a functional level.

**Genome modification tools for Platynereis**

Compared to other well-established model species, such as Drosophila and C. elegans, in the last years there were very limited molecular techniques available on such an emerging and multi-disciplinary research model like Platynereis. Previous studies only focused on gene expression using techniques such as quantitative RT-PCR and in-situ hybridization, but there were no established tools that could be used to study the function of a gene in vivo.

Notably, at the time I joined, the very first genomic tools had been set up by colleagues in the lab, which allowed transposon-based transgenesis: Tol2-mediated transgenesis for transient analysis of the expression patterns and Mos1-mediated transgenesis for creating stable transgenic lines in Platynereis (Backfisch et al., 2013) (Backfisch et al., 2014). At this point, the very first project I followed and which constituted the main efforts of my PhD study was the pioneering of a genetic tool that would induce site-directed mutations on a selected locus and generate animal knockouts. Together with the joint efforts of colleagues in the lab, we succeeded in this by developing for the first time working Transcriptional Activator-Like Effector Nucleases (TALENs) in Platynereis.
**What are Transcriptional Activator-Like Effector Nucleases (TALENs)?**

TALENs, together with Zinc finger nucleases (ZFN) and CRISPRs, are a class of engineered nucleases that cut the genomic double-stranded DNA by fusing the protein FokI nuclease domain to a DNA binding domain (Christian et al., 2010). Unlike ZFNs technology, TALENs can target basically any given DNA sequence, by designing a specific DNA binding domain, which anchors to both sides of the double-strand, leaving a spacer region where the nuclease will create a nick. The DNA binding domain is based on the transcription activator-like effector (TALE) proteins that were first discovered in the plant pathogen Xanthomonas. In the host, these proteins can bind promoter sequences of the plant genome activating the expression of plant genes that help bacterial infection.

The TALE domain is composed of a central repeated highly conserved 33–34 amino acid sequence bearing two critical and adjacent amino acids, the 12th and 13th. These two positions, referred to as the Repeat Variable Di-residue (RVD), are highly variable and show a strong correlation specifically with one of the 4 nucleotide bases (Boch et al., 2009). This straightforward correlation between amino acids and DNA recognition means that TALE domains can be custom-designed to recognize any desired sequence of the gDNA.

Figure 3 shows the binding of two paired TALEs separated by a short spacer (15-30bp) to a target sequence that enables the dimerization and activation of the two FokI nuclease domains, resulting in a double-strand break of DNA within the spacer region. As a result, the cellular machinery will then try to repair this DNA damage primarily by non-homologous end joining (NHEJ). This process is not error-free and as a consequence small insertions or deletions (indels) are introduced around the cleavage site, but even a single nucleotide deletion leads to frame-shifted mutations that can result in premature stop codons leading to truncated and non-functional proteins (Moscou and Bogdanove, 2009) (Sander et al., 2011).
Figure 3. Schematic drawing of TALEN-mediated mutagenesis. Each Left and Right TALENs are composed of repeated variable domains (RVDs) binding to the DNA and a FokI nuclease domain. FokI dimers are able to induce double-strand breaks in the spacer region. (Drawing modified from Sanjana, N. E. et al. 2012)

TALENs have been reported for successful targeted mutagenesis in many model organisms, such as mice (Qiu et al., 2013), zebra fish (Zu et al., 2013), Xenopus (Lei et al., 2012) and Drosophila (Liu et al., 2012). Recently, more evidences are also showing the functionality of TALENs across other less conventional invertebrate models such as crickets (Watanabe et al., 2016), mosquitos (Aedes aegypti) (Aryan et al., 2013), silkworm (Bombyx mori) (Sajwan et al., 2013) and even the Echinodermata sea urchin (Hosoi et al., 2014).
Neuropeptides are among the largest class of signaling molecules used across all species to communicate intercellularly and regulate many fundamental physiological processes, meaning their biological role has been essential throughout evolution. My thesis aims at characterizing two of these relevant families: GnRH-like and OT/AVP-like families, which are known from vertebrate reproduction. Though they are conserved between vertebrates and protostomes, they have remained largely unexplored in Lophotrochozoa. I therefore took advantage of the suitability of Platynereis dumerilli (Pdu) as an invertebrate marine model organism for evolutionary comparisons and explored this species with respect to its highly interesting reproductive biology. In particular, my work focuses on:

1) characterizing the Vasotocin/Vasotocin Receptor system in Platynereis
2) contributing to provide the first molecular tool for site-directed mutagenesis of endogenous genes through the establishment of TALENs in Platynereis
3) placing Platynereis GnRH-like and AKH-like peptides in the context of recent phylogeny, through bioinformatic analysis and the validation of their cognate receptors
4) shedding first light on Pdu-crz/invgnrh1 gene function using a TALEN-engineered mutant strain.
Results

1. Characterising the Vasotocin (VTN) and Vasotocin Receptor signaling system in Platynereis

In 2007, a study by Tessmar-Raible et al. showed that Platynereis posses a single ortholog of the OT/AVP family, named Vasotocin, which is composed of the nonapeptide and a longer neurophysin protein, similarly to the vertebrate-type precursors. Moreover, the neurosecretory cells expressing Vasotocin in the annelid’s larval brain were found to be the same cell types constituting the vertebrate hypothalamus, when compared to Zebrafish. This finding supported the idea that these vasotoninergic cell types must have an ancient role, since they were present in the common ancestor of annelids and vertebrates, the Urbilateria (Tessmar-Raible et al., 2007). So far, the role of OT/AVP system across protostomes is still unclear, since functional studies link the invertebrate peptide orthologs to reproductive behavior, memory formation and water homeostasis.

Identification of two putative Platynereis Vasotocin receptors

Given these premises, it was interesting for my study to further characterize this signaling system in our invertebrate model, starting from its receptors. Therefore, I began by assembling cDNA fragments belonging to 2 putative Pdu-vasotocin receptor genes, that had been obtained and cloned previously by degenerated and RACE-PCR experiments in the lab. These fragments, however, failed to give a full coding sequence for both genes, either because the 5’ or 3’ end were missing. To elongate and obtain a complete coding sequence for both genes, I performed bioinformatic analyses first by blasting them against the Platynereis transcriptome EST available data. Then, to confirm that the sequences I had assembled were full 7-transmembrane domains proteins, like all classes of GPCRs, I used the online TMHMM Server 2.0 prediction tool. This analysis, as shown in Figure 4, predicted that both Pdu-Vasotocin receptor 1 and 2 (else referred to as VtnR1 and VtnR2) had 7 transmembrane domains and very likely belong to the GPCR class family. Having a full cDNA sequence of 2 putative vasotocin receptor genes, which have been clustered by
phylogenetics with other orthologs of closely related species (not shown), meant that I could further proceed in validating them.

**Figure 4. Transmembrane domain analysis of putative Vasotocin receptors VtnR1 and VtnR2** TMHMM probabilities for the VtnR1 and VtnR2 candidates calculated with the translated open reading frame. The x-axis shows the position along the N to C terminus and the y-axis the probability for a transmembrane domain or an inner/outer domain.

**Vasotocin Receptor candidates bind specifically to Vasotocin**

To validate the binding of Vasotocin ligand to these 2 orthologous receptor proteins, I performed a GPCR deorphanisation assay in collaboration with the Schoofs Lab, KU Leuven, according to their established method in the latest publication (Beets et al., 2012).
I first cloned the coding sequences for Pdu-VtnR1 and Pdu-VtnR2 into a pEGFP-N1 plasmid, fused to eGFP protein to test their expression as a transmembrane protein in a mammalian cell system. HEK cells were transfected with a single receptor plasmid and monitored after 24h. GFP protein was visible across the whole cytoplasm and cellular membrane, indicating the transfection had worked and the protein might still be in the process of being modified in order to be carried to the outer membrane (not shown). After cloning each Pdu-receptor into a pCDNA3.1 expression vector, Chinese hamster ovary (CHO) cells, stably overexpressing apo-aequorin and the promiscuous Ga16 subunit, were transiently transfected with a single receptor. The GPCR, when binding to its extracellular agonist, through a well characterized cell signaling pathway (see Fig. 5A) triggers the release of calcium intracellularly, which in turn binds to aequorin and emitted light can be measured through a photometer at 469nm.

I therefore challenged cells that expressed either one of the two receptors, with the active cyclic VTN ligand (CFVRNCPPGa) and measured the half-maximal effective concentration (Ec50), in a dose-dependent way. The results showed that both receptors were able to bind specifically VTN peptide at two extremely low nanomolar concentrations. In fact, VtnR1 responded with an Ec50 of 5.8nM and VtnR2 with Ec50=1.3nM. Both concentrations are clearly within the physiological range of receptor-ligand activation and comparable to values obtained in other GPCR deorphanisation studies (Beets et al 2012). Experiments were run in triplicates to confirm the Ec50, but only one dose-response curve per receptor is shown in Fig. 5B. As negative controls, empty pcDNA3.1 plasmid-transfected cells were also challenged with the same ligands, showing no response. We can therefore confirm that the predicted ortholog candidates VtnR1 and VtnR2 are Pdu-Vasotocin specific receptors and this further corroborates the presence of an OT/AVP orthologous peptidic hormone in Platynereis.
Figure 5. VtnR1 and VtnR2 are activated by nanomolar concentrations of Pdu-VTN. (A) Scheme of CHO cell membrane is shown, expressing the GPCR (dark green), which binds to both the extracellular ligand (yellow) and the intracellular G-16 protein (red). Upon receptor activation, the α subunit of G-16 dissociates from the β/γ subunits and activates phospholipase C (PLCγ) which initiates an IP₃/Ca²⁺ cascade. The increased Ca²⁺ concentration stimulates aequorin to emit light of 469 nm (bioluminescence).
which is detected by a. Drawing from H. Bai and S.R. Palli “Advanced Technologies for Managing Insect Pests”. (B) Using the Ca\(^{2+}\) mobilitation assay shown above, in both experiments, CHO cells were transfected with either the VtnR or an empty pcdna3.1 vector (negative control). Each data point is shown as a relative % to the highest value (100% activation) after normalization to the maximum calcium response elicited. Ec50=half maximal effective concentration. Each experiment represents one replicate.

