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Platynereis dumerilii

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1. ABSTRACT

Biological clocks are fundamental mechanisms that help the livings to anticipate external environmental change and adjust their physiology and behavior accordingly. However, only the solar-related clocks have been intensively investigated at the molecular level. Molecular mechanisms of lunar-related clocks remain elusive.

In this study, I used the marine worm *Platynereis dumerilii* (*Nereididae*, *Annelida*) as model organism to investigate the circalunar timing mechanism. *Platynereis* exhibits lunar periodicity in its reproduction, a phenomenon that has been shown to be controlled by an endogenous circalunar clock. Although circalunar reproductive cycles are widespread in the marine environment, little is known about the molecular mechanisms involved in these processes. My work focused on the molecular output of the circalunar clock, to identify candidate factors that might mediate the state of the clock to synchronize maturation or behavior.

The main part of my thesis focused on the small molecule hormone melatonin. Melatonin is a tryptophan derivative involved in seasonal timing of vertebrates whose production depends on the two rate-limiting enzymes arylalkylamine N-acetyltransferase (AANAT) and Hydroxyindole O-methyltransferase (HIOMT). The orthologs of vertebrate-type *aanat* and *hiomt* were identified in *Platynereis*, and endogenous melatonin and its precursor N-acetylserotonin (NAS) were detected for the first time in annelids, suggesting a common origin of this pathway in bilaterians. I have established methods to quantify endogenous melatonin levels from *Platynereis* heads, along with mRNA levels for the enzymes present in the same samples. Melatonin as well as the mRNA level of *Platynereis* (*Pdu*-) *aanat* and *hiomt* was measured under distinct lunar conditions (new moon, full moon and free-running full moon). Daily temporal profile of melatonin level was found to be under endogenous circalunar clock control, while nocturnal light suppressed the overall level of melatonin. Similarly, the temporal profile of *Pdu-hiomt* transcript level was also found to be regulated by the circalunar clock; however the influence from nocturnal light is very limited.

With the help of custom raised antibodies, I localized Pdu-Hiomt to small groups of neurons in the brain region, supporting the notion of melatonin as a neuro-hormone. The approximate co-localization of Pdu-Hiomt with the circadian gene expressing center and ciliary photoreceptor cells suggests a connection between melatonin production and the circadian clock as well as photoreceptors.

Efforts were also made to further characterize Aanat and Hiomt proteins in *Platynereis*, and to establish tools for the functional dissection of the melatonin biosynthesis pathway using transposon-mediated transgenesis and transcription activator-like effector nucleases (TALENs). TALENs with high mutagenesis efficiency were designed and generated for *Pdu-hiomt* locus that can be used in gen-

erating knock-out mutation lines for in-depth functional analysis in order to explore the relevance of this pathway for circalunar reproductive timing.

To complement the candidate-based approach focusing on melatonin, I also engaged into a mid-throughput pioneer screen with enhanced sensitivity for circalunar clock-regulated genes. Around 500 genes predicted to encode transcription factors, neuropeptides or hormone-synthesizing enzymes were analyzed for differences in circalunar expression profiles, and a subset of 50 genes were selected and subjected to a second round of analysis. Sixteen genes, mostly encoding presumptive neuropeptide precursors, were identified as candidates for circalunar clock-controlled genes, as they consistently showed distinct regulation under new moon and free-running full moon conditions. Deeper investigation of these genes including their spatial distribution will broaden our understanding of the molecular output of the circalunar clock.

2. ZUSAMMENFASSUNG

Biologische Uhren sind grundlegende Mechanismen, die Lebewesen helfen, externe Umweltveränderungen zu antizipieren und ihre Physiologie und ihr Verhalten entsprechend anzupassen. Bisher wurden jedoch nur dem Tag-Nacht-Rhythmus folgende biologische Uhren auf molekularer Ebene untersucht. Molekulare Mechanismen der dem Mondzyklus folgenden Uhren sind wissenschaftlich weitgehend unerforscht.

In dieser Dissertation verwende ich den marinen Borstenwurm *Platynereis dumerilii* (Nereididae, Annelida) als Modellorganismus, um eine zirkalunare biologische Uhr zu untersuchen. *P. dumerilii* zeigt eine Mond-Periodizität in seiner Fortpflanzung. In früheren Studien wurde gezeigt, dass dieses Phänomen durch eine endogene zirkalunare Uhr gesteuert wird. Obwohl zirkalunare Reproduktionszyklen bei marinen Organismen weit verbreitet sind, ist wenig über die molekularen Mechanismen bekannt, die an diesen Prozessen beteiligt sind. Meine Arbeit konzentriert sich auf den molekularen Output der zirkalunaren Uhr, um mögliche Faktoren zu identifizieren, die den Zustand der Uhr vermitteln und so die Geschlechtsreife und das Verhalten der Würmer synchronisieren.

Der Hauptteil meiner Arbeit konzentriert sich auf das Hormon Melatonin. Melatonin ist ein Tryptophan-Derivat, das an der Steuerung von saisonalen Zyklen in Wirbeltieren beteiligt ist. Die Produktion von Melatonin hängt von den beiden Enzymen Arylalkylamin N-Acetyltransferase (AANAT) und Hydroxyindol O-Methyltransferase (HIOMT) ab. Die *Platynereis*-Orthologe zu den *aanat*- und *hiomt*-Genen wurden identifiziert, und endogenes Melatonin sowie dessen metabolischen Vorläufer N-Acetylserotonin (NAS) wurde in Ringelwürmern nachgewiesen. Dies lässt auf einen gemeinsamen Ursprung dieses Stoffwechselweges in Bilaterien schließen. Diese Arbeit etabliert Methoden, um den endogenen Melatoninspiegel zusammen mit den vorhandenen mRNA-Mengen für die beiden Biosynthesenzyme in *Platynereis*-Köpfen zu quantifizieren. Melatonin sowie die mRNA-Spiegel von *Platynereis* (*Pdu*-) *aanat* und *hiomt* wurden unter unterschiedlichen lunaren Konditionen gemessen (Neumond, Vollmond und „free-running“ Vollmond). Es wurde gezeigt, dass der tägliche zeitliche Verlauf des Melatoninspiegels von einer endogenen zirkalunaren Uhr gesteuert wird, wohingegen nächtliches Licht das Gesamtniveau von Melatonin senkt. In ähnlicher Weise wird der zeitliche Verlauf des Spiegels an *Pdu-hiomt* Transkripten durch die zirkalunare Uhr reguliert, jedoch bleibt hier der Einfluss von „Nachtlicht“ sehr begrenzt.

Das Auftreten von *Pdu-Hiomt* in der Hirnregion, in der sowohl Photorezeptoren als auch Schlüsselmoleküle der zirkadianen Uhr exprimiert werden, legt das Zusammenspiel der Melatonin-Produktion mit der zirkadianen Uhr und Photorezeptoren nahe, und wirft ein Licht auf den gemeinsamen evolutionäre Herkunft der neuro-sekretorischen und lichtempfindlichen Zellen, aus denen sich möglicherweise auch die Zirbeldrüse der Wirbeltiere entwickelt hat.

Weiters wurden Versuche unternommen, um Aanat- und Hiomt-Proteine in *Platynereis* weiter zu charakterisieren und Werkzeuge für die Funktionsanalyse des Melatonin-Biosynthese-Pathways mittels Transposon-vermittelter Transgenese und „Transcription Activator-Like Effector Nucleases“ (TALENs) zu etablieren. TALENs mit hoher Mutagenese-Effizienz wurden für den *Pdu-hiomt* Locus entwickelt, die bei der Erzeugung von Knock-out- Linien verwendet werden können, um so die Relevanz dieses Stoffwechselweges für den zirkalunaren Fortpflanzungszyklus zu erkunden.

Um den Kandidaten-basierten Ansatz zu ergänzen, der sich auf Melatonin fokussiert hat, habe ich auch einen Pionier-Screen für zirkalunar regulierte Gene durchgeführt. Etwa 500 Gene, die meistens für Transkriptionsfaktoren, Neuropeptide und Hormon-synthetisierende Enzyme kodieren, wurden auf Unterschiede in ihren zirkalunaren Expressionsprofilen untersucht, eine Teilmenge von 50 Genen wurde einer tiefergehenden Analyse unterzogen. Sechzehn Gene, einschließlich vermuteter Neuropeptid-Vorläufer, wurden durch die durchweg unterschiedliche Regulierung unter Neumond- und „free-running“ Vollmond-Konditionen als Kandidaten für unter zirkalunare Kontrolle stehender Gene identifiziert. Weitere Untersuchungen dieser Kandidatengene einschließlich ihrer räumlichen Verteilung wird unser Verständnis des molekularen Outputs der zirkalunaren Uhr erweitern.

3. INTRODUCTION

It is proposed that life on earth originated in the sea. Since this time, living organisms have been exposed to alternating light conditions and fluctuating temperatures. The rotation of the earth results in the bright day and the dark night, forming a day. The orbiting of the moon around the earth results in the moon phases with different nocturnal illumination, resulting in a lunar month. The orbiting of the earth around the sun, together with the earth's axial tilt results in the changes in the day length, giving rise to the different seasons. The period of exposure to light affects organisms in various ways. For example, light is a source for energy, but also contains destructive UV radiation. Light also increases visibility for certain animals, which may allow animals to gather food or find mates more easily, but at the same time may enhance their visibility to predators.

Living organisms have evolved to adapt their metabolism, behavior, and entire life cycles to the periodic fluctuations in illumination levels and the accompanying environmental changes, resulting in various biological rhythms. More interestingly, they also use these predictable changes as a means to anticipate the coming environmental changes, using so-called “biological clocks”. Animals have developed mechanisms to set these clocks to particular entrainment stimuli (Zeitgeber), and also to distribute the time information to other parts of the body, using e.g. hormones. Studies aimed at understanding biological rhythms and the underlying biological clocks gave rise to the rapidly developing field of chronobiology [1].

Most research in chronobiology has focused on the solar-related rhythms (e.g. daily and seasonal rhythms), especially circadian (Latin: around a day) rhythms due to their ubiquitous nature and ease of study. Non-circadian rhythms, including lunar-related rhythms are comparatively less well understood. What is more, the only studies focused on lunar periodicities are descriptive and consist of classical behavioral observations. Thus, the molecular mechanisms underlying lunar rhythms remain largely unknown.

Platynereis dumerilii is a marine annelid worm that has fascinated biologists for more than a century, due the lunar rhythmicity of their mass spawning reproductive events [2]. Early laboratory studies demonstrated that *P. dumerilii* indeed possesses an endogenous circalunar clock (i.e. around 29.5 days), that can be entrained by moonlight and synchronizes reproductive timing [3]. The recent establishment of a powerful molecular toolkit has made *Platynereis* an emerging model organism for chronobiology, evolutionary-developmental biology, neurobiology and marine biology [4]. These capabilities now permit more detailed investigations of the molecular mechanisms that constitute a circalunar clock, in a model organism with a well-established lunar rhythm.

3.1 Biological rhythms

Biological rhythms are the oscillating of behaviors and physiology of living organisms to adapt themselves to the periodic environmental changes. These rhythms typically reflect the oscillations of geophysical cycles such as the day-night cycles. A biological rhythm can be the result of immediate response to the rhythmic environmental change, e.g. light intensities between day and night, or temperature differences between seasons. However, organisms often rely not only on direct responses to environmental stimuli, but have evolved internal oscillators that are in phase with the external cycles, hence enabling the organisms to anticipate the external oscillations, and therefore prepare to expectable changes ahead of time. Such endogenous oscillators are commonly referred to as biological clocks. Biological clocks differ from simple biological rhythms, in that they maintain rhythmic oscillations in the absence of external stimuli (also called “free-running”). This feature of clocks to free-run can also be used to experimentally distinguish if an observed rhythm relies only on environmental cycles or on an endogenous oscillator.

In order to adjust to the local environment, a clock needs to be synchronized to an external stimulus, called “Zeitgeber” (a German word meaning “time giver”) [5]. The setting of the clock to external stimuli is called “entrainment”. Various external stimuli can serve as Zeitgebers, including: light, temperature, food intake, or tides [5–7].

A biological clock is a biological rhythm that fulfills three criteria: 1. The rhythm persists in the absence of external cues (endogenous); 2. The rhythm can be adjusted to match the local environment (entrainable); 3. The rhythm maintains over a range of physiological temperatures (temperature compensating). Given that many organisms live under a broad range of temperatures, a true biological clock needs to be able to maintain period length under this wide range of temperatures to keep track of time. To investigate if a biological rhythm is endogenous, “free running” assays are performed, whereby organisms are first entrained to a specific rhythmic Zeitgeber, which is subsequently removed. In presence of an endogenous clock, the resulting oscillations should be retained [8].

3.1.1 Solar-related biological clocks

Sunlight is the main source of light and energy for life on earth. It has numerous impacts on animals, including the synchronization of solar-related rhythms. Among all the solar-related biological clocks, circadian clocks (or day/night clocks with a period of 24 hours) are the most common and intensively studied. Circadian clocks have been characterized in animals, plants, fungi and cyanobacteria [9], and have been reported to regulate various behavioral and physiological processes; the best known example being the sleep-wake rhythms in mammals.

Circadian clocks consist of a set of highly conserved genes: the “clock genes” that comprise a core transcription and translation feedback loop. This has been well studied in vertebrates, where the core circadian oscillator is composed of positive elements: *clock* and *bmal*, and negative elements: *period* (*per1*, *per2*, *per3*) and *cryptochrome* genes (*cry1*, *cry2*) [10]. The heterodimerization of CLOCK and BMAL proteins drives the transcription of the three *period* genes, *cry1*, and *cry2* by binding to the E-box (CACGTG) in their promoter region. Accumulated period and cryptochrome proteins (PERs and CRYs) will in turn negatively regulate the CLOCK-BMAL induced transcription of *per* and *cry* genes [11] (Figure 1A). The completion of the transcription and translation feedback loop takes around 24 hours, resulting in the rhythmic oscillation of the clock-related genes.

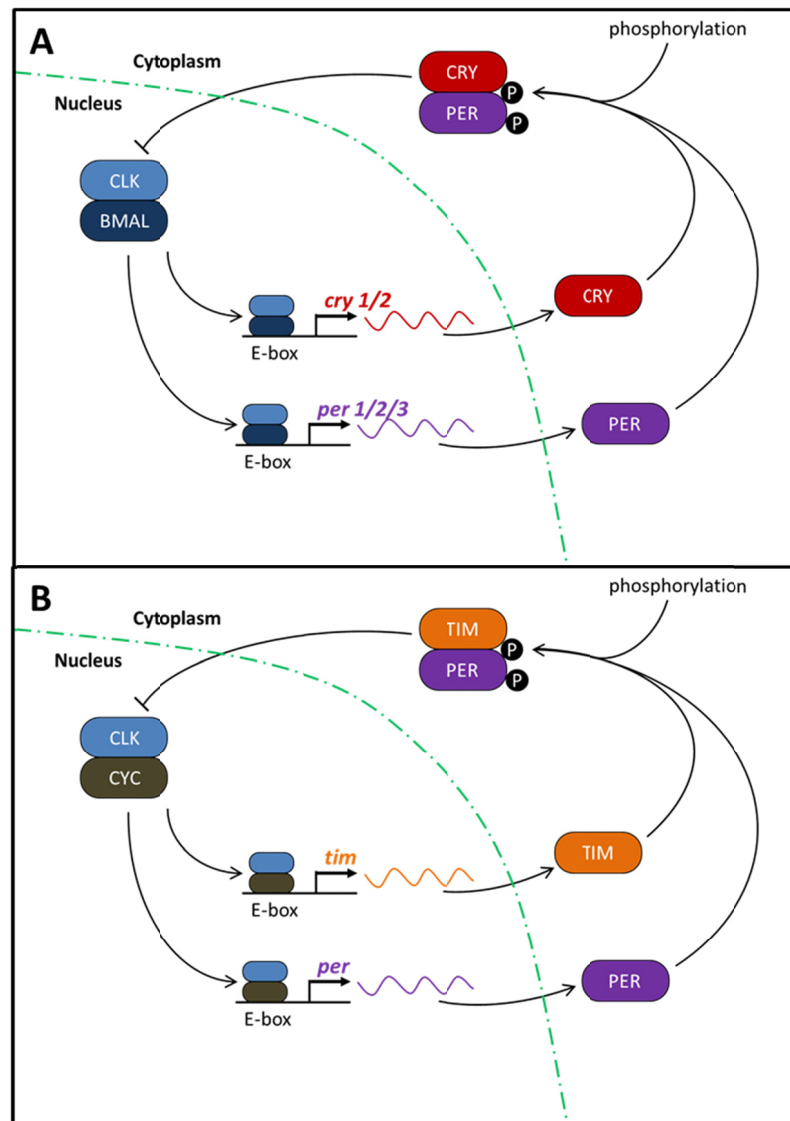


Figure 1. Schemes of core circadian oscillators of A) mammals and B) *Drosophila melanogaster*
 CLK: CLOCK, *cry*: cryptochrome, *per*: period, CYC: CYCLE, *tim*: timeless; green dash-dot lines: nuclear membrane. P in black circle represents phosphorylation. Graphics simplified from A) Ref. [10] and B) Ref. [12].

Aside from vertebrates, circadian clocks have also been widely studied in the fruit fly *Drosophila melanogaster* [13]. *Drosophila* possesses a similar central circadian clock with *clock* and *cycle* as major positive elements and *period* and *timeless* as major negative elements [14] (Figure 1B). Expression of circadian clock genes was also studied in other insect species but considerable differences have been found in the molecular oscillatory components of different species [12]. No master clock has been discovered yet in crustaceans [15], and circadian clocks in other invertebrate taxa remain largely unexplored. Recently, orthologs of core clock genes of both vertebrates and *Drosophila* were discovered in the bristle worm *Platynereis dumerilii*, which were also shown to be co-expressing in a specific brain region, shedding light on the common origin of this clockwork in the animal kingdom [16].

In vertebrates, the central circadian clock is located in the suprachiasmatic nucleus (SCN), a distinct group of cells located in the hypothalamus [17]. External light information received by a subset of the retinal ganglion cells located in the eyes is transmitted through the retino-hypothalamic tract directly to the SCN to entrain the central circadian clock. Time information from the SCN in turn regulates neuronal and hormonal activities that regulate a variety of biochemical, physiological and behavioral functions. In insects, orthologs of known core circadian clock components were found to be expressed in the central brain or the optic lobes, indicating that centralized circadian clocks also exist in diverse invertebrates [17–21].

Apart from circadian rhythms, the other major solar-related rhythms that have been studied are seasonal rhythms. Seasonal rhythms are typified by changes in the daylight period across the calendar year. Annual migration or reproductive cycles are reported to be clock-controlled, for example, the annual migration of birds [23], the seasonal reproduction of sheep [24] and the hibernation of chipmunks [25]. Compared to circadian rhythms, little is known about the molecular nature of the core oscillators of these infradian (longer than a day) clocks. However, molecular investigations studying the activation of output pathways reveal the impact of circadian clocks on annual rhythms. One of the best-known hormones that is regulated as an output of the circadian clock in vertebrates is melatonin (5-methoxy-N-acetyl-serotonin), a small molecule hormone produced mainly in the pineal gland [26]. Melatonin is widely considered to play a critical role in measuring day lengths to encode seasonal information, which will be described in detail in Section 3.2.

3.1.2 Lunar rhythms

For the moon to appear in the same place relative to any particular point on the earth takes 24.8 hours, or one lunar day. Every 29.5 days, the moon orbits once around the earth and comes back to its original position, which is called a lunar month. On the one hand, every lunar day the position of the moon with respect to the seawater drives the tides to rise and fall two times (every 12.4 hours). On the

other hand, the orbit of the moon around the earth puts the moon, the earth and the sun in one axis twice every lunar month (every 14.8 days), giving rise to the “spring tides”, where the tide’s range reaches its maxima driven by the gravity of the moon and the sun [27].

The light intensity changes during the lunar cycles and the related tidal conditions cause rhythms in living organisms. For example, nocturnal light from the moon can influence the visibility of both the predator and the prey, thus altering their activity levels (reviewed in Ref. [28]); the water coverage as well as the light condition at the intertidal region affects the locomotor activity of its inhabitants, e.g. that of the shorecrab [29]. Moonlight itself is also used as a time cue for synchronization of reproduction in many marine species. The most well-known example of this is the mass spawning of the coral communities within the Great Barrier Reef (QLD, Australia), where corals across the different species spawn on the same night once a year [30]. Unlike the tidal rhythms that have a rather short period length and mostly affect the behavior of animals, lunar light-regulated cycles are more commonly involved in the synchronization of reproductive events [4].

While solar-related rhythms are intensively studied, lunar rhythms have received considerably less attention from chronobiologists, despite the fact that lunar periodicity in the reproduction of marine species has been observed since as far back as ancient Greek and Roman times. In the 4th century BC, Aristotle noticed that the gonads of sea urchin were largest at full moon, as the gonad was the part eaten and considered a delicacy. In the last century lunar periodicity started to attract attention of researchers again, mainly of marine animals (reviewed in Ref. [27, 30]). Among these species, some are shown to possess an endogenous circalunar clock that is entrained by moonlight. The egg-laying and zoeae-releasing behavior of female *Sesarma haematocheir*, a terrestrial crab in Japan was shown to be free-running under constant light-dark conditions (L:D 14:10) , and could be re-entrained by artificial moonlight [32]. The eclosion of the marine midge *Clunio marinus* only occurs during low tide of spring tides, and the adult emergence event was shown to be under circalunar clock control that is also temperature compensated [33]. The synchronized mass spawning of the bristle worm *Platynereis dumerilii* has fascinated researchers for more than a century, and the synchronization can be maintained in the laboratory for several months under constant light-dark conditions [3].

The presence of an endogenous circalunar clock has the advantage over immediate light or tide triggered responses, in that it allows animals to anticipate coming events or favorable environmental conditions. For marine species, especially those with external fertilization, it is essential to ensure the encounter of the gametes, for which the moon serves as the most obvious and trustworthy indicator of time.

While lunar periodicity of reproductive cycles has been observed for a broad range of marine animals, the molecular mechanisms of the proposed underlying circalunar clocks are largely unknown.

Elements of the circadian clock have been observed to be influenced by moonlight. Melatonin as the major output of circadian clocks in vertebrates was shown to be of more elevated levels during new moon nights comparing to full moon nights in the rabbitfish *Siganus guttatus*, while the pineal mRNA level of putative core clock component *per2* was upregulated at the full moon period [34]. However, in the absence of experiments conducted under free-running conditions, it is not clear if the difference was the result of endogenous circalunar clock regulation, versus an immediate response to the external light stimulus. To date, no gene was identified as core element of the circalunar clock in any species.

Lunar rhythms are more commonly observed in marine animals, and less reported for terrestrial species, but their presence and importance might be underestimated. Despite little previous evidence of endogenous circalunar clocks in mammals, it was recently shown that human sleep and melatonin levels were differently regulated during different moon phases even in absence of light cue during the night [35]. As life likely originated in the sea, one hypothesis could even be that the common ancestor of human and the modern marine species might have already possessed a circalunar clock that has been retained in some terrestrial species. To address hypotheses like this, a better understanding of circalunar clock mechanisms in an experimentally tractable model system is required. Such an experimental approach will yield molecules and mechanisms that can subsequently be tested in other systems to address if there are molecularly related processes in other systems.

3.1.3 Interaction between the different biological rhythms

As the alternating light regimes from the sun and the moon are co-existing and interwoven, animals are compelled to adjust themselves to both the solar-related and the lunar-related environmental changes simultaneously. The arising challenge for chronobiologists is to decipher the molecular interaction between the different types of rhythms and biological clocks: Do all the biological clocks share the same core molecular components, or are they completely independent of each other? To which extent do different clocks share the same molecular components? How do molecular clocks interfere with each other?

Due to the lack of understanding of the core biological clock components other than the circadian clock, the interconnectedness of different clocks at the molecular level remains rather obscure. However, different rhythms are shown to be interconnected in many species. The moonlight-synchronized mass spawning behaviors mostly occur at certain circadian time, e.g. at night for the coral reefs, between sunset and midnight for *Platynereis dumerilii* [36]. In this case it could be hypothesized that the circadian and circalunar clocks interact to fine-tune the reproductive timing to specific hours. Likewise, lunar reproductive cycles also show seasonality: for example, mass spawnings of *Platynereis dumerilii* only occur during the summer months from May to September [36]. The intricate regime of different rhythms on the same biological process, primarily reproduction,

argues for the existence of a shared molecular regulator. Current hypotheses propose that hormones may fulfill this function by coordinating signaling between different clocks and their downstream molecular processes [31].

3.2 Melatonin

3.2.1 Production and metabolism of melatonin

Melatonin, or N-acetyl-5-methoxytryptamine, is a tryptophan derivative hormone that is found in animals, plants and bacteria. Melatonin was first extracted from bovine pineal gland, and known for its activity of changing the skin color of tadpoles by contracting the epidermal melanophores [37], and hence got its name [26]. It was later on demonstrated to perform circadian fluctuations in humans [38] and was hence more widely studied also in other species. To date, it is found ubiquitously present in all main taxa of vertebrates, and has also been reported to be detected in representatives of various non-vertebrate taxa, including insects [38, 39], crustaceans [41], cnidarians [42], plants [43], bacteria [44] and many more, with only few exceptions such as the Antarctic krill *Euphausia superba* [45]. Although melatonin seems widespread, information on important invertebrate taxa, including annelids, is still scarce [46]. Moreover, as outlined below, it is currently also unclear if fluctuations in melatonin production represent an ancient chronobiological feature of animal nervous systems, or if these represent a more recent evolutionary innovation.

In mammals melatonin as a small molecule with high hydrophobicity is secreted into the blood stream by passive diffusion, and is rapidly metabolized mainly by hydroxylation to 6-hydroxy-melatonin and excreted in the urine after conjunction with sulfuric or glucuronic acid [38]. The half-life of intravenously administered melatonin is 0.5-5.6 minutes in humans [47]. Therefore the presence of melatonin in mammals chiefly depends on its production. The concentration of 6-sulfatoxymelatonin (the chief metabolite of melatonin) in the urine closely parallels the serum melatonin concentrations and therefore can be used to estimate melatonin production [38]. To date, little is known on the metabolic pathway of melatonin in invertebrates.

In vertebrates, melatonin is primarily produced in the pineal gland composed of pinealocytes and glial cells. Peripheral melatonin production sites include photosensitive organs, especially the retina [48], and individual studies also demonstrate possible synthesis of melatonin in reproductive tissues, e.g. human ovaries [49]. While the retina organization resembles each other in all vertebrates, the structure of the pineal gland from lower vertebrates to mammals has greatly changed [50]. In lower vertebrates, the pineal gland contains the circadian clock as well as photosensitive cells resembling the retinal cones, and is mostly positioned to facilitate light perception [51], whereas in mammals it does not have the photosensitivity and is situated in the central part of the brain. Instead, the mammalian pineal

gland receives light information from the retina transmitted through the retino-hypothalamic tract via the SCN. The pinealocytes producing melatonin in the mammal pineal gland are phylogenetically derived from photoreceptor cells, but direct photosensitivity was gradually replaced by an indirect photosensitivity via neuronal transmission [50].

Such a scenario would be compatible with the notion that the melatonin pathway can also be found in simpler nervous systems which do not possess a specialized pineal organ. Consistent with the notion, melatonin was found to be produced in the brain in certain invertebrate species, as in the face fly *Musca autumnalis*, the crayfish *Pacifastacus leniusculus*, or the head region of *Drosophila melanogaster* and the planarian *Dugesia dorotocephala* (reviewed in Ref. [52]). More frequently, melatonin was found in the photoreceptive organs, especially in crustaceans and some insect species [52], such as in the eyes of the crayfish *Procambarus clarkii* [41], in the retina of *Octopus vulgaris* [53]. Localization of melatonin in the neuronal system of anthozoans, *Renilla koellikeri* and *Nematostella vectensis* goes in line with the neuronal origin of melatonin [52, 41]. Additionally, immuno-labeling of melatonin highlighted reproductive tissues in these species, indicating a potential involvement of melatonin in reproduction. Despite these lines of biochemical evidence, however, it has remained unclear if melatonin in these invertebrate model systems is produced by enzymes orthologous to vertebrate Aanat and Hiomt, the enzymes responsible for the last two steps of melatonin biosynthesis (see Section 3.2.3). This uncertainty has made it difficult to conclude to which extent the occurrence of melatonin in invertebrate and vertebrate models reflects deeper evolutionary relationships between the producing cell types or rather parallel evolutionary paths.

3.2.2 Circadian regulation of melatonin by both clock and light

Melatonin was intensively studied in vertebrates, especially mammals because of its involvement in the circadian rhythm (as outlined below), and is widely known for its characteristic higher level at night. It is called the “hormone of darkness” as the night peak applies not only for diurnal animals, but also for nocturnal animals [55]. In vertebrates, melatonin is regulated by both the endogenous circadian clock and the external light stimulus. Free running experiments in Syrian hamsters have shown that plasma and pineal melatonin concentrations maintained the nocturnal peak even under constant darkness, confirming that the melatonin rhythm is driven by endogenous circadian clock[56]. In fact in mammals for which the regulation of melatonin was most thoroughly studied, external light stimuli are sensed by a subset of photosensitive retinal ganglion cells and the light/dark information is transmitted to the SCN before reaching the pineal gland. Light entrained circadian clock in the SCN in turn regulates the melatonin production[57, 58]. Melatonin is one of the most well studied outputs of the circadian clock in vertebrates, and was also reported to have an important feedback role in circadian timing[59, 60]. However, the production of melatonin is also suppressed acutely in response to light exposure during night [61].

Unlike vertebrates, the regulation of melatonin with regard to the photoperiod (i.e. the period of daylight in every 24 hours) in invertebrates is controversial. In some species, such as in *C. elegans* [62], the planarian *Dugesia japonica* [49], the crayfish *Procambarus clarkii* [63], as well as several insects (reviewed in Ref. [52]), melatonin was reported to have the highest level during the dark period just as in vertebrates. However, there is enough evidence showing variable species-dependent melatonin regulation towards the photoperiod. In *Renilla koellikeri* no circadian fluctuation of melatonin was observed, but melatonin was found to exhibit seasonal difference, with high values corresponding to the first stage of sexual maturation [54]. In some species even fluctuation with the opposite phase of vertebrates was observed, peaking during the light phase, as in the freshwater prawn *Macrobrachium rosenbergii* [64] and the crayfish *Procambarus clarkii* [41]. In addition, care must be taken while interpreting these melatonin rhythms, as no free-running experiment was carried out, it was also not clear if the present melatonin rhythm detected is endogenously driven or a direct response to the external stimuli. In summary, although melatonin being a circadian time messenger has been well characterized in vertebrates, its regulation towards the circadian photoperiod doesn't seem to be conserved throughout the animal kingdom.