**Vasotocin peptide is expressed in the central-ventral area of the brain**

Since previous studies showed Vasotocin-neurophysin transcripts were expressed in the larval brain area and qPCR experiments on worm heads confirmed the presence of *vtn-neurophysin* mRNA, I wanted to localize more precisely Vasotocin expressing domains in the adult worm brain. Although I repetitively tried through whole mount *in situ* hybridization, a specific expression pattern could never be detected for *vtn*. This might have been due to low transcript levels. Nevertheless, I took advantage of anti-Pdu-VTN antibodies which had been obtained by our lab and performed immunostainings (IS) on adult heads, by optimising the IS protocol to the new antibodies (described in Materials and Methods). As a result, I was able to detect VTN active peptide using two different sera, the FRKT-anti-VTN (raised against the cyclic form of VTN, from our FR-KT Lab) and the Jekely-anti-VTN (independently made by Jekely's Lab, raised against the CPPGa epitope). Figure 6 nicely shows that both antibodies, independently tested, gave the same characteristic W-shaped pattern localized in the central-ventral area of *Platynereis* brain (Fig. 6 A-B). These specific structures are not the size of a cellular body but rather represent the vasotocin-enclosing vesicles spread along the axon projections of neurosecretory cells located very deep within the worm’s brain. As a comparison, serotonin immunostainings on another polychaete also revealed small pointed-like structures that define axon projections (Forest and Lindsay, 2008). As a negative control a pre-immune serum (PIS) of the same rabbit used for producing the FRKT anti-VTN serum was tested and it gave no specific staining (not shown).

I also included the commonly used neuronal marker for acetyled-tubulin, which stains for acetylated microtubules in the axons (Tessmar-Raible et al., 2007), but I could not co-localise vasotocin and acetylated-tubulin due to technical limitations. In fact, I tried both performing immunostainings on thin head sections and slicing pre-stained heads. Results
on 5um sections of pre-stained heads showed that anti-acetylated tubulin antibody could not penetrate deep in the tissue where the vasotocin-positive region lays (Fig.6 C-D), while it nicely stains the dorsal ciliated neurosensory structures, two posterior lobes and the photoreceptive cells of the eyes (Veedin-Rajan et al., 2013). Moreover, the optical slicing performed with a laser confocal microscope also revealed that in the deepest tissue the anti-acetylated signal was lost, compared to anti-vtn staining. Therefore it was not probably due to light being strongly scattered in a thick biological tissue.

**Figure 6. Localisation through IS of Vasotocin peptide in Platynereis adult brain.** (A-B) VTN staining performed with two independent antibodies: FRKT-anti-cyclic-VTN on an eyeless adult head (A) and Jekely-anti-VTN (B). (C) Overlap of anti-acetylated tub (green), DAPI (blue) and VTN (red) and (D) acetylated tubulin and VTN on 5um sections of prestained heads. Yellow arrows indicate specific VTN staining.
Through qPCR experiments I tested the expression of the *vasotocin receptors* mRNA across different worm tissues and, though mRNA relative levels were very low, mature adult heads had a higher abundance. When trying to also localize the receptors expressing cells to further understand which are the vasotocin-responsive tissues, I could not detect any specific staining through WMISH. The reason is most likely the low transcript levels (confirmed by previous attempts and two other RNA expression screens, Nanostring and RNA sequencing data).

Oxytocin and Vasopressin-like peptides were shown to have different functions across invertebrate species. Most of the studies, however, were carried out using gain-of-function experiments, cross-species interactions, injections of the active compound. In order to study the function of the gene, using the cleanest type of molecular approach, we first had to establish a method for the generation of a knockout line.

2. Creating a *Pdu-vtn* knockout strain through TALENs-mediated mutagenesis

As indicated above, one of the techniques that has recently emerged as a powerful tool to introduce lesions at specific regions in the genome is the use of TALEN constructs. As previous work in *Platynereis* had demonstrated that zygotes can be successfully microinjected with nucleic acids, together with colleagues in the lab, we joined in an effort to pioneer the use of TALENs to mutagenise targeted endogenous genes (Bannister et al., 2014). Specifically, I created working TALENs targeting the *Pdu-vtn* gene. To ensure efficient binding of TALENs to the genomic DNA region and avoid possible SNPs, I genotyped the *Pdu-vtn* locus of two commonly used laboratory, VIOs and PINs. I chose to PCR-amplify genomic DNA of the region comprising the exon 2 and 3, which includes the vtn peptide coding sequence. PCR products from 8-10 animals per strain were analysed through a restriction digest for size polymorphisms and different profiles, indicating allelic variations, were sequenced and compared to the BAC reference sequence. Results showed that *vtn* locus has a 6kb size polymorphism in intron 2 in PIN strain worms, but no SNPs were detected in the exonic sequences of either VIOs and PINs. Therefore we concluded that the exonic BAC reference sequence could be used as a template for TALENs design.
Potential TALEN target sites were selected using certain criteria: a) target sites did not overlap with known SNPs or exon–intron boundaries; (b) target sites were 5' upstream or within the mature vtn peptide region; (3) a “T” residue preceded the first nucleotide in each TALEN-binding site; and (4) spacer regions contained a single restriction enzyme site. To design possible TALEN constructs we used the online prediction tool TAL Effector nucleotide targeter v1.0 and 2.0 (https://tale-nt.cac.cornell.edu/) (Doyle et al. 2012), following these criteria and according to Bannister et al. From the predictions, I selected 3 different TALEN pairs, named A, B, C pairs (Figure 7).

**Figure 7. Three pairs of TALENs binding to the vtn_ex2 target DNA.** Vtn_exon2 (green) codes for the signal peptide (blue) and the VTN peptide (purple). This is where 3 pairs of TALENs were designed and tested (orange and red). Pair B, TAL_BFwd and TAL_BRev (red) binds across the peptide region, where a single restriction enzyme site (MfeI) is also located.
The TALENs constructs were first generated based on Cermak et al. protocol, which had been freshly published by the time we wanted to start. As explained in Methods and Materials, I assembled TALENs in a backbone featuring a wild-type FokI domain and TALEN insertion site from the pTAL3 vector (Cermak et al., 2011) and a pCS2+ N- and C-terminal architecture (N287/C230) created by Stephanie Bannister and named pCS2+_TAL3-WT.

Validating TALENs constructs efficiency with TnT assay

To test whether the different TALENs pairs were able to recognize and cleave the specific target sequence, I performed an in vitro Transcription-N-Translation cleavage assay (TnT assay). As explained in Material and Methods, to each reaction mix, one pair of TALEN expression plasmids together with the target DNA or non-target DNA was added. As negative control, only Left TALEN construct was added. Results in Figure 8 show that pair A has the highest ratio of cut vs. uncut target DNA after incubation, followed by pair B, while pair C gives a fully uncut band comparable to the ‘uncut’ negative control, where only Left-TALEN construct was added. Given the fact that pair B binds right across the VTN peptide region, where there is a higher chance that any kind of mutation will disrupt the mature peptide form, compared to pair A which binds further upstream across the signal peptide, I decided to proceed with testing the mutation rate of TALEN B pair, named ‘Bf’ and ‘Br’ constructs, in vivo.

![Image of gel electrophoresis showing DNA target region and cut efficiency](image-url)
**Figure 8.** TnT assay was used to test the cutting efficiency of TALEN pairs (A,B,C) Agarose gel of TnT reactions, green arrows indicate size of uncut target DNA, red arrows show the cut target DNA bands. The ‘uncut’ reaction contains only Left TALEN (negative control). Cut efficiency is calculated as the % of cut band intensity compared to the uncut band intensity. On the right is shown the same target DNA amplicon when non-digested/digested by MfeI restriction enzyme (positive control).

Before doing that, I sub-cloned these individual constructs into another backbone vector featuring heterodimeric DD/RR FokI domains (Dahlem et al. 2012) and shorter N-and C-terminal (N136/C63), since this architecture was reported to mediate better mutation efficiencies and reduce the chance of unspecific genome cuts (Miller et al., 2011) (Dahlem et al., 2012). When comparing also the cutting efficiency, in vitro, of these two backbones bearing the same vtn-targeting TALENs (Bf and Br), it was clear the shorter version was more efficient by 30%. (not shown, Figure S3D Bannister et al., 2014).

**TALENs are able to induce genomic lesions on microinjected embryos**

Based on these findings, I proceeded with testing whether these new Bf_DD and Br_RR TALENs constructs were active in vivo, by injecting Platynereis embryos (at zygote up to 4-cells stage) with various concentrations of mRNA encoding the Forward and the Reverse TALENs, together with a fluorescence dye TRITC to monitor the injection procedure. To screen for TALEN-induced mutations, we established a method for digesting single or small pools of larvae and using this lysate as template for PCR (see Materials and Methods). This facilitated the mutation screening, as we could analyse TALEN-injected animals at a larval stage as early as 24 hours post fertilization (24hpf).

The PCR-based mutation screening consisted in the amplification of the target genomic DNA region, followed by the restriction digestion of such amplicon using the selected restriction enzyme (MfeI), which cuts specifically once in the spacer region. If mutations are induced that disrupt the restriction site, the PCR product should be either completely or partially resistant to digestion (‘uncut’). If big deletions or insertions are present these would result in a size difference in the band. I tested the mutation rate using an assay that amplified a 257bp region comprising exon2 and part of intron2 specifically on injected and
non-injected larvae, used as controls. When the amplicon is cut by MfeI it gives a 90bp and a 167bp distinctive band (Fig. 9A). After sequencing subcloned uncut PCR products, I found evidence of a variety of indel mutations, ranging from small deletions up to a 109bp insertion site. Interestingly, Blastn analysis revealed that this inserted sequence had exact homology with part of intron 3 of the vtn locus. (Fig. 9B).

To estimate a mutation frequency of the TALEN Bf/Br pair and also understand whether there was a concentration-dependent effect on larvae, I calculated the percentage of larval samples for which evidence of mutant PCR products could be detected. The efficiency on pooled (n=4-5) injected larvae ranged from 5-75%, and it was not concentration-dependent (Fig. 9C). Therefore, I could conclude that the injected TALEN pair targeting vtn gene did not have a lethal effect on larvae, neither it affected negatively the development of Platynereis embryos. In fact, the number of normal vs. mis-developed embryos did not differ between injected (TRITC+) and un-injected (TRITC-) samples (not shown, Figure S6B Bannister et al., 2014) nor did the larval survival rate increase at higher concentrations of TALEN mRNAs. Further observations also suggested that injecting TALEN mRNA up to 400ng/ul, the highest used concentration in this study, into Platynereis is generally nontoxic.
Figure 9. Evidence of TALEN-induced mutations on injected larvae. (A) Restriction digest screening on pools of 24hpf larvae injected with vtn_Bf/Br TALEN mRNA at concentration of 200 ng/μl. Black arrow indicates uncut PCR product (also present in NI control); sample 71 shows a digestion-resistant PCR product, red arrow indicates a 109bp insertion in sample 72. (B) Sequencing analysis of uncut and insertion bands from samples in A. “Δ” indicating deletions and “+” indicating insertions. Restriction site is shown in bold; TALEN-binding sites in yellow, spacer in grey, inserted nucleotides in green, nucleotides differing from wild type in blue. (C) Mutation frequency measured as a % of mutation-positive samples/total on pooled larval samples injected with 40, 100, 200ng/μl of TALENs mRNA.