3.2.3 Critical enzymes in melatonin biosynthesis: Aanat and Hiomt

Melatonin is a tryptophan derivative. Tryptophan is hydroxylated by a tryptophan hydroxylase (EC 1.14.16.4) to 5-hydroxy-tryptophan (5-HTP), which is then decarboxylated by aromatic amino acid decarboxylase (EC 4.1.1.28) into 5-hydroxy-tryptamine (5-HT) or serotonin [65]. In vertebrates, serotonin can be N-acetylated by Aralkylamine-N-acetyltransferase (Aanat, EC 2.3.1.87) resulting in N-acetyl-serotonin (NAS), the direct precursor of melatonin. Finally, a methyl group is added by hydroxyindole-O-methyltransferase (HIOMT, also termed N-acetylserotonin methyltransferase or ASMT, EC 2.1.1.4) to form melatonin [63, 64].

In vertebrates, the role of the last two enzymes in the melatonin biosynthesis pathway has been intensively studied, due to the characteristic night peak of melatonin. Hiomt was suggested by the earliest investigations to determine the quantity of melatonin produced at night [68]. Later, however, it was suggested that Aanat was the rate-limiting enzyme controlling the rhythmic production of melatonin [69]. This idea was widely accepted and Aanat was also termed the “timezyme” [70]. It was shown in numerous species including human that the activity of Aanat increases rapidly and dramatically at the onset of night and decreases even faster at the night to light transition [66, 68], with little difference in the Hiomt activity throughout the day. The serum melatonin levels parallels Aanat activity due to the quick diffusion to the blood stream and the rapid elimination. In line with this idea, the *aanat* gene in rats was found to possess a functional E- box in the first intron, where the BMAL-CLOCK heterodimer could bind and mediate the transcription, suggesting *aanat* as a direct transcriptional target of the core circadian clock oscillator [72]. The expression of *aanat* was also

shown to be altered in *perl* (one of the “negative elements” of the core circadian clock) deficient mouse [73], further indicating the circadian clock regulation of *aanat* transcription. Aanat activity was also shown to be regulated by light perception in the retina. The neuronal system is activated during dark period, releasing norepinephrine, which in turn activates the α 1- and β 1-adrenergic receptors in the pineal gland, causing the rise of cyclic AMP (cAMP) and calcium concentrations. Aanat enzyme is activated by cAMP-dependent phosphorylation on Ser and Thr residues, resulting in the recruitment of 14-3-3 ζ that increases the catalytic activity and stabilizes the protein [74]. Inversely, the exposure to light induces rapid decrease of both Aanat activity and protein [75]. These observations supported the role of Aanat in shaping the daily rhythmic melatonin production.

However, by carefully examining the concentration of melatonin and its precursors NAS and serotonin, as well as the activity of Aanat and Hiomt at light-dark transition time and at night in an integrated manner, recent findings again suggested that Hiomt might indeed be the one controlling the melatonin amount produced at night, at least under certain conditions. In Siberian hamsters (*Phodopus sungorus*), the nocturnal melatonin peak amplitude was observed to correlate with the Hiomt activity but not the activity of Aanat [76]. In the same species, it was furthermore observed that the activation of β -adrenergic receptors stimulated Aanat activity, but the production of melatonin was disconnected with the Aanat activity, whereas Hiomt activity was correlated with melatonin production [77]. In a mutated rat strain with reduced Aanat activity, melatonin levels remained the same as in wildtype animals; and the authors further noticed that the Aanat product NAS was present in vast excess compared to melatonin at night, arguing for a downstream limiting factor in melatonin production [78]. In summary, which enzyme plays a bigger role in regulating the melatonin synthesis is still under debate and clearly needs further investigation. Indeed, which enzyme determines melatonin production might be species- and situation-dependent, and both could have a role in regulating melatonin.

In invertebrates, the relative importance of Aanat- and Hiomt-like enzyme activities for the temporal regulation of melatonin is even less clear. As mentioned, biochemical evidence suggests that several invertebrate groups also possess melatonin biosynthesis capacity, by showing Aanat/Hiomt-like enzymatic activities in animal tissues (reviewed in Ref. [46]), but the identity of the respective enzymes – and, if available, the equivalence to their vertebrate counterparts – has remained unclear. As Aanat was more accepted as the responsible enzyme for the melatonin night peak in vertebrates, it also attracted more attention in invertebrates, especially in the context of circadian rhythms. While in some species Aanat activity was found to be fluctuating in a circadian manner, sometimes also coinciding with melatonin levels [59, 47], the orthology of the underlying enzymes with the vertebrate pathway is still debated. The Aanat family is part of the large GCN5-related acetyltransferase (GNAT) superfamily that uses acetyl-CoA to acetylate their substrates [79]. Indeed, the only genetic studies of Aanat in invertebrates revealed that the invertebrate-type Aanats (ivAanats,

excluding the insect-type Aanats) belong to a different sub-family of the Aanat family than the vertebrate-type Aanats (vtAanats) [77, 78]. ivAanat lacks the E-box in the regulatory region, neither its expression nor the activity was found to fluctuate in a circadian pattern. It was rather believed to have a detoxification function by acetylating various toxins. What is more, the only enzymes that have been demonstrated to be able to catalyze the acetylation of serotonin in insects and nematodes don't belong to the Aanat family but to another remotely-related family of acetyltransferase within the GNAT superfamily [79, 59, 80]. Aanat family members (ivAanats and vtAanats) were found in Gram-positive bacteria, fungi and vertebrates but not in nematodes and insects [84], thus it has even been speculated that the pathway may have been independently acquired in vertebrates and fungi through horizontal gene transfer from bacteria [84].

Hiomt has been even less studied in invertebrates. Itoh *et al.* [47, 82] used biochemical methods to detect Hiomt-like activity in the silkworm *Bombyx mori* and the freshwater planarian *Dugesia japonica*. In both species Hiomt-like activity fluctuates in a circadian pattern, and in the silkworm it even persisted under constant darkness. At the molecular and genetic level, a putative gene possibly encoding Hiomt in *C. elegans* was reported, without details of the gene identification method [86]. In the genome of *Nematostella vectensis*, where Aanat is lacking, three Hiomt transcripts and three related EST sequences were found [87], however the proof of true orthology of these genes to other Hiomt encoding genes still require further investigation. In summary, to date information on Aanat and Hiomt in invertebrates, especially at the molecular and genetic level, is still largely lacking, leaving the question open for the possible common origin of melatonin biosynthesis pathway in the animal kingdom.

3.2.4 Melatonin and reproductive timing

In the polar or temperate climate, winter is associated with lower temperature and scarce food supply, thus animals living in these regions must adapt their reproduction to the seasonal changes to ensure the survival of their offspring. Many long-living organisms therefore display seasonal reproductive behaviors. Siberian hamsters and Syrian hamsters are two typical long-day breeding animals which only breed during summer, in contrary, reproduction in the Soay sheep is simulated by exposing to short days. As melatonin level are rising during the dark period in vertebrates, the duration of its elevated level can be used as an indicator of night length. Indeed, in Siberian hamsters the duration of elevated levels of pineal melatonin was longer in days with short photoperiod [88]. By daily infusion of melatonin into pinealectomized animals with different length and amount, it was found that the duration of the infusion was by far the most important factor triggering the responses associated with day length, e.g. short infusion duration evoked long day responses including testis growth, and the elevation of reproduction-related hormones [89]. Similar effects were also reported in Syrian hamsters [90], as well as in short-day breeding female sheep, with reproductive response evoked by long

duration of melatonin infusion [88, 89]. Correlations of melatonin levels with the maturation and reproduction of various vertebrates were reported, including the one-humped male camel (*Camelus dromedarius*) [93], the European starlings (*Sturnus vulgaris*) [94] and even humans (reviewed in Ref. [95]), but in these cases the cause-effect relationship remains to be determined. The coincidence of a rise of the melatonin levels with the sexual maturation in *Renilla koellikeri* [54] suggested its possible involvement in the reproduction of invertebrates. Recently, plasma melatonin, ocular melatonin and the mRNA level of *aanat* were reported to be affected by the exposure to moonlight in lunar-synchronized spawner fish species, e.g. Seagrass Rabbitfish (*Siganus Canaliculatus*) [93, 94] and goldlined spinefoot (*Siganus guttatus*) [98], indicating the potential role of melatonin as a time cue even in lunar rhythms. However, care must be taken in distinguishing the effect of endogenous circalunar clocks and acute light-response. In absence of free-running experiments, melatonin cannot yet be claimed to be an output signal of the circalunar clock.

Summarizing the existing knowledge on melatonin and its biosynthesis pathway in the context of circadian rhythms and seasonal reproduction it must be stated that these are more thoroughly studied in vertebrates than in invertebrates. But studies on invertebrate melatonin and its involvement in reproduction leads to more interesting questions open to be addressed: Is melatonin biosynthesis pathway a conserved ancient pathway throughout the animal kingdom, or is it a result of a recent evolutionary event? Can melatonin be a unifying molecule serving to reset the clock for various biological rhythms, or are there different molecules involved in different biological rhythms or species? To answer these questions, the choice of a proper model organism is essential.

3.3 *Platynereis dumerilii*: model organism for evolution and chronobiology

3.3.1 Model for evolution and developmental studies

Platynereis dumerilii is a marine bristle worm that belongs to the Polychaeta Class of the Annelida Phylum. Unlike the most well-established invertebrate model organisms, e.g. the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* that belong to the Ecdysozoa, *Platynereis* represents the other clade of the protostomes, the Lophotrochozoans. Protostomes along with the deuterostomes where vertebrates belong to, are the two main branches of the Bilateria that diverged about 600 million years ago. The phylogenetic position is illustrated in the simplified evolutionary tree of bilaterians in Figure 2. As a large superphylum of the animal kingdom, the lophotrochozoans are greatly under-represented in the molecular biology research. Lophotrochozoans are phylogenetically distant to arthropods, nematodes and vertebrates, therefore could provide new insights into the evolution of bilaterians [99].

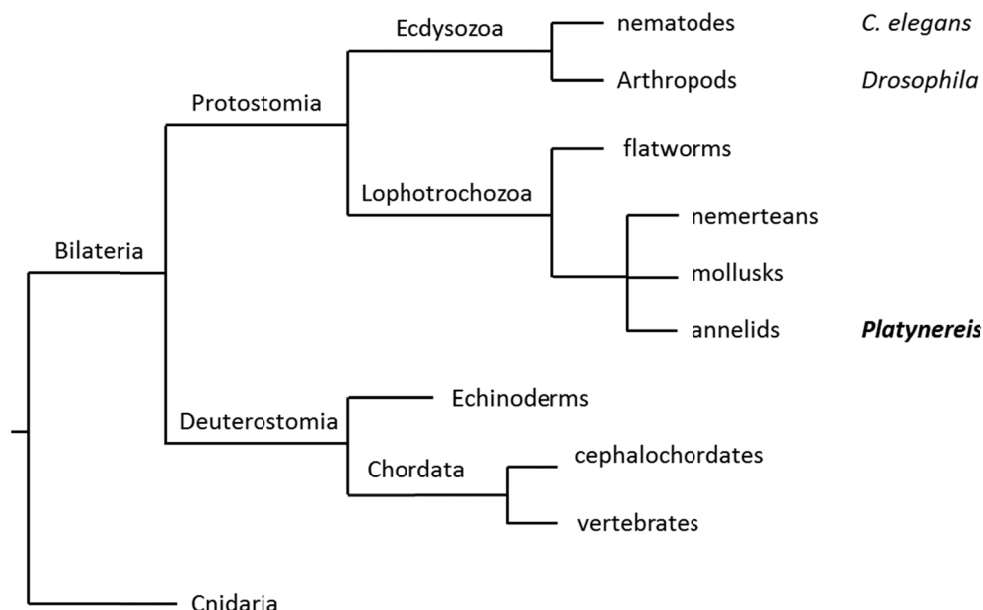


Figure 2. Simplified evolutionary tree of the Bilateria

Graphic modified from Ref. [99]

Platynereis is also considered a living fossil. It has been living in the same environment as its ancestors millions of years ago and retained many ancestral features. It was shown to be a molecularly slow-evolving species, as its protein sequences, as well as the intron number and positions in the genome show more similarity to vertebrates than to other protostomes as nematodes and insects [99]. It also possesses the orthologs of many vertebrate-like protein coding genes that have been lost in the fast-evolving model organisms as *D. melanogaster* and *C.elegans* [96, 97]. On the cellular level, *Platynereis* was shown to possess more ancestral-type cell types than other vertebrate and invertebrate model organisms based on molecular fingerprints and cell morphologies and positions [98–102]. Its central nervous system, body plan and developmental style also come closer to the ancestral morphology [103–105].

Recently, with the joint efforts from different research groups, different datasets of expressed sequenced tags (EST), transcriptome sequences, individual genomic BAC clones [99] and a high-coverage reference genome have been generated (<http://4dx.embl.de/platy/>). Together, these provide valuable data sources for studies with *Platynereis* at the genetic and genomic level.

What also makes *Platynereis* advantageous as a model organism for scientific research is that unlike most other marine species, it can be bred in the laboratory. *Platynereis* is small in size, with the length of adult worms up to 6cm, but normally only 2-3 cm. Carl Hauenschild established a laboratory culture of *Platynereis* in 1953, and the animals have been bred ever since in laboratories without interruption [109]. Furthermore, one pair of worms can produce several thousands of offspring in a single spawning event, facilitating genotyping and breeding of transgenic and mutated animals.

Together, these features make *Platynereis* a useful model organism for molecular biological research in evolution, neurobiology, developmental biology and evo-devo.

3.3.2 Lunar reproduction: orchestration of clocks and light

Platynereis typically inhabits coastal waters no deeper than 3m. Atokous animals (Figure 3A) live in a silk tube on the algal thalli at the bottom of the sea [109]. They have a semelparous life cycle that lasts from 3 months up to 2 years. Mature male and female animals undergo different epitokous metamorphosis (Figure 3B) and perform “nuptial dances” at the water surface. At the end of the mating performance, sperm and eggs are released synchronously, and the adult worms die soon after. Fertilized eggs develop into trochophora larvae after one day and live a planktonic and positively phototactic life-style for a few days. At 3-4 days after fertilization the three-segmented juveniles settle down and start building living tubes, and later grow into atokous adults [109].



Figure 3. *Platynereis dumerilii*

A) Atokous *Platynereis*; B,C) Epitokous *Platynereis* specimens of both sexes have enlarged eyes and the trunk is divided into two parts with different shapes of parapodia and body color. B) Mature males are white in the anterior part and red in the posterior part due to the great number of blood capillaries and the body cavity filled with sperm. C) Mature females are a yellow because the body cavity is filled up with oocytes.

For these animals with external fertilization, a synchronized spawning event among individuals is essential to ensure the success of reproduction. Like many other marine species, *Platynereis*' mass spawning is synchronized to the moon phase. The lunar synchronized emergence and mass spawning of these animals have been documented and studied for more than a century [2]. Later on, it was

shown that laboratory cultures of *Platynereis* under artificial illumination also displayed lunar reproductive behavior, which could be entrained by periodic dim nocturnal illumination mimicking the full moon [3, 107]. Most interestingly, once the culture is entrained, the spawning synchronization can be retained for more than 3 months even without the nocturnal light stimulation (i.e. free-running), showing the existence of an endogenous circalunar clock that can be entrained by dim nocturnal illumination [3]. The emergence of epitokous animals in nature also happens only during night [111], which could be under circadian clock control. Consistently, previous work from our lab identified and confirmed the core circadian genes in *Platynereis*, and showed that the locomotion of *Platynereis* is under circadian and circalunar clock control [16]. Along with the other advantageous features discussed in Section 3.3.1, *Platynereis dumerilii* re-emerges as a unique model organism in chronobiology for studying the molecular mechanisms of the circalunar clock and its interaction with other biological clocks.

Previous work in our lab has indicated that the circadian clock might be influenced by the circalunar clock on the molecular level: the transcript levels of three circadian clock genes – *pdp1*, *clock* and *period* – are significantly elevated in free-running full moon light regimes, compared to new moon conditions [16]. This shows that the circalunar clock acts – at least in this case – on distinct molecular targets that can be measured by quantitative methodology. However, as these circadian clock genes are supposed to have cell-autonomous functions, the molecular mechanism by which the circalunar clock signal is transmitted to other tissues, and influences reproductive processes, still remain enigmatic.

Classical studies have shown that the oogenesis and spermatogenesis of *Platynereis* occur in a highly synchronized manner [109]. Hormones diffuse in the body way and were shown to play a critical role in the regulation of maturation [112], making it an attractive assumption that hormones might transmit the signal from endogenous clocks to the actual maturation and reproductive timing. A key incentive of my study therefore was to identify and characterize hormones under control of the circalunar clock.

3.3.3 Genome modification tools for *Platynereis*

Although *Platynereis* seems to be a useful emerging model organism for multiple biological research fields, available genetic and genomic tools were very limited compare to other well-established model organisms for molecular biology, such as *Drosophila* and *C. elegans*. Previous studies mainly focused on the gene expression using technique such as quantitative RT-PCR, in-situ hybridization to study the pattern of gene regulation, but for in-depth analysis of gene function *in vivo*, there were no functional genomic tools established for *Platynereis* at the beginning of my PhD study. In the last few years, along the course of my study, our lab has pioneered in developing genetic and genomic toolkits for this fascinating animal. With the joint effort of many lab members, we have established protocols for

transposon-based transgenesis and for mutagenesis with transcriptional activator-like effector nucleases (TALENs).

3.3.3.1 Transposon-mediated transgenesis

A transposable element (or transposon) is a DNA sequence whose position can be changed in the genome (“transposition”). There are two classes of transposons: Class I or retrotransposons involve the transcription of DNA into an RNA intermediate and the reverse-transcription to copy the DNA before being inserted (copy-and-paste). Class II or DNA transposons function by direct excision and insertion of the DNA that are catalyzed by various transposase enzymes. Various DNA transposons have been engineered into specific gene integration systems and became a powerful tool for genetic analysis and transgenesis in both vertebrate and invertebrate model organisms. Recently, the Tc1/mariner-type element Mos1 and the hAT/Tol2 element have been shown to mediate transposition in *Platynereis* [113]. By co-injection of the transposon vector containing the donor DNA insert and the synthetic mRNA of the corresponding transposase, Tol2-mediated integration of the transgene exhibited already robust expression in the injected generation (G0), while Mos1-mediated transgenesis only produced mosaic expression. However, no transgene expression was observed in the G1 with Tol2-mediated integration, while Mos1-mediated transgene integrations could be stably expressed in the following generations. Therefore, Tol2-mediated transgenesis is useful for transient analysis of the expression patterns and Mos1-mediated transgenesis would be the method of choice for creating stable transgenic lines in *Platynereis* [113].

3.3.3.2 Transcriptional Activator-Like Effector Nucleases

Using transcriptional activator-like effector nucleases (TALENs) for targeted mutagenesis has rapidly emerged as a technique of choice for precision genome modification. TALENs are engineered nucleases that are created by fusing a DNA binding domain to a monomeric, C-terminal FokI nuclease domain [114]. The DNA binding domain is based on the transcription activator-like effector (TALE) proteins from the plant pathogene *Xanthomonas*, which contains a central repeat domain with each repeat recognizing one target base. Each repeating peptide sequence harbors two critical and adjacent amino acids, also called “repeat variable di-residue” (RVD), that preferentially recognizes one of the four bases [115], [116]. Thus the TALE domain can be custom-designed to recognize and bind to user-defined sequences in the genomic DNA. The binding of paired TALEs to tandem sequences separated by a short spacer (15-30bp) enables the dimerization and activation of the FokI nuclease, resulting in a double-strand break of DNA within the spacer region. As a result of errors during DNA repair by non-homologous end joining (NHEJ), small insertions or deletions (indels) are introduced around the cleavage site, sometimes resulting in frame-shifted mutations that often lead to premature stop codons

(Figure 4). By designing TALEN binding sites near the 5' end of the protein coding sequence the mutations can lead to the production of non-functional or truncated proteins [115–117].

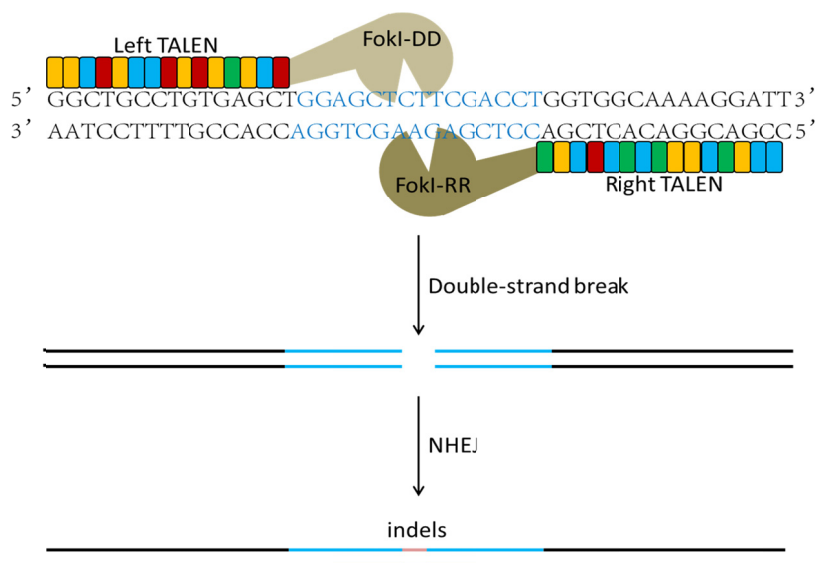


Figure 4. Schematic drawing of TALEN-mediated mutagenesis

Colored rectangles: repeating peptide sequences in the DNA binding domain; FokI-DD and FokI-RR: FokI nuclease domain. Genomic sequences shown at the upper part are represented with solid lines in the same colors at the lower part of the graphic. See text for detailed description.

TALENs have been reported for successful targeted mutagenesis in many conventional model organisms, such as mice [118–120], zebra fish (*Danio rerio*) [121–123], *Xenopus tropicalis* [116, 124, 125] and *Drosophila melanogaster* [128]. There were also evidences showing the functionality of TALENs in other insects, such as mosquitos (*Aedes aegypti*) [129], *Anopheles gambiae* [130], silkworm (*Bombyx mori*) [129–131] and cricket (*Gryllus bimaculatus*) [134]. Recently reports also appeared for sea urchin [135] and newt [136].

Another promising aspect is to use TALEN as an accurate genome editing tool. By co-introduction of a custom-designed repair template with homology arms to the genomic locus, the homology directed recombination (HDR) could be mediated resulting in deletion, point mutation or insertion into the TALEN cleavage site. With single-stranded DNA oligonucleotides containing short homology arm (18-53nt) as repair template, single restriction site or short custom-designed sequence was inserted into zebrafish genome [124], and precise short deletions were introduced in human cells [137]. Larger deletion of 7.8kb was also precisely introduced by co-transfecting single-stranded DNA oligonucleotides with two pairs of TALENs in human cells [137]. By co-introduction of donor plasmid containing larger insertion cassette with longer homology arms (385-800bp) surrounding the TALEN target site, a mCherry expression cassette was successfully integrated in mammalian cells [138], and GFP was likewise inserted into a specific locus on mouse Y chromosome [122].

In summary, TALENs have been shown to be a powerful tool for generating site-specific mutations in various species, and our lab has pioneered in using TALENs for targeted gene mutagenesis in *Platynereis* [139]. Therefore using TALEN to generate gene-specific mutated lines provides a promising approach in deciphering the function of circalunar clock target genes in *Platynereis*.

3.4 Aims of the thesis

Lunar periodicity plays an important role in synchronizing spawning events in marine species, and might also have unknown impacts on terrestrial animals including humans. Despite the long-standing knowledge of its existence, research at the molecule level is just emerging. This study is among the first that attempts to decipher this complex and largely unknown phenomenon, for which the lunar reproduction of *Platynereis dumerilii* serves as a perfect model. The main aim of the present study is to identify and characterize the targets of the circalunar clock.

Melatonin was shown to play a prominent role as a target of the circadian clock in vertebrates, and was reported to be regulating the seasonal reproductive cycles in mammals, with reports hinting at its involvement in lunar periodicity. As a starting assumption, I therefore hypothesized that the melatonin pathway might serve as a target of the circalunar clock of *Platynereis*. As discussed above, the level of melatonin mainly reflects the production of this small molecule, which is dependent on the last two steps of its biosynthesis pathway. Therefore as the first step to understanding the circalunar clock targets I used a candidate-based approach focusing on the melatonin biosynthesis pathway.

With first data showing the regulation of circalunar clock on the melatonin biosynthesis pathway at the transcript level, we further hypothesized that more circalunar clock target genes could be regulated at the transcript level. Therefore as the second approach I was engaged in a pioneer screening for further circalunar clock regulated transcripts.

I conducted my study in four parts: Firstly, in order to study melatonin in the context of lunar periodicity in *Platynereis* I started with characterizing the melatonin biosynthesis pathway and located the presumptive melatonin producing cells. These results also allowed me to elucidate the evolution of melatonin pathway, which in turn will help to understand its conserved role in time-keeping. Secondly, I looked into the regulation of melatonin as well as the expression profile of *Platynereis aanat* and *hiomt* with regard to moon phases to assess if the production of melatonin and the regulation of its pathway components were under control of the circalunar clock or nocturnal light. Thirdly, I sought to create transgenic animals and mutation lines for in-depth analysis of the function of this pathway as well as to expand the genomic toolkit for *Platynereis*, which should not only solidify the findings of the present study but also of benefit follow-up studies and the research community. Finally, I was screening for and validating more circalunar clock regulated genes to broaden the understanding of circalunar clock downstream signals.

4. RESULTS

4.1 Discovery of a melatonin biosynthesis pathway in *Platynereis dumerilii*

In order to investigate the role of melatonin in lunar periodicity, the first question to be addressed is that of the existence of melatonin and its biosynthesis pathway in *Platynereis*. Therefore, the first part of my project consisted of detection of melatonin and its precursors as well as the identification of the genes coding the melatonin producing enzymes. These include the immediate melatonin precursors serotonin (5-hydroxytryptamine, 5-HT) and N-acetyl-serotonin (NAS), as well as enzymes arylalkylamine N-acetyltransferase (AANAT, EC 2.3.1.87) and Hydroxyindole-O-Methyl Transferase (HIOMT, EC 2.1.1.4)(Figure 5).

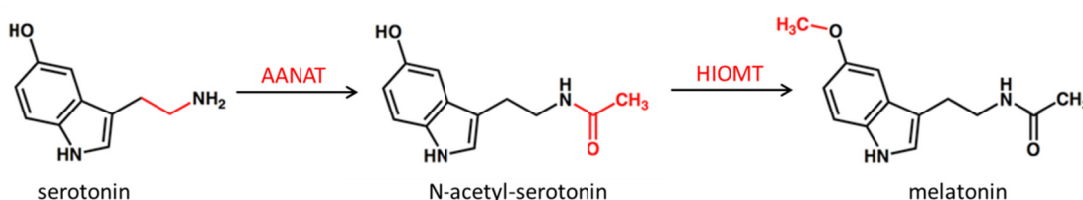


Figure 5. The last two steps of melatonin biosynthesis pathway

Serotonin is acetylated on the primary amine group by Aanat with acetyl coenzymeA as the acetyl group donor to produce N-acetyl-serotonin (NAS); NAS is methylated by Hiomt on the hydroxyl group with S-Adenosyl methionine as methyl group donor to produce melatonin.

4.1.1 Establishment of a quantitative extraction and detection method for melatonin

In order to address the question of melatonin regulation experimentally, it was critical to first establish a robust and sensitive quantification method for melatonin. Moreover, as I aimed at putting the measured melatonin levels in the context of gene expression dynamics, it was important to develop a method that would be compatible with both melatonin quantification and quantification of RNA levels of specific genes. I established a melatonin extraction method based on liquid-liquid extraction with chloroform that is also compatible with total RNA extraction from the same tissue. Melatonin as a small lipophilic molecule would be recovered in the chloroform fraction, whereas RNA should reside in the aqueous fraction.

As outlined in the introduction, biochemically, melatonin has been reported from samples of various taxa, but regarding the expected amounts, the reported concentrations of melatonin in invertebrates vary dramatically among different species. For example, the freshwater crayfish *Procambarus clarkii* was reported to have around 100-700 pg melatonin per eye or around 100-500 pg/ml hemolymph [41];

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in *Octopus vulgaris*, melatonin was reported to be around 35-50 pg/ml hemolymph or around 150-250pg/g retina [53]; the sea pansy *Renilla koellikeri* was reported to have 100-460pg melatonin/g dry weight [54]. From these examples, I reasoned that in *Platynereis*, a concentration of 1pg/mg tissue or less could be expected. Taking into account that an adult *Platynereis* weighs only a few milligrams, with the head weighing less than 1mg, I concluded that the detection method would need to be able to detect sub-picogram levels of melatonin. Different methods have been used for detecting small amount of melatonin in animal tissues, including competitive radioimmunoassay (RIA)[40, 52, 137], Enzyme-Linked Immunosorbent Assay (ELISA)[45] as well as liquid chromatography combined with tandem mass spectrometry [41, 138]. Of these, RIA and ELISA are the most promising methods to detect melatonin at sub-picogram levels. A detailed review about earlier reports of melatonin detection in invertebrates has been previously published by Vivien-Roels *et al.* [52]. I chose to use the competitive ELISA kit (Bühlmann) that was validated by Pape *et al.* [45], and that is based on the Kennaway G280 anti-melatonin antibody, based on which I also developed a modified protocol for maximized sensitivity (detection limit 0.04 pg/well).

I first tested the efficiency of the extraction method with chloroform by extracting standard solutions with extraction time from 2min to 30min under vigorous shaking. The recovery rate of standard solution extractions is shown in Figure 6A. Within 2min of extraction, the recovery rate already reached 95%, with no significant improvement when extended to 30min. For following melatonin extractions, the liquid-liquid extraction step was carried out under vigorous shaking for 5min.

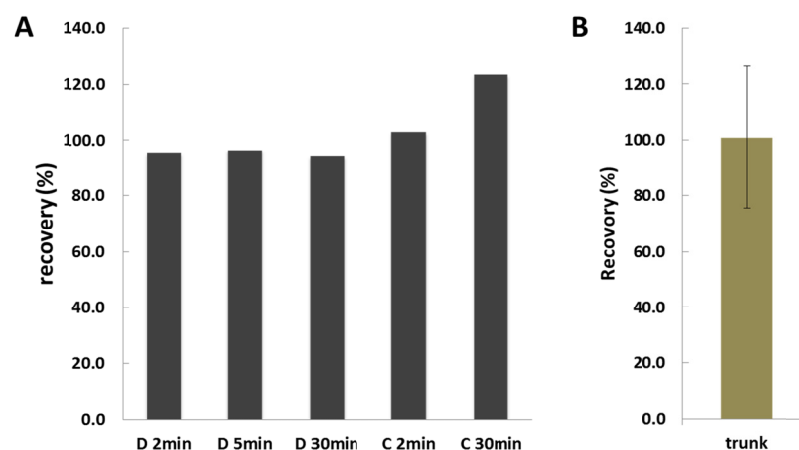


Figure 6. Extraction efficiency of melatonin using liquid-liquid extraction with chloroform

A) Recovery of standard solutions with different extraction time. Starting melatonin amounts: Standard D contained 1pg of melatonin, standard C contained 0.4pg of melatonin. B) Recovery of spiked melatonin in *Platynereis* tissue homogenates. Error bar: standard deviation.

The extraction method was also validated for animal tissues by spiking 1 pg of melatonin into homogenates of equivalent of 7.5 *Platynereis* bodies. The recovery rate of spiked melatonin in animal tissues is shown in Figure 6B. The recovery rate of spiked melatonin was 101.0±25.5% (mean ±

standard deviation), similar to the precision range reported by the kit manufacturer. Therefore a reproducible melatonin detection method with high extraction efficiency was established, which allowed the quantification of mRNA levels from the same animal tissues.

4.1.2 The critical metabolites of the melatonin pathway can be detected in *Platynereis* heads

After having established a quantitative extraction method for melatonin from worm tissues, I next addressed if melatonin naturally occurred in *Platynereis dumerilii*.

As melatonin production in vertebrates was reported to be primarily in the brain region and optic organs, I hypothesized that the melatonin production in *Platynereis* was also mainly in the head region. Sixty *Platynereis* heads were sampled at the same time and melatonin was extracted with chloroform. Although the ELISA kit was tested for cross-reactivity, we also have adopted HPLC-MS/MS in collaboration with the company Pharma-Analyt to confirm the presence of melatonin as well as NAS, and the assess quantification quality of ELISA. For this, the same biological sample was divided into two equal aliquots and measured with the two above-mentioned techniques. Similar melatonin concentrations of between 0.035 and 0.043 pg per head were measured, confirming the presence of melatonin and validating the reliability of the ELISA kit (Figure 7). As melatonin has previously been detected in certain Gram-positive bacteria, I treated 5 adult *Platynereis* with antibiotics (ampicillin 110µg/ml and streptomycin 390µg/ml) for 24 hours before decapitation, and measured melatonin amount with ELISA alongside with 5 untreated animals decapitated under the same conditions. The experiment was carried out with 4 biological replicates under two different conditions (full moon, new moon) respectively, and antibiotics-treated samples did not show to contain less amount of melatonin. Therefore I concluded that melatonin detected with our methods reflected the endogenous melatonin from *Platynereis*, that melatonin can indeed be found in the *Platynereis* head, and that the level of melatonin in the worms is in the range of 30-40 fg/head.

Taking advantage of the mass spectrometry approach which also allowed for the distinction of the melatonin precursor N-acetylserotonin, NAS was also detected by HPLC-MS/MS with an estimated amount of 1-10 pg/animal. Whereas serotonin levels were not assessed by mass spectrometry, serotonin is known as a common neurotransmitter present in almost all vertebrate and invertebrate nervous systems including *Platynereis* [139, 140].

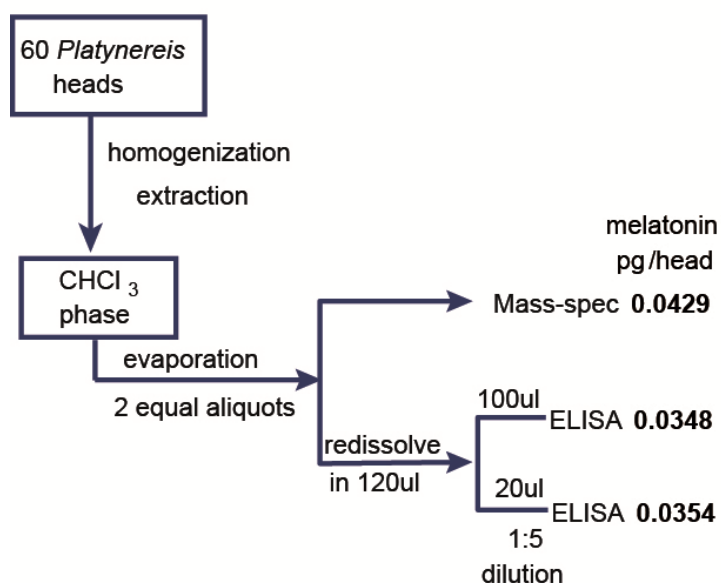


Figure 7. Detection of melatonin from *Platynereis* head tissue with two different methods

Together, therefore, I concluded that the critical metabolites of the melatonin pathway are all present in *Platynereis dumerilii*. This is compatible with the existence of a functional biosynthetic melatonin pathway in the worms. From these data, I next turned towards the characterization of the possible biosynthesis enzymes Aanat and Hiomt.

4.1.3 *Platynereis* possesses orthologs of the vertebrate-type melatonin biosynthesis enzymes Aanat and Hiomt

Whereas the previous results demonstrated the presence of the key metabolites of the melatonin pathway in the worms, they left open if also the enzymes for the biosynthesis of melatonin could be detected. In addition, as outlined in Section 3.2.3, it was not clear if melatonin biosynthesis enzymes as they are known from vertebrates were actually already present in the last animal ancestor. To address both questions, I therefore turned towards the analysis of candidate melatonin biosynthesis enzymes in *Platynereis*.

Two genes encoding putative *Platynereis* correlates of Aanat and Hiomt were previously identified in the lab (unpublished data). In order to investigate how these molecules related to the known enzymes of the Aanat and Hiomt families, I carried out phylogenetic analyses using representative sequences of other Aanat and Hiomt members as well as suitable outgroups.

Iyer *et al.* [84] carried out searching for Hiomt orthologs, but only found Hiomt in vertebrates and bacteria. Consistently, BLASTP searches using the Pdu-Hiomt sequence against the non-redundant protein sequences at NCBI (<http://blast.st-va.ncbi.nlm.nih.gov>) did not detect any significant similarity in yeasts and insects, but instead only in vertebrates and bacteria. I therefore combined the *Platynereis*

Hiomt sequence together with vertebrate and bacteria Hiomt sequences were incorporated in a phylogenetic analysis for Hiomt. For this analysis, plant OMTs were used as outgroup. Similarly, phylogenetic analysis for Aanat included *Platynereis* Aanat sequence alongside with representatives of other Aanat family members from chordates, lophotrochozoans and fungi, with bacteria NATs as outgroup. Phylogenetic analyses group Pdu-Aanat (Figure 8A) and Pdu-Hiomt (Figure 8B) with high branch support with other Aanat and Hiomt members respectively.

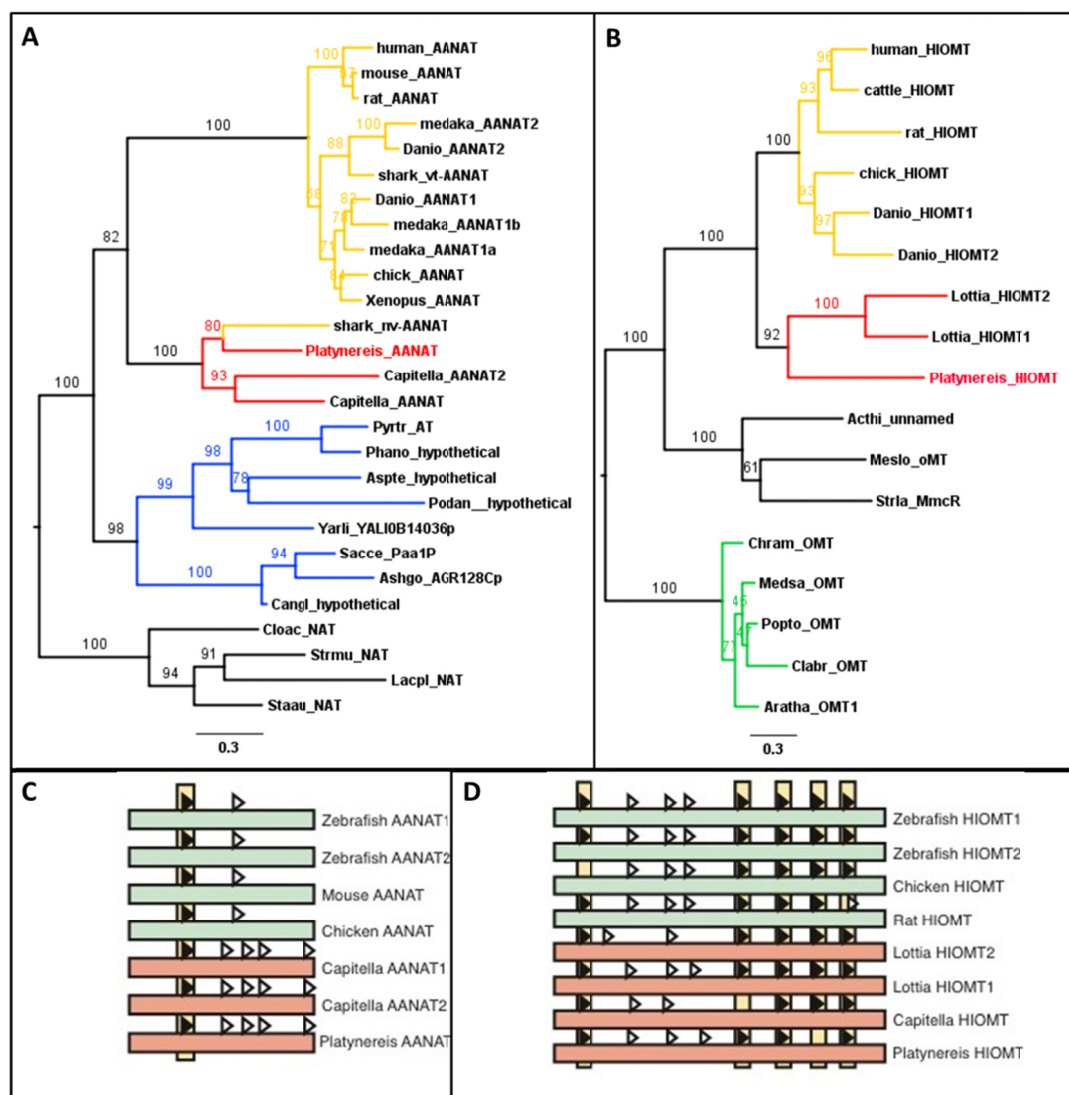


Figure 8. Phylogenetic trees of Pdu-Aanat and Pdu-Hiomt

A) Rooted maximum likelihood (ML) tree of Aanat, bacterial NAT was taken as outgroup. B) Rooted ML tree of Hiomt, plant OMT was taken as outgroup. Phylogenetic analyses were carried out for both enzymes using IQ-TREE [144] and visualized in FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>). Numbers indicate support values for the branch. Branch colors: yellow- chordates, red- lophotrochozoans, blue- fungi, black- bacteria, green- plants. C,D) Shared introns analyses for Aanat (C) and Hiomt (D) (Raible, unpublished data). Introns in deuterostomes (green) and protostome (red) representatives are labeled by triangles. Boxed black triangles indicate conserved introns and white triangles indicate non-conserved introns.

Figure 8. Phylogenetic trees of Pdu-Aanat and Pdu-Hiomt (continued)

Species and NCBI accession numbers for the proteins are as follows:

human_Aanat: *Homo sapiens*, AAH92430; mouse_Aanat: *Mus musculus*, NP_033721; rat_Aanat: *Rattus norvegicus*, sp_Q64666.1; Danio_Aanat1: *Danio rerio*, Q6V7J8; Danio_Aanat2: *Danio rerio*, Q6PCA1; medaka_Aanat1a: *Oryzias latipes*, Q2L4U8; medaka_Aanat1b: *Oryzias latipes*, Q2L4U7; medaka_Aanat2: *Oryzias latipes*, Q2L4U6; shark_vt-Aanat: *Callorhinchus milii*, NP_001279701 (vertebrate-type Aanat); shark_nv-Aanat: *Callorhinchus milii*, AGX26123 (non-vertebrate Aanat); chick_Aanat: *Gallus gallus*, NP_990489; Xenopus_Aanat: *Xenopus laevis*, Q7T2T4; Capitella_Aanat: *Capitella teleta*, JGI Protein Id: 185754; Capitella_Aanat2: *Capitella teleta*, JGI Protein Id: 220811; Cagl_hypothetical: *Candida glabrata*, XP_446590; Sacce_PaalP: *Saccharomyces cerevisiae*, NP_010356.1; Ashgo_AGR128Cp: *Ashbya gossypii*, NP_986794.1; Yarli_YALI0B14036p: *Yarrowia lipolytica*, XP_500865.1; Aspte_hypothetical: *Aspergillus terreus*, XP_001208792; Podan_hypothetical: *Podospira anserine*, XP_001909214.1; Phano_hypothetical: *Phaeosphaeria nodorum*, XP_001803735.1; Pyrtr_AT: *Pyrenophora tritici-repentis*, XP_001941583.1; Cloac_NAT: *Clostridium acetobutylicum*, WP_010890795.1; Lacpl_NAT: *Lactobacillus plantarum*, WP_011100964.1; Strmu_NAT: *Streptococcus mutans*, WP_002262546.1; Staa_NAT: *Staphylococcus aureus*, WP_000244266.1.

human_Hiomt: *Homo sapiens*, UniProtKB/Swiss-Prot: P46597.1; cattle_Hiomt: *Bos taurus*, P10950.2; rat_Hiomt: *Rattus norvegicus*, NP_653360.2; chick_Hiomt: *Gallus gallus*, Q92056.1; Danio_Hiomt1: *Danio rerio*, AAI54030.1; Danio_Hiomt2: *Danio rerio*, NP_001103947.1; Lottia_Hiomt1: *Lottia gigantea*, ESP00347.1; Lottia_Hiomt2: *Lottia gigantea*, ESO89631.1; Capitella_Hiomt: *Capitella teleta*, JGI Protein Id: 173689; Strla_MmcR: *Streptomyces lavendulae*, AAD32742.2; Acthi_unnamed: *Actinomadura hibisca*, BAA23149.1; Meslo_omT: *Mesorhizobium loti*, WP_010915321.1; Chram_OMT: *Chrysosplenium americanum*, AAA80579.1; Clabr_OMT: *Clarkia breweri*, O04385.2; Popto_OMT: *Populus tomentosa*, AAF63200.1; Medsa_OMT: *Medicago sativa*, P28002.1; Arath_OMT1: *Arabidopsis thaliana*, AAB96879.1.

These data already provide good evidence that the sequences identified from *Platynereis* indeed represent orthologs of vertebrate Aanat and Hiomt proteins, respectively. To further test this hypothesis, we compared the position of introns with respect to the aligned proteins. As intron positions are a slowly evolving feature, they represent a second measure to test if two proteins derive from a common evolutionary precursor or not. Indeed, the comparison of intron positions showed that *Platynereis* Aanat and Hiomt both have shared introns with vertebrate Aanats and Hiomts respectively, thus shared introns supported the ancestry of Aanat and Hiomt (Figure 8C, D),

In conclusion, both the protein-based phylogenetic analyses and the intron analysis support the notion that the *Platynereis* Aanat and Hiomt candidates are true orthologs of the vertebrate enzymes involved in melatonin biosynthesis.

4.2 Localization of melatonin biosynthesis pathway in *Platynereis dumerilii*

4.2.1 *Platynereis* Hiomt is expressed in distinct neurons of the brain

Melatonin being a small molecule with high hydrophobicity, it can easily penetrate the cell membranes and diffuse through tissues, whereas Hiomt is the enzyme catalyzing the last step of the melatonin biosynthesis pathway, its localization reflects where the catalytic reaction takes place, in other words, the production site of melatonin. In *Nematostella vectensis* it was already shown that immunostaining against melatonin gave broad staining pattern and *hiomt* expression correlated with intensified signal of melatonin distribution [42]. Therefore to study the localization of melatonin producing cells in *Platynereis*, I performed immuno-staining against *Platynereis* Hiomt. Customized polyclonal antisera against partial Pdu-Hiomt containing the substrates binding site were raised in two rabbits, and the serum showing specific staining was chosen for further immunohistochemistry (IHC) analysis.

In order to perform immuno-staining on adult worms, I tested different fixatives and permeabilization methods, and developed an IHC protocol which included a permeabilization step with acetone that permits a better penetration of the antibodies. Comparing to IHC on larvae, the protocol for adult worms also involves longer incubation time and more intensive washings. As internal references, I included anti-acetylated tubulin antibodies to highlight stable microtubules, as they occur in axons [142, 104] and DAPI to highlight the nuclei (Figure 9). As a result, I was able to perform IHC on whole adult animals with the newly established protocol.

As negative control, the primary antibody was preadsorbed with excess of recombinant full-length Pdu-Hiomt protein before using it in immuno histochemistry (protein expression and production see Section 4.4.1). The total absence of green fluorescent signal in the negative controls confirms the specificity of the antibody. IHC analysis revealed specific stainings in the brain region of adult *Platynereis*, highlighting three distinctive types of staining (Figure 9): 1. two groups of large-sized neurons with respective visible projections located dorsally posterior to the eyes (yellow arrow heads); 2. Two groups of small neural cell bodies near the posterior eyes (red arrow heads); 3. A group of small dots between the posterior eyes (yellow arrow).

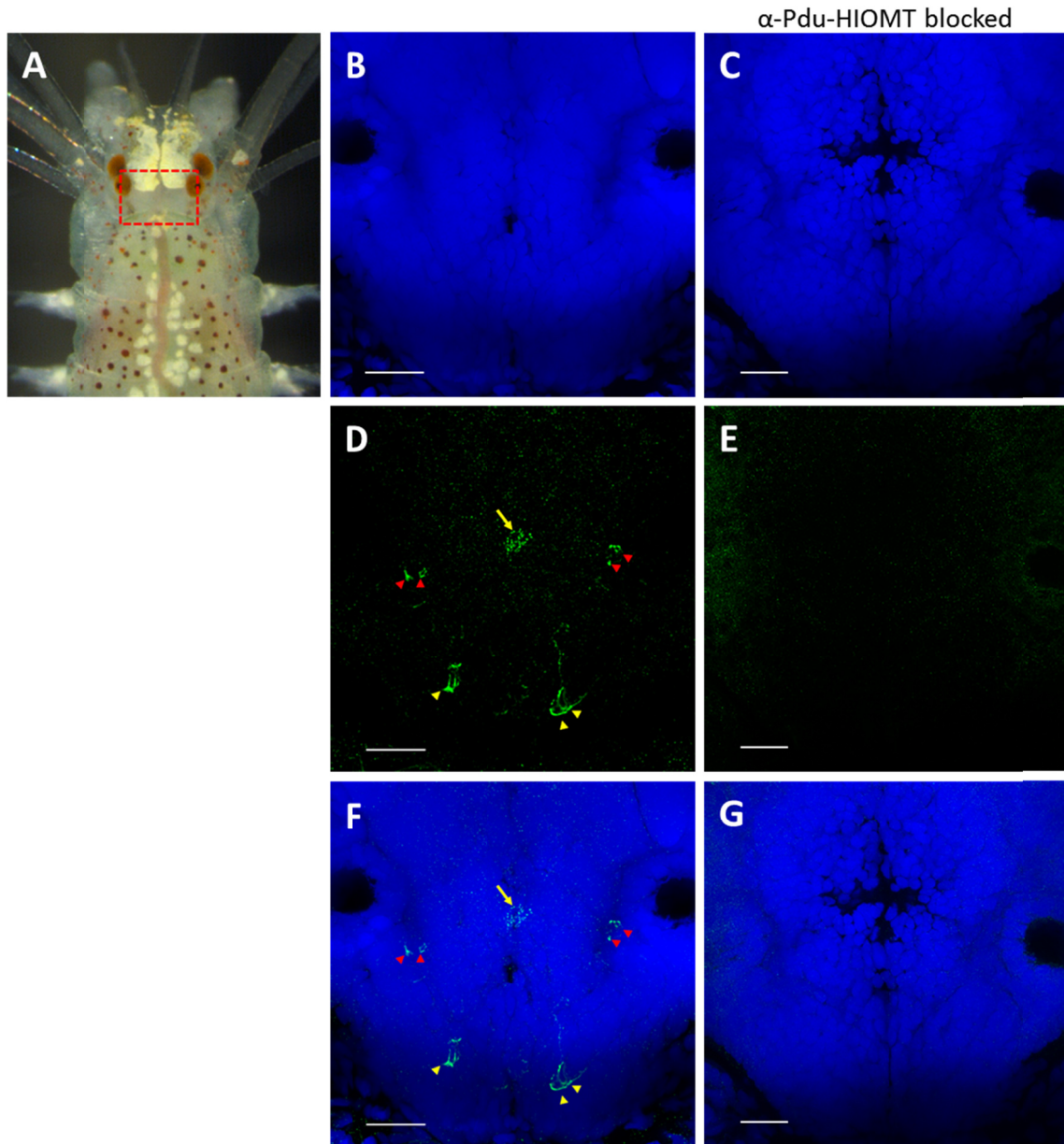


Figure 9. Hiomt expression in the brain region of adult *Platynereis*

A) Head of *Platynereis* adult animal, red dashed square shows the region scanned in B) – G). B, C) nuclei (blue); D, E) Pdu-Hiomt (green). F, G) merge. C, E, G) Primary antibody blocked with recombinant Pdu-Hiomt. Scale bar: 20µm. All images are in dorsal view. Arrows and arrow heads point to the specific staining identified, see text for detail.

Visible neurites stained together with the cell bodies support the neuronal nature of the highlighted cells. Absence of co-localization with DAPI staining indicates the localization of this enzyme in the cytoplasm. Interestingly, the third type of staining consists of small punctuated patterns too small to be cell bodies. These stainings are located deeper from the dorsal side, in the region of the infra-cerebral gland. As this region is also believed to contain endings of longer projections that belong to cell bodies in the dorsal medial brain, it is well possible that these signals reflect sub-localized Hiomt protein in the neurites of some of the Hiomt-positive cell bodies.

4.2.2 Localization of *Platynereis* Aanat

Another important element of the melatonin biosynthesis pathway is Aanat, catalyzing the acetylation of serotonin to produce N-acetyl-serotonin, the direct precursor of melatonin. Therefore I also carried out IHC analysis using antibodies against Pdu-Aanat. The first trials using polyclonal antibodies against the N-terminus of Pdu-Aanat (aa1-94) raised in rabbit did not give specific staining in IHC. Subsequently we engaged to raise monoclonal antibody in collaboration with the Ogris group (Max F. Perutz Laboratories) against N-terminus of Pdu-Aanat (aa1-85) in mouse. Antisera from each bleed and each separated clone were tested both with western blot on recombinant Pdu-Aanat as well as *Platynereis* head lysate and IHC on adult whole animals. The clone tested positive in western blot and showed specific staining in IHC was chosen for generating the final monoclonal antiserum. Preliminary IHC data against Pdu-Aanat revealed single neuronal cells (Figure 10, yellow arrow) as well as neurites in the brain regions between the eyes (Figure 10, yellow arrow heads). However, further experiments need to be carried out in order to confirm the specificity of the staining and to draw further conclusions.

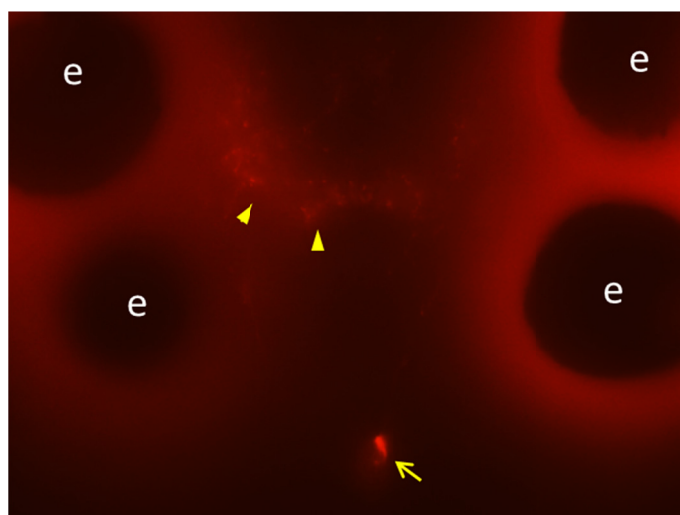


Figure 10. Aanat expression in the brain region of adult *Platynereis*

Dorsal view of IHC against Pdu-Aanat in the brain region of *Platynereis*. Red: Pdu-Aanat. Yellow arrow and arrow heads point to the identified stainings. e: adult eyes.

4.3 Lunar regulation of melatonin biosynthesis pathway

4.3.1 Melatonin fluctuation under different moon phases

One of our hypotheses is that melatonin serves as a time-measuring molecule also for lunar periodicities, encoding the nocturnal light intensity from different moon phases. To test this hypothesis, I sampled heads of *Platynereis* at 6 different time points distributed throughout a 24-hour day, under new moon (NM), full moon (FM) and free running full moon (FR-FM) illuminations

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respectively. In order to compensate individual size differences, melatonin amount was normalized to the total RNA of the tissue.

To test if melatonin is regulated by the circalunar clock, I compared the endogenous melatonin profile under NM and FR-FM conditions. By applying one-way ANOVA test, I found melatonin showing tendency of fluctuation during a NM day (ANOVA test $p=0.08$, $\text{mean}\pm\text{SD} = (0.040\pm0.016 - 0.085\pm0.022)$ pg/ μgRNA , Figure 11A, blue), reaching its highest concentration at the end of the scotophase and the beginning of photophase, whereas during FR-FM, melatonin concentration remained constant throughout the day (ANOVA test $p=0.92$, $\text{mean}\pm\text{SD} = (0.042\pm0.012 - 0.067\pm0.002)$ pg/ μgRNA , Figure 11A, red). From these data, it can be concluded that the daily fluctuation pattern of melatonin is under endogenous control of the circalunar clock.

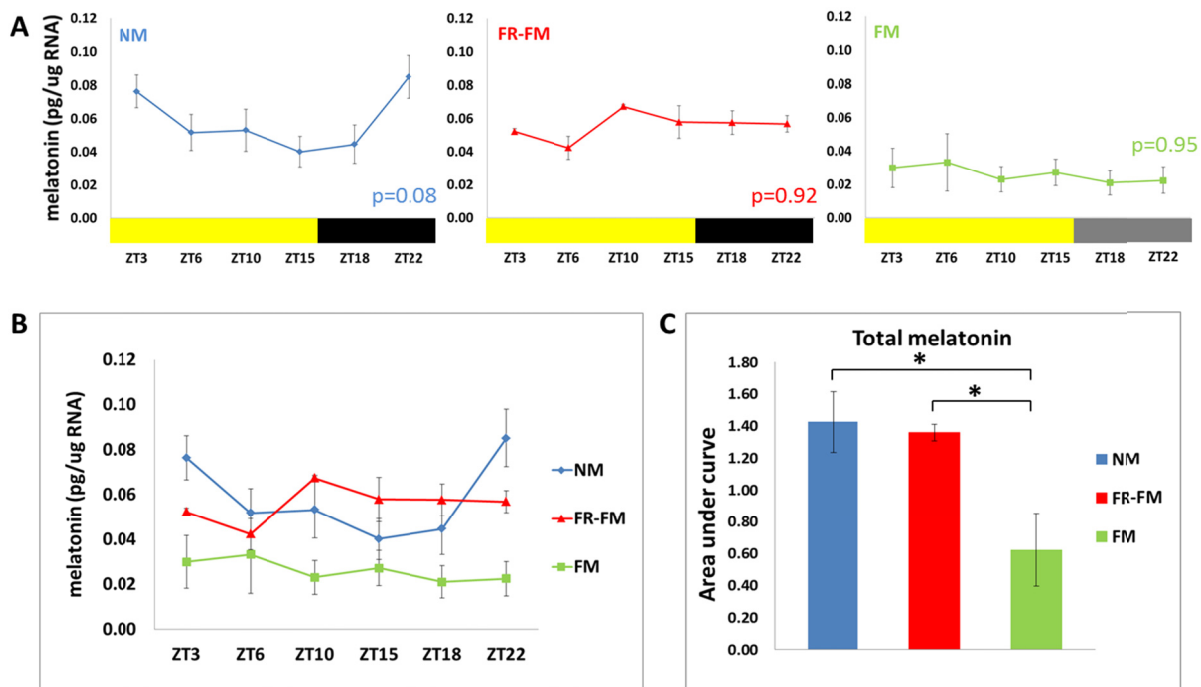


Figure 11. Endogenous melatonin in *Platynereis* is under circalunar control and influenced by light.

A) Daily melatonin concentration profile under NM (blue), FR-FM (red) and FM (green) illumination. Y-axis: normalized melatonin concentration (pg/ μg total RNA). ZT: *Zeitgeber* Time. Yellow bars and black bars below indicate the photophase and scotophase respectively. Grey bar indicates dim-light illuminated night during artificial FM. The p values were calculated with one-way ANOVA test. B) Integrated view of the three graphs in (A). C) Overall daily melatonin amount calculated as area under the curve based on the data shown in (A). Values are means $\pm$ SEM. NM: n=3; FM: n=4, FR: n=3. Each replicate contained 15 heads. * $p<0.05$.

Comparably, melatonin levels were also found to be constant under FM through the course of the day (ANOVA test $p=0.95$, $\text{mean}\pm\text{SD} = (0.021\pm0.015 - 0.033\pm0.035)$ pg/ μgRNA , Figure 11A, green). However, the overall level of melatonin is significantly lower under FM conditions (Figure 11B). I

estimated the overall production of melatonin by calculating the area under the curve of the melatonin concentration, and observed a more than 2-fold decrease of melatonin under FM conditions, whereas no significant difference was observed between NM and FR-FM melatonin amount (Figure 11C). These observations indicate an influence from the nocturnal light on melatonin production in addition to the endogenous circalunar clock control.

4.3.2 Endogenous enzymatic activity of *Platynereis* Hiomt

In order to further understand the regulation of melatonin synthesis, I attempted to measure the endogenous enzymatic activity of Pdu-Hiomt for the methyl transfer from SAM to NAS. As a positive control, I used a cell lysate containing human Hiomt (Hs-Hiomt) (described in detail in Section 4.4.2). *Platynereis* heads were sampled and homogenized on ice, and the supernatant of the homogenate was equally divided into two aliquots. Hs-Hiomt cell lysate was spiked into one aliquot of the supernatant of the extracted *Platynereis* heads to control for protein degradation, and enzymatic reactions were carried out under same conditions. The resulting melatonin amount was measured with HPLC-MS/MS and calculated as percentage of melatonin resulting from the positive control (Figure 12). Positive control showed significant amount of melatonin yield, and spiked samples also retained most of the enzymatic activity of Hs-Hiomt, indicating the intactness of the enzyme. No melatonin was detected from *Platynereis* head homogenates. Thus under my experiment conditions I was not able to measure the enzymatic activity of endogenous Pdu-Hiomt activity, leaving it inconclusive if the circalunar clock regulation of melatonin production happened at the enzyme activity level.