Generating the first knock-out lines for Pdu-vtn gene

Subsequently, I raised adult worms from embryos injected with TALENs against Pdu-vtn gene. As TALEN mRNAs first need to be translated in the embryo in order to generate active nucleases, worms are generally expected to be chimera of cells carrying wild-type
loci and cells with one or two mutant copies of the gene, possibly also their germline. This is the reason why, when analyzing electrophoresis gels, the uncut bands have different intensities compared to their respective fully-cut bands. Figure 10A reports 3 different types of mutations recovered by sequencing uncut PCR products amplified from the gDNA of 3 individual adult worms (#26, #28, #39). Even by looking at the nucleotide sequence, and when translating into their amino acids, it is clear how each one of these mutations induced across the peptide region will lead to a non-functional mature peptide. In fact, worm F0#26 had a 5nt insertion, F0#28 a 100bp deletion and F0#39 a 8nt deletion. These animals, that were found to be mutation-positive (called ‘founders’), were eventually outcrossed to another WT worm in the culture in order to generate and set out their F1 offsprings. To validate that TALENs can also mediate germline transmission of the mutation in *Platynereis*, like it has been shown in other organisms (Cade et al. 2012; Aryan et al. 2013; Qiu et al. 2013; Sajwan et al. 2013), I analysed the F1 offspring generation. The resulting offsprings will be a pool of either WT (+/+) or heterozygous mutants (+/-), carrying one of the mutations detected in the founder. I genotyped several different F1 batches for evidence of mutation transmission and eventually restricted to 3 mutation-positive batches. I named them according to the number given to the ‘founder’ parent: vtnTM26, vtnTM28, vtnTM39. Sequencing revealed that the same mutations were present in the respective parent (Fig. 10A). This time the PCR assay was performed amplifying from young animals’ tail clips a 458bp DNA band. After MfeI restriction digestion of the amplicon, WT homozygous samples (+/+) give a 90bp and 368bp bands. On the other hand, the presence of heterozygous animals (+/-) is revealed by detecting uncut PCR products. Figure 10B shows the genotyping gel of 1 of the 3 different F1 generations I raised (vtnTM26 offsprings). In order to keep more than one knock-out strain so that further functional experiments can be validated on them independently, I raised those three *Pdu-vtn*(+/−) lines in parallel. I was able to obtain the first knockout animals by the third generation (F3). Evidence of homozygous worms for *Pdu-vtn* is shown in Figure 10C (red arrows). VtnTM28 strain has a 100bp deletion mutation, therefore genotyping using the same PCR assay is made easy. The mutated allele, in fact, will be detected as a smaller size deletion band and there is no need to digest the amplicon with MfeI enzyme. These results demonstrate that TALENs represent a highly suitable approach for generating stable and heritable genomic modifications in *P. dumerilii*. 
**Figure 10. Pdu-vtn mutations are transmitted to the offsprings.** (A) Sequencing analysis of uncut and deletion bands from 3 mutation-positive founders (F0) and their offsprings (F1s) compared to WT allele (top). TALEN-binding sites are shown in red, peptide region in purple, restriction site in dark green; different residues are colored in pink. N residues are sequencing errors. (B) Genotyping of the F1 generation on single larvae. The heterozygous animals of vtnTM26 strain are detected by the presence of MfeI-resistant
bands (orange arrow). NI is non-injected control, WT. (C) Genotyping of the F3 generation on single animals. Homozygous (-/-) individuals of vtnTM28 strain are detected by a smaller-size band bearing a 100bp deletion (red arrow). Heterozygous (+/-) have both size alleles, WT animals show only a higher band.

3. Deciphering the evolutionary relationship between CRZ/invGnRH and AKH systems in Platynereis

We know that Platynereis transcriptome encodes for 4 peptides belonging to this wide AKH/ACP/CRZ/GnRH superfamily. These genes have also been predicted by another neurotranscriptomic screen study where they have been named GnRH1, GnRH2, AKH1, AKH2 (Conzelmann et al., 2013). The predicted peptides are downstream of the signal peptide sequence and end with a dibasic-cleavage site, a hallmark of all peptidic hormonal coding sequences across all different species. The grouping of these candidates into the respective orthologs, however, is still an open question because of their short peptide sequence and difficult evolutionary history.

**Phylogenetic analysis suggests Platynereis possesses two CRZ/invGnRH, one AKH, one AKH/invGnRH-type ligands, one CRZ/invGnRH receptor and two AKH receptors**

In light of Hauser et al. latest subfunctionalisation hypothesis, I therefore first wanted to compare Platynereis peptide sequences with the known orthologs across different species through a manual bioinformatic alignment. To do this, I manually aligned sequences based on Roch et al. study, according to their N-term pyroglutame (pQ¹); the most highly conserved residues like W, F or S; and their dibasic cleavage site KR. This alignment (shown in Figure 11) can be further subdivided into clear invertebrate AKH, ACP, CRZ, CRZ/invGnRH subgroups and a less-characterized AKH/GnRH group, whose amino acidic residues would cluster into the AKH-like group but are not AKHs in sensu strictu (Hauser and Grimmelikhuijzen, 2014). Results suggest that Platynereis possesses 2 CRZ/invGnRH ligands, one AKH-type peptide (Pdu-AKH2) and one belonging to the AKH/GnRH subgroup (Pdu-AKH1) (Fig. 11). Given the findings, we renamed Platynereis genes using a more accurate nomenclature compared to the study of Conzelmann et al. (Conzelmann et al., 2013), who used a BLAST-based clustering algorithm to group peptides into larger families. In this study I will therefore refer to: Pdu-crz/invGnrh1 and 2, Pdu-akh1 and Pdu-akh2.
Figure 11. Analysis of mature peptide sequences suggests the presence of two CRZ/invGnRH-type ligands, one AKH-type ligand and one AKH/invGnRH-type ligand in Platynereis. Peptide sequences, including novel Pdu mature peptides (indicated by grey arrow) have been manually aligned and then subdivided according to Roch et al, 2014 study on this wide superfamily. Identical amino acids are in blue, most conserved in black, less conserved in red.
As stated, the evolutionary relationship between these different classes of neuropeptides are hard to analyse from their short prohormone sequences, but it becomes more clear when looking at their relative GPCR receptor sequences. For this reason we scanned through the transcriptomic available dataset of *Platynereis* to look for putative candidates of these receptors. By blasting receptor sequences of closely related Lophotrochozoa species, like *Capitella, Aplysia, Helobdella*, we retrieved 3 invGnRHR-like candidate sequences. Through phylogenetic analysis, I included these putative receptor sequences into the latest and current AKH/GnRH/CRZ/ACP receptors tree by Hauser et al., 2014 and complemented with other recently discovered invertebrate receptor sequences to see where they would actually cluster. Differently from Hauser et al., I used a more robust outgroup, the GPCRs from the vertebrate OT/AVP system. As already proposed by previous literature, my output tree confirms: a) that arthropods and Lophotrochozoa have both two distinct receptor clusters, an AKHR-type and CRZR-type; b) more in line with the study from Roch et al., that the invertebrate CRZR/GnRHRs are a sister group of the clade combining vertebrate GnRHRs and protostome AKHR/ACPRs, indicating it has originated in the common bilaterian ancestor from the very first duplication of the GnRHR superfamily member. Most importantly for our study, this analysis shows *Platynereis* has rather 2 AKHR-type receptors and 1 which clustered into the actual CRZ/invGnRH-type family. Another *Pdu-GnRH-like* receptor sequence taken from (Bauknecht and Jékely, 2015) and referred to as ‘GnHR_Jekely2015’ also seems to have been mis-named and would actually be a predicted AKH-like receptor.
Figure 12. Phylogenetic tree of the GnRHR superfamily and clustering of Pdu-receptor sequences. The tree was generated using maximum likelihood with bootstrap values (CLC Mainworkbench7). Mammalian OTR/VPR receptor family was used as an outgroup. Yellow: CRZR/invGnRHR subgroup (including Pdu-GnRHR3); Green: vertebrate GnRHRs; Blu: AKHR and ACPR (including Pdu-GnRHR1, 2 and GnRHR_Jekely2015).
The predicted ‘Pdu-GnRHR’ receptors are activated by *Platyneris CRZ/invGnRH* and AKH peptides

Given the phylogenetic finding that hints at a common evolutionary branching of Pdu-invGnRH and AKH systems, we tested whether the predicted 3 Pdu-CRZ/invGnRHRs proteins would bind either one or more of the proposed CRZ/invGnRH or the AKH ligands. To do this, Karim Pyarali Vadiwala with the collaboration of colleagues from the Schoofs Lab in Leuven, carried out the same GPCR deorphanisation assay I mentioned before.

We therefore challenged these cells, expressing a single receptor, with the predicted active CRZ/invGnRH1, CRZ/invGnRH1, AKH1, AKH2 ligands (pQAYHFSNGWMPa; pQHAFSTGWTPGF1a; pQFSLSLPGKWNa; pQLTQLGWGSAGSSa) and measured the half-maximal effective concentration (Ec50) in a dose–dependant way. The results showed that two of the receptors, initially named as GnRHR-like 1 and 2, were able to bind specifically only AKH2 peptide, at two different nanomolar concentrations. In fact, GnRHR1 responded with an Ec50 of 11nM, while GnRHR2 was activated at higher concentrations (Ec50=131nM), but both are clearly within the physiological range of receptor-ligand activation (Figure 13). We can therefore confirm the above predicted phylogenetic classification of these two receptors as proper AKHRs. More interestingly, the third receptor (GnRHR3) was activated by both CRZ/invGnRH1 and CRZ/invGnRH2 (Ec50=0.03nM and 0.49nM), but also by the AKH1 peptide, though with lower affinity and uM concentrations (Figure 13).

<table>
<thead>
<tr>
<th>Pdu Receptor</th>
<th>Activating peptide</th>
<th>Dose response curve (Ec50)</th>
</tr>
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<tbody>
<tr>
<td>GnRHR1</td>
<td>AKH-2</td>
<td>(11nM)</td>
</tr>
<tr>
<td>GnRHR2</td>
<td>AKH-2</td>
<td>Lower affinity (131nM)</td>
</tr>
</tbody>
</table>
**Table 3.** Predicted Pdu-GnRHRs and binding affinities to their ligands.

| GnRHR3 | CRZ/invGnRH-1, CRZ/invGnRH-2, AKH-1 | CRZ/invGnRH-1 (0.03nM), CRZ/invGnRH-2 (0.49nM), Lower affinity AKH-1 (1.6uM) |

**Figure 13. Validation of predicted Pdu-GnRHRs and binding affinities to their ligands.**

The table summarises the receptor-ligand activation results of three candidate Pdu-GnRHRs, when tested for the binding to Pdu-CRZ/invGnRH1, 2 and AKH1 and AKH2 active compounds. The validation and dose-response curves were performed by colleagues and collaborators with the same Ca+2 mobilisation assay used to test VtnRs.