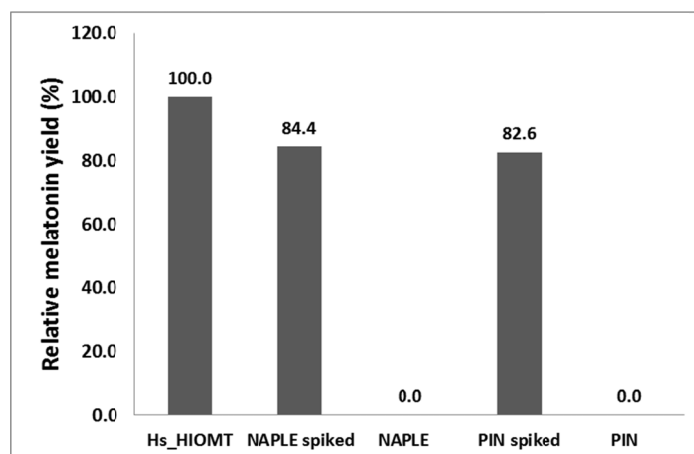


Figure 12. Relative enzymatic activity of endogenous Pdu-Hiomt

Melatonin yield in enzymatic assays with *Platynereis* head lysate was calculated as percentage of the yield from the positive control assay with Hs-Hiomt. NAPLE: wildtype worms collected in Ischia, PIN: inbred worms strains from our *Platynereis* culture. Spiked: Animal homogenate spiked with Hs-Hiomt cell lysate.

4.3.3 Gene expression of *Pdu-aanat* and *Pdu-hiomt*

A change of melatonin dynamics, as observed between NM and FR-FM, could be caused on different levels. One of the levels is the transcription of the melatonin producing enzymes. I sought to explore if the melatonin synthesis was regulated by altering the transcript level of the two critical enzymes Aanat and Hiomt, and to investigate in *Platynereis*, which one plays a more critical role in regulating melatonin production. Therefore I measured the transcript level of *Pdu-aanat* and *Pdu-hiomt* with quantitative RT-PCR, using the total RNA extracted from the samples for melatonin measurement (described in Section 4.3.2). Prostomia and peristomia samples were analyzed separately and daily variances were analyzed with one-way ANOVA test. To test if the transcript levels of these two genes are under endogenous circalunar clock control, I first compared the mRNA levels of *Pdu-aanat* and *Pdu-hiomt* under NM and FR-FM conditions respectively. While the transcript level of *Pdu-aanat* showed similar mild fluctuation profile under both NM and FR-FM conditions (Figure 13A, B), the transcript level of *Pdu-hiomt* showed steady fluctuation under NM condition (ANOVA test $p < 0.005$, Figure 13C, D, blue curve) reaching the highest level at ZT 15 and lowest level at ZT 6, but remained constant throughout the day under FR-FM (ANOVA test $p > 0.5$, Figure 13C, D, red curve).

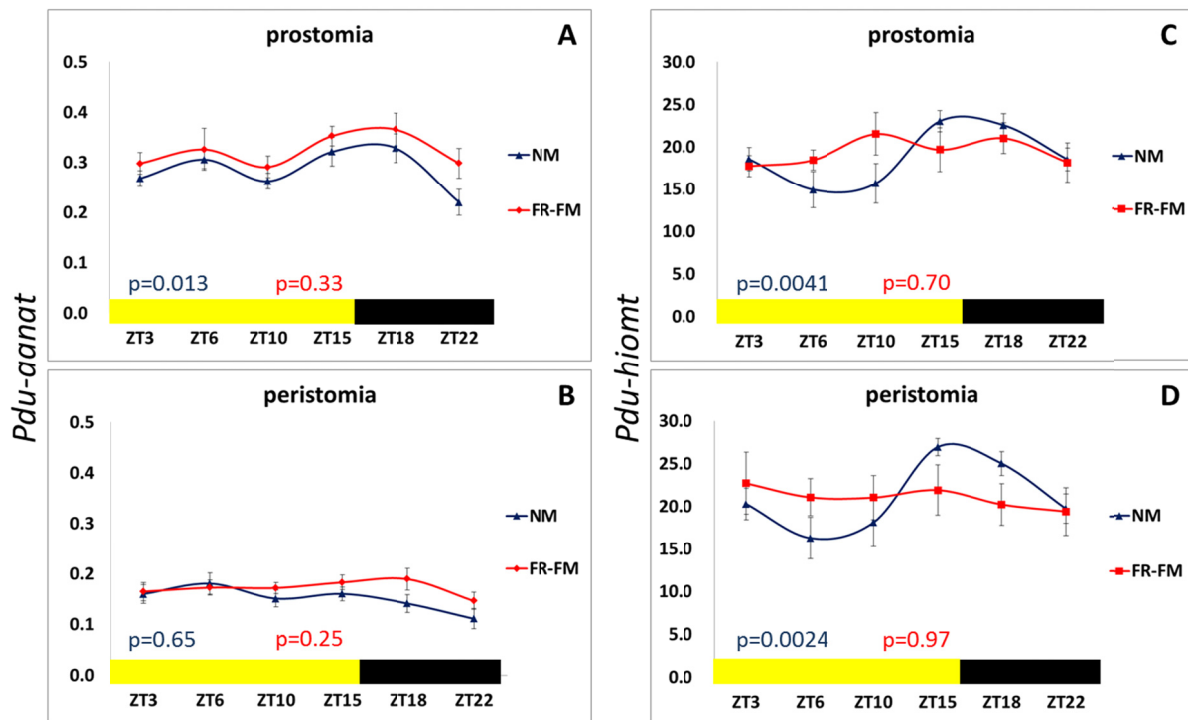


Figure 13. *Pdu-hiomt* transcript level is under circalunar control.

Temporal profiles of A,B) *Pdu-aanat* and C,D) *Pdu-hiomt* RNA expression in *Platynereis* heads during NM (blue) and FR-FM (red) at indicated ZT. ZT: *Zeitgeber* Time. Y-axis: relative RNA levels normalized to house-keeping genes. *Pdu-aanat* was normalized to *rps9* and *Pdu-hiomt* was normalized to *cdc5*. Yellow bars and black bars below indicate the photophase and scotophase respectively. Values are means $\pm$ SEM. P values represent the significance from ANOVA test for the curve in the same color. NM: n=9; FR-FM: n=9. Each replicate contained 5 heads.

Evidently, the transcript level of *Pdu-hiomt* is under endogenous circalunar clock control, whereas a circalunar clock regulation on *Pdu-aanat* is disputable. For this reason, more emphasis was placed on characterizing *Hiomt* at genetical, transcriptional and protein levels.

In vertebrates, melatonin is regulated by circadian clocks, but direct light exposure also suppresses melatonin immediately, by an acute decrease in *Aanat* activity [70]. In *Platynereis*, melatonin level was also found to be influenced by nocturnal light in addition to the endogenous circalunar clock regulation (Section 4.3.2). I therefore sought to investigate if *Pdu-aanat* and *Pdu-hiomt* gene expressions were also influenced by nocturnal light exposure. I compared the FM and FR-FM transcript levels of *Pdu-aanat* and *Pdu-hiomt*. For *Pdu-aanat*, the transcript level under FM was found to be mildly fluctuating especially in the prostomia (ANOVA test $p=0.042$, Figure 14A, green curve), with the highest value at the beginning of the dim-light illuminated night. For *Pdu-hiomt*, the transcript level in prostomia remained relatively constantly during a FM day, comparable with FR-FM profile. However, the expression profile of *Pdu-hiomt* in the peristomia under FM conditions showed steady fluctuation (ANOVA test $p=0.0058$, Figure 14D), with a decrease in the artificially illuminated night period.

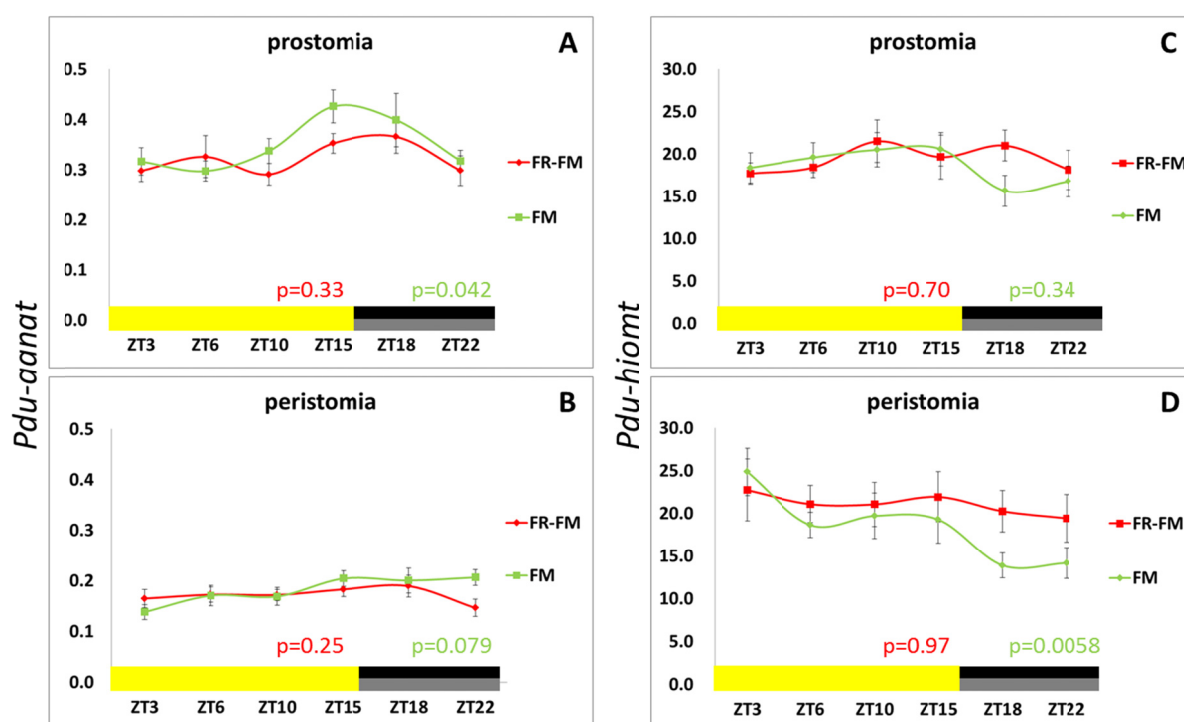


Figure 14. Light impact on *Pdu-aanat* and *Pdu-hiomt* transcript level

Temporal profiles of A,B) *Pdu-aanat* and C,D) *Pdu-hiomt* RNA expression in *Platynereis* heads during FR-FM (red) and FM (green) at indicated ZT. ZT: Zeitgeber Time. Y-axis: relative RNA levels normalized to house-keeping genes. *Pdu-aanat* was normalized to *rps9* and *Pdu-hiomt* was normalized to *cdc5*. Yellow bars and black bars below indicate the photophase and scotophase respectively. Grey bar indicates dim-light illuminated night during artificial FM. Values are means $\pm$ SEM. P values

represent the significance from ANOVA test for the curve in the same color. FM: n=12, FR-FM: n=9. Each replicate contained 5 heads.

These results indicate a potential influence of light on the transcription of *Pdu-aanat* and *Pdu-hiomt*, with differentiated regulation among *Pdu-hiomt* expressing cells, predominately in the peristomium.

4.4 Further characterization of *Platynereis* Aanat and Hiomt

The orthology of *Platynereis aanat* and *hiomt* was confirmed *in silico* with various methods (see Section 4.1.3), but this orthology does not, by itself, prove that the encoded proteins have the respective enzymatic activity. Measuring the enzymatic activity, however, could be an important way to study regulatory principles beyond the regulation of transcript abundance. I therefore attempted to study the enzymatic activity of both enzymes *in vitro*. According to my previous observation, gene expression of *Pdu-hiomt* is regulated by circalunar clock (see Section 4.3.3), indicating the potential involvement of Hiomt in regulating melatonin production, thus more effort was dedicated to the enzymatic activity of Pdu-Hiomt.

4.4.1 Expressing recombinant Aanat and Hiomt in bacteria

Numerous systems exist for protein expression, including bacteria systems, yeast systems, insect cell systems, mammalian cell systems, plants and other eukaryotic systems. A detailed analysis of the expression and purification techniques of over 10,000 different proteins was carried out with the joint efforts of various research institutes and published in a review in 2008 [146]. As suggested by the review article, I have chosen *E. coli* bacteria as the recombinant host to express Pdu-Aanat and Pdu-Hiomt.

As expression vector, I used a glutathione-S-transferase (GST) gene fusion system to express a recombinant fusion protein with a N-terminal GST moiety. *Schistosoma japonicum* GST has a molecular mass of 26kDa and can be expressed in *E. coli* with full enzymatic activity. It has a high affinity to glutathione thus can be purified from cell lysate using immobilized glutathione. The GST tag can be cleaved later on or during the purification with a site-specific protease. The pGEX-4T-2 vector that I used offers multiple cloning sites for inserting the gene of interest and the expression of the fusion protein is only under induction of isopropyl β -D thiogalactoside (IPTG), the fusion protein can be cleaved with thrombin [147].

Coding sequences of both *Pdu-aanat* and *Pdu-hiomt* were cloned into pGEX-4T-2 vector and transformed into Rosetta(DE3)pLysS Competent Cells. First I induced small-scale protein expressions with IPTG at three different temperatures and analyzed the cell lysates with SDS-PAGE. By inducing at 18°C I observed the most Pdu-Aanat and Pdu-Hiomt protein in the soluble fraction (Figure 15). So protein expression of larger amounts was carried out with induction at 18°C.

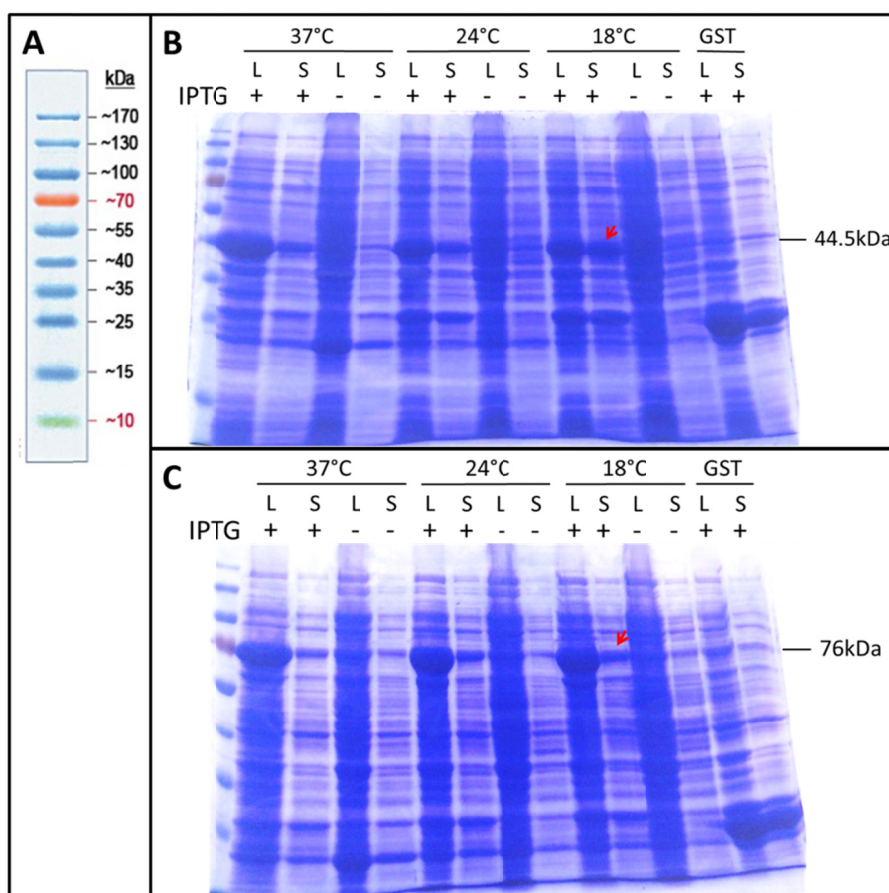


Figure 15. Optimization of the inducing temperature for Pdu-Aanat and Pdu-Hiomt expression in *E. coli*

A) Molecular masses of PageRuler Prestained Protein Ruler (Thermo Scientific). B, C) SDS-PAGE analysis stained with Coomassie Brilliant Blue with bacteria lysates (L) and supernatants (S) for B) GST tagged Pdu-Aanat (44.5kDa) and C) GST tagged Pdu-Hiomt (76kDa). IPTG (+/-): bacteria culture induced (+) or not induced (-) with IPTG. GST: pGEX-4T-2 vector without insert induced at 37°C, over-expressing GST. Protein amount loaded in each lane: 1/200 of total volume. Red arrow points at the band of the most soluble over-expressed protein.

Recombinant proteins were affinity purified using Gluthathione Sepharose 4B beads in a batch procedure and samples were taken at each step of purification to be analyzed with SDS-PAGE (Figure 16). Both Pdu-Aanat and Pdu-Hiomt were successfully expressed and purified, with a yield of around 6.7mg of recombinant Pdu-Aanat and 2.2mg of recombinant Pdu-Hiomt per one liter of bacteria culture. Enzymatic assays with both recombinant proteins were carried out, but no enzymatic activity was observed.

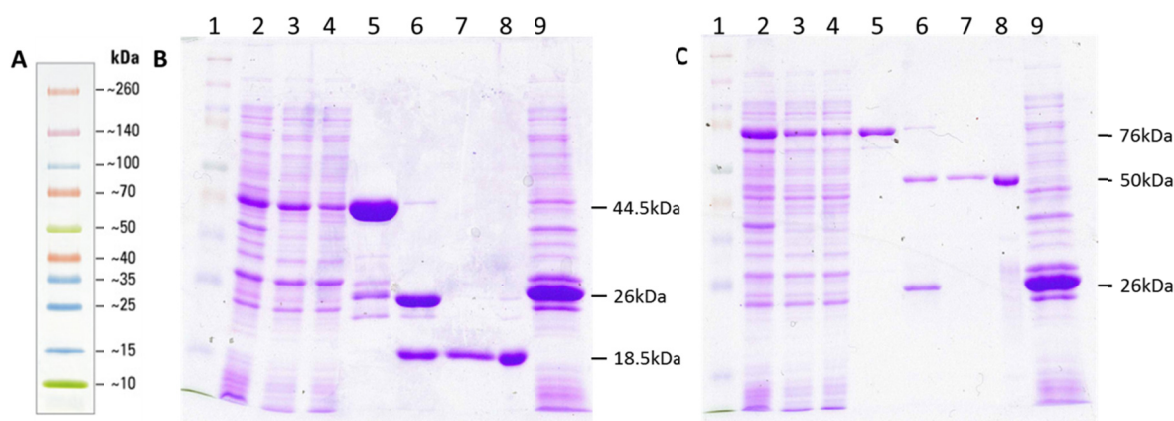


Figure 16. Purification of recombinant proteins expressed in *E. coli*

A) Molecular masses of Spectra Multicolor BR Ladder (Thermo Scientific). B,C) SDS-PAGE analysis stained with Commassie Brilliant Blue with samples reserved at different purification steps for affinity-purification of recombinant B) Pdu-Aanat (18.5kDa); C) Pdu-Hiomt (50kDa). 1. Ladder; 2. Bacteria lysate (1/80000 of total volume); 3. Supernatant of bacteria lysate (1/80000); 4. Supernatant not bound to glutathione beads (1/80000); 5. Protein bound to glutathione beads (1/2500); 6. Glutathione beads cleaved with thrombin (1/5000); 7. Supernatant after cleavage (1/5000); 8. Concentrated supernatant after cleavage (Pdu-Aanat: 1/4000; Pdu-Hiomt: 1/2200); 9. Bacteria lysate expressing GST (26kDa).

4.4.2 Using Hiomt expressed in mammalian cell culture

In the absence of enzymatic activity with bacterially expressed Pdu-Aanat and Pdu-Hiomt under our assay conditions, I sought to include a positive control into the enzymatic assay to validate the assay conditions. Botros *et al.* [148] have used a mammalian cell expression system with COS-7 cells to produce small amount of active human Hiomt (Hs-Hiomt) protein for enzymatic assays. Upon my request, the research group kindly sent us the pcDNA-DEST47 vector containing active Hs-Hiomt CDS for expression in mammalian cells. I have cloned Pdu-Hiomt into the pcDNA-DEST47 vector using the Gateway TOPO cloning technology and expressed Pdu-Hiomt in COS-7 cells. The pcDNA-DEST47 vector offers the possibility of expressing GFP-fusion proteins, so I have expressed both the native Pdu-Hiomt protein by including the stop codon in the CDS and the Pdu-Hiomt-GFP fusion protein by omitting the stop codon. The GFP-fusion protein served to monitor the protein expression. As negative control, I used a C-terminal GFP-fused oxytocin receptor in pEGPF-N3 vector which should not possess methyl-transferase activity but could serve as a monitor of the transfection rate (Figure 17).

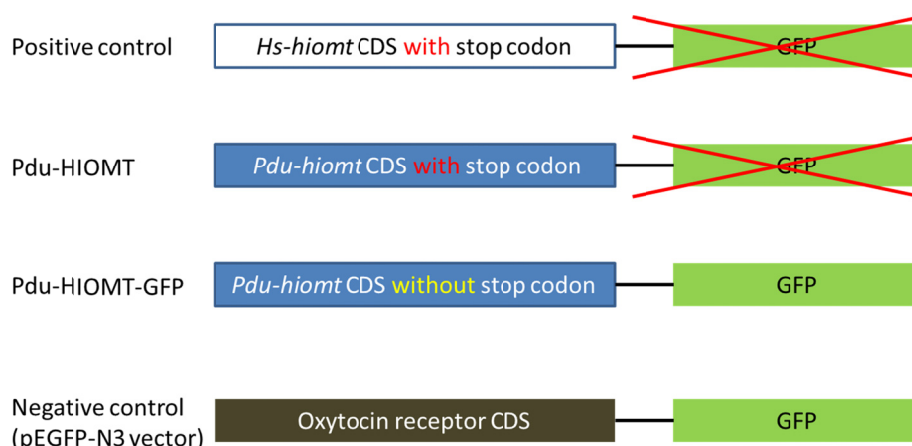


Figure 17. Scheme of the constructs cloned into pcDNA-DEST47 vector for expression in mammalian cells

Positive control: *Hs-hiomt* CDS including the stop codon and an extra nucleotide to cause frame-shift in GFP coding sequence, generating native Hs-HIOIMT protein. Pdu-Hiomt: *Pdu-hiomt* CDS including the stop codon and an extra nucleotide to cause frame-shift in GFP coding sequence, generating native Pdu-HIOIMT protein. Pdu-Hiomt-GFP: *Pdu-hiomt* CDS excluding the stop codon and in frame with the GFP coding sequence, generating C-terminal GFP fused Pdu-HIOIMT protein. Negative control: C-terminal GFP-fused oxytocin receptor in pEGFP-N3 vector.

GFP signal was recorded for GFP-fused Pdu-Hiomt (Figure 18D) and GFP-fused oxytocin receptor (Figure 18B) 24 hours after transfection, confirming the successful transfection and the expression of GFP-fused proteins. Western blot analysis with aliquots of cell lysates using polyclonal antibody against Pdu-Hiomt confirm the integrity of enzyme proteins used in the enzymatic assays (Figure 18E). Cell lysates of COS-7 cell culture transfected with different constructs were used as catalyst in the enzymatic assays to detect Hiomt activity. Whereas Hs-Hiomt converted significant amount of NAS to melatonin (Figure 19), no melatonin was detected in either Pdu-Hiomt or GFP-fused Pdu-Hiomt catalyzed reactions.

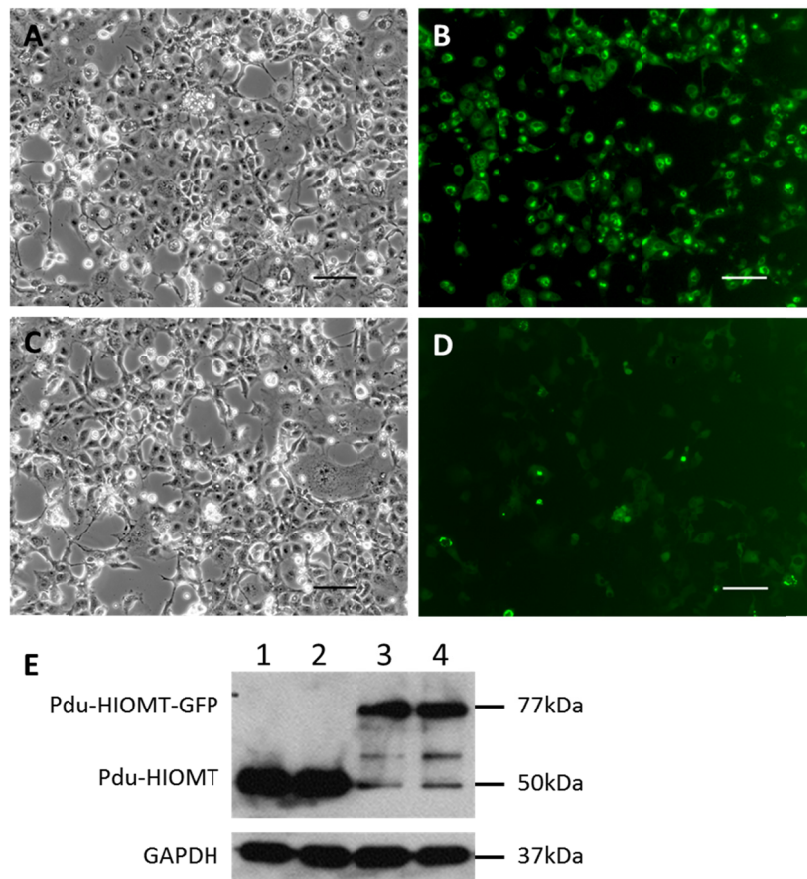


Figure 18. Expression of Pdu-Hiomt in mammalian cells

A-D) GFP expression in transfected COS-7 cell culture 24 hours after transfection. A, B: Oxytocin receptor-GFP. C, D: Pdu-Hiomt-GFP. A,C: light field; B,D: GFP. Scale bar: 100 μ m. E) Western blot with custom raised Pdu-Hiomt antibody on COS-7 cell lysate transfected with Pdu-Hiomt constructs, the transfections were carried out in duplicates. 1,2: Pdu-Hiomt (50kDa); 3,4: Pdu-Hiomt-GFP (77kDa).

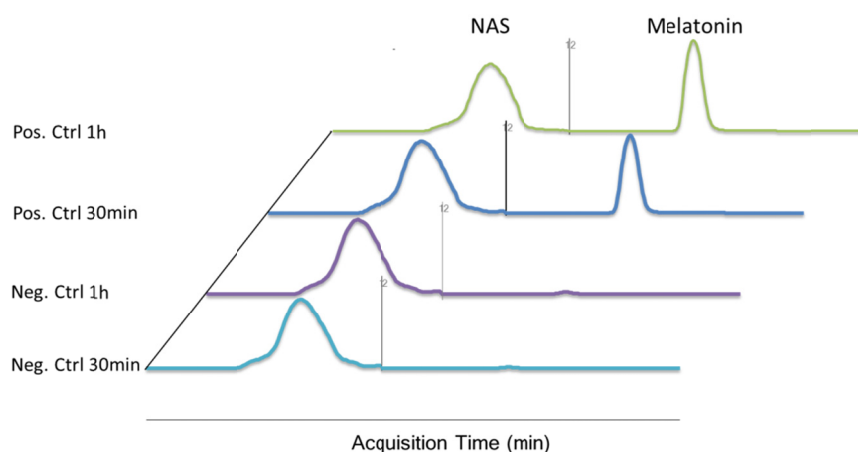


Figure 19. HPLC-MS/MS detection of NAS and melatonin from positive and negative control-reactions

Positive control: Hs-Hiomt, negative control: oxytocin receptor. 30min, 1h: reaction time. Reactions with Pdu-Hiomt resemble the negative control (not shown).

4.4.3 Using chimeric proteins expressed in mammalian cell culture

In absence of any detectable enzymatic activity with Pdu-Hiomt expressed in mammalian cells, I sought to understand the structural difference between Hs-Hiomt and Pdu-Hiomt. The crystal structure of the active isoform of Hs-Hiomt has been published in the beginning of 2013 [148] (PDB ID: 4A6E, 4A6D), allowing a structure prediction of Pdu-Hiomt. A threading model of Pdu-Hiomt was generated by SWISS-MODEL (<http://swissmodel.expasy.org/>) by Gang Dong (Max F. Perutz Laboratories) based on the published structure of Hs-Hiomt (PDB ID:4A6E), which shares 31% identities in the N-terminal domain and 34% in the C-terminal domain (Figure 20A).

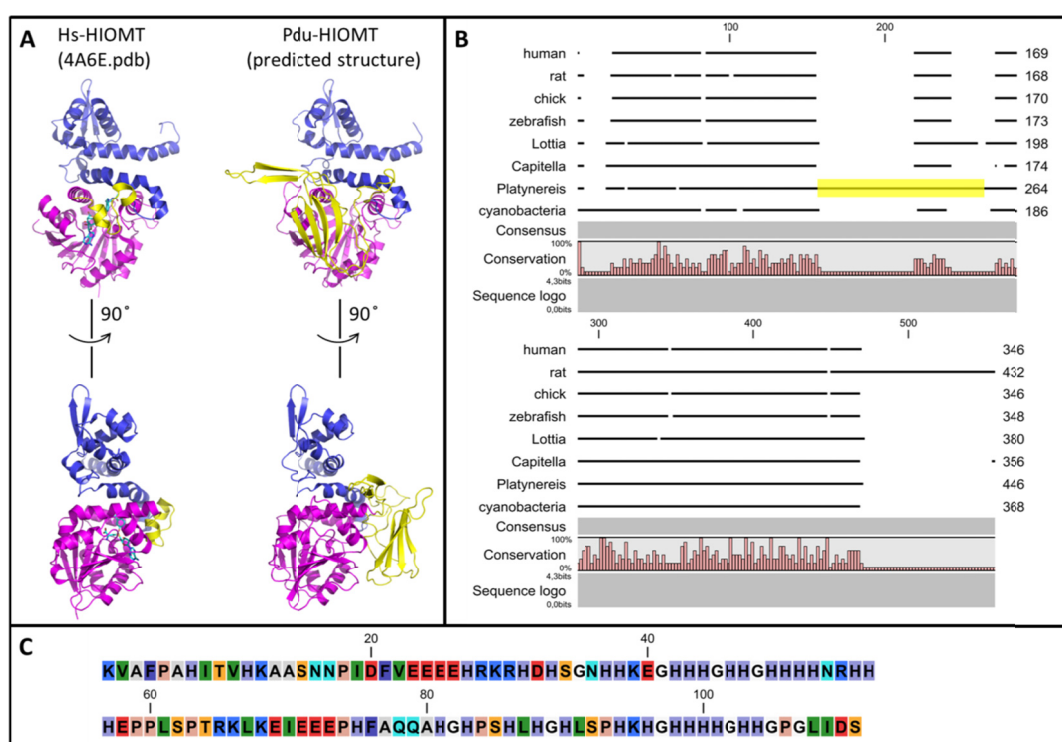


Figure 20. *Platynereis* Hiomt possess a long linker domain rich in acidic amino acids and histidine.

A) Crystal structure of human Hiomt in complex with S-adenosyl methionine (SAM) and NAS (left) and the predicted *Platynereis* Hiomt (right). Blue: N-terminal domain; pink: C-terminal domain; yellow: linker domain. B) Alignment of Hiomt sequences. Yellow bar highlights the linker domain of Pdu-Hiomt. Annotated sequences (human, rat, chick, cyanobacteria) were retrieved from NCBI database (<http://www.ncbi.nlm.nih.gov/>): human (*Homo sapiens*) P46597.1; rat (*Rattus norvegicus*) B3GSH5.1; chick (*Gallus gallus*) Q92056.1; zebrafish (*Danio rerio*) NP_001103947.1; cyanobacteria (*Trichodesmium erythraeum* IMS101) YP_722726.1. *Lottia* sequence were retrieved by BLASTP against NCBI database (<http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi>) using human Hiomt isoform 1 sequence P46597.1, his with highest score were taken: *Lottia* (*Lottia gigantea*) ESP00347.1; *Capitella* (*Capitella capitata*) sequence was retrieved by BLASTP against online Capitella resource (<http://genome.jgi-psf.org/pages/blast.jsf?db=Capca1>), hit with the highest score was taken: 173689. C) Pdu-Hiomt linker domain, amino acid residues are color-coded with Rasmol colors. Bright red: Asp, Glu; pale blue: His.

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Whilst the predicted structure of Pdu-Hiomt showed great similarity to Hs-Hiomt in the N-terminal and C-terminal domains containing the substrate binding sites and active center, the linker between the N-terminal and C-terminal domain of Pdu-Hiomt (110aa) was found to be much longer than in the human ortholog (19aa). By aligning the Pdu-Hiomt sequence to its orthologs in other organisms, the long linker domain was found to be specific to *Platynereis*. Neither any vertebrate ortholog nor the ortholog in *Capitella capitata* or cyanobacteria possess a similar linker domain (Figure 20B). While looking in detail into the long linker domain, it was found that it is rich in acidic amino acids (Asp, Glu) and Histidine (His) (Figure 20C).

I therefore sought to eliminate the linker domain of Pdu-Hiomt to test the functionality of the conserved enzymatic active center, thus a chimeric protein was designed by exchanging the specific linker domain of Pdu-Hiomt with the short linker of Hs-Hiomt (Figure 21), resulting in a *Platynereis-homo-Platynereis* chimera (PHP chimera). Chimeric protein sequence was generated by two round of PCR and cloned into pcDNA-DEST47 vectors for mammalian cell expression. GFP-fused protein was also generated to monitor the transfection rate and the integrity of the upstream protein. GFP signal was observed 24 hours after transfection, and protein expression was also confirmed by western blot analysis. But no melatonin was detected from the enzymatic reactions using the chimeric proteins. In summary, to date I was not able to show the enzymatic activity of *Platynereis* Aanat and Hiomt *in vitro*.

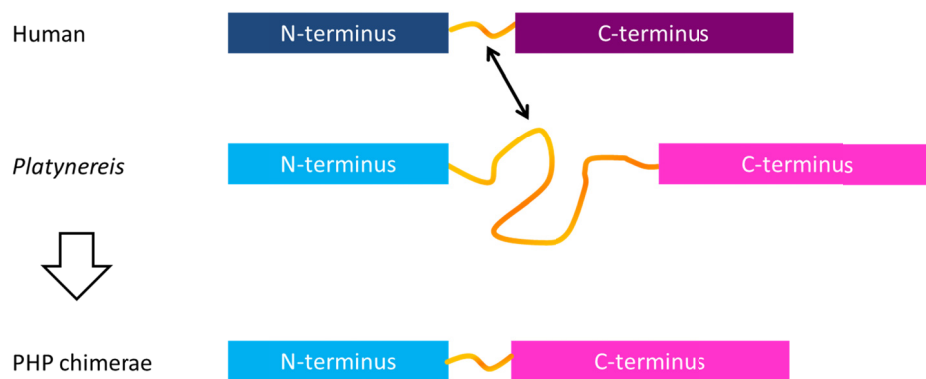


Figure 21. Design of *Platynereis*-human chimeric Hiomt protein

4.5 Generating genetic strains to dissect the role of the melatonin biosynthesis pathway

As outlined, my efforts to reconstitute the melatonin biosynthesis pathway *in vitro* appeared to be less successful, but from this negative result, it was not possible to make a statement if or if not Aanat and Hiomt were involved in producing melatonin in the worm. As an alternative approach, I therefore decided to assess if ablation of the respective genes – or chemical ablation of the respective cells –

abrogated melatonin production in the worm. This approach was made possible by the recent establishment of transposon-based transgenesis [113] and TALEN-mediated mutagenesis techniques [139] for *Platynereis* in our lab.

4.5.1 Efforts in generating transposon-based *Pdu-aanat* and *Pdu-hiomt* transgenic lines

I used EGFP-F2A-NTR-SV40pA-rpsLneo cassette to generate reporter constructs for *Pdu-aanat* and *Pdu-hiomt*. The cassette contains an EGFP coding sequence linked to a bacterial nitroreductase (NTR). The EGFP serves to label the cells expressing *Pdu-aanat* or *Pdu-hiomt* and NTR moiety encodes the enzyme that converts metronidazole to a cytotoxic DNA-crosslinking agent [149]. BACs containing *Pdu-aanat locus* (CH305-102P16) and *Pdu-hiomt locus* (CH305-201G23) were identified [99] and the targeting cassette was inserted into the first exon of both genes by BAC recombineering.

In order to trigger the expression of the reporter cassette genes, it is essential to include the promoter region into the reporter construct. For *Pdu-aanat*, genomic regions of 7kb upstream and 6.6kb downstream of the coding sequences were initially designed to be included in the reporter construct. However, the resulting whole construct is too big in size (19kb) and was difficult to be amplified and sub-cloned. Alternative constructs were designed including less sequences up- and downstream of the coding sequence resulting in reporter constructs of 12kb and 6.4kb were successfully cloned into the transposon vector. Similarly for *Pdu-hiomt*, constructs including a genomic region of 1kb upstream and different lengths downstream of the coding sequence were designed with the size of 14kb, 11.6kb and 9.8kb respectively. The longest construct could not be amplified and sub-cloned thus only the two smaller constructs were cloned into the transposon vector. The construct of 11.6kb was further tested for transgenic efficiency which included a region of 385bp downstream of the coding sequence.

Tol2 and Mos1 transposon vectors were both successfully used for generating reporter constructs for other genes in *Platynereis* [113]. While Tol2 transposon induced genome integration demonstrated decent transient expression, the Mos1 transposon induced genome integration was shown to be advantageous for germ line transmission and thus creating a transgenic line [113]. I sub-cloned the reporter constructs into both Tol2 and Mos1 transposon vectors at the I-SceI site and injected the plasmid along with synthetic mRNA of the corresponding transposase into *Platynereis* zygotes and embryos at 2 to 4-cell stage.

To confirm the functionality of the reporter construct, 5 days after injection, the Tol2 transposon injected animals were screened under a stereomicroscope with a FITC fluorescence filter (Figure 22A). While the reporter construct for *Pdu-aanat* (6.4kb) never showed any GFP signal, the larvae injected with *Pdu-hiomt* construct not only displayed GFP signal, but the signal highlighted specific cells in the brain region between the juvenile eyes. Therefore further efforts were attributed to generating trans-

RESULTS

genic animals in *Pdu-hiomt* locus. The larger constructs for *Pdu-aanat* (12kb) were not yet tested *in vivo*.

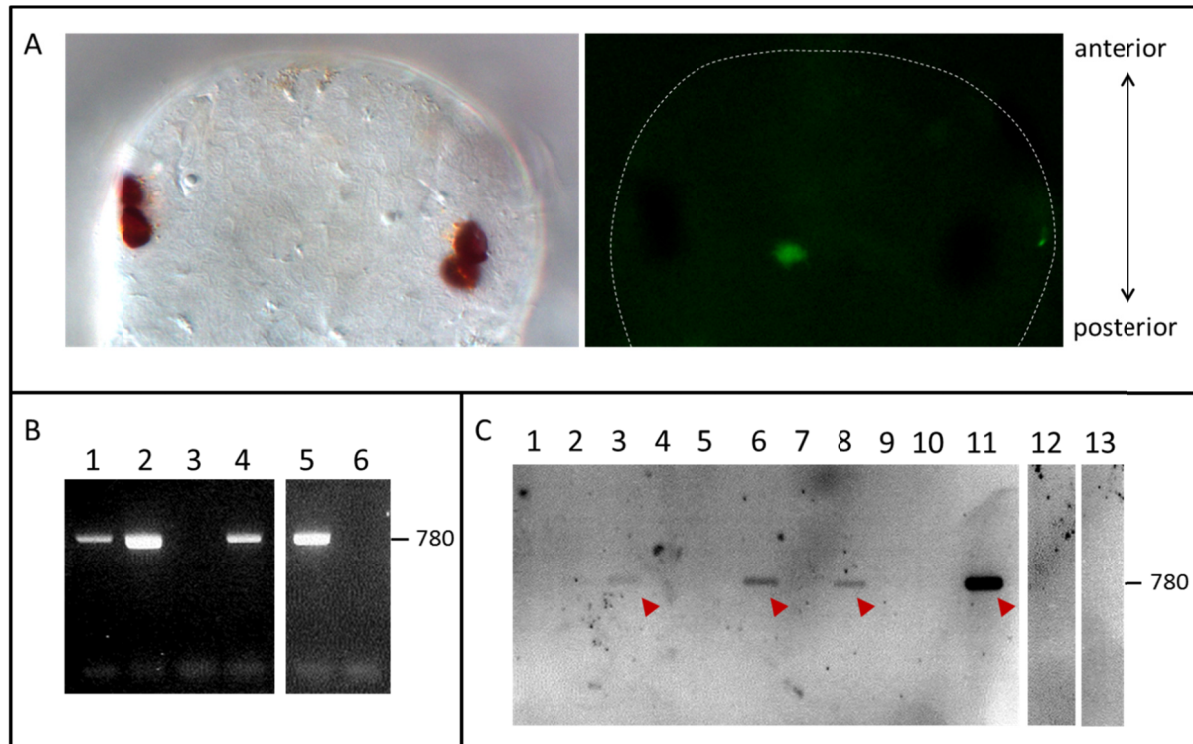


Figure 22. Tol2 and Mos1 transposon-mediated transgenesis in *Platynereis*

A) Tol2 transposon-mediated transient GFP expression in 5dpf injected larva. Dorsal view, left: light field; right, FITC. Dotted line outlines the head. B) Amplification of Tol2 transposon-mediated integration of *egfp* from genomic DNA of adult injected *Platynereis*. Lane 1-4: Tol2 transposon vector injected animals; lane 5: positive control; lane 6: H₂O. C) Amplification of Mos1 transposon-mediated integration of *egfp* from genomic DNA of adult injected *Platynereis*. Lane 1-11: Mos1 transposon vector injected animals; lane 12: uninjected animal; lane 13: H₂O. Red arrow heads highlight positive integration of *egfp*.

To compare the spatial distribution of EGFP expressing cells with Pdu-Hiomt positive cells from immunohistochemistry, injected larvae were also raised into adult animals and genomic integration of the reporter construct was assessed with PCR amplification of the integrated *egfp* moiety. The upper primer was placed in the *Pdu-hiomt* locus and lower primer was in the integrated *egfp* region. Genomic DNA from three out of the four Tol2 transposon vector injected animals analyzed were found to be *egfp* positive (Figure 22B), confirming the genomic integration of the reporter construct. However, no GFP signal was observed under microscope, which could result from weak or mosaic expression in the founder animals.

I also intended to generate a stable transgenic line with Mos1 transposon. Four out of 38 Mos1 transposon vector injected animals were also tested positive for reporter construct integration (Figure 22C), and the offspring from both positive and negative animals were raised and tested. However from the 8

batches of 94 animals tested, none showed *egfp* integration. Mos1-based reporter constructs have been successfully used to generate *alpha-tubulin* and *r-opsin* transgenic lines in *Platynereis* [111, 148], where the germline transmission rates in *Platynereis* have been observed to be between 3% and 9%. Therefore, using the existing reporter constructs, a transgenic line could be eventually recovered by further injections and genotyping.

4.5.2 Generating *Pdu-hiomt* knock-out animals with TALEN

Recently, transcription activator-like effector nucleases (TALENs) has been rapidly developed into a tool for customized genome engineering, and our lab has pioneered in using TALEN for targeted gene mutagenesis in *Platynereis* [139]. I have adopted this genomic tool to target and mutate *Pdu-hiomt*.

To ensure an efficient binding of the TALENs to the targeted genomic DNA, it is important to avoid SNPs in the TALEN binding region, therefore I genotyped the potential TALEN targeting region of *Pdu-hiomt* locus. Genomic DNA of 13 animals of B32134 strain (ancestors of PIN and VIO strain) as well as gDNA from one WT animal were used as template to amplify the genomic region including the first two exons of *Pdu-hiomt*, and the resulting PCR products were sub-cloned and further digested with restriction enzymes, resulting in two different patterns (Figure 23A). Two different alleles with 16 SNPs in the first two exons were identified by direct sequencing of selected clones, corresponding to the BAC sequence and the cDNA sequence previously cloned in pJET1.2 vector. The two alleles are therefore mentioned as “BAC allele” and “pJET allele” in this section.

Possible TALEN targeting sites were predicted by using the online TALEN designing tools [151] for both alleles separately. In order to facilitate following genotyping steps, only TALEN pairs with a restriction site in the spacer region were considered. Ideally, SNPs should be excluded from the TALEN binding site as well as the restriction site. As it was not possible to avoid SNPs completely, I have designed different right TALENs targeting the same region of the two different alleles, sharing the same left TALEN recognition site (Figure 23C). The injection solution consisted of mRNA for all three TALENs to maximize binding efficiency. Primers for the different alleles were also designed and used for genotyping (Figure 23D). An *EcoRI* restriction site was included in the spacer region of the TALEN binding sites. A TALEN-induced mutation in the spacer region could disrupt the restriction site, thus could be detected by incomplete digestion with *EcoRI* (Figure 23B).

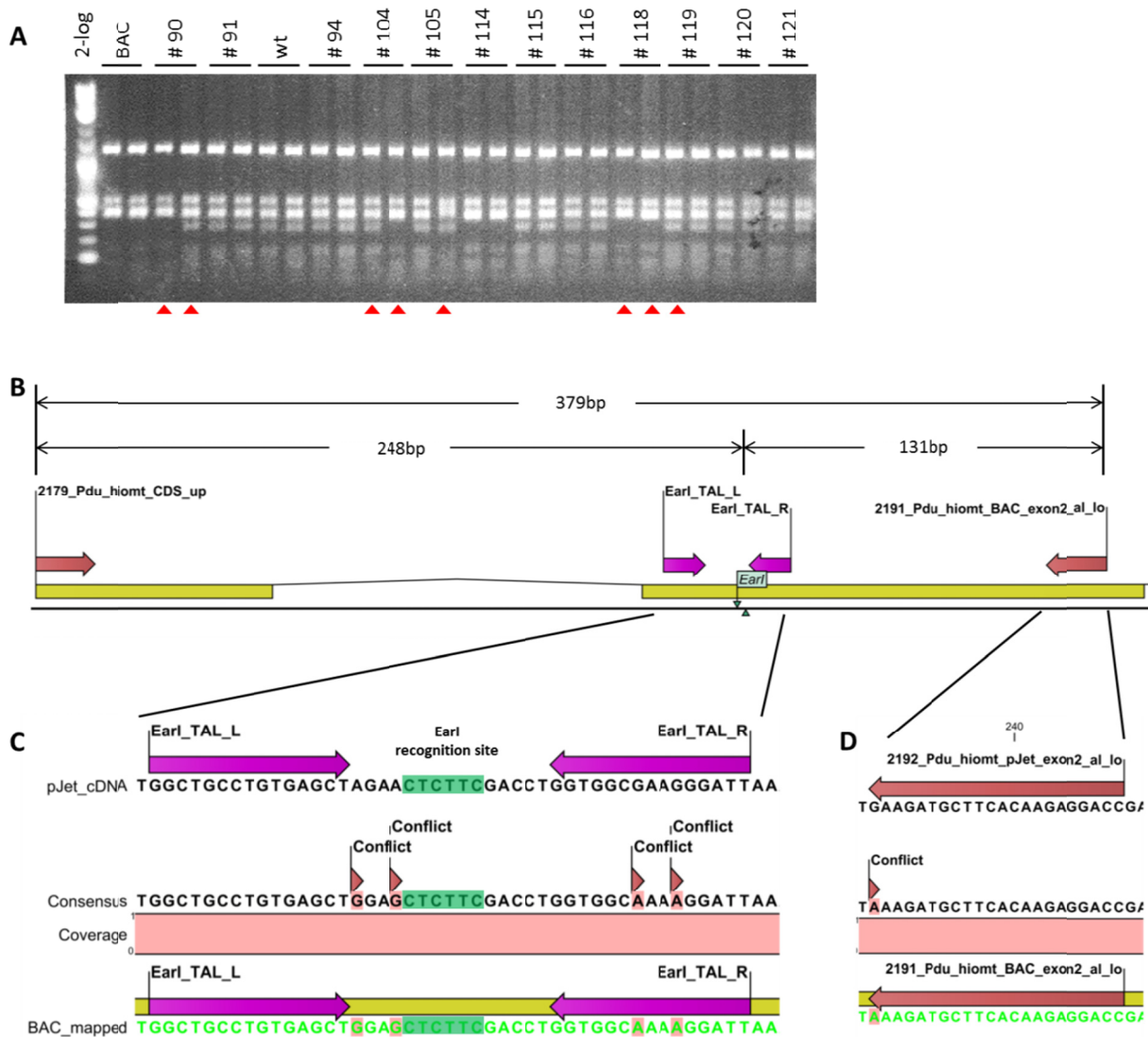


Figure 23. Design of TALENs for *Pdu-hiomt*

A) Restriction digest of plasmids inserted with *Platynereis* genomic sequences resulted in two different patterns. Red arrow heads indicates clones subjected to sequencing. B) Overview of TALEN design for *Pdu-hiomt*. Violet arrows: TALEN binding sites; red arrows: genotyping primers. Position of EarI restriction site and the sizes of genotyping PCR product before and after restriction digestion are indicated. C) Detailed view of TALEN binding sites. Violet arrows: TALEN binding sites. One left TALEN and two right TALENs were designed. Green block indicates EarI recognition site. Red arrow heads indicate SNPs. D) Detailed view of the reverse primer design for genotyping. 3' end of the primers are different by one nucleotide, enabling allele specific PCR.

PCR products from 36 samples of 2 embryos each all showed a clear uncut band while the PCR product from control samples of uninjected embryos were completely digested, indicating a very high mutagenesis efficiencies of the TALEN pair (Figure 24A). Judging from the intensities of cut and uncut bands, the estimated mutagenesis efficiency ranged between 21% and 38%. A selection of 10 uncut bands was sub-cloned and mutations were confirmed by sequencing (Figure 24B). One out of the 10

mutations showed a 5 nt deletion, leading to a premature stop codon. The TALEN pair was thus confirmed as highly efficient and could be used to generate *Pdu-hiomt* mutated animals.

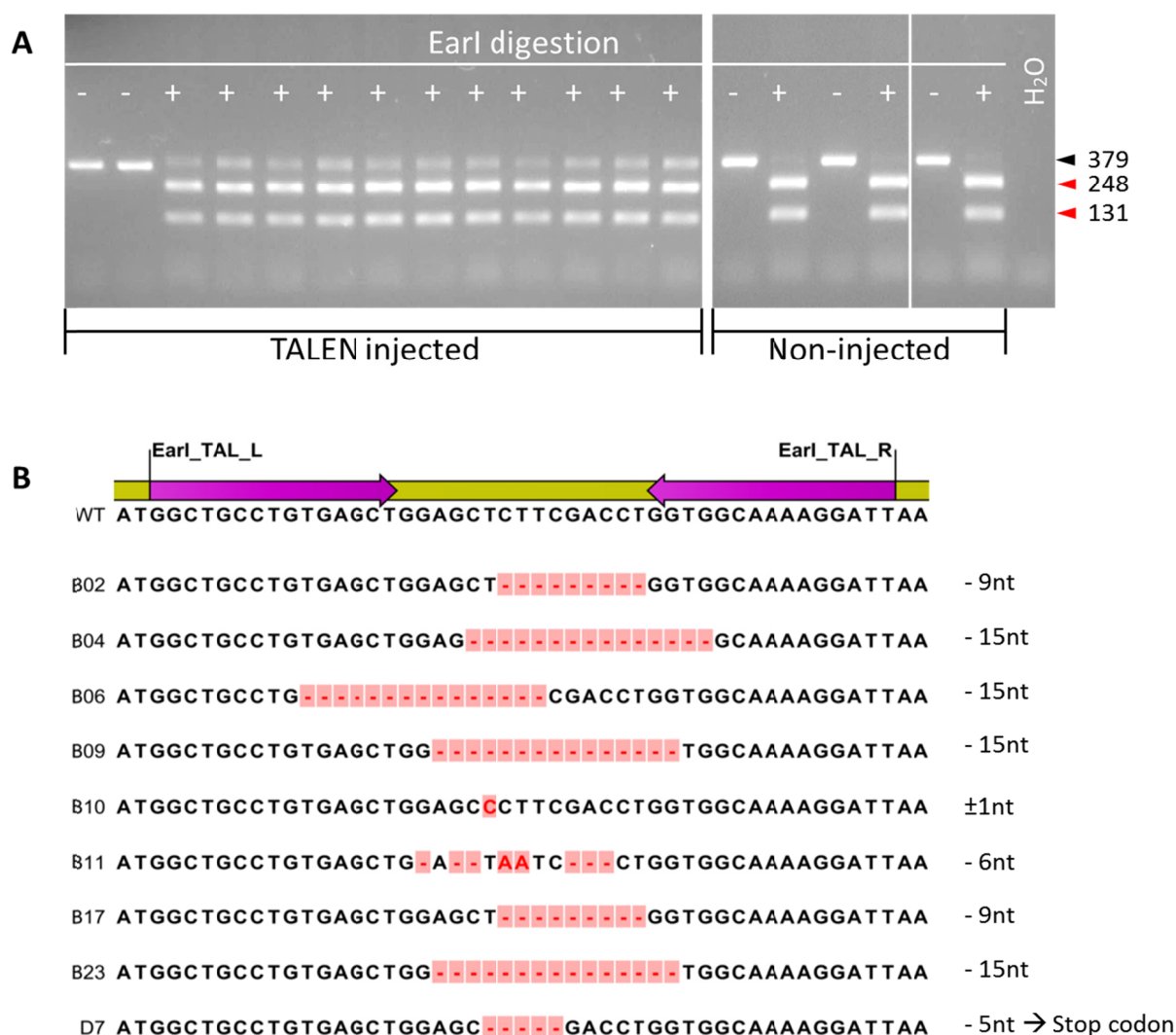


Figure 24. TALEN-mediated mutagenesis of *Pdu-hiomt*

A) Example gel image of restriction digest screening on 24hpf larvae, samples contain pools of 2 larvae, black arrow head indicates the size of uncut PCR product, red arrow heads indicate the size of EarlI restriction digest products; B) Mutations detected by sequence analysis of uncut and insertion bands. WT: wildtype sequence of BAC allele.

Further embryos were injected resulting in a total number of around 960 injected embryos. Around 373 larvae were swimming 24 hours after fertilization (39%), and 224 survived 7 days after fertilization (23%). Injected larvae were raised for breeding and screening for potential carriers, and 50 reached adulthood (5%). Part of these animals matured during the last months and were crossed with wild-type animals, and the remaining animals are expected to mature over the coming months. By analyzing resulting batches 4 batches out of the 25 batches contained heterozygote F1 animals with the germline transmission rate of 3-33%. These batches will be raised, from which later-on confirmed F1

heterozygotes can then be used for systematic incrosses to generate offspring. A comparison of homozygous (*pdu-hiomt*^{-/-}) individuals with heterozygous or wildtype siblings will then allow me to assess if *Hiomt* is indeed required for melatonin biosynthesis and if *Hiomt* (and by inference melatonin) is required for resetting the circalunar clock, and/or for mediating circalunar behavior.

4.6 A systematic approach to identify novel circalunar clock-regulated genes

The effect of the circalunar clock could be mediated by a variety of factors. Melatonin stood out as a candidate based on its role in circadian biology and in photoperiodic input into seasonal timing mechanisms, and the regulation of *Pdu-hiomt* demonstrated that the circalunar clock does target transcript levels of this gene. This result lead to the hypothesis that circalunar clock might have further target genes regulated at the transcript level. In order to test if this hypothesis, I participated in a pioneer screen in which we predicted a series of putative neuropeptide-encoding genes, along with transcription factors and hormone-synthesizing enzymes, and investigated their circalunar expression profiles in a massive parallel approach using the NanoString nCounter gene expression system.

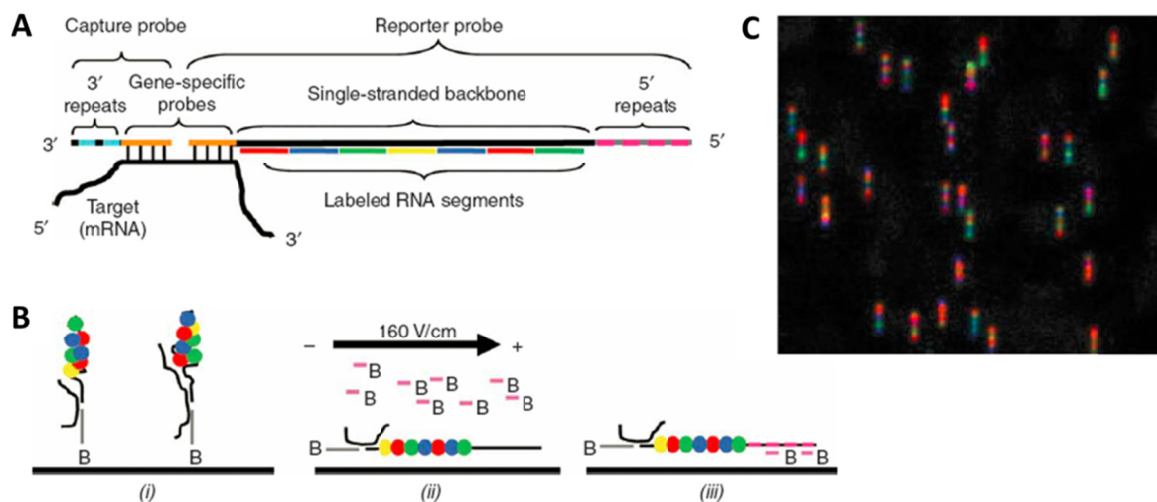


Figure 25. Schematic overview of NanoString nCounter gene expression system working principles

A) Scheme of the hybridized target-probe complex. After hybridization, excessive reporter and capture probes are removed with affinity-purification first by the 3' repeats then by the 5' repeats respectively. B) Scheme of binding, aligning, and immobilization of the hybridized complex. The purified complexes are (i) attached to a streptavidin-coated slide via biotinylated capture probes; (ii) elongated and aligned by voltage application, where 5' repeats are hybridized by adding biotinylated complementary oligonucleotides; (iii) immobilized by the binding of the biotin-labeled 5' repeats to the slide surface. After turning off the voltage the slide with immobilized reporters is prepared for imaging and counting. C) False-color image of a representation of immobilized reporter probes. (Schemes rearranged from Ref. [152])

The NanoString nCounter gene expression system is a hybridization-based technique to measure mRNA expression levels without enzymatic reactions. For each gene of interest, two sequence specific probes were generated: one Capture probe serving to fix the target-probe complex onto a surface and one Reporter probe serving to provide the detection signal. Every target mRNA is barcoded by a fluorescent tag with ordered colors and by counting the number of codes for each mRNA a direct digital readout of expression level can be generated. A schematic overview of the NanoString nCounter gene expression system is shown in Figure 25. It was reported to be more sensitive than microarrays and to have a similar sensibility to real-time PCR [152].

For the first experiment with NanoString system, immature *Platynereis* heads were sampled under NM and FR-FM conditions over 24 hours in 3-hour intervals, with 3 biological replicates per time point. Total RNA was extracted and gene expressions of the 500 candidate genes were measured by the NanoString system. The list of candidate genes mainly consisted of around 250 predicted transcription factors and around 250 predicted secreted peptide hormones. By comparing the gene expression profile between NM and FR-FM, we selected 50 potential circalunar clock regulated genes which are subjected to a second round of NanoString analysis. The expression level of each candidate gene was normalized to the total counts of each sample to compensate differences induced by input total RNA amount. To validate the data obtained with the NanoString systems, I sought to recapitulate the results with different technique and samples. I have selected eight genes and analyzed the mRNA level with quantitative RT-PCR using a different set of samples. Out of the eight genes, *gene1* (isotig 12505) showed consistent higher level of expression during NM than during FR-FM, with the lowest level at the beginning of the photophase (Figure 26A-C); *gene8* (isotig 10193) exhibited daily fluctuations with phase shift between NM and FR-FM (Figure 26D-F), indicating the endogenous circalunar clock regulation of these two candidate genes. Both candidates were predicted as genes coding neuropeptide hormones, whole-mount in-situ hybridization (WMISH) was carried out by Susanna Leiter for *gene8*, and it was found to be expressed in the nuchal organ as well as in the neurosecretory center between the posterior eyes (Figure 26G).

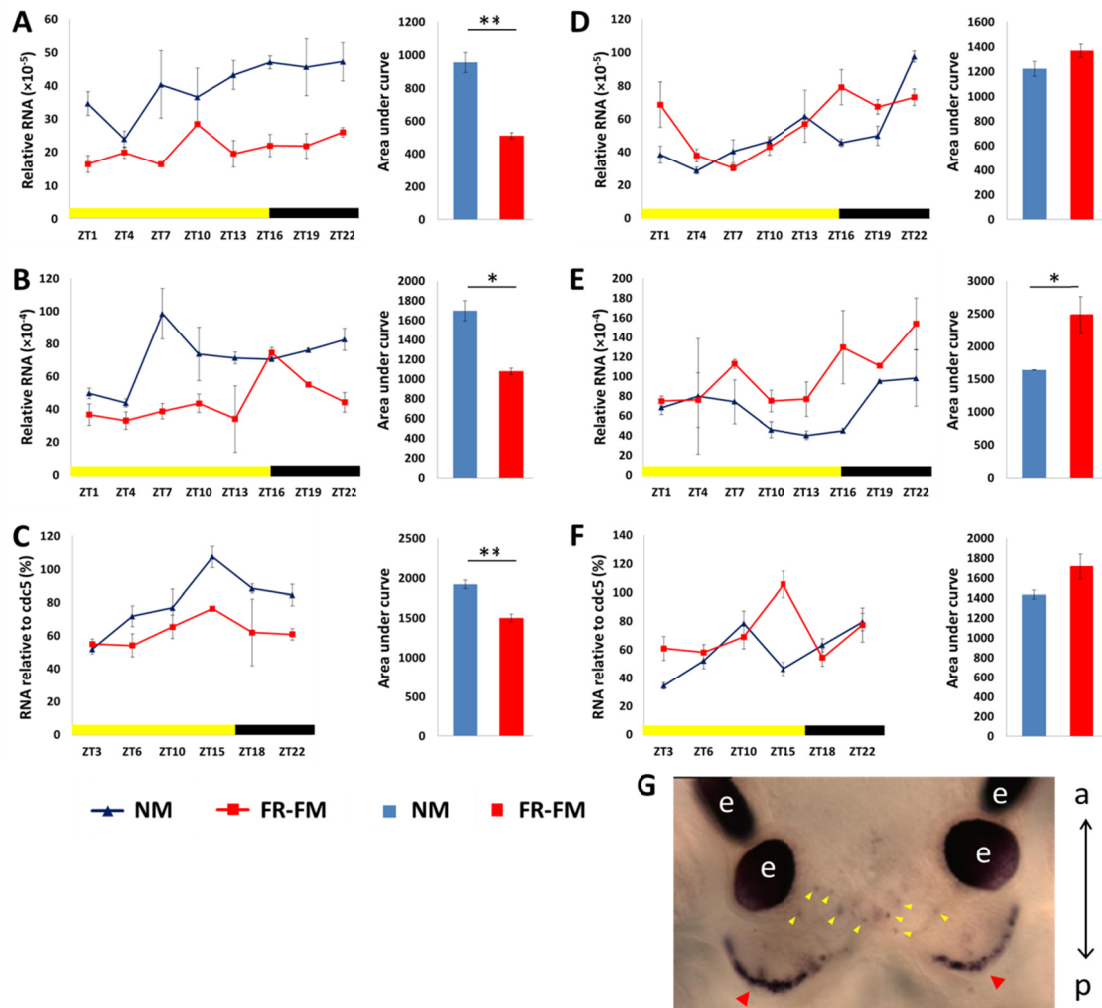


Figure 26. *Gene1* and *gene8* are the first validated candidate genes under circalunar clock control.