4. Investigating for the first time the role of Pdu-CRZ/invGnRH1 neuropeptide using the mutant line

As stated in the introduction, the ancient function of invGnRHs is still a puzzle. Though some studies on less-evolved Protostomia point at a reproductive role, like for OctGnRH, evidence on other mollusks like Aplysia show contrary results. Moreover, phylogenetic studies suggest that invGnRHs do not cluster together with verGnRHs, rather are grouped to CRZ class of peptides found in protostomes. Among these is Pdu-crz/invGnRH1, which I showed to have a cognate orthologous receptor belonging to the same class and therefore is the right candidate if one wants to investigate their enigmatic role. I decided to do this using the mutant line ‘crz/invgnrh1TM1’, which was set up by Vitaly Kozin in the lab.

The maturation timing of *Platynereis* CRZ/invGnRH1<sup>(+/−)</sup> mutant females is anticipated compared to wild types within the same culture

In order to investigate whether CRZ/invGnRH1 has an impact on the reproductive timing of *Platynereis*, we monitored the normal spawning rhythm in the mutant line. Animals from different crz/invgnrh1TM1 set out batches (male +/− x female +/−), each bearing all three different genotypes (+/+: +/−: −/−), were raised according to normal breeding conditions in the lab, as explained in Materials and Methods. Two months after the first batch was set
out, the first animals started maturing and we recorded this ‘spawning data’ till the boxes were over, meaning over a period of time of more than a year. Animals, therefore, were registered on their spawning day according to their sex and genotype. Overall, the mutant strain maturation was timed to the expected lunar rhythmicity, peaking once a month during new moon phase. Importantly, Fig. 14A shows that among the total of animals registered in this experiment (329 individuals), the mendelian segregation ratio was maintained. In fact, by calculating the $X^2$ square test on the % of all three genotypes obtained, in both males and females population, the p-value is higher than 0.05, therefore rejecting the null hypothesis.

In Fig. 14B I summarized the results of this analysis, by grouping mature animals that spawned within the same week (data normalized to the number of weeks post fertilization of the batch) and separating the data between males and females. This is because we were able to notice a difference in the spawning rhythm of WT vs. mutants between the two groups. In fact, homozygous females (-/-) spawned significantly earlier compared to the WT females (+/+), within the same culture, while males did not show this same difference. To better understand whether there was a significant change in the maturation time of mutant females vs. WT females, I represented the results using the cross correlation function, which compares the similarity between two data distributions. Fig. 14C shows that among females, there is a lag of -3 (3 weeks), meaning that the two spawning distributions would be most correlated if one is shifted of -3 weeks ($cc = 0.676$) with respect to the other, compared to non-shifting the two distributions. (Lag 0 cc = 0.617). In male the correlation coefficient of 0.529 does not improve when distributions are shifted. The correlation results did not change when I normalised the number of animals to the total number of worms per sex and genotype.

<table>
<thead>
<tr>
<th></th>
<th>-/-</th>
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<th>+/+</th>
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<th>-/- (%)</th>
<th>+/- (%)</th>
<th>+/+ (%)</th>
<th>$X^2$ test</th>
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<tr>
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<td>36</td>
<td>150</td>
<td>26.7</td>
<td>49.3</td>
<td>24</td>
<td>0.887</td>
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329
Figure 14. Spawning timing of males and females of crz/invgnrh1TM1 strain. (A) Table reports the total number of animals used for the experiment, the sex and % of genotypes recovered (X² test performed online at http://www.socscistatistics.com/tests/chisquare/) (B) Spawning record of females and males from the same culture over a period of 20 weeks (normalized to weeks post fertilization). Each graph
shows the distribution of WT vs. (-/-) animals. New moon phases, which coincide with the spawning peaks, are indicated by black circles. (C) Cross correlation function of the spawning distribution between WT and (-/-) for females and males. Correlation coefficient (ACF) is indicated on the Y-axis. X-axis lags refer to the week number. Correlation factors above/under the dotted line are significant. In females it is highest at Lag -3, in males is highest at Lag 0.

**Pdu-AKHS and CRZ/invGnRH1 precursors localize in different brain domains**

From receptor-ligand binding data, AKHs and invGnRHs in *Platynereis* seem to be more related as signaling systems than what expected. Through whole mount *in situ* hybridization I tried to localize the expression domains of their precursors in the worm adult head (overview in Fig.15 A). To confirm the staining pattern was real and investigate whether the expression changed across later stages of maturation of the animal, I sampled both mature and premature worm heads. Results reported in Figure 15 demonstrate that *Platynereis* crz/invgnrh1, akh1 and akh2 precursors are located within different domains of the brain, constituting a small set of giant-neuropeptidic-expressing cells. The pattern is conserved across premature and mature animals for the new genes I tested: akh1, akh2 (Fig. 15C-F). Given that crz/invgnrh1 had been tested for expression in adult heads before, I included only a mature head to confirm its localization in a subset of central gigantic neurosecretory neurons (Fig. 15B). Moreover, in accordance with the function of prohormone convertases in processing peptide precursor proteins, *Platynereis* phc2 enzyme was also found to colocalise within the same peptide expression regions, especially the posterior lobes and the central dorsal brain (data not shown). It would have been interesting to know the expression domain of the validated receptor *Pdu-GnRHR3*, but repeated WMISH trials failed to give a staining due to detection limits and same applies for crz/invgnrh2 mRNA. These findings still indicate that the different peptides might have distinct roles, but could still signal to the same receptive cells.
Figure 15. Expression of Pdu-crz/invgnrh1, akh1, akh2 in the adult brain through WMISH. (A) Dorsal view of adult mature head. The brain region is between the 2 sets of eyes and other neuronal structures are the sensory appendices (10x) an, antennae, pa, palp, pc, posterior cirri, ac, anterior cirri, no, nuchal organ, e, adult eyes (anterior up). WMISH staining of Pdu-crz/invgnrh1 on mature head (B), akh1 in premature and mature head (C-D), akh2 in premature and mature head. Specific staining is indicated by white arrows either centrally or on one side of the brain symmetry. All pictures in dorsal view, anterior up (20x)
mRNA expression profiling shows peptide precursors are upregulated during maturation

It is known from studies in *Platynereis* that hormone transcript levels change drastically during maturation. To find whether these hormones have a role in the maturation of the animal, I investigated the mRNA expression of *vtn, crz/invgrh1* and other peptide hormone genes at different stages of development, by sampling animal heads. Quantitative RT-PCR analysis repeated on three or more independent samplings, showed that transcript levels of *vtn* and *crz/invgrh1* peptide genes are upregulated at later stages of development, specifically when testing fully mature stage (animals are actively swimming and ready to release germ cells into the water by spawning) compared to premature stage (animals germ cells have started maturing and they still lay in tubes) (Fig. 16A-B).

This finding also holds true for other peptide hormone genes we tested by performing a semi-unbiased screen, aside from this candidate-specific analysis. In fact, during my study, I contributed to the set up and analysis of the Nanostring nCounter gene expression system. In this experiment, for each gene of interest, two sequence specific probes are generated: one Capture probe that fixes the target-probe complex onto a surface and one Reporter probe serving to provide the detection signal. Every target mRNA is barcoded with a unique fluorescent tag code and the readout signal is generated by counting the number of different codes for each mRNA. The system has a similar principle to real-time PCR, but does not require the use of any mRNA amplification signal and has been shown to be more sensitive than microarrays (Geiss et al., 2008). The bioinformatics pipeline used to fish for over 250 putative neuropeptide-encoding genes in *Platynereis* transcriptome has been set up by Florian Raible. Specifically, criteria included the presence of a signal peptide and dibasic cleavage site, the absence of any transmembrane domains. The first round of Nanostring analysis was conceived in order to screen for circalunarly-regulated genes. This means that immature *Platynereis* heads were sampled under new moon (NM) and free-running full moon (FR-FM) conditions at 8 time points during a 24h period, with 3 biological replicates per time point. To perform a second round of validation, we compared the gene expression profile between NM and FR-FM and selected 50 potential circalunar clock regulated genes. The expression level of each candidate gene was normalized to the total counts of each sample to compensate differences induced by the total RNA input. This
time, however, I also included mature heads from males and females under NM at two
different time points of the day (zeitgeber time ZT+1 and ZT+7) in the new sampling, which
was performed as described above. This way I was able to compare the mRNA levels of
those 50 candidate secreted peptides also across maturation.

Evidence, first of all, confirmed that vtn gene is upregulated also between immature and
mature stages, though levels change between different time points of the day (Fig. 16C).
Crz/invgnrh1 at the time of the Nanostring setup was not included in the gene list for
further validation as it did not show up as a possible circalunar gene. More interestingly,
results showed that, among those candidates selected from the circalunar clock Nanostring
screen, there are additional secreted peptides that are differently expressed during
maturation. I chose to report a few examples of these genes in Fig. 16D-F, such as LYamide,
gene1 (isotig 12505) and gene 8 (isotig10193). These candidates stood out for their
significantly higher (LYamide and gene8) or lower (gene1) levels of expression when
comparing mature with immature stage at both time points (NM1 and NM7).
Figure 16. mRNA expression of peptide precursor genes is differently regulated during maturation. (A-B) qPCR analysis of vtn and crz/gnrh1 mRNA on Platyneis head samples at premature and mature stage. Values represent means ± standard error bars of 3BRs (vtn) and 5BRs (crz/gnrh1). Each replicate contained 5 heads. (*) p value <0.05. (C-F) Nanostring analysis of vtn, LY amide, gene 1 and gene 8 mRNA on Platyneis head samples at immature and mature stage on new moon (NM) at two different time points of the day (NM1, NM7). Values represent means ± standard error bars of 3BRs. Each replicate contained 5 heads.
Conclusions and Discussion

As outlined before, the key question I tried to answer in this study is the role of ancient neuropeptides belonging to two families, which are both involved in reproductive functions in vertebrates. Across evolution, these families have been well conserved between vertebrates and protostomes, though there have been several gene duplication events and loss of ligand-receptor groups, which make it hard to draw clear functional conclusions. *Platynereis*, given its tightly-regulated reproductive rhythm and its ancient ‘fossil’ history was the most suited comparative model. This is why I attempted to infer the role of Vtn and invGnRH system, using different approaches, and above all I succeeded in the establishment of TALENs as the first molecular tool for site-directed mutagenesis of endogenous genes in *Platynereis*. With the creation of working TALENs constructs, I was able to generate knockout lines for Pdu-vtn gene.