A-C) Temporal profile (line chart) and overall RNA expression (bar chart) estimated by calculating the area under curve in the line chart of *gene1* (isotig12505) under NM (blue) and FR-FM (red) detected with A) first NanoString run, B) second NanoString run and C) quantitative RT-PCR.

D-F) Temporal profile (line chart) and overall RNA expression (bar chart) estimated by calculating the area under curve in the line chart of *gene8* (isotig10193) under NM (blue) and FR-FM (red) detected with D) first NanoString run, E) second NanoString run and F) quantitative RT-PCR.

Legends apply for A-F. NanoString data were normalized to total counts of the samples, quantitative RT-PCR data were normalized to *cdc5*. Yellow and black bars indicate sampling time points during photophase and scotophase respectively. Notice the different sampling time points between samples analyzed with NanoString system and quantitative RT-PCR. A,C,D,F: $n=3$, values are means $\pm$ SEM; B,E: $n=2$, values are mean $\pm$ SD. * $p<0.05$, ** $p<0.005$.

G) WMISH staining of *gene8* in juvenile adult *Platynereis* head region (picture modified from bachelor thesis Figure 23A of Susanna Leiter). a: anterior, p: posterior, e: eye. Red arrow heads: staining in the nuchal organ; yellow arrow heads: staining between the posterior eyes (not exhaustively).

Further 14 genes showing consistent difference between NM and FR-FM were selected. Validation of these genes and studying the spatial distribution of their expression will give us further insights into

the circalunar clock downstream signaling pathway. First results from the on-going WMISH showed that *gene15* (isotig 11375) was expressed in a bilaterally symmetric region behind the posterior eyes resembling a third pair of eyes (Figure 27A). As this remained the exclusive site of expression detected in a naturally occurring mutant without eye pigments (Figure 27B), this gene appeared to be absent from the eyes. *Gene17* (isotig 28605) by contrast, showed strong staining around the eyes in the juvenile adult animals (Figure 27C), which could already be observed at early larval stages (48hpf, Figure 27D). As nine out of the of 16 candidate genes were predicted to be secreted peptides, including *gene1*, *gene8*, *gene15*, *gene17*, these results indicated that besides the melatonin biosynthesis pathway, also putative secreted factors were under circalunar clock control, although to which extent each of these factors contributes to the lunar reproductive behavior remains to be investigated.

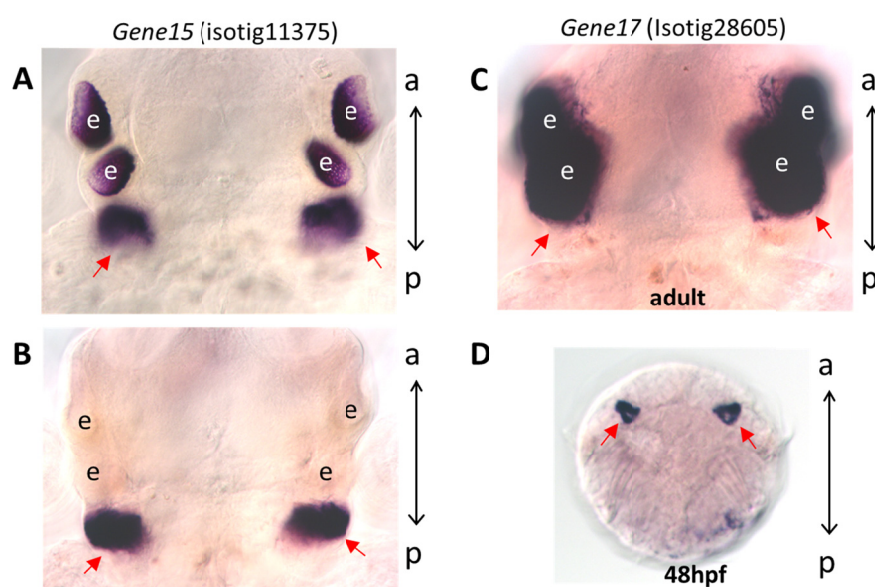


Figure 27. WMISH staining of circalunar clock target genes revealed different target tissues.

WMISH staining of A) *gene15* (isotig11375) in juvenile adult *Platynereis* head region. B) *gene15* (isotig11375) in juvenile adult *Platynereis* head region without eye pigment. C) *gene17* (isotig28605) in juvenile adult *Platynereis* head region. D) *gene17* (isotig28605) in whole 48hpf *Platynereis* larva. All pictures in dorsal view. a: anterior, p: posterior, e: eye. Red arrows point to the specific staining.

5. DISCUSSION

As outlined in the introduction, the key question to be answered by this study is to identify and characterize the targets of the circalunar clock. In the present study I was able to show that melatonin serves as a first identified output molecule of the circalunar clock in *Platynereis*, and the regulation of melatonin production at the transcript level of *hiomt*, the gene encoding the enzyme catalyzing the last step in melatonin biosynthesis. In addition, the localization of the presumptive melatonin producing cells suggests a connection between melatonin production and the circadian clock as well as the photoreceptors. Besides *hiomt*, I have also identified further candidate genes regulated by the circalunar clock at the transcript level. Together, this study provides a first inside into the downstream signaling of the circalunar clock in *Platynereis dumerilii*.

5.1 Placing melatonin into the framework of the circalunar biology of *Platynereis dumerilii*

5.1.1 A brain-located melatonin biosynthesis pathway is likely an ancient condition of bilaterians

Melatonin is the first candidate I have investigated as one of the targets of the circalunar clock. In order to assess the regulation of melatonin as well as elements of its biosynthesis pathway in *Platynereis dumerilii*, I first characterized the melatonin biosynthesis pathway in this particular species. Melatonin has been intensively studied in vertebrates, and its presence was also reported in various invertebrate species as well as in bacteria, fungi and plants [46]. However, it was still not explored in some important taxa, including annelids. In this study, endogenous melatonin was detected for the first time in an annelid worm, *Platynereis dumerilii*. The synthesis of melatonin depends on the enzymes Aanat catalyzing the N-acetylation of serotonin and Hiomt catalyzing the O-methylation of N-acetylserotonin (NAS). Genes encoding these two enzymes were also identified in *Platynereis*. Although melatonin seems to be ubiquitous, the evolutionary origin of its biosynthesis pathway was still largely unknown. Our results shed new light on the common origin of this biosynthesis pathway in bilaterians.

N-acetyltransferases in insects (i-NATs) don't belong to the Aanat family (including vt-Aanats and nv-Aanats except for i-NATs), and Aanat family members were also absent in nematodes, giving rise to the speculation of horizontal gene transfer from bacteria to chordates [84]. The phylogenetic analyses indicate that, unlike i-NATs, *Platynereis* Aanat belongs to the Aanat family and is closely related to vt-Aanats (Figure 8A, Figure 28A), thus an Aanat family member is also present in protostomes. This observation is direct evidence against the horizontal gene transfer theory and argues for a common ancestral gene encoding Aanat family members. Recently, Falcon *et al.* [153] suggested that vertebrate-type *aanat* (*vt-aanat*) was a duplication of non-vertebrate *aanat* (*nv-aanat*) that underwent

rapid evolution and specialized in rhythmic NAS production. Consistent with the observation of Falcon *et al.*, *Platynereis* Aanat also groups with other nv-Aanats, notably nv-Aanat of cat shark (*Calorhynchus milii*) which possesses both vt-Aanat and nv-Aanat [153] (Figure 8A).

As Aanat was believed to be the enzyme responsible for the daily fluctuation of melatonin in vertebrates [69], less studies have focused on Hiomt. Iyer *et al.* [84] equally searched for Hiomt orthologs, and only found Hiomt in vertebrates and bacteria, and also suspected for horizontal gene transfer to be responsible for the genes encoding vertebrate Hiomt. Previously a putative gene for Hiomt was reported in *C. elegans* [86], but our phylogenetic analysis reveals that it is only very distantly related to other metazoan, plant and bacterial Hiomts (Figure 28B). In contrast, we have identified the ortholog of Hiomt in *Platynereis*, which is supported by shared intron analysis (Figure 8D). Phylogenetic analysis groups *Platynereis* Hiomt with the other vertebrate and invertebrate Hiomt members (Figure 8B), suggesting a common origin of this gene in bilaterians.

In vertebrates, melatonin is predominantly produced in the pineal gland with peripheral production in the retina. In invertebrates, evidences also indicate that melatonin is produced in the neural system, especially in photosensitive tissues. However, studies on the spatial distribution of melatonin in invertebrates remain scarce. The identification of the enzymes for melatonin biosynthesis in *Platynereis* therefore provided a tool for me to investigate the possible sites of melatonin biosynthesis. The understanding of the localization of melatonin production in *Platynereis* brings more insights into the origin of this pathway and the evolution of the pineal gland in Bilateria. A comparison of the localization of melatonin producing cells with known photoreceptor cells and the expression pattern of circadian clock genes shed light on the interaction between these components.

Immunohistochemistry (IHC) using custom-raised antibody against Pdu-Hiomt revealed at least three distinctive groups of staining in the brain of adult *Platynereis*: Group 1. Large-sized neurons in the posterior medial brain; Group 2. Small neural cells near the posterior eyes; Group 3. Small dots between the posterior eyes (Figure 9D).

The posterior dorsomedial brain of *Platynereis*, particularly in a paired oval shaped structure was found to express the core circadian clock gene orthologs (Figure 29A) [16]. Group 1 staining of Pdu-Hiomt also falls into this particular circadian clock center (Figure 29B, C), suggesting a possible interaction between the circadian clock and melatonin production. Indeed, in addition to lunar regulation, both melatonin and the transcript level of *Pdu-hiomt* showed fluctuation at a daily basis during NM. However, in order to confirm the circadian clock regulation on melatonin production, circadian free-running experiments need to be carried out. Moreover, Group 1 staining of Pdu-Hiomt suggests that Hiomt-positive cells are in close proximity to the ciliary photoreceptor cells that are highlighted by staining with anti-acetylated tubulin antibody (Figure 29D,E).

The ciliary photoreceptor cells in *Platynereis* were reported to be homologous to the vertebrate ciliary photoreceptor cells, which diversified into various deep brain photoreceptors including the photoreceptor cells in the pineal gland [102]. The observed proximity might therefore reflect an ancient link between photosensitive functions and the production of melatonin, and is consistent with the observation that light has direct impact on melatonin production. However, cells revealed in the Group 2 staining are also in close proximity to light sensitive cells, whereas these (in the adult eyes) are supposed to be of a fundamentally different class (rhabdomeric photoreceptors) that is not related to the pinealocyte photoreceptors. At present, I have not attempted to dissect which of the two light receptors is responsible for mediating the light-dependent reduction of melatonin production, or if there are additional photoreceptors that might have this function. Of note, for testing if the adult eyes are responsible for mediating the effect of light, a conditional cell ablation strategy can be employed that abrogates the eyes at defined time points of development [154].

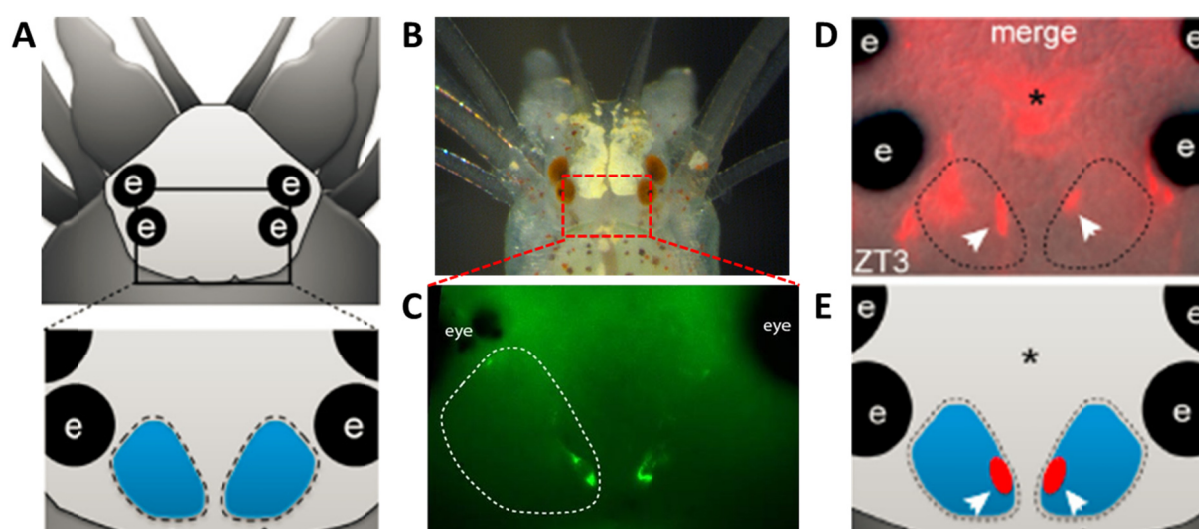


Figure 29. Pdu-Hiomt expressing cells are close of the core circadian clock as well as photosensitive cells.

A) Schematic drawing of *Platynereis* head indicating the paired oval shaped structure (blue) hosting the circadian gene expressing center. e, adult eyes. B) Light-field picture of *Platynereis* head. Red dashed square outlines the region shown in (C). C) Co-localization of Group 1 staining in IHC against Pdu-Hiomt (green) with the circadian gene expressing center. White dotted line circles the oval shaped circadian gene expressing center as in (A) [Zantke *et al.* unpublished data]. D) Staining with anti-acetylated tubulin antibody (red) highlights the prominent cilia of the ciliary photoreceptors. E) Scheme of the location of the ciliary photoreceptor cells in relationship with the circadian gene expressing center. White arrow heads point at the mentioned cilia. A, D, E: Graphic taken from Ref. [16].

Interestingly, Group 3 staining displayed the sub-localization of Pdu-Hiomt in the ends of neurites in the infra-cerebral gland, with the cell bodies located elsewhere, e.g. in the cells revealed in the Group 1 and Group 2 stainings. The infra-cerebral gland in polychaetes consists of neurosecretory cells, also

previously called the cerebra-vascular complex [155] because of its near connection with the blood vascular system. Cells in this region share similarity to cells in neurosecretory organs of other invertebrates and the pituitary gland in vertebrates [104]. It harbors neurosecretory endings and is believed to be an endocrine center [153–155], consistent with the neuro-hormone nature of melatonin. Moreover, the region between the posterior eyes were also found to express various hormones important for the development and maturation including the brain hormone [159], insulin-like peptides [Benjamin Backfisch, unpublished data] and the predicted secreted peptide *gene 8* (Section 4.6) identified in this study. It is worth noticing that, consistent with my study, anti-chicken Hiomt antibody was used to label Hiomt in a nemertean worm *Myoisophagos lacteus* belonging to another clade of the Lophotrochozoa. Similarly, the antibody labelled a few cells in the dorsal region of the dorsal cerebral ganglia in the same region of the photoreceptor-like cells [160].

Preliminary data from IHC analysis of Pdu-Aanat localization also revealed single neuronal cells in the posterior dorsomedial brain region (Figure 10). However, in order to confirm the co-localization of Aanat and Hiomt in *Platynereis*, double IHC with both antisera need to be carried out.

5.1.2 Challenges in characterizing the enzymes

The enzymatic activities of Pdu-Aanat and Pdu-Hiomt were not successfully recapitulated with bacterially expressed recombinant proteins. As bacteria are prokaryotes, they lack the full protein folding machinery or the enzymes to accomplish the required post-translational modifications. Hence, eukaryotic proteins expressed in bacteria are often non-functional or insoluble [146]. Indeed, I also tried to express human Aanat and Hiomt in the bacterial expression system which could serve as positive controls in the enzymatic assays, but only obtained insoluble proteins. Consistently, human Aanat was reported to be difficult to be purified [161] and attempts to express human Hiomt in *E. coli* only yielded insoluble or degraded proteins [162]. In this case the apparent non-functionality of the recombinant Pdu-Aanat could be the different working conditions required for these enzymes in different species, as I have adopted the most widely-used enzymatic assay conditions. In fact, while most studies adopted the same conditions in enzymatic assays to measure Aanat and Hiomt activities from various animal tissues including mammalian pineal glands, crustacean optic lobes or even bacteria and fungi, the only studies comprising orthology analyses revealed that invertebrate-type Aanats sometimes have different pH preference than the vertebrate-type Aanat [77, 78]. This could also attribute to the difficulties in detecting endogenous Pdu-Aanat and Pdu-Hiomt activities.

To overcome the drawbacks of bacterial expression systems and the lack of a positive control, I additionally expressed Pdu-Hiomt and Hs-Hiomt in mammalian cells, but no O-methylation of NAS was observed with Pdu-Hiomt, while Hs-Hiomt showed robust O-methyltransferase activity (Figure 19). The structural prediction of Pdu-HIOIMT revealed a large Aspartate, Glutamate and Histidine-rich

linker domain specific to *Platynereis* (Figure 20). The acidic amino acids and histidines are more susceptible to pH changes and might serve as metal binding site, and in turn divert the active condition for the enzyme from the orthologs of other species, resulting in the non-functionality under our experimental conditions. In addition, the human *hiomt* gene gives rise to 3 different protein isoforms resulting from alternative splicing of exon 6 and 7 [163], among which only the isoform 1 (345aa) exhibits enzymatic activity [148]. Alternative splicing of the *hiomt* gene was also observed in other species [164] but the gene regulation mechanism is still to be elucidated. I have intended to search for evidence of alternative splicing of *Pdu-hiomt* by amplifying the exons harboring the linker domain from cDNA and search for PCR product with different size, but did not observe any additional band on the gel electrophoresis. Yet, in absence of sufficient studies on this topic, alternative splicing remains an attractive possible mechanism in *hiomt* gene regulation, and *Pdu-Hiomt* might as well have other active isoforms than the one that we identified.

5.2 Melatonin: time cue for lunar periodicity?

5.2.1 Melatonin level is controlled by circalunar clock and affected by nocturnal light

The main hypothesis of this study is the involvement of melatonin in the lunar periodicity in *Platynereis dumerilii*, possibly as an output signal as in the case of circadian and annual rhythms. Therefore I tested the endogenous melatonin level in *Platynereis* heads under different light regimes simulating NM, FR-FM and FM conditions. Melatonin, while fluctuating under NM conditions, remains constant throughout the day under FR-FM and FM conditions. As under FR-FM the light regime is exactly the same as under NM, the difference of melatonin level between these two conditions can only result from an endogenous signal, namely the circalunar clock. In other words, the melatonin level was circalunar “free-running”. In mammals, the night peak of melatonin is controlled by the circadian clock, however the production of melatonin can be repressed by acute light exposure [61], indicating a direct regulation through light. In line with this, the overall melatonin levels under FM conditions are less than half of the levels under FR-FM conditions. As the only difference between FM and FR-FM light regime is the nocturnal dim light exposure, I therefore conclude that the overall expression level of melatonin is also influenced by nocturnal light exposure.

5.2.2 *Platynereis hiomt* transcript level is under circalunar clock control

As discussed in the introduction (Section 3.2.3), in vertebrates Aanat was more widely accepted as the enzyme responsible for melatonin’s night peak, while recently the important role of *Hiomt* in determining melatonin level also started to attract researchers’ attention again. The debate of relative im-

portance between the two enzymes in vertebrates has not stopped, and their role in influencing melatonin level in invertebrates remains even more elusive.

In my studies I looked into the regulation of *Pdu-aanat* and *Pdu-hiomt* mRNA levels in the context of lunar periodicity. *Pdu-aanat* mRNA levels showed only a mild fluctuation or remained constant throughout the day at all artificial moon phases, inconsistent with the temporal patterns of melatonin level. This result argues against a circalunar clock regulation of the transcript level of *Pdu-aanat*. However, care must be taken to draw further conclusions, because the enzyme activity of Aanat and Hiomt could also be regulated after transcription and translation, e.g. at the post-translational level. In fact on one hand, in rodents, Aanat activity is regulated at the transcription level, but in ungulates and primates including human, aanat mRNA remains constantly high throughout the day and melatonin is controlled mainly by Aanat activity [162–165]. However on the other hand, in the case of invertebrates, it was reported that non-vertebrate *aanat* (*nv-aanat*) lack the regulatory regions important for clock and light regulation, and the function of *nv-aanat* was not limited to melatonin synthesis, thus was not supposed to be involved in time-keeping [153]. In summary, Aanat's role in melatonin rhythmic production in *Platynereis* is to be further investigated.

The transcript level of *Pdu-hiomt* by contrast was steadily fluctuating under NM conditions but not under FR-FM conditions, consistent with the temporal pattern of melatonin level, therefore I conclude that the circadian profile of *Pdu-hiomt* mRNA level is under circalunar clock control. The highest transcript level of *Pdu-hiomt* under NM conditions was observed at the end of the photophase and the beginning of scotophase (between ZT15 and ZT18, light off at ZT16), and the highest level of melatonin under same conditions was measured at the end of scotophase (ZT22-ZT3). As the synthesis of melatonin only occurs after several biochemical steps downstream of the transcript, including translation, post-translational modification and enzymatic reaction, the delay of the melatonin peak compared to the *Pdu-hiomt* transcript peak could be expected. Interestingly, in nocturnal mammals such as rats and hamsters, the melatonin onset was also observed to be markedly delayed compared to the dark onset (up to 6.5 hours) and the serotonin onset [169].

As melatonin levels in *Platynereis* are also influenced by nocturnal light, I would like to investigate if the light impact acts at the transcript level of *Pdu-hiomt*. Comparable with FR-FM, the mRNA level of *Pdu-hiomt* under FM remained relatively constant along the day course in the prostomia, but fluctuation was observed in the peristomia. By analyzing the qRT-PCR data of *Pdu-hiomt*, the C_T values in the peristomia is generally higher than in the prostomia, with an average of 1.33 cycles. With the same starting total RNA amount, the fraction of *Pdu-hiomt* comparing to the total RNA in prostomia was estimated to be 2.5 fold as in peristomia, so prostomia harbors the majority of *Pdu-hiomt* expression. Taking into account of the Pdu-Hiomt localization revealed by immunostaining, Pdu-Hiomt positive cells were only found in the prostomia. The RNA level detected in the peristomia might be due to the

cutting errors during sampling or additional neurites not detectable by microscopy. In any case, it is plausible to consider the data from prostomia representative of the total transcript of *Pdu-hiomt*, thus light only has, if any, minor influence on *Pdu-hiomt* transcript level.

5.2.3 Lunar regulation of melatonin: a proposed model

Melatonin is mostly known as a circadian output signal in vertebrates, but it also receives external light information and in turn resets the circadian oscillators [170]. In mammals with seasonal breeding, melatonin was found to encode seasonal information by serving as a signal of day length, and it was also suggested to reset the annual reproductive cycles [168, 169]. Analogously in *Platynereis*, on one hand, melatonin is controlled by the endogenous circalunar clock, in other words, it is downstream in the signaling cascade of the circalunar clock oscillators. On the other hand, the reaction to direct nocturnal light input makes it a possible time cue to reset the circalunar clock oscillators in phase with the external environment.

The coinciding temporal pattern of the *Pdu-hiomt* transcript with melatonin suggests that the rhythmic melatonin production could be regulated by the circalunar clock by periodically altering the transcription of *Pdu-hiomt*. At the same time, nocturnal light was found to markedly down-regulate the overall melatonin level while its impact on *Pdu-hiomt* mRNA level was minimal, thus the down-regulation of the melatonin overall level by nocturnal light is not realized at the transcript level of *Pdu-hiomt*. Possible mechanisms of melatonin down-regulation include the down-regulation of Pdu-Aanat or Pdu-Hiomt activity at the post-translational level.

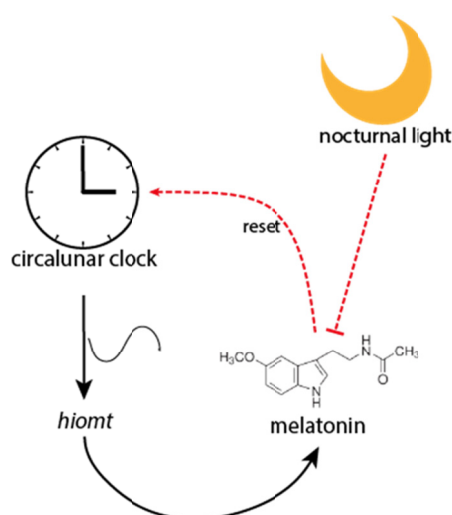


Figure 30. Schematic drawing of the proposed model of melatonin lunar regulation in *Platynereis*

Melatonin fluctuation is controlled by endogenous circalunar clock via *Pdu-hiomt* transcription, and could serve in turn as a time cue to reset the circalunar clock.

In future follow-up studies, the establishment of methods to measure endogenous NAS amount as well as Aanat and Hiomt activities from *Platynereis* tissue would be of great help to further understand the molecular mechanism of melatonin regulation. In summary, a model of melatonin regulation in context of lunar periodicity is proposed and illustrated in Figure 30.

5.3 Directions for testing the role of the melatonin biosynthesis machinery in reproductive periodicity

In order to rigorously assess where *Pdu-aanat* and *Pdu-hiomt* are expressed, especially to assess if the cells expressing each of the two genes co-localize and their spatial relationship with other factors, e.g. photoreceptors and circadian clock genes, I applied transposon-mediated transgenesis to label *Pdu-aanat* and *Pdu-hiomt* *in vivo*. For the in-depth functional analysis to assess if Pdu-Aanat and Pdu-Hiomt are required for melatonin biosynthesis I also used transcription activator-like effector nucleases (TALENs) to generate mutated animals expected to have non-functional Pdu-Hiomt. Whereas the transposon-mediated transgenesis approach was less successful, TALEN-mediated mutagenesis successfully gave rise to batches containing F1 heterozygous *Pdu-hiomt*^{+/-} animals.

5.3.1 Challenges in using transposon-mediated transgenesis for *in vivo* labeling

A transposon-mediated transgenesis protocol has been recently developed for *Platynereis*, and was adopted to label genes such as *rps9*, *maf* *in vivo* as well as to generate transgenic lines for *Platynereis* *r-opsin* and *alpha-tubulin* [111, 148]. In my studies I have attempted to label *Pdu-aanat* and *Pdu-hiomt* genes with EGFP and to generate transgenic lines with integrated EGFP and NTR expression for potential use of cell ablation driven by *Pdu-aanat* or *Pdu-hiomt* promoters.

As no information is available on the location of the regulatory region of these genes in *Platynereis*, different constructs have been designed for *Pdu-aanat* and *Pdu-hiomt* including various length of the regions upstream and downstream of the coding sequence, and larger constructs should more probably contain the promoter region. However, as DNA fragments larger than 10kb are difficult to be amplified by PCR and to be sub-cloned into a vector, only shorter constructs were obtained. The shortest reporter construct for *Pdu-aanat* only included a few hundred base pairs up- and down-stream of the coding sequence, and did not show any GFP signal, probably due to the omission of the regulatory region.

The reporter construct for *Pdu-hiomt* by contrast triggered specific GFP expression in Tol2 transposon injected larvae, therefore could be considered functional. However, no GFP-labelled cell was observed in adult animals positively tested for integration of the reporter construct. To date, in *Platynereis* only ubiquitously or abundantly expressed genes as *rps9*, *alpha-tubulin* [113] and *r-opsin* [150] have been labeled, sometimes partially [113], at adult stage. Evidence is still lacking in labeling weakly ex-

pressed genes in adult animals. At the same time, *Platynereis* possesses numerous pigment cells, also in the head region between the eyes, which are strongly autofluorescent upon exposure to UV light, rendering it difficult to distinguish signal from the reporter construct.

5.3.2 Efficient targeted genome editing using TALEN

Previously, no efficient genome editing tool was available for *Platynereis*. As transcription activator-like effector nucleases (TALENs) were recently shown to be able to efficiently generate mutations in various organisms, I decided to use this emerging tool in *Platynereis*. In the first attempts, TALENs were designed for *Pdu-aanat* and cloned into pTAL3 vectors with homodimeric FokI domains as used by Cermak *et al.* [151], but the TALENs generated with this vector failed to give rise to any mutations in *Platynereis*. Later on I designed new TALENs for both *Pdu-aanat* and *Pdu-hiomt* with restriction sites in the spacer and cloned them into new TALEN expression vectors pCS2TAL3-DD and pCS2TAL3-RR that were shown to be able to cause targeted mutations in *Platynereis* [139]. PCS2TAL3-DD and -RR vectors contain complimentary heterodimeric FokI nuclease domains as well as shorter N- and C-termini to increase mutation induction. In comparison, I used RVD NH instead of NN for the recognition of the nucleobase G, as it was suggested to bind G more specifically [170, 171]. However, after intensive trials of injections and genotyping, no mutation was observed. Similarly, other attempts using NH for G recognition for TALEN design also failed to generate mutations, for different genes in both *Platynereis* and zebrafish. Apparently, the NH RVD while possibly increasing the specificity of G recognition might decrease the overall binding efficiencies. Finally, after optimizing the TALEN design and adopting NN for G recognition, I have successfully generated TALENs targeting the second exon of *Pdu-hiomt* with high mutagenesis efficiency. This TALEN construct can be now used to generate *Pdu-hiomt* mutated line of *Platynereis*, which in turn will greatly help in understanding the role of *Pdu-hiomt* in circalunar clock regulation of melatonin production.