1. Vasotocin is an active cyclic neuropeptide located in *Platynereis* posterior-ventral brain

Though phylogenetic analysis of the Vasotocin prohormone coding gene (comprising neurophysin) had already suggested that Vasotocin belongs to the Oxytocin/Vasopressin nonapeptide family (Tessmar-Raible et al., 2007), the validation of 2 orthologous GPCR receptors has once again confirmed their orthology to the OT/AVP system. From immunostainings and ligand-receptor binding assay I was able to confirm that *Platynereis* posses an endogenous Vasotocin neuropeptide that is active in its cyclic form (disulfide bond formation). Like the oxytocin and vasopressin orthologs in mammals, active cyclic Vasotocin is also stored in vesicles along the axon projections, given the subcellular staining pattern, similar to serotonin. Unfortunately, I could not colocalise the neuropeptide with the acetylated-tubulin neuronal marker, but the depth of the anti-vtn staining compared to anti-a-tub suggests that Vasotocin is found is the ventral-central region of the worm’s brain. Importantly, it has been proposed by autoradiographical methods that *Platynereis* brain, like other Nereids species, possesses groups of neurosecretory cells located in the anterior-dorsal, posterior-ventral brain and,
additionally, in the posterior lobes (Müller, 1973). In particular, Müller and Hofmann refer to this posterior-ventral area as to the ‘infracerebral gland’. The infracerebral organ is a neurohemal endocrine gland and is known as a release site for neuropeptides in annelid species, which develop elaborate neuroendocrine brain centers (Dhainaut-Courtois et al., 1986) (Baskin, 1970) (Golding, 1970). My findings supports the idea that active short neuropeptides in *Platynereis*, such as Vasotocin, are present in a ventrally-positioned gland, close to the blood vessel, where they may be released to act as endocrine factors. Unfortunately, I failed in localising the validated VTN receptors to understand whether the system in more neuromodulatory or paracrine. The raising and testing of antibodies against these two GPCR proteins could help in further characterizing this system.

What is the ancient role of OT/AVP signaling system? It is still a puzzle. In fact, studies on other invertebrate orthologs have so far given many diverse hypotheses: a possible neuromodulatory, reproductive and osmoregulatory function. The fact that *Platynereis vtn* transcripts are upregulated during later stages of development is more compatible with a role of the nonapeptide in the maturation of the animal, rather than having a homeostatic function. Moreover, *Platynereis* is a marine invertebrate and these species often reach their osmotic equilibrium passively, without the expense of energy or are subject to a weak osmoregulation, making the hypothesis that Vasotocin could be a water-balance signaling factor less attractive. Therefore in this context, *Pdu-vtn* knock-out strains I generated become a suitable and ideal system where to test these functional hypothesis and perform loss-of-function experiments. In particular, *Pdu-vtn* lines are not lethal, animals reach full maturation and give raise to living batches. Moreover, from preliminary observations, the majority of worms spawned during new moon phases, in line with normal spawning rhythms observed under our laboratory conditions (Zantke et al. 2013). This indicates that Vasotocin does not impact directly on the reproductive capacity of the animal, but rather it might still act as a neuromodulator on its reproductive behavior. Given this hypothesis, the first follow-up experiments should include behavioral assays on the mutant strain aimed at testing the mating behavior (spawning) of the animals. Moreover, given that expression at a transcriptional level is not always analyzable and regulations may occur also at a translational level, peptidomics between mutants and wild types could bring to light other downstream targets of the system.
2. TALENs are a valid and efficient tool for site-directed mutagenesis in *Platynereis*

A big achievement of my work was the contribution to the establishment of the TALENs-induced mutagenesis technique in our model species, specifically against *Pdu-vtn* gene. More generally, the assembly of tailored TALENs into the pCS2TAL3-DD and pCS2TAL3-RR vectors, bearing heterodimeric FokI domains and a shorter N/C-terminals (N136/C63), increased the mutation rate and resulted in an efficient genome mutagenesis tool also for other *Platynereis* genes (Bannister et al., 2014). Not only TALENs induced site-directed mutations that disrupted the coding region of a targeted gene and its translated protein, but these mutations were also found to be stably transmitted to the F1 and following generations. Importantly, by having outcrossed each founder animal (F0) to a wild-type animal, the risk of off-target mutations being transmitted was also reduced. In fact, though I designed TALENs specifically to target exon2 of *vtn* locus and have assembled them using heterodimeric FokI domains, their nuclease activity on other genomic sites cannot be excluded. Nevertheless, even if TALENs would mediate unspecific mutations else in the genome, these would likely be lost after outcrossing for two consecutive generations. This is the case especially for the ‘vtn-TM28’ mutant strain. Moreover, having created three different knock out lines (*vtn-TM26*, TM28 and TM39) assures the possibility of generating trans-heterozygous animals, reducing the transmission of off-target mutations. This also allows the possibility of repeating the same loss-of-function experiment on 3 independently mutagenized strains.

We conclude that TALENs represent a highly suitable approach for generating stable and heritable genomic modifications in *P. dumerilii* and resulting mutants are the cleanest system where to study the loss-of-function of an endogenous gene. This is an important mile-stone since it allows studies also on non-conventional invertebrate model species, representing the big Lophotrochozoa branch, where *Drosophila* or *C.elegans*, though more advanced in molecular techniques, would not help.

3. *Platynereis* has a CRZ/invGnRH and AKH signaling system sharing a common evolutionary origin

The new phylogenetic analysis I performed was aimed at placing pdu-GnRH-like system
into the right evolutionary context given recent literature on this wide AKH/ACP/CRZ/invGnRH invertebrate-specific superfamily (Hauser and Grimmelikhuijzen, 2014) (Roch et al., 2014a). First of all I was able to notice that the previously identified Pdu-specific peptides: gnrh1, gnrh2, akh1, akh2 have been mis-named. In fact, it is not uncommon that phylogeny of pro-neuropeptides can be rather challenging. This is because, peptide genes have repetitive sequences and conserved sequence stretches often constitute only a few residues corresponding to biologically active short peptides (Jekely, 2013). Therefore, by choosing to manually align these peptide sequences and comparing them to the known lophotrochozoan and arthropod orthologs, I classified them into other more specific subfamilies. The analysis revealed that Platynereis posses 2 CRZ/invGnRHs (previously named gnrh1 and 2), 1 proper AKH (previously AKH2), and 1 AKH/GnRH type ligand (previously AKH1). It was fundamental to also make a phylogenetic analysis of putative Pdu-GnRHsR based on a robust alignment of long GPCRs orthologous receptors belonging to this wide GnRH/AKH/ACP/CRZ family. This assignment, which was confirmed by the receptor-ligand validation assay, suggested that Platynereis has rather 1 CRZR/invGnRH-type (previously GnRHR3) and 2 AKH-type orthologous receptors (previously GnRHR1 and 2). Moreover, I could conclude that the identified GnRH-like peptides are real orthologs of this ancient signaling system. It also might explain why the other only predicted Pdu-GnRHR by Jekely's group (Gnrh_Jekely2015) remains without a cognate ligand. The tree, in fact, suggests it is rather an AKH-type receptor and the receptor-ligand interaction has been over-looked by their high-throughput assay (Bauknecht and Jékely, 2015). On the other hand, our validation assay seems to be more accurate and can be extended also to a library of peptides (Beets et al., 2012).

Today, the common evolutionary view agrees on the fact that vertebrates have lost the GnRH related peptides that invertebrates have conserved: insects have 3 independent systems (AKH, ACP, CRZ), while Lophotrochozoa have retained only an AKH and CRZ/GnRH system. Interestingly, the binding of Pdu-GnRHR3 to both CRZ/invGnRHs and AKHs type ligands adds rather complexity to the system. This is in line with the existence of an enigmatic intermediate class of peptides found in Lophotrochozoa, the AKH/invGnRHs proposed by Hauser et al. study, to which also Pdu-AKH1 belongs. Ligands belonging to this subgroup seem to cluster within the AKH lineage, but have properties found in the invGnRHs. This unclear subfunctionalisation between these two signaling systems had
already been suggested by a study on *C. elegans*. In fact, Lindemans et al. have characterised an AKH/GnRH-like peptide which is more similar to AKH structurally, but has a role in reproduction (Lindemans et al., 2009). Taken together, these results indicate that the invGnRH and AKH may not be two independent hormonal systems in *Platynereis*, but rather there’s a crosstalk between them and the signaling pathway is more complex than what suggested by other phylogenetic literature.

4. Loss-of-function experiments on the mutant strain suggest Pdu-CRZ/invGnRH1 has a role compatible with the maturation timing of *Platynereis*

The function of the ancient GnRH system is still an open question: CRZs and invGnRHs have been linked to diverse roles such as energy metabolism, reproduction and memory formation. Moreover, from phylogenetic analysis, the vertebrate GnRHRs are a sister group of the invertebrate AKHR/ACPRs, rather than the CRZR/invGnRHR, meaning they do not belong to the same ancient group. Therefore, the functions of the invertebrate GnRH-like peptides are not necessarily related to reproduction. When trying to investigate the role of such orthologs in other non-model organisms, functional studies have been carried out using mostly gain-of-function experiments, cross-species interactions and injections of the active compounds. Given the possible cross-reactivity of peptides with related receptors, functional studies that are just based on the addition of peptides to an animal are potentially misleading. My work on *Platynereis*, therefore, provides for the first time a functional analysis on an invertebrate GnRH system that takes advantage of the availability of genetic mutants (Pdu-crz/invGnrh1<sup>−/−</sup>).

Homozygous parents reach complete maturation and are fertile once crossed, therefore, the effect of Pdu-CRZ/invGnRH1 does not directly impact on the reproductive capacity of the species. On the other hand, when trying to monitor the normal spawning rhythm of such strain, I was able to show that Pdu-crz/invGnRH1 knockout female population reaches full maturation and consequently spawns significantly earlier compared to wild types, while males did not show any difference within the same culture. This female-specific maturation timing phenotype is actually compatible with a role of the neuropeptide in energy-switch processes of females undergoing maturation. Moreover, like
for vtn gene, the upregulation of the GnRH-like prohormone mRNA in fully mature worms is consistent with a possible function on the maturation of the animal. Given these first functional clues, the mutant strain could be used to test the abundancy of female (like oogenesis or vitellogeneis-specific) and male-specific factors and more unbiased screens between homozygous and wild types, like RNA sequencing or peptidomics, could offer new candidate downstream genes that are differently regulated and therefore interesting to investigate further. We do not propose simply a reproductive role, but rather hypothesize that Pdu-crz/invGnRh1 regulates the energy-switch and metabolism in females, ultimately affecting the reproductive timing of the animal. Since the pdu-crz/invgnrh1(−/−) phenotype might still be compensated by the other ligands acting on the same receptor, other functions could be unmasked by knocking out Platynereis invGnRHR3 receptor gene.

Finally, the hypothesis that peptide hormones are involved in the reproductive timing of Platynereis is also supported by the Nanostring experiment, which found the presence of circalunarly-regulated peptide candidates in the transcriptome. Further characterization of such genes might bring us closer to the understanding on circalunar regulation of the spawning rhythm in Platynereis.
Methods and Materials

**Platynereis animal culture**

The animals were maintained under the same laboratory conditions described by Hauenschild and Fischer (Hauenschild and Fischer, 1969), at temperature of 18°C and in a 1:1 mix of Natural Sea Water (NSW, from the Alfred Wegener Institute, Bremerhaven) and Artificial Sea Water (ASW, 35 ‰ Tropic Marine sea salt dissolved in distilled water). In the culture room the light regime consists of constant 16 hours of light and 8 hours of darkness and during ‘full moon’ period a dim light is kept switched on for 8 consecutive days, simulating moonlight. In order to facilitate sampling of animals, worms were also kept in another room which had inverted moonlight conditions, called ‘outphase’. Special shelves were also used to recreate an inverted circadian system, with animals receiving a light regime 12 hours shifted compared to the normal culture. The worms in the culture have been inbred for years into different strains. If not otherwise mentioned, PIN or VIO strains were used.

**Sampling of animal tissues**

During sampling, worms were removed from the culture or their special light regime, placed into the same mixture of water (NSW:ASW=1:1) and decapitated immediately. Decapitation means the prostomium (head with 1-2 parapodia) and peristomium (the second anterior part in an annelid) of the animal were quickly separated with a surgical blade (Schreiber Instrument, No.22) and stored in an Eppendorf tube on ice. For qPCR and Nanostring sampling, tubes containing 3 to 5 pulled animals heads were snap-frozen in liquid nitrogen and stored at -80°C until further treatment. For each sample, 3 or more different biological replicates were collected.
**Immunostainings on Platynereis whole heads and sections**

*Primary and secondary antibodies:*

The antisera used in this study to detect Platynereis Vasotocin were differently produced. The anti-cyclic Vasotocin (#7975) and anti-linear Vasotocin (#7976) used by the Raible Group (named as 'FRKT') are polyclonal antibodies purified from immunized rabbits through affinity chromatography by Squarix Biotechnology. A third antibody against Pdu-Vasotocin was produced and gently offered by the Jekely’s Group (named 'Jekely'), raised against the truncated peptide antigen CPPGa. The antisera were used at 1:500 dilution if not otherwise stated. A standardly tested monoclonal anti-acetylated tubulin antibody produced in mouse (Sigma-Aldrich) was used to stain for neuronal axonal scaffold at 1:200 dilution. For Secondary antibodies, two fluorophore-coupled antibodies were used. A goat anti-rabbit IgG antibody conjugated to Cy3 (Invitrogen) and a goat anti-mouse IgG antibody conjugated to Alexa488 dye (Invitrogen). Both were used at 1:200 dilutions.

*Whole heads*

The protocol used on whole heads was optimized for anti-vtn antibody in the following steps:

- Animals’ heads were prepared by decapitation and immediate fixation into Bouins’ fixative (1.3% picric acid, 35% formaldehyde, acetic acid, 15:5:1) for 2h at 4°C on a belly dancer.
- Wash 5 × 10min in 1ml PTW
- Store at 4°C in PTW up to one week
- Permeabilise the tissue by adding 1ml pre-chilled Acetone and store at -20°C overnight.
- Wash 4 x 5min in PTW
- Digest using Proteinase K (80μg/ml) (150μl/40ml PTW) without shaking for 6min at RT
- Rinse two times shortly in 2mg/ml glycine in PTW
- Wash 1-2-5-10-20min in PTW
- Block with 10% sheep serum in PTW in 300ul for 2h-4h at RT
- Remove the blocking solution
- Incubate with 300ul primary antibody in 5% sheep serum in PTW at 4°C for 48h
Wash 3 × 15 min and every hour with PTW at RT, wash overnight at 4ºC
Incubate with 300ul the secondary antibody in 5% sheep serum in PTW at 4ºC for 24-48h
Wash 3 × 15 min and every hour with PTW at RT
Transfer to sterile DABCO-glycerol and store at 4ºC, protected from light

Cryosectioning of stained heads

Heads were removed from glycerol and washed 5x5 min in PTW. Incubated in 20% Sucrose/PBS overnight at 4 degC. A single head was mounted in O.C.T. Compound (TissueTek) in squared molds and immediately frozen in liquid nitrogen. Slicing was performed with a cryotome (Leica Cryostat). The chamber and the specimen were cooled down to -20ºC and samples were cut into 10µm and 20µm thick slices. Each single slice was collected on optimized coated glass slides (SuperFrost Plus, MENZEL-GLASER). The slides were washed with ddH2O. When completely dry, they were mounted with DABCO/glycerol and a coverslip was put on top.

WMISH on Platynereis adult heads

Whole-mount in-situ hybridization (WMISH) procedure on adult Platynereis heads is based on the protocol described by (Backfisch et al., 2013) with minor modifications.

Platynereis head sampling and fixation

Animals were decapitated as described above and the heads were collected and fixed in a solution made of 4% Paraformaldehyde (PFA) in 2×PTW (2×PBS with 0.1% Tween-20) for 2 hours at RT. The heads were washed 3 times for 5 min in methanol and stored in methanol at -20ºC at least overnight till further use.

Antisense RNA probe generation

Linearized DNA template of the different genes was obtained by cloning the coding sequence of each gene of interest into a vector that contains a transcription promoter sequence, like Sp6 or T7. In order for the transcription reaction to transcribe anti-sense RNA, clones with an anti-sense orientation of the coding sequence were selected. If sub-
cloning in such a vector was not possible, the second way to generate linearized DNA template used was the amplification of the coding sequence using a Reverse primer bearing the SP6 sequence at its end and a Forward primer at the beginning of the CDS. Anti-sense RNA probes were generated using Digoxigenin (DIG)-labeled UTP (Roche). The reaction set up was the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearized DNA template</td>
<td>3-4 μg</td>
</tr>
<tr>
<td>100 mM DTT</td>
<td>2 μl</td>
</tr>
<tr>
<td>DIG RNA Labeling Mix (Roche)</td>
<td>2 μl</td>
</tr>
<tr>
<td>Ribolock RNase Inhibitor (Fermentas)</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>10xTranscription buffer (NEB)</td>
<td>2 μl</td>
</tr>
<tr>
<td>Sp6 or T7 RNA polymerase (Fermentas)</td>
<td>1 μl</td>
</tr>
<tr>
<td>H₂O</td>
<td>Add up to 20 μl volume</td>
</tr>
</tbody>
</table>

The reaction was incubated at 37°C for 3 hours in a thermoblock, then 1μl of RNase-free DNase I (Roche) was added and the reaction was further incubated for 15min. The RNA probe was purified using the RNeasy Mini Kit (Qiagen) and eluted in 20μl volume. After checking for probe size and RNA quality using 2μl of the eluate + 2μl of RNA loading dye in electrophoresis, the rest was diluted in 30-50μl of Hyb Mix (50% formamide, 5×SSC (0.75M NaCl, 0.075M sodium citrate), 50μg/ml Heparin, 0.1% Tween-20, and 0.5mg/ml torula (yeast) RNA, all from Sigma-Aldrich) and stored at -20°C.

**Proteinase digestion of the head samples and WMISH protocol**

The samples were rehydrated in PTW and digested using proteinase K (from where) according to these steps performed at RT, if not otherwise stated.

- Rehydrate for 3min in 75% MeOH in PTW (v/v)
- Rehydrate for 3min in 50% MeOH in PTW(v/v)
- Rehydrate for 3min in 25% MeOH in PTW(v/v)
- Wash 2 x 5min in PTW
- Digest in 100μg/ml proteinase K without shaking for 5min -
Rinse two times shortly in 2mg/ml glycine in PTW, freshly prepared
Fix in 4% PFA in PTW for 20min
Wash 5 × 5min in PTW

Samples were hybridized according to these steps at 65°C in a water bath.

Prehybridize samples in 1ml Hyb Mix for 2h
Dilute 8-20ul of probe in final 200μl volume of Hyb Mix and denature RNA at 80°C for 10min
Replace prehybridization solution with denatured probe
Hybridize at 65°C overnight

The next day samples were washed using 20 x SSCT solution (3M NaCl, 0.3M sodium citrate, pH 7.0, 0.1% Tween-20) and following these steps at 65°C in the water bath:

Wash 2 × 20min in 50% formamide in 2 × SSCT (formamide: 4 × SSCT = 1:1 (v/v))
Wash 20min in 2 × SSCT (4 × SSCT with 1:2 dilution)
Wash 2 × 20min in 0.2 × SSCT (4 × SSCT with 1:20 dilution)

Samples were incubated with primary antibody and detection reagents according to these steps:

Block samples for 2 hours in 1ml 5% sheep serum (SIGMA??) in PTW at RT, shaking
Replace blocking solution with Anti-Digoxigenin-Fab fragments antibody (Roche, 1:2000) in 2.5% sheep serum in PTW and incubate overnight at 4°C
Wash 5 × 1-2-5-10-20min in PTW and then every hour at RT and leave overnight in PTW at 4°C shaking
Equilibrate samples 2 × 5min in SB1 (100mM Tris-Cl (pH 9.5), 100mM NaCl, 50mM MgCl₂, 0.1% Tween-20)
Transfer samples to a 6-well dish, replace SB1 with SB2 (100mM Tris-Cl (pH9.5), 100mM NaCl, 50mM MgCl₂, 0.1% Tween-20, 5% polyvinylalcohol) containing the staining reagents 450μg/ml NBT (Roche) and 175μg/ml BCIP (Roche)
Stain in darkness at RT and then 4°C to slow down the reaction if needed till best signal/background ratio is obtained
To stop the reaction, wash 3 × 5min in PTW
Postfix in 4% PFA in PTW at RT for 1 hour
Transfer samples to 100% glycerol and store in darkness at 4°C for further mounting
Microscopy

Samples from IS and WMISH, after mounting on glass slides in DABCO/glycerol were documented under brightfield or fluorescent widefield microscopy. Images were taken using an Axioplan2 microscope (Zeiss) with a 10x, 20x objective or a 40x oil immersion objective and a Zeiss Axiocam MR5 camera. For a better documentation of whole heads stainings, also confocal microscopy was used. Z-stack confocal images were taken using a Zeiss LSM 710 confocal microscope with a 40x oil immersion objective. An excitation wavelength of 405nm was used for DAPI staining, 488nm was used for Alexa488, and 561nm was used for Cy3. Images were recorded with the software Zen2010 (Car Zeiss Microscopy) and deconvolved with the Huygens deconvolution software (Scientific Volume Imaging). Further image processing including the generation of z-projection images was effectuated with the ImageJ software package (http://imagej.nih.gov/ij/).