The TALENs with high mutagenesis efficiency also offers the possibility of precise genome editing. By co-introducing genome-repairing templates with homology arms to the genomic locus, custom-designed sequence could be inserted into the TALEN target site. It was shown that a short phiC31 Integrase landing site (attP) could be successfully inserted into the TMT-opsin 1b locus of medaka fish (*Oryzias latipes*) by co-injection of an ssDNA donor with short homology arms [Fischer *et al.* unpublished]. In the next step, transgene vectors containing an attB site could induce site-directed transgenesis into the landing site by co-introduction of a phiC31 integrase coding mRNA. With this method, reporter genes such as *egfp* could be inserted into the endogenous locus and solve the problem of *in vivo* labeling. Similarly, I have designed ssDNA donors to insert attP site into *Pdu-hiomt* locus with the highly efficient TALEN pair. Eventually, the detection of attP insertion will greatly enlarge our genome modification toolkit for *Platynereis* as to date no evidence of TALEN-mediated insertion has been shown.

5.4 Further circalunar clock regulated candidate genes will shed light on lunar reproduction

In addition to the candidate-based approach focusing on melatonin, I have also taken part in screening for and identifying other circalunar clock regulated candidate genes with NanoString nCounter gene expression system, an emerging technique with high-throughput and high sensitivity. After narrowing down the 500 candidate genes analyzed in the first round to 50 in the second round, we have identified 16 genes with consistently different expression between NM and FR-FM, two out of which I have validated by qRT-PCR analysis (Figure 26). The amplification regions in qRT-PCR were different from the probe binding region in NanoString measurement for either gene respectively, ruling out the possibility of sequence-specific artifacts. Thus NanoString nCounter gene expression system has proved to be a powerful tool for high-throughput screening for conditional gene regulations.

Whole-mount In-situ Hybridization (WMISH) of selected genes has already revealed different regions targeted by the circalunar clock. Stainings in the neurosecretory center between the posterior eyes (*gene8*, Figure 26G) also coincide with the circadian clock center (Figure 29A), again indicating a connection between the different clocks. Stainings in the nuchal organ (*gene8*, Figure 26G) and behind the posterior eyes (*gene15*, Figure 27A, B) are consistent with the secretory function that these regions host, although experiments need to be carried out to further confirm the co-localization of these genes with secreting cells. Most interestingly, stainings in the eye region (*gene17*, Figure 27C, D) indicates an possible light regulation on other circalunar clock target genes than *Pdu-hiomt*. A co-localization with the photoreceptor cells could be confirmed by a potential decrease of the expression of this gene by the ablation of the r-opsin expressing cells [154].

In the next steps it would be interesting to undertake in-depth analysis the 16 selected candidate genes: Firstly, similar qRT-PCR analyses should be carried out to confirm the regulation from endogenous circalunar clock for the other 14 selected candidate genes. Secondly, on-going bioinformatics analysis on all of the 16 selected candidate genes will help to find out their nature and potential function. The majority of the selected candidate genes were predicted secreted peptides, possibly neuropeptide hormones. Taking into account of the important role that neuropeptide hormones play in the reproductive timing in other species [175], these genes might bring us nearer to the understanding on the circalunar clock regulation of lunar spawning behavior in *Platynereis*. Thirdly, further *in-situ* hybridization analysis on juvenile adult heads for studying the spatial distribution of the validated candidate genes will not only reveal the expression pattern of these genes, but also shed light on their interaction with the core clocks and light receptors. Finally, the expression level between juvenile and mature animals will be compared to uncover their involvement in the maturation. In summary, rather than readily elucidate

the mechanism of circalunar clock downstream signaling, this part of work opens vast perspectives for follow-up studies.

5.5 Conclusions

My study intended to decipher the molecular output of the circalunar clock, with the model organism *Platynereis dumerilii*. I used both a candidate-based approach focusing on the small molecule hormone melatonin and a high-throughput screen for circalunar clock regulated genes. In the aspect of melatonin, the discovery of the whole melatonin biosynthesis pathway in *Platynereis* suggests the existence of common ancestral genes encoding Aanat and Hiomt respectively in bilaterians, and the spatial distribution of Pdu-Hiomt sheds light on clocks and light regulation of melatonin production. Melatonin was found to be an output signal of the circalunar clock, whose production is regulated at the transcription level of *Pdu-hiomt*, but at the same time it might also serve to reset the clock in phase with the external environment. In order to in-depth analyze the function of melatonin biosynthesis pathway I generated TALENs targeting the *Pdu-hiomt* locus with high efficiency that can be used in creating mutated animals. In the aspect of screening for further circalunar clock regulated genes I have identified a list of genes consistently showing distinct expression profiles under new moon and free-running full moon condition, and validated the first two candidate genes by analyzing their expression profile in different sample sets using alternative techniques.

6. MATERIALS AND METHODS

6.1 *Platynereis dumerilii* culture

The animals were maintained under laboratory conditions described by Hauenschild and Fischer [176] at constant 18°C in a mixture 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) with a volume ratio of 1:1. Standard light regime in the culture room consisted of 16 hours of light (6h to 22h UTC+1) and 8 hours of darkness (22h to 6h UTC+1) every day, with a dim light lit only 8 days per lunar month during the normal dark period simulating moonlight. In order to facilitate studying animals with late night time points, a special “circadian inverted shelf system” was also used with animals receiving a light regime of 12 hours shift compare to the normal culture. The worms were inbred into different stains. If not otherwise mentioned, PIN or VIO stains were used.

6.2 Measurement of gene expression and melatonin concentration

6.2.1 Sampling

All animals were taken out of the habitat and translocated into fresh sea water (NSW:ASW=1:1) at the same time prior to sampling. Animals were kept under previous light regime until decapitation. At time of decapitation, the prostomium and peristomium of the animal were quickly removed and separated with a surgical blade (Schreiber Instrument, No.22) and shortly stored on ice in an Eppendorf tube. Every sample containing five prostomia or peristomia was then snap-frozen in liquid nitrogen and stored at -80°C until further treatment. For each time point, 3 biological replicates with a total amount of 15 animals were sampled.

6.2.2 Extraction of total RNA from animal tissue

Frozen animal tissue samples were transferred into liquid nitrogen before being proceeded. 350 µl of RLT buffer (RNeasy Mini Kit Qiagen, containing 1% β-mercaptoethanol) was added into each sample. The samples were disrupted and homogenized manually with sterilized pestles then kept on ice. Total RNA was extracted with RNeasy mini Kit following manufacturer’s protocol, and the concentration was measured with NanoDrop ND-1000 Spectrophotometer (NanoDrop).

6.2.3 Compatible extraction method for total RNA and melatonin

Frozen animal tissue samples were disrupted and homogenized as described in Section 6.2.2 then transferred to 2 ml Eppendorf tubes containing 1.5 ml chloroform. Liquid-liquid extraction was performed by shaking at 1400rpm on a Thermomixer (Eppendorf) for 5 min at RT. Phases were separated by centrifuging at 17,000g for 3 min at RT. The upper, aqueous layer was then carefully removed and

total RNA extraction was performed immediately with RNeasy mini Kit following manufacturer's protocol. The interphase was discarded. All three replicates of the lower organic layer from the same time point were pooled and evaporated to dryness in a vacuum concentrator (Univapo 100H, UniEquip) with a refrigerated condensation Trap (Savant) and kept at -80°C until melatonin measurement.

6.2.4 cDNA synthesis

Template RNA was thawed on ice, and 400ng of total RNA was used for each reaction. cDNA was generated with QuantiTect Reverse Transcription Kit (Qiagen) following manufacturer's protocol. The same amount of ddH₂O was added to each sample template (typically 25-45µl) to reach the final volume needed.

6.2.5 Quantitative Real-Time PCR

Freshly generated cDNA was used as template. The PCR mixture contains 5µl cDNA template, 1µl forward primer (10µM); 1µl reverse primer (10µM); 10µl Power SYBR Green PCR master mix (Applied Biosystems) and 3µl ddH₂O.

Quantitative RT-PCR was performed on StepOne Plus Real-Time PCR system using MicroAmp optical 96-well reaction plates sealed with MicroAmp optical Adhesive Film (all from Applied Biosystems).

Quantitative RT-PCR program: 95°C 10min; 40x {95°C 15sec; 60°C 1min}

6.2.6 Data analysis

Data was collected with StepOne Software v2.2 (Applied Biosystems), and the threshold of C_T value of the target genes was calculated automatically. The same threshold was applied for all the genes tested on the same plate. C_T values were exported to Excel (Microsoft) and the fold change in comparison to the reference gene was calculated. Each sample with the same target gene was measured in duplicate and the average was taken for calculation. Gene expression level of the target genes was calculated using the following formula as percentage of the reference gene:

$$\text{Relative RNA concentration}\% = 1 / (2^{(C_T \text{Target gene} - C_T \text{Reference gene})}) * 100\%$$

Statistical calculations including the 2-tailed Student's t-test were carried out in Excel, and One-way ANOVA tests were performed using the online statistics tool under:

<http://www.physics.csbsju.edu/stats/anova.html>.

6.2.7 ELISA for melatonin concentration

Vacuum-dried samples (see Section 6.2.3) were redissolved in 100µl Incubation Buffer from Direct Saliva Melatonin ELISA kit (Bühlmann) overnight at 4°C, 1000rpm shaking on a Thermomixer (Eppendorf). The Melatonin amount was measured following the manufacturer's assay protocol with minor modifications, omitting the samples pretreatment step designed for removing the protein residue from the saliva samples. The absorbance of each well was read with a VICTOR³V 1420 multilabel plate reader (PerkinElmer) at 450nm.

6.2.8 Data analysis

Standard curve: The absorbance at 450nm was recorded for each standard solution including Incubation Buffer (containing 0pg melatonin) and Blank (containing saturated melatonin) in duplicate. The absorbance values of each standard and the Incubation buffer were subtracted with the average of the Blank to get the corrected average absorbance. The binding (B) of each standard solution was calculated as a percentage of the binding of the Incubation Buffer (B₀) with the corrected absorbance of the Incubation Buffer taken as 100%:

$$B/B_0 (\%) = \text{percent bound} = \frac{\text{corrected absorbance of standard}}{\text{corrected absorbance of Incubation Buffer}} \times 100\%$$

A standard curve was thus generated by plotting the percent bound on the y-axis versus the concentration of melatonin in pg/ml on the x-axis using a four-parameter non-linear regression algorithm. The regression was done with Matlab R2011a (see script in Appendix) using the formula

$$y = (A - D)/(1 + (x/C)^B + D.$$

A typical standard curve is shown in Figure 31.

Samples and Controls: The absorbance at 450nm was recorded for each sample in duplicate and controls in single replicate. Each absorbance was subtracted with the average of the Blank to get the corrected average absorbance. The percent bound ($B/B_0=y$) was calculated as described above and the melatonin concentration (x) was calculated using the formula

$$x = C \left((A-D)/(y-D) - 1 \right)^{1/B}.$$

Correspondingly, the melatonin amount in each sample is $x(\text{pg/ml}) \times 0.1\text{ml} = 0.1x(\text{pg})$. The total melatonin amount in the prostomia and the peristomia were added to estimate the total melatonin amount in the head. In order to normalize the melatonin concentration with the tissue amount, the total RNA in microgram was calculated and the melatonin concentration was expressed in (pg melatonin/µg total RNA). Statistical calculations including the 2-tailed Student's t-test were carried out in

Excel, and One-way ANOVA tests were performed using the online statistics tool under: <http://www.physics.csbsju.edu/stats/anova.html>.

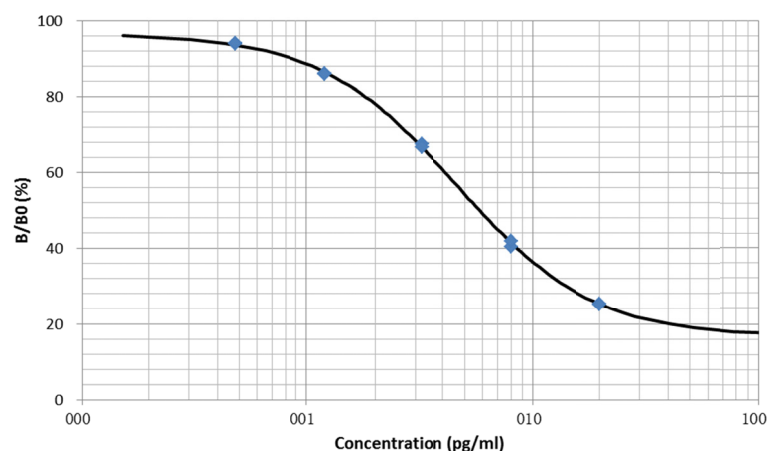


Figure 31. A typical standard curve of melatonin ELISA

X-axis: logarithmic scale of melatonin concentration in pg/ml, Y-axis, calculated B/B₀ value in percentage. Blue dots represent the measured data from standards used for generating the standard curve. Black line represents the fitted curve. Fitted parameters in this example: A=96.58; B=1.44; C=4.6; D=16.65; R²=0.9996.

6.3 Expression and purification of recombinant protein in bacteria

6.3.1 Cloning into pGEX-4T vector

The coding sequence of *Platynereis dumerilii aanat* (*Pdu-aanat*) and *hiomt* (*Pdu-hiomt*) were previously cloned into pJET1.2 vector (Thermo scientific). The plasmids were linearized with BglII (Promega) and diluted 1:10000 (*Pdu-aanat*) or 1:20000 (*Pdu-hiomt*) with H₂O to be used as template for the subsequent PCR reaction. A BamHI restriction site was added by PCR to the 5' end and an XhoI restriction site to 3' end of *Pdu-aanat* and *Pdu-hiomt* CDS (primers #1808, #1809, #1810, and #1811) using Phusion DNA polymerase (Thermo scientific) with the following recipe:

Reagent	Stock	Pdu-aanat	Pdu-hiomt
HF buffer	5×	10μl	10μl
dNTPs	10mM	2μl	2μl
Primer F	5μM	2μl	2μl
Primer R	5μM	2μl	2μl
DNA template		2μl	1μl
Phusion polymerase	5U/μl	0.5μl	0.5μl
ddH ₂ O	ad 50μl	31.5μl	32.5μl

PCR program: 98°C 30sec; 35 x {98°C 10sec; 60°C 1min ; 72°C 1min}; 72°C 7min.

Both genes were cloned into pGEX-4T-2 vector (GE Healthcare Life Sciences,) by sticky end cloning into BamHI and XhoI sites. The correct insert with the right direction was confirmed by sequencing with PGEX5' and PGEX 3' primers. Empty vector as well as plasmids with insert genes was propagated in One Shot TOP10 Chemically Competent *E. coli* (Invitrogen).

6.3.2 Optimization of inducing conditions

pGEX-4T-2 plasmid containing the CDS of either *Pdu-aanat* or *Pdu-hiomt* CDS as an insert or empty pGEX-4T-2 vector was transformed into Rosetta(DE3)pLysS Competent Cells (Novagen). Fresh starting cultures for each fusion protein of interest, as well as for GST were grown at 37°C overnight. For each protein, 6 flasks of 25ml LB medium (containing 50µg/ml ampicillin and 30µg/ml chloramphenicol) were inoculated with the starting culture at a ratio of 1:50 or 1:100, and grown at 37°C until an optical density at 600 nm (OD600) of 0.6-0.8, was reached. Once the cell density was reached, the cultures were cooled down to the inducing temperature before adding IPTG to a final concentration of 0.5mM, and grown further at inducing temperature with vigorous shaking at 160-180rpm. For the optimization of growth condition, 3 different inducing temperatures with corresponding protein expressing time were used (37°C 4h, 24°C 5-6h, 18°C overnight), and at each temperature one culture without IPTG induction was included as negative control. The GST-only expressing culture (control) was only induced at 37°C. At the end of the induction period at each given temperature, the cultures were pelleted at 4°C, 4000rpm for 15min, and each pellet was resuspended thoroughly on ice in 2ml lysis buffer (50mM HEPES pH=7.5, 300mM NaCl, 1mM MgCl₂, shortly before use: add 10µg/ml DNase I and 1mM Pefabloc SC protease inhibitor). The cell suspension was then snap frozen in liquid nitrogen and stored at -80°C. To lyse the bacteria cells, frozen bacteria suspensions were thawed in a water bath at RT, gently mixed and stored on ice. One aliquot of 10µl from each lysate was taken for analysis. The rest of all lysates was centrifuged at 4°C, 20000xg for 5min, the supernatant was taken into another tube and 10µl from each supernatant was taken for SDS-PAGE analysis. The temperature with the most soluble target protein was chosen for large-scale protein expression and purification.

6.3.3 SDS-PAGE with Coomassie stain

Polyacrylamide gels were prepared according to protein size: 12% gel was used for GST fusion protein of Pdu-Aanat and 10% gel for GST fusion protein of Pdu-Hiomt. To every 10µl of sample, 10µl of 2xLaemmli buffer (containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH approx. 6.8, premixed by Sigma-Aldrich) was added [177]. The samples were boiled at 98°C for 5min, cooled down and loaded onto gel. After running the SDS-PAGE, the gels were stained with Coomassie Brilliant Blue R (0.25% (w/v) Brilliant Blue R, 40% (v/v) methanol and 7% (v/v) acetic acid) for 5min, rinsed 2 times with ddH₂O then the excessive stain was removed with destaining buffer (67.5% H₂O, 7.5% acetic acid and 25% Methanol, v/v). Pictures

were taken by scanning the gel on an image scanner between two transparent plastic foils. Alternatively, a faster and less toxic destaining method was also used: the Coomassie stained gel was shortly heated to boil in a microwave in ethanol destaining buffer (45% (v/v) ethanol and 10% (v/v) acetic acid) to remove the staining background.

6.3.4 Large scale protein expression

Larger amount of bacteria culture (1-3 liters) was used to express target protein under optimized inducing conditions. The cells were harvested by centrifuging at 4°C, 5000rpm for 12min in an Avanti J-26XP centrifuge (Beckmann Coulter) with a JLA-8.1000 rotor. Each liter of culture was thoroughly resuspended in 25ml lysis buffer (see Section 6.3.2, in addition, one tablet of cOmplete Protease Inhibitor Cocktail, EDTA free (Roche) was added to 150ml of lysis buffer immediately prior to use), snap frozen in liquid nitrogen and stored at -80°C.

The bacteria suspensions were thawed in a water bath at RT with magnetic stir on the bottom. The suspensions were kept for further 10min after thawed up, and sonicated for 30sec on ice with 50% cycle with Sonopuls HD200 (Bandelin electronic) to ensure complete lysing. The lysates were centrifuged at 4°C, 40000rpm for 40min in an Optima L-80XP ultracentrifuge (Beckman Coulter) with a Ti45 Rotor and the supernatant was kept for affinity purification.

6.3.5 Affinity purification of GST-tagged recombinant protein

Gluthathione Sepharose 4B beads (GE healthcare) were used to purify GST fusion protein with a batch proceeding method.

Buffers used: Two types of buffers were used during the protein purification procedure:

Low salt binding buffer (LSBB): 50mM HEPES (pH=7.5), 300mM NaCl, 4mM DTT.

High salt binding buffer (HSBB): 50mM HEPES (pH=7.5), 1000mM NaCl, 4mM DTT.

Equilibrating beads: On milliliter of beads was taken for each liter of protein expressing culture. The glutathione beads were spun at 4°C, 500g for 5min and the supernatant was discarded. The beads were equilibrated 2 times with 5ml LSBB each, spun down at 4°C, 500g for 5min and the supernatant was discarded each time.

Binding of GST-tagged protein: For binding the target protein to the beads, the lysate supernatant was added to the equilibrated beads, very gently rotated at 4°C for 1h. The beads were spun down at 4°C, 500g for 5min and an aliquot of the supernatant was taken for SDS-PAGE analysis to ensure complete binding.

Washing beads: The beads were washed 5 times with LSBB by adding 5ml of LSBB; each time the beads were gently mixed, spun down at 4°C, 500g for 5min and the supernatant was discarded. The beads were then washed two times with HSBB by adding 10ml of HSBB; each time the beads were gently rotated for 15min at 4°C and spun down at 4°C, 500g for 5min with the supernatant discarded. The beads were further washed two times with LSBB as previous LSBB washes. After washing 10ml of LSBB was added and the beads were stored on ice before on-beads cleavage.

Cleavage of GST: 250U of Thrombin (Serva Electrophoresis) was dissolved in 1ml LSBB and added to each batch of beads bound with GST tagged fusion protein. The beads were gently rotated at 4°C overnight. An aliquot of the beads suspension was taken and analyzed with SDS-PAGE to check the completion of cleavage and the degradation of target protein. The beads were spun down at 4°C, 500g for 5min when cleavage was completed. The supernatant was collected and stored on ice. The beads were washed twice with 2ml LSBB each and the supernatants were collected. The supernatants were pooled together and filtered with a 0.2 µm Polyethersulfone syringe filter (VWR) to eliminate beads and an aliquot was taken for analysis. The A280 absorbance of the protein was measured with NanoDrop ND-1000 Spectrophotometer (NanoDrop).

Concentrating protein: To concentrate the protein solution, the filtered supernatant was transferred to an Amicon Ultra-15 Centrifugal Filter (Millipore) with a proper molecular weight cut-off. For Pdu-Aanat (18.5kDa) a 10kDa filter was used and for Pdu-Hiomt (50kDa) a 30kDa filter. The filters were centrifuged at 4°C 4000rpm for 10-15min until the desired final volume was reached. The absorbance at 280 nm (A280) was measured with a Nanodrop and an aliquot was taken for analysis. Purified protein was snap frozen and stored at -80°C.

Calculating protein concentration: The extinction coefficient of each protein was calculated using the online ProtParam tool: <http://expasy.org/tools/protparam.html>. The corrected protein concentration was calculated using the following formula:

$$\text{concentration}(\text{g} \cdot \text{L}^{-1}) = \frac{\text{A280 absorbance}}{\text{light path}(\text{cm}^{-1}) \times \text{extinction coefficient}(\text{M}^{-1} \cdot \text{cm}^{-1})} \times \text{Molecular mass}(\text{Da}).$$

Regenerating beads: The beads bound with GST were washed 3 times with 6M urea, 3 times with 70% EtOH and stored in 20% EtOH in the fridge. The beads could be reused only for purification of the same protein to avoid cross contamination.

6.4 Expression of proteins in mammalian cell culture

6.4.1 Maintenance of COS-7 cell culture

COS-7 cells (American Type Culture Collection, ATCC) were cultured in complete growth medium (DMEM (Sigma) with 10% fetal bovine serum (Sigma) and 1x Pen/Strep) at 37°C and 5% CO₂. When

reaching 90%-100% confluency, cells were trypsinized and re-plated at 1:5-1:8 dilution. For storage, 1×10^6 cells were suspended in freezing medium (90% FCS, 10% DMSO) slowly frozen and stored at -80°C or in vapor phase of liquid nitrogen. Frozen cells were rapidly thawed up in a water bath set to 37°C , suspended in complete medium and plated onto tissue culture dish (BD Falcon).

6.4.2 pcDNA-DEST47 plasmid

The pcDNA-DEST47 plasmid (Invitrogen) was used for the expression of target proteins with or without GFP fusion in mammalian cells. Due to the *ccdB* gene for negative selection, the empty vector was only propagated in *ccdB* Survival *E.coli* strain.

6.4.3 PCR products for protein expression in mammalian cells

Upper primers: Primers were designed to add a Kozak consensus sequence (CACC) to the 5' of each gene. As G at position +4 was critical for function, an extra sequence of ATGGGA was added before the starting ATG if needed. (Primer #2139)

Lower primers: In order to generate a C-terminal GFP fusion protein, primers were designed without a stop codon and in frame with the Cycle 3 GFP gene after recombination (primer #2141). In order to generate an untagged native protein, primers were designed to include the stop codon as well as an additional G to be out of frame with the Cycle 3 GFP gene (primer #2140).

PCRs were carried out with Phusion DNA polymerase (Thermo scientific) for blunt end PCR products with diluted linearized plasmid containing *Pdu-hiomt* CDS as template (see Section 6.3.1) using the following recipe:

Reagent	Stock	Volume (μl)
HF buffer	5×	5
dNTPs	10mM	1
Primer F	5 μM	2.5
Primer R	5 μM	2.5
DNA template		1
Phusion polymerase	5U/ μl	0.25
ddH ₂ O	ad 25 μl	12.75

PCR program: 98°C 30sec; 35 x { 98°C 5sec; 64°C 20sec ; 72°C 30sec} ; 72°C 7min

6.4.4 Generating genes for chimeric proteins

Chimeric genes were generated using two PCR rounds. In the first round matching sequence to *Pdu-hiomt* were added to 5' and 3' of the human *hiomt* (*Hs-hiomt*) CDS linker region (primers #2147, #2148), and the two *Pdu-hiomt* CDS regions excluding the linker region were amplified separately,

with 5' end and 3' end compatible for protein expression in mammalian cells (primers #2139, #2145, #2146, #2141, #2140). In the second round same primers as in Section 6.4.3 were used, with gel purified PCR products from the first round as template. PCR was carried out as in Section 6.4.3.

6.4.5 Cloning genes of interest into pcDNA-DEST47 plasmid

The PCR products containing the coding sequence of interest were cloned into a pENTR/D-TOPO vector using the pENTR Directional TOPO Cloning Kits following the manufacturer's instructions. The entry clones in the pENTR/D-TOPO plasmid were recombined into pcDNA-DEST47 using Gateway TOPO cloning procedure. A recombination reaction mixture contained 1µl pENTR plasmid with entry clone (150ng/µl), 1µl pDEST-47 vector (150ng/µl), 6µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0), and 2µl Gateway LR Clonase II enzyme mix (Invitrogen). The reaction mixture was incubated at 25°C for 2 hours, then 1µl of proteinase K (20mg/ml) was added and the reaction was incubated for a further 10min at 37°C and cooled down on ice. One µl of each reaction was transformed into One Shot® TOP10 Chemically Competent E. coli (Invitrogen), and clones with the right insert were confirmed by sequencing with primers for #1372, rev: #2100.

6.4.6 Transfection

The plasmids used for transfecting COS-7 cells were prepared with EndoFree Plasmid Maxi Kit (Qiagen) following the manufacturer's protocol and diluted to 1µg/µl. Approximately 24 hours before transfection, COS-7 cells were trypsinized and counted with Countess automated cell counter in a Countess cell counting chamber slide (Invitrogen). Dead cells were stained with Trypan Blue (0.4%). In each well of a 6-well plate (tissue culture treated, CytoOne), 5×10^5 cells were plated in 2.5ml of complete growth medium. Transfection was carried out with TransIT-LT1 Transfection Reagent (Mirus) following the manufacturer's instructions, GFP expression was confirmed by fluorescence microscopy 24 hours after transfection. One well was transfected with a C-terminal GFP-fused human oxytocin receptor in pEGPF-N3 vector to monitor the transfection rate and serve as negative control for enzymatic assays.

6.4.7 harvesting cells

Cells were harvested 48 hours after transfection. Growth medium was removed by aspiration and the cells were gently washed once with 2.5ml PBS per well. Cells were harvested on ice with a 25cm cell scraper (Sarstedt) in 200µl cold CHAPS buffer (50mM sodium phosphate pH 7.9, 4% CHAPS). Cell lysates were centrifuged at 4°C 20238g for 5min and the supernatant was used in enzymatic assays and for western blot.

6.5 Enzymatic assays for Aanat and Hiomt activity

Aanat and Hiomt enzymatic activities were measured with methods previously described by Champney *et al.* [178]. Aanat activity was carried out in 0.25 M potassium phosphate buffer (pH 6.5) containing 1mM serotonin and 1mM acetyl-Coenzyme A. Hiomt activity was carried out in 0.05M sodium phosphate buffer (pH 7.9) containing 1mM of N-acetyl serotonin (NAS) and 1mM of S-Adenosyl methionine (SAM). If not otherwise specified, the reactions were carried out at 37°C for 1 hour and stopped by adding 20µl of 6M perchloric acid to each 100µl of reaction. Stopped reactions were spun at 20000g for 5min and the supernatant was analyzed in our collaborator Prof. Christopher Gerner's lab (University of Vienna) with HPLC-MS/MS for serotonin, NAS and melatonin. Special conditions are mentioned below.

6.5.1 Aanat and Hiomt enzymatic assay with purified recombinant protein

Each reaction was carried out in 100µl with 10ug purified protein. The stopped reactions were spun at 20000g for 20min.

6.5.2 Hiomt enzymatic assay with mammalian cell lysate

Each reaction was composed of 50µl of cell lysate (Section 6.4.7) and 50µl of reaction concentrate resulting in reaction solutions of 0.05M sodium phosphate buffer (pH 7.9) containing 5µM NAS and 30µM SAM. The reactions were carried out at 37°C or RT, and stopped by adding 20µl of perchloric acid.

6.5.3 Hiomt enzymatic assay with *Platynereis* head lysate

The animals were decapitated and the anterior part containing both prostomia and peristomia were collected and stored on ice until tissue disruption. Each sample of 47-65 animals was disrupted and homogenized in 150-300µl 0.05M sodium phosphate buffer (pH 7.9) with 4% CHAPS on ice, and centrifuged at 4°C 20000g for 5min. An aliquot of the supernatant was taken for the measurement of endogenous NAS and melatonin. Each reaction was composed of 50µl of animal tissue lysate supernatant and 50µl of reaction concentrate resulting in reaction solutions of 0.05M sodium phosphate buffer (pH 7.9) containing 2µM NAS and 12µM SAM. The reactions were carried out at RT for 1-2 hours and stopped by adding 20µl of perchloric acid.

6.6 Western blot

6.6.1 Extraction of total protein from animal tissue

Platynereis were decapitated and the anterior part containing both prostomia and peristomia was collected and snap-frozen in liquid nitrogen. Frozen tissue was disrupted and homogenized in RIPA buffer (50mM Tris-HCl (pH 7.4), 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 150mM NaCl, 2mM EDTA, 50mM NaF, one cOmplete Mini Protease Inhibitor Cocktail Tablet (Roche) was added immediately before use).

6.6.2 Measurement of protein concentration

Protein concentrations were measured with BCA protein assay using the Pierce BCA Protein Assay Kit (Thermo Scientific). BSA standards were prepared according to the manufacturer's instructions. Working reagent (WR) was prepared freshly before the reactions by mixing BCA Reagent A and B at a ratio of 50:1. For the use of the microtiter plate procedure was used by adding 10µl of sample or standard and 200µl of WR to each well. Samples and standards were all measured in duplicates. The plate was incubated at 37°C for 30min and the absorbance of 550nm was measured on a VICTOR³V 1420 multilabel plate reader (PerkinElmer). The average 550nm absorbance of the Blank standard was subtracted from the measurement of all other standards and samples. By plotting the Blank-corrected 550nm absorbance for each BSA standard on the x-axis and its concentration in µg/ml on the y-axis, a standard curve was generated with a 2-parameter regression $y=ax^2+bx$. The concentration of samples was determined by taking the Blank-corrected 550nm absorbance as x value and calculating the y value using $y=ax^2+bx$.