Phylogenetic analysis

All phylogenetic analysis was performed using CLC Mainworkbench v7. Peptide and GPCR sequences were downloaded from Hauser et al., 2014 supplementary or retrieved at NCBI. Trees were generated using maximum likelihood phylogeny, UPGMA construction method with bootstrap values (1-100).

GPCR Receptors validation assay

The putative Pdu-GPCRs were validated for the binding to their cognate ligand in collaboration with Liliane Schoofs Lab, KU Leuven. I tested the Pdu-VtnR1 and Pdu-VtnR2 receptor candidates that we previously fished from the transcript data by using the EST454DX database. VtnR1 and R2 full coding sequences were first cloned into a CMV mammalian expression vector (pCDNA3.1) and adding the Kozak sequence at the N terminus. The deorphanisation assay was performed in accordance to the protocol described in (Beets et al., 2012). Briefly, Chinese hamster ovary (CHO) cells K1, stably overexpressing the mitochondrial targeted apo-aequorin (mtAEQ) and the human Gα16 promiscuous subunit, were cultured and transfected with either pcDNA3.1(+vtnr1 or pcDNA3.1(+vtnr2. Cells for negative control experiments were transfected with an empty pcDNA3.1(+) vector. Vasotocin, Arg-Vasopressin peptides (BACHEM) and all other peptides
HPLC purified by IMP, were first dissolved in Acetonitrile at 1M concentration and stored at -20C. The protocol used was:

CHO-WTA11 cells are cultured in T75 flask with complete DMEM and 0.25% trypsin-EDTA (Sigma) in a 37C incubator.

Day before transfection, remove culture medium and add 3 ml trypsin-EDTA to detach cells.

Collect cells in 5ml complete DMEM and transfer 1,5ml into new flask containing 13ml fresh complete DMEM.

Next day transfect cells using transfection mix made of 45µl Ltx Plus (Invitrogen), 3ml serum free DMEM and adding 7-8µg plasmid DNA.

Incubate at RT for 30min.

Remove all cell culture medium except for 3ml, add the transfection mix and incubate for 24h.

Add 20ml complete DMEM and incubate cells again for 24h.

Dry peptides and store overnight at -20C.

Remove cell culture medium, wash 1x with PBS and add 3ml 0,2 % EDTA in PBS to detach cells.

Collect cells with 10ml complete DMEM in a falcon tube and count cells.

Centrifuge cells at 800 rpm for 4 min, resuspend cells in 0,1% BSA (Sigma) in DMEM without phenol red (Gibco) to a final density of 5.000.000 cells/ml.

- Add coelenterazine h in methanol (500µM, Invitrogen) to final concentration of 5µM (10µl/ml) and incubate cells on shaker for 4h at RT.

- Prepare compound plate meanwhile, by resuspending the dried peptides in BSA and prepare all dilutions from this stock. Pipet 50ul of peptide solution into each well.

- Dilute cell suspension 10x (with BSA medium) and incubate for an additional 30 min.

- Monitor calcium response for 30sec by placing the 15ml falcon tube cell suspension into the luminometer, dispense 50ul onto each well of the compound plate.

- Lysate cells using 0,1% Triton X-100 in BSA medium to register the full calcium responses.

- Use ATP (3µl) as a positive control.
They were initially tested at a concentration of 10^{-5}M and final concentrations from 10^{-4} to 10^{-12}M for dose-response curves. Calcium responses were monitored for 30 seconds on a MithrasLB 940 luminometer (Berthold Technologies). Dose-response measurements were always conducted in triplicate and for at least two independent experiments. Half maximal effective concentrations (EC_{50} values) were calculated from dose-response curves that were constructed using a computerized nonlinear regression analysis with a sigmoidal dose-response equation (SigmaPlot 12.0).

**TALENs design and constructs**

Generation of the *Platynereis* TALENs constructs was carried out as published in Bannister et. at, 2014 (Bannister et al., 2014). The two genes knocked out in this study are *Pdu-vtn* and *Pdu-crz/invgnrh1*. For *Pdu-crz/invgnrh1* TALENs were designed and constructed by Vitaly Kozin, in a similar manner. Here I will describe the methods used to generate *vtn* TALENS.

*Genomic DNA extraction from adult worm samples*

Genomic DNA was extracted from whole adult worms or only a tail sample using the NucleoSpin Tissue kit (#740952, Machery-Nagel) and following the animal tissue protocol, except that tail samples were extracted using half-volumes of buffers. DNA was eluted using 50μl of elution buffer for tails and 100μl from whole worms. Samples were stored at -20°C.

*Platynereis genes and genomic sequences used in this study*

The coding sequences used as references for designing primers, were previously discovered both for *vtn* (EF544399: Tessmar-Raible et al., 2007) and *gnrh1* (unpublished, Florian Raible). The genomic sequences of these genes were obtained from assembly of shotgun sequence reads from BAC clones identified to include the coding sequences of each gene (*vtn*: CH305_55J15 and CH305_88I16).

*Genotyping for allelic differences among Platynereis strains*

Primers were designed to amplify genomic regions for each target gene. For primer sequences see Appendix. For *Pdu-vtn*, primers #1806 and #1807 were designed to cover exon 2 and exon 3. Genomic regions were amplified from gDNA samples of worms from 2
different inbred strains, VIO and PIN. PCRs were carried out with Phusion DNA polymerase (Thermo scientific) using the following recipe:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF buffer (5X)</td>
<td>5ul</td>
</tr>
<tr>
<td>dNTPs (10mM)</td>
<td>1ul</td>
</tr>
<tr>
<td>Primer Fwd (5uM)</td>
<td>1ul</td>
</tr>
<tr>
<td>Primer Rev (5uM)</td>
<td>1ul</td>
</tr>
<tr>
<td>gDNA template</td>
<td>2ul</td>
</tr>
<tr>
<td>Phusion polymerase (5U/ul)</td>
<td>0.25ul</td>
</tr>
<tr>
<td>ddH2O</td>
<td>up to 25μl</td>
</tr>
</tbody>
</table>

PCR programme: 98°C 30sec; 30x {98°C 30sec; 62°C 1min; 72°C 2min;} ; 72°C 10min

Bands corresponding to the size expected from BAC reference, used as a control, and other different size bands corresponding to putative different size polymorphisms were subcloned into pJET1.2 vector using the CloneJET PCR Cloning Kit (Thermo scientific). For each product, multiple clones were double digested with BglII to excise the cloned insert and HinfI, a frequent cutter, (fast digest enzymes, Thermo scientific) to look at the different restriction enzyme profiles. The different subcloned sequences were confirmed by direct sequencing (LGC genomics).

**TALEN design and construction**

All TALENs were designed using the TALEN Targeter prediction tool (TAL Effector nucleotide targeter v1.0 and 2.0; https://tale-nt.cac.cornell.edu/) (Doyle et al., 2012). The protocol and criteria used are published in Bannister et al. 2014. Exon sequences were given as input and we selected for equal repeat variable diresidue (RVD) lengths, spacer length of 15–20bp, NN for G recognition, and the presence of a unique restriction enzyme site in the spacer. In particular for vtn gene, TALENs binding either upstream or across the
mature VTN peptide region were strictly chosen.

TALENs were constructed according to the assembly protocol published by (Cermak et al., 2011) using the GoldenGate plasmid kit (v1.0, v2.0 Addgene: 1000000024). The mixture recipes were generated by giving as input the RVD sequences into the Golden Gate TAL Assembly form (Excel file available from: https://tale-nt.cac.cornell.edu/protocols). In brief, the following protocol is the one we adapted and established for Platynereis TALENs as published in Bannister et al., 2014.

Step1: Generating pFUS-RVD vectors

The first step requires the generation of pFUS vectors containing maximum 10 RVDs at once. Mix in 1.5ml tubes on ice the following reagents for each single reaction:

1 µl pFUS-A or B (150ng/ul)
1 µl BsaI
1 µl BSA 2mg/ml
1 µl T4 Ligase
2 µl 10x T4 LigaseBuffer

1 µl Of each individual RVD plasmids (150ng/ul)

4 µl Water up to 20ul volume

Digestion/Ligation Reaction Incubation

- Transfer each reaction to a 0.2ml PCR tube.
- Incubate reactions in PCR machine running this cycle: 10x (37°C/5min + 16°C/10min) + 50°C/5min + 80°C/5min.
- Cool tubes on ice and add 1ul of Plasmid Safe Nuclease and 1ul of ATP solution (25mM).
- Incubate tubes at 37°C for 1h
**Transformation**

Transform 5µl of each ligation reaction into home made XL1-blue cells and plate onto Spectinomycin plates together with 40µl X-gal (20mg/ml) and 40µl of IPTG (0.1M) for a blue/white colony screening assay. Incubate plates at 37°C overnight and pick white colonies, as these will be the ones carrying recombinant bacteria. Perform colony PCR to check for the right cloning of the insert using primers pCR8_F1 and pCR8_R1.

**Step2: Generating final destination pTAL vectors**

pTAL vectors are generated by ligating more pFUS vectors together per TALEN. To enable further validation of the TALEN mRNA using the SP6 promoter of transcription, we previously cloned a final destination vector that features the cloning site of pTAL-3 with a pCS2+ backbone, which will be referred to as pCS2+_TAL3 (GoldenGate TALEN Construction kit v1.0) (Bannister et al., 2014). pLR vectors are chosen depending on the last RVD of the sequence and contain the last “half-repeat”. Mix in a 1.5ml tube the following reagents:

1 µl pCS2+_TAL3 (75ng/ul)
1 µl pLR-ng (150ng/ul)
1 µl Esp3I
1 µl T4 Ligase
2 µl 10x T4 LigaseBuffer

1 µl Of each pFUS plasmid (from step1) (150ng/ul)

Water up to 20ul volume

**Digestion/Ligation Reaction Incubation**

Transfer each reaction to a 0.2ml PCR tube. Incubate reactions in PCR machine running this cycle: 10x (37°C/5min + 16°C/10min) + 37°C/15min + 80°C/5min. Cool tubes on ice and add 1ul of Plasmid Safe Nuclease and 1ul of ATP solution (25mM). Incubate tubes at 37°C.
for 1h. As described in step1, transform bacterial cells and plate onto Ampicillin plates. Pick only white colonies for checking the cloning of the insert. Use primers TAL_F1 (#1823) and TAL_R2 (#1824) for colony PCR.

The final backbone plasmid used to assemble each TALEN has a wild-type FokI domain from the pTAL3 vector and a shorter N- and C-terminal (N136/C63) and was created by Stephanie Bannister. As mentioned in Results, TALENs for vtn were reconstructed into complementary heterodimeric FokI expression plasmids: pCS2TAL3-DD (for Left TALEN) and pCS2TAL3-RR (for Right TALEN) (Dahlem et al. 2012), obtained from Addgene (plasmids #37375 and #37376). All final TALEN expression vectors were sequence-verified using seqTAL_1-5 forward and TAL_R2 primers (Cermak et al., 2011).

**Transcription/translation in vitro cleavage assay**

TALENs efficacy in cutting the target DNA sequence was tested using an in vitro cleavage assay which we refer to as a transcription/translation (TnT) assay, similar to that previously described (Mussolino et al., 2011). Reactions were assembled using the following recipe:

Left TALEN pDNA 1ug (1-2ul)
Right TALEN pDNA 1ug (1-2ul)
Target DNA fragment 225ng (1-5ul)
Methionine (1mM) 20uM 1ul
Sp6 quick-coupled TnT master mix 40ul

add RNase-free water up to 50ul volume

Negative control reactions contained only left TALEN plasmid DNA. Positive control reactions contained 225ng of egfp-coding sequence, L/R TALENs for eGFP and T7 TnT master mix. Reactions were incubated for 3h at 30C. DNA was purified by phenol/chloroform extraction according to the following steps, using phase-lock gel tubes:

- Add 150ul of RF water to each reaction
- Add your 200ul TnT reaction sample to the Phase-lock tube.
- Add 200ul of phenol-chloroform-isooamyl alcohol (25:24:1) on top of the TnT sample
- Mix by inverting the tubes several times.
- Centrifuge the tube at 16,000xg for 5 min
- Collect the aqueous phase (~150-200ul) above the gel and transfer to a second Phase-lock tube.
- Add an equal volume of chloroform and mix like before.
- Centrifuge the tube at 16,000xg for 5 minutes.
- Collect the aqueous phase and transfer to a fresh 1.5ml eppendorf.

To precipitate the DNA the following steps were:

- Add 300ul 100% ethanol to the aqueous phase.
- Add 10ul of 3M Sodium Acetate (NaAc)
- Incubate for at least 30min at -80 deg or overnight at -20 deg.
- Centrifuge for 15 minutes at maximum speed in 4deg. centrifuge
- Wash DNA pellet with 500ul ice cold 70% ethanol
- Centrifuge for 15 min at max speed at 4 deg.
- Air-dry the pellet for 10-15 minutes
- While the pellet dries pour a 1.2%-2% Agarose gel (1.2% should be enough for resolving products >500bp. Go higher if your cut products will be closer to 200bp.
- Resuspend the DNA pellet in 8ul of NF water.
- Make RNase A/H solution by diluting RNase A (or RNase H) from QIagen midi prep kit to 1:1000 in P1 buffer.
- Add 2ul of RNase A/H solution
- Incubate for 5 min at room temperature
- Incubate at 70 deg. C for 5 minutes to inactivate the RNase A/H

Our established protocol is available online at ResearchGate: https://www.researchgate.net/publication/261331928_TnT_in_vitro_assay_protocol-_Tessmar-Raible_Labs_2012)
**TALEN mRNA transcription**

Plasmids encoding full-length TALENs were linearized by NotI-HF (New England BioLabs), gel-purified (Gel Extraction kit, Qiagen), and used as templates for in vitro transcription using mMESSAGE mMACHINE Sp6 Kit (AM1340, Life Technologies). mRNA was cleaned up using RNEasy Kit, Qiagen.

**Embryo microinjection**

Fertilized embryos were injected with TALEN mRNA at the one- to four-cell stage. Microinjections in embryos were performed following a well-established protocol in the lab as described previously (Backfisch et al., 2013). Injection solutions were prepared in a final volume of 10ul containing 1ul of 3% TRITC-dextrane in 0.2M KCl (Invitrogen) and equal concentrations of each TALEN mRNA (from 40-400ng/ul) in RNase free water. Injection solutions were filtered using 0.45mm PVDF centrifugal filters (Ultrafree-MC-HV, Millipore) and centrifugation at 12,000 3g for 1–3 min. Average injection volumes were estimated to range from 25 to 60pl per embryo.

**PCR assay and restriction digest mutation screening on larvae**

A first mutation screening was carried on injected larvae. Pools of larvae (n= 4-5) were harvested at 24 hpf or later and digested in a solution containing Proteinase K (NucleoSpin Tissue, Machery-Nagel) and 1xPCR buffer (10x Qiagen PCR buffer with 1ul Proteinase K solution per 10ul of solution). The reaction was incubated at 56°C for 2–3hr and terminated at 95C for 10 min. Samples were used directly or stored at 4°C.

PCR reaction was carried out using HotStar Taq Plus (Qiagen), the primers #1806 and #2072 and 2ul of larvae lysate as gDNA template, according to this protocol:

- 10x buffer (10X) 2.5ul
- dNTPs (10mM) 1ul
Primer Fwd (5uM) 1ul
Primer Rev (5uM) 1ul
gDNA template 2ul
HotStar polymerase (5U/ul) 0.25ul
ddH2O up to 25μl volume

PCR programme: 95°C 5min; 35x {94°C 30sec; 62°C 1min; 72°C 1min;} ; 72°C 10min

Half of the PCR product was then further digested using the specific enzyme MfeI (ThermoScientific) and incubated at 37C for 1.5 hours. Digested and non-digested products were run on electrophoresis gel, using 2.5% Agarose gels and 0.01% (v/v) SYBR Safe DNA gel stain (Life technologies). Undigested bands were subcloned into pGEM-T vector (Promega) and mutations were confirmed by direct sequencing (Microsynth) with primer #2072.

**Genotyping of F1 animals**

Mutation-positive founder worms were raised and outcrossed with wildtype animals. Single offsprings of F1 generation were sampled at either early larval stages or later young age with clipping of a small piece of tail. Samples were treated using either gDNA extraction protocol or proteinase K digestion and screened for heterozygous genotype using a revised PCR protocol:

NEB 2x mix 10ul
Fwd #1806 1ul
Rev #3237 1ul
gDNA 2ul
H2O up to 20ul volume
PCR programme: 95°C 5min; 35x {94°C 30sec; 62°C 1min; 72°C 1min;} ; 72°C 10min

The restriction digestion of PCR product and sequencing was performed as mentioned above.

**Scoring of Platynereis crz/invgnrh1TM1 strain spawning timing.**

Animals from the knock out strain were maintained under same breeding conditions (like described above) and under the same moon phase regime. 4 batches were taken into consideration for the analysis given their close birthdate (date of fertilization of the offsprings). One week after fertilization, batches were set up into larger boxes and the larvae were left to settle down and construct tubes. After 3 months the first worms started maturing. Everyday the batches were checked for maturing worms and these were removed into separate cups, like usually done during our worm-duties, since they are inevitably committed to maturation for the last few days of their life cycle and can be eaten up by the other animals if left in the box. On the day of full maturation, they were entered on a record specifying quantity, sex, batch and genotype.

**RNA extraction for qPCR and Nanostring**

To the frozen animal tissue samples, 350μl of RLT buffer (RNeasy Mini Kit Qiagen, containing 1% β-mercaptoethanol) were added, together with a metal bead and the tissue was disrupted mechanically using the Tissue Lyser (Qiagen) for 3 min 20Fz. Total RNA was extracted with RNeasy mini Kit following the manufacturer’s protocol, and the concentration was measured with NanoDrop ND-1000 Spectrophotometer (NanoDrop). RNA samples were stored at -80C.

**Quantitative Real-Time PCR**

For each sample, 400ng of total RNA was used to generate cDNA following the QuantiTect Reverse Transcription Kit (Qiagen) and its manufacturer’s protocol and cDNA was eluted in
30μl. Depending on the final volume of cDNA needed for qPCR analysis, from 20-50μl of RNase-free water was added to each template and stored on ice. The final qPCR mixture contained 5μl cDNA template, 1μl forward primer (10μM); 1μl reverse primer (10μM); 10μl Power SYBR Green PCR master mix (Applied Biosystems) and 3μl RNase-free water. Quantitative Real-Time PCR was performed using StepOne Plus Real-Time PCR system on MicroAmp optical 96-wells plates sealed with MicroAmp optical Adhesive Film (Applied Biosystems). Quantitative RT-PCR program: 95°C 10min; 40x {95°C 15sec; 60°C 1min}

_data analysis_

Data was generated with StepOne Software v2.2 (Applied Biosystems) by setting an automatic threshold of CT values which was applied to all the target genes tested on the same plate. After exporting on an Excel file, the fold change mRNA was calculated in comparison to the reference gene (Pdu_cdc5). Gene expression level of the target genes was calculated using the following formula as percentage of the reference gene:

Relative RNA concentration% = 1/(2^(CT target gene – CT reference gene))*100%

Statistical calculations including the 2-tailed Student’s t-test were carried out in Excel.

Nanostring sample preparation

Samples for Nanostring analysis were collected under NM and FR-FM days in 3 replicates of 5 immature animal heads each, at 8 time points over a 24h period. For the second round of Nanostring also mature heads were collected at NM in 3 biological replicates of 5 heads each. After having extracted mRNA, RNA quality was confirmed with qPCR by comparing the circadian profile for certain circadian genes like Pdu-bmal, Pdu-period and Pdu-clock with published data. The candidate gene list contained over 250 putative secreted peptide hormones, circadian clock genes and Pdu-cdc5, used as a house-keeping gene. Probe generation, hybridization and counting of transcripts were carried out by Nanostring Technologies, Inc.

_data analysis_

Positive and negative control probes were included in the hybridization of each sample. Counts for positive controls were summed up and averaged. The normalization factor was
calculated by dividing the sum of counts for each sample with the average number of counts from all samples. Normalized data was generated by dividing the counts of each candidate gene as well as the negative controls with the normalization factor. Mean and Standard deviation (SD) of the negative controls of each sample was calculated and counts lower than mean+2×SD of the corresponding negative control were eliminated. Finally, positively and negatively corrected data of each gene were calculated as percentage of the total counts of the corresponding sample. All calculations and statistics were done with Excel (Microsoft). Candidates were selected on the basis of the most significantly higher or lower RNA levels at NM compared to FR-FM, estimated by calculating the area under each curve. The 50 most differently regulated genes were validated with a second round of Nanostring.

*List of primers used*

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<tr>
<th>#</th>
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<tr>
<td>#1</td>
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hemimetabolous insect, the two-spotted cricket gryllus bimaculatus, using TALENs. In Methods in Molecular Biology, pp. 143–155.