6.6.3 Antibodies

Primary antibodies:

Polyclonal antisera against the C-terminus of Pdu-Hiomt (aa196-445) were raised in rabbit by injection of a 10x His-tagged partial protein by a commercial provider (Primm srl, protein sequence MGSDKIHHHHHHHHHHGVSPTRKLKEIEEPHFAQQAHHGHPSHLHGHLSPHKHGHHHHGH-HGPGGLIDSHALHERYRAFDTVGKLQAPHLMTAYDLSSFQSAVDLGAGTGCMGFELARVYD-NMQVTLM DVPRVTEL VKHHFMPPDINALKIEFLPGDFFITELPEADLYLLSKIVHDWNDEQV-EKLLHLCFEKINPGGGILLFEKCLNDHKDGPVSAHMWDTFIMTLCAGRLRSGKEYRHLEN-VGFTQVQIHRTQEQTDFDIYARKP, N-terminal tag underlined). Sera from immunized rabbits were affinity purified in the lab using an on-blot purification protocol and used at 1:60 dilution. Pre-immune serum from the same rabbit (1:100) and antibody pre-incubated with a 10-fold excess (by weight) of recombinant full-length Pdu-Hiomt were used as control.

Polyclonal antisera against the N-terminus of Pdu-Aanat (aa 1-94) was raised commercially in rabbit by injection of a 10x His-tagged partial protein (Primm srl, protein sequence MGSD-KIHHHHHHHHHHHGVMALTGEFRLLTPQDVQTAFAQLELDGYPSDEAATLEIMQMRQREAPQLCMGYFECSGLFGFILATRAVSEKFTHEMSAHEPDGESVCIHSVCVD, N-terminal tag underlined). Sera from immunized rabbits were affinity purified by Primm srl and was used at 1:200.

Monoclonal antisera against the N-terminus of Pdu-Aanat (aa 1-85) was raised in collaboration with the Ogris lab (Max F. Perutz Laboratories) in mouse by injection of a 6x His-tagged partial protein (protein sequence MALTGEFRLLTPQDVQTAFAQLELDGYPSDEAATLEIMQMRQREAPQLCMGYFECSGLFGFILATRAVSEKFTHEMSAHEPDGESLEHHHHHHH, C-terminal tag underlined). For screening for immunized mouse, blood sera were used at a dilution of 1:500-1:1000. Cell culture media from oligoclonal were directly used as antisera.

A polyclonal anti-actin antibody produced in rabbit was purchased from Sigma-Aldrich and used at a dilution of 1:5000.

A polyclonal anti-GAPDH antibody recognizing the C-terminus of human GAPDH produced in rabbit (Millipore) was used at a dilution of 1:10000.

Secondary antibody:

Horseradish peroxidase (HRP) linked polyclonal Anti-Rabbit IgG (whole molecule) antibody produced in goat was purchased from Sigma-Aldrich and used at a dilution of 1:3000.

6.6.4 Western blot

Clarified lysates of the transfected COS-7 cells (3µg), extracted protein from animal tissue (4.5µg) or purified recombinant protein (1µl, 1:6 dilution) were loaded into each well. Proteins were resolved on SDS-PAGE gels (10-15% depending on the size of the protein) in a Bio-Rad Mini Protean Tetra Cell (Bio-Rad) with PageRuler Prestained Protein Ladder (Thermo Scientific) as standard and electrotransferred onto polyvinylidene difluoride (PVDF) membrane (PeqLab) in a Bio-Rad Trans-Blot SD semi-dry transfer cell (Bio-Rad). Blots were blocked in 5% skimmed milk powder in PTW (1×PBS containing 0.1% Tween-20) at RT for 1 hour and incubated in the primary antibody diluted in 2.5% skimmed milk powder in PTW at 4°C overnight. An anti-actin antibody was used for normalization for animal tissue samples, and anti-GAPDH antibody was used for COS-7 cell lysates. Excess of antibody was removed by washing in PTW for 3-4 times. The blots were incubated in the secondary antibody diluted in PTW at RT for 1 hour and excess antibody was removed by washing 3-4 times in PTW. Chemiluminescence reaction was started by adding the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and detection was carried out after 5min of incubation in the dark room on a Kodak BioMax MR Film (Sigma-Aldrich).

6.7 Immunohistochemistry (IHC)

6.7.1 Antibodies

Primary antibodies:

The Same polyclonal antiserum against Pdu-Hiomt as used for western blot (Section 6.6.3) was affinity purified by Primm srl and used at a 1:500 dilution. Antibody pre-incubated with a 50-fold excess (by weight) of recombinant full-length Pdu-Hiomt was used as control.

The blood sera from immunized mice against Pdu-Aanat for screening for monoclonal antiserum as used for western blot were used at dilutions of 1:50 and 1:200. Cell culture media from oligoclones were directly used as antisera without dilution.

A monoclonal anti-acetylated tubulin antibody produced in mouse was purchased from Sigma-Aldrich and used at a dilution of 1:200.

Secondary antibodies (fluorophore coupled):

An affinity purified goat anti-mouse IgG antibody conjugated to Cy3 and an affinity purified goat anti-rabbit IgG antibody conjugated to the green-fluorescent Alexa Fluor 488 dye were purchased from Invitrogen, and used at a 1:200 dilution.

6.7.2 IHC on larvae

Fixation:

- Fix *Platynereis* larvae in Bouin's fixative (saturated picric acid: 35% formaldehyde: glacial acetic acid = 15:5:1, v:v:v) for 1 hour RT
- Wash 5 times in PTW (1,2,5,10,20min)
- Stored at 4°C in PTW up to one week till IHC

Digestion:

- Rinse 2 × 5 min in PTW
- Digest in Proteinase K (80µg/ml) at 20°C without shaking; with a length depending on the stage of the larvae as described in the table below.

Stage	Time of ProteinaseK digestion
16h-72h	45sec – 1min
72h-5d	2min

1 week to 6 weeks	3min
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- Rinse two times shortly in 2mg/ml glycine in PTW
- Wash 5 times in PTW (1,2,5,10,20min)

Antibody incubations:

- Block in 5% sheep serum (Sigma-Aldrich) in PTW for 1 hour at RT
- Remove the blocking solution
- Incubate in the primary antibody in 2.5% sheep serum in PTW at RT for 1 hour or at 4°C overnight
- Wash 5 × 10min in PTW
- Incubate in the secondary antibody in 2.5% sheep serum in PTW at RT for 1 hour or at 4°C overnight, protected from light
- Wash 5 × 10min in PTW
- Transfer into 6-well plates and store in DABCO-glycerol (2.5mg/ml DABCO(Roth), 90% glycerol) at 4°C, protected from light

6.7.3 IHC on adult animals

Fixation:

- Choose small adult *Platynereis* animals and fix in Bouin's fixative for 2 hours at 4°C
- Wash 5 × 10min in PBT (1×PBS with 1% Triton X-100)
- Stored at 4°C in PBT up to one week till IHC

Permeabilization:

- Discard storing liquid, add 1ml prechilled acetone and permeabilize at -20°C for 30min
- Wash 4 x 5min in PBT

Digestion:

- Digest in Proteinase K (80µg/ml) without shaking for 6min at 20°C
- Rinse two times shortly in 2mg/ml glycine in PTW
- Wash 5 times in PTW (1,2,5,10,20min)

Antibody incubations:

- Block in 10% sheep serum in PBT for 2h at RT
- Remove the blocking solution
- Incubate in the primary antibody in 5% sheep serum in PBT (containing 0.05% NaN₃) at 4°C for 3 days
- Wash 3 × 30min and 3 × 1hour in PBT
- Incubate in the secondary antibody in 5% sheep serum in PBT (containing 0.05% NaN₃) at 4°C for 3 days, protected from light
- Wash 3 × 30min and 3 × 1hour in PBT
- Transfer into 6-well plates and store in DABCO-glycerol at 4°C, protected from light

6.8 Whole-mount in-situ hybridization

The protocol for whole-mount in-situ hybridization (WMISH) on adult *Platynereis* head is based on the protocol described by Backfisch *et al.* [150] with minor modifications.

6.8.1 *Platynereis* head sampling and fixation

Animals older than 3 months with very small sized were selected and decapitated, the anterior part containing 2-3 pairs of parapodia were collected and fixed in 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 -20°C at least overnight till further treatment.

6.8.2 RNA probe generation

Digoxigenin (DIG) – labelled RNA probe reaction mixture was assembled by adding the components in the following order:

Linearized template	1 µg
100 mM DTT	2 µl
DIG RNA Labeling Mix (Roche)	2 µl
Ribolock RNase Inhibitor (Fermentas)	0.5 µl
10xTranscription buffer (NEB)	2 µl
RNA polymerase (SP6 or T7)	1 µl
H ₂ O	ad 20 µl

The reaction was incubated at 37°C for 3 hours, then 1µl of RNase-free DNase I (Roche) was added and the reaction was further incubated for 15min. The probe was purified with RNeasy Mini Kit (Qiagen), diluted in 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. Aliquots were taken before addition of Hyb+ mix for electrophoresis analysis with Ethidium Bromide staining and Dot-Blot quality test.

6.8.3 Dot-Blot quality test of RNA probe

- Resolve RNA by electrophoresis on 1% agarose gel
- Blot RNA on Nylon membrane (Peqlab) for 1 hour
- Cross-link RNA onto membrane in a UV crosslinker (Stratalinker 2400, Stratagene)
- Dry membrane till complete dryness
- Equilibrate membrane for 1-2min in PI (100mM Tris-Cl (pH 7.5), 150mM NaCl)
- Block membrane for 30min in PII (1×PBS with 0.1% Triton X-100) by gentle shaking
- Incubate membrane for 30min in Anti-Digoxigenin-Fab fragments antibody (Roche, 1:2000) in PIII
- Wash 2 × 15min in PI
- Incubate membrane for 2min in PIII (100mM Tris-Cl (pH9.5), 100mM NaCl, 50mM MgCl₂)
- Stain membrane in PIII containing 450µg/ml NBT (Roche) and 175µg/ml BCIP (Roche)
- Wash 1-2min in PII and take picture

6.8.4 Proteinase digestion and post-fixation

All steps were performed at RT on a shaker except otherwise indicated.

- 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 × 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 5×5 min in PTW

6.8.5 Hybridization

All steps were performed at 65°C in a water bath.

- Prehybridize samples in Hyb+ mix for 2 hours
- Meanwhile, dilute proper amount of probe in 200 μ l Hyb+ mix and denature at 80°C for 10min then immediately put on ice
- Replace prehybridization solution with denatured probe
- Hybridize at 65°C overnight

6.8.6 Washing at 65°C

Prepare $4 \times$ SSCT by diluting $20 \times$ SSC (3M NaCl, 0.3M sodium citrate, pH 7.0) 1:5 and add Tween-20 to 0.1%.

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

6.8.7 Antibody incubation

- Block samples for 2 hours in 5% sheep serum in PTW at RT, shaking
- Replace blocking solutions with Anti-Digoxigenin-Fab fragments antibody (Roche, 1:2000) in 2.5% sheep serum in PTW, incubate overnight at 4°C
- Wash 3×40 min, 3×1 hour at RT, then wash overnight at 4°C

6.8.8 Detection

- Equilibrate samples 2×5 min in SB1 (100mM Tris-Cl (pH 9.5), 100mM NaCl, 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, 10% polyvinylalcohol) containing 450 μ g/ml NBT (Roche) and 175 μ g/ml BCIP (Roche)
- Stain in darkness till best signal-background ratio obtained

6.8.9 Post-fixation and storage

- Wash $3 \times 5\text{min}$ in PTW to stop reaction
- Post-fix in 4% PFA in PTW at RT for 1 hour
- Replace fixative with glycerol and store in glycerol in darkness at 4°C till mounting and microscopy

6.9 Optical microscopy

6.9.1 Common microscopy

Samples from immunohistochemistry, *in-situ* hybridization were mounted onto glass slides in their storing solution respectively. For live imaging of *Platynereis* older than 5dpf, animals were paralyzed in 0.37M MgCl_2 mixed 1:1 with sea water and mounted in the same solution. Fluorescent as well as light field images were taken on an Axioplan2 microscope (Zeiss) with a $20\times$ objective or a $40\times$ oil immersion objective, and a Zeiss AxioCam MR5 camera was used for documentation.

6.9.2 Confocal microscopy

Samples were mounted onto glass slide in ProLong Gold antifade reagent (Life technologies) and cured overnight before imaging. Z-stack confocal images were taken using a Zeiss LSM 710 confocal microscope with a $40\times$ 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/>).

6.10 Generating transposon-based transgenic lines

6.10.1 Generating reporter constructs of *Pdu-aanat* and *Pdu-hiomt*

Reporter constructs for *Pdu-aanat* and *Pdu-hiomt* were generated by BAC recombineering and sub-cloning the recombined locus into transposon vectors. A plasmid containing an EGFP-F2A-NTR-SV40pA-rpsLneo construct was generated by Andrea Brezovich (Figure 32), by cloning F2A-NTR-SV40pA from p3E-E2N into S006-R6K-2xTY1-GFP-FNF-3xFLAG via a BsrGI restrictions site. The targeting cassette to be inserted into the first exon of *Pdu-hiomt* locus was generated by PCR using this plasmid as template, with the primers containing homology arms matching the first exon of *Pdu-hiomt*(primers #1388, #1389) using the following recipe:

Reagent	Stock	Volume (μl)
HF buffer	5×	10
dNTPs	10mM	1
Primer F	5μM	2
Primer R	5μM	2
DNA template		1
Phusion polymerase	5U/μl	0.5
ddH ₂ O	ad 50μl	33.5

PCR program: 98°C 5min; 35 x {98°C 10sec; 60°C 30sec ; 72°C 1min50sec} ; 72°C 7min

A *Platynereis* BAC library was generated by Raible *et al.* [99]. The BAC CH305-201G23 containing the *Pdu-hiomt* locus and CH305-102P16 containing the *Pdu-aanat* locus were used for recombineering.

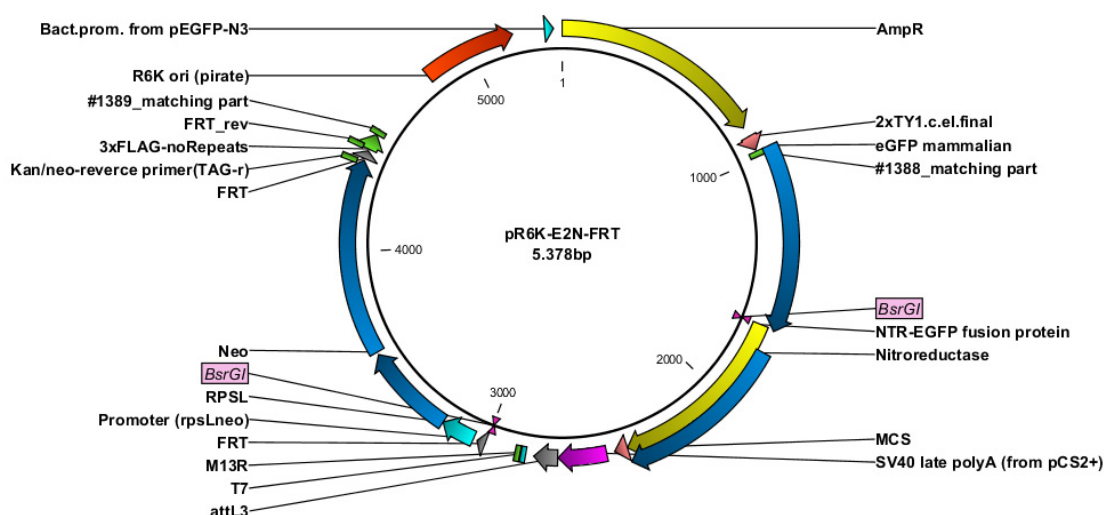


Figure 32. Map of the plasmid containing an EGFP-F2A-NTR-SV40pA-rpsLneo construct

BAC recombineering was performed with the Counter-Selection BAC Modification Kit (Gene Bridges) following the protocol of the manufacturer. Successfully recombined BAC clones were confirmed by comparing the restriction digestion product patterns and direct sequencing into the targeting cassette region.

The Mos1-based transposon vector pMosSce^{frkt318} and the Tol2-based transposon vector pTol2Sce^{frkt761} were previously generated in the lab and have already been successfully used in generating the transgenesis vector pMos{r-opsin::egfp}^{frkt890} [150] and pTol2{rps9::egfp}^{frkt868} [113]. The recombined *Pdu-aanat* and *Pdu-hiomt* locus were PCR amplified and a restriction site for I-SceI was added using the recombined BAC as template (primers for *Pdu-aanat*: #1343, #1368, #1369, #1371, primers for *Pdu-hiomt*: #1505, #1506, #1507, #1508). The following recipe was used:

Reagent	Stock	Volume (μl)
HF buffer	5×	10
dNTPs	10mM	1
Primer F	5μM	2
Primer R	5μM	2
DNA template		0.5
Phusion polymerase	5U/μl	0.5
ddH ₂ O	ad 50μl	34

PCR program: 98°C 5min; 35 x {98°C 10sec; 60°C 30sec ; 72°C 3min15sec-10min (depending on the amplicon size)} ; 72°C 7min

The amplicon was sub-cloned into both transposon vectors via the I-SceI site. The final transposon plasmid for injection was then prepared with EndoFree Plasmid Maxi Kit (Qiagen) following manufacturer's instructions.

6.10.2 Generating transposase mRNA

Linearized pCS2+ vectors containing Mos1 and Tol2 transposase were used as template and transposase mRNA was generated by *in vitro* transcription with the mMESSAGE mMACHINE SP6 Transcription Kit (Life Technologies). The reactions were cleaned up with RNeasy Mini Kit (Qiagen).

6.10.3 Microinjection into *Platynereis* zygotes

Microinjection into *Platynereis* zygotes or 2-cell stage embryos was carried out as described [113]. The injection solution contained 300ng/μl transposon plasmid DNA, 300ng/μl corresponding transposase mRNA and 0.6% (w/v) TRITC-Dextran (MW 70kDa, Invitrogen), and was filtered using 0.45μm PVDF centrifugal filters (Ultrafree-MC-HV, Millipore) prior to use by centrifugation at 12,000g for 3 min at 4°C.

6.10.4 Monitoring of GFP expression

Live monitoring of GFP expression was performed on a Zeiss Lumar V.12 Stereomicroscope with a FITC fluorescence filter. Images were taken on a Zeiss Axioplan2 microscope with a 20× objective or a 40× oil immersion objective and a Zeiss AxioCam MR5 camera.

6.10.5 Screening for transgenic carrier or offspring

Pdu-hiomt construct injected *Platynereis* embryos or offspring of the potential transgenic carriers were raised for more than 2 months and the last third of a single animal (tail) was collected. Genomic DNA was extracted from the tails with NucleoSpin Tissue kit (Machery-Nagel) and was used as template to PCR amplify the integrated eGFP (primers #1324, #1200) with the recombined BAC as positive con-

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trol. HotStarTaq Plus DNA Polymerase (Qiagen) was used in a total volume of 25 μ l with the following recipe:

Reagent	Stock	Volume (μ l)
ddH ₂ O		14.3
CoralLoad PCR buffer	10 $\times$	2.5
dNTPs	10mM	0.5
Primer F	5 μ M	2.5
Primer R	5 μ M	2.5
MgCl ₂	25mM	1.5
Taq	5U/ μ l	0.2
DNA template		1

PCR program: 95°C 5min; 35 x {94°C 30sec; 63°C 45sec; 72°C 1min}; 72°C 10min.

The PCR products were resolved on an agarose gel and stained with Ethidium bromide. The animals in which eGFP could be amplified from the gDNA were tested as having integrated the reporter construct.

6.11 Generating knock-out lines with TALEN

6.11.1 Genotyping for allelic differences

Tails from worms of PIN or VIO strain were collected and genomic DNA was extracted from single tails. Primers were designed with the amplicon covering most of the first two exons of *Pdu-hiomt* (primers #1324, #1828). PCRs were carried out with Phusion DNA polymerase (Thermo scientific) with gDNA as template using the following recipe:

Reagent	Stock	Volume (μ l)
HF buffer	5 $\times$	5
dNTPs	10mM	1
Primer F	5 μ M	2.5
Primer R	5 μ M	2.5
DNA template		2
Phusion polymerase	5U/ μ l	0.25
ddH ₂ O	ad 25 μ l	11.75

PCR program: 98°C 30sec; 35 x {98°C 10sec; 72°C 45sec} ; 72°C 7min

The amplicons were sub-cloned into pJET1.2 vector with the CloneJET PCR Cloning Kit (Thermo scientific) and the plasmids were double digested with BglII and HinfI (fast digest enzymes, Thermo

scientific) with the resulting digestion profile indicating the allelic differences. The genomic sequences were confirmed by direct sequencing (LGC genomics).

6.11.2 Prediction and design

TALENs were designed as previously described [139]. TALEN target sites were predicted using the online tool TAL Effector Nucleotide Targeter 2.0 (<https://tale-nt.cac.cornell.edu/node/add/talen>) by entering the CDS sequence of the first 3 exons. TALENs spanning over different exons were excluded and only TALENs with a restriction site in the spacer were considered, and NN for G recognition. One TALEN pair with the restriction site of EarI in the spacer was selected for *Pdu-hiomt*. All selected TALENs have 15RVD and a 15bp spacer. Care was taken to avoid SNPs in the TALEN target sites. As this was impossible for *Pdu-hiomt*, a region with the least SNPs was chosen and two different right TALENs were designed to target the two different alleles.

6.11.3 Constructing TALENs

TALENs were constructed using the GoldenGate plasmid kit (Addgene) with the protocols published by Cermak *et al.* [151]. The heterodimeric FokI expression plasmids pCS2TAL3-DD and pCS2TAL3-RR (Addgene) [179] were used to assemble the final TALEN expression plasmid. Final TALEN expression vector sequences were confirmed by direct sequencing using TAL_F1 and TAL_R2 primers [151] (primers #1823, #1824).

6.11.4 Preparation of TALEN mRNA

TALEN expressing plasmids were digested with NotI-HF (New England BioLabs) and used as a linear template for in vitro transcription with the mMESSAGE mMACHINE SP6 Transcription Kit (Life Technologies). The concentration of yielded mRNA was measured with a NanoDrop.

6.11.5 Injection into *Platynereis* zygotes

The TALEN mRNA was diluted to the final concentration for injection without further purification. The injection solution contained 400ng/μl of each TALEN mRNA and 0.6% TRITC-Dextran (MW 70kDa, Invitrogen) in RNase free water. Further treatment of the injection solution and the microinjection method was already described in Section 6.10.3.

6.11.6 Genotyping of injected larvae

Pools of two or five embryos were harvested between 20hfp and 24hfp and were digested with Proteinase K as described by Bannister *et al.* [139]. One microliter of the reaction was used as PCR template for genotyping. As it was not possible to avoid SNPs when designing primers, two different re-

verse primers were designed matching the two different alleles (primers #2191, #2192). HotStarTaq Plus DNA Polymerase (Qiagen) was used in a total volume of 25µl with the following recipe:

Reagent	Stock	Volume (µl)
ddH ₂ O		7.3
CoralLoad PCR buffer	10×	2.5
Q-solution	5×	5
dNTPs	10mM	0.5
Primer F (#2179)	5µM	2.5
Primer R1 (#2191)	5µM	2.5
Primer R2 (#2192)	5µM	2.5
MgCl ₂	50mM	1
Taq	5U/µl	0.2
DNA template		1

PCR program: 95°C 5min; 35 x {94°C 30sec; 64°C 1min ; 72°C 40sec} ; 72°C 10min.

A restriction digest mixture contained 10µl PCR product, 2µl CutSmart buffer (NEB), 1µl EarI (NEB), ddH₂O ad 20µl and was carried out at 37°C for 1 hour. Digestion reactions were resolved on 2% agarose gel with 0.01% (v/v) SYBR safe DNA gel stain (Life technologies) and an undigested band was taken as evidence for TALEN-induced mutation. Undigested bands were sub-cloned into pGEM-T vector (Promega) and mutations were confirmed by direct sequencing (Microsynth) with primer #2179.

6.11.7 Genotyping of F1 offspring

The injected larvae were raised and outcrossed with wildtype animals. Thirty single embryos per batch were harvested between 24hfp and 7dpf were digested with 10µl Proteinase K solution each as described by Bannister *et al.* [139]. One microliter of the reaction was used as PCR template for genotyping as described in Section 7.11.6.

A restriction digest mixture contained 5µl PCR product, 1µl CutSmart buffer (NEB), 0.5µl EarI (NEB), ddH₂O ad 10µl and was carried out at 37°C for 2 hours. Digestion reactions were resolved on 2% agarose gel with 0.01% (v/v) SYBR safe DNA gel stain (Life technologies) and an 50% undigested band was taken as evidence for heterozygous offspring and germline transmission of the founder animal. Undigested bands were sub-cloned into pGEM-T vector (Promega) and mutations were confirmed by direct sequencing (Microsynth) with primer #2179.

6.12 Nanostring

6.12.1 Sample preparation and raw data generation

RNA samples for nanostring were prepared by Olga Antonova and Alessandra Polo. Animals were decapitated at Zeitgeber Time (ZT) 1,4,7,10,13,16,19,22 under NM and FR-FM in 3 replicates of 5 animals each. Total RNA was extracted using RNeasy Mini Kit (Qiagen) and the concentration was measured with a NanoDrop. RNA quality was confirmed with RT-qPCR by comparing circadian profile for *Pdu-bmal*, *Pdu-period* and *Pdu-clock* with published data [16]. The candidate gene list contains around 250 putative transcription factors and around 250 putative secreted peptide hormones predicted by Paola Oliveri (University College London), circadian clock genes and *cdc5* as house-keeping gene. Probe generation, hybridization and counting of transcripts were carried out by Nanostring Technologies, Inc.

6.12.2 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 $\text{mean} + 2 \times \text{SD}$ 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).

6.12.3 Cloning of candidate genes

cDNA was generated from total mRNA extracted from *Platynereis* heads using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) following the manufacturer's instructions. One microliter of the cDNA was taken as template and PCRs were carried out with Phusion DNA polymerase (Thermo scientific) followed with sub-cloning into pJET1.2 vector (Thermo scientific) or with HotStarTaq Plus DNA Polymerase (Qiagen) followed with sub-cloning into pGEM-T vector (Promega). Gene 15 (isotig11375) was cloned with primers #2440 and #2441, and gene 17 (isotig 28605) was cloned with primers #2468 and #2469. The cloned sequences were confirmed by direct sequencing (Microsynth) and used as template for the generation of RNA probes for in-situ hybridization.

6.13 List of primers

Cloning, genotyping and sequencing primers:

ID#	Name	Sequence
1200	EGFP rev	CTCGTCCATGCCGAGAGTGATCC
1324	Pdu_hiomt_pred_up1	ATGTTGGACGTGGACAGACATGACAC
1343	Pdu_aanat_full_rev	GTTCTATGTGTAATTGGCCTTTTG
1367	SceI_aanat_up1	TAGGGATAACAGGGTAATGTTGTGCTCCTGTCTAGACCG
1368	SceI_aanat_up2	TAGGGATAACAGGGTAATGAGAATGTCAGCAGATTTGTGTTG
1369	SceI_aanat_up3	TAGGGATAACAGGGTAATGCCGAGGGTAAAAAACTTTT-GACTG
1370	SceI_aanat_lo1	TAGGGATAACAGGGTAATGGAAGATCACCGAGCACTGTC
1371	SceI_aanat_lo2	TAGGGATAACAGGGTAATGACAACGCTTTAGAAATCTTCAG
1372	T7	TAATACGACTCACTATAGGG
1388	Pdu_hiomt_1st_ex_MSL_eGFP_up	TGTTGGACGTGGACAGACATGACACGGGCTCCAAGGACTT-GGATTACACTATGGTGAGCAAGGGCGAGGAGC
1389	Pdu_hiomt_cas_1st_exon_lo	TTTATTTAAGTAACACATACCAGAGATGCTCTG-TAGCCATCGAGCAATGATAACAGATGAGGGCAAAGCGCT
1390	hiomt_recomb_lo	CAGAGATGCTCTGTAGCCATC
1455	Pdu_hiomt_locus_lo2	CTCTATTATATTTCAAATACAGTACTCCGC
1505	Pdu_hiomt_SceI_up1	TAGGGATAACAGGGTAATCTCTGTTTCCCATGTTAG-TTATTGCG
1506	Pdu_hiomt_SceI_lo1	TAGGGATAACAGGGTAATAGGCCACTTAAACATACCCGACTC
1507	Pdu_hiomt_SceI_lo2	TAGGGATAACAGGGTAATCTCTATTATATTTCAAATACAG-TACTCCGC
1508	Pdu_hiomt_SceI_lo3	TAGGGATAACAGGGTAATCTCTATTATATTTCAAATACAG-TACTCCGC
1808	Pdu_aanat_cds_up_BamHI	TCAGGATCCATGGCACTGACAGGAGAATTTCAG
1809	Pdu_aanat_cds_lo_XhoI	ACTCTCGAGCTATACGAGCATCACCATTTCAAAC
1810	Pdu_hiomt_cds_up_BamHI	TCAGGATCCATGTTGGACGTGGACAGACATGAC
1811	Pdu_hiomt_cds_lo_XhoI	ACTCTCGAGTTAAGGTTTTCTTGCGTAGATGATGTC
1823	TAL_F1	TTGGCGTCGGCAAACAGTGG
1824	TAL_R2	GGCGACGAGGTGGTCGTTGG
1828	Phio_exon2_allele_lo	GACTCGGTCCTCTTGTAAGCATCTT
2100	cycle_3_GFP_R	CCATCTAATTCAACAAGAATTGGGACAAC
2139	Pdu_hiomt_TOPO_for	CACCATGGGAATGTTGGACGTGGACAGACA
2140	Pdu_hiomt_TOPO_rev	GTTAAGGTTTTCTTGCGTAGATGATG
2141	Pdu_hiomt_TOPO_rev_non Stop	AGGTTTTCTTGCGTAGATGATGTC
2145	PHP_hiomt_P1_lo	TTGGTGCTTACCTGATTTGATTTC
2146	PHP_hiomt_P2_up	TCCCATGCGTTGCATGAG
2147	PHP_hiomt_H_up	GAAATCAAATCAGGTAAGCACCAATACCTGGAGACGTTT-GGCG
2148	PHP_hiomt_H_lo	CTCATGCAACGCATGGGA GGACCTGTAGATGGCCGTAA

ID#	Name	Sequence
2179	Pdu_hiomt_CDS_up	ATGTTGGACGTGGACAGACAT
2191	Pdu_hiomt_BAC_exon2_al_lo	GGTCCTCTTGTGAAGCATCTTT
2192	Pdu_hiomt_pJet_exon2_al_lo	GGTCCTCTTGTGAAGCATCTTC
2440	gen15_up1	CCAGTTGACCTTCACCATAGC
2441	gen15_lo1	GGCTTGATTTTAATTACAACAGATACG
2468	gen17_up1	GAAAGCCATGAAGCCCATAA
2469	gene 17_lo1	AGGACTGAGAGTCCCTCCAAG
	pGEX5'	GGGCTGGCAAGCCACGTTTGGTG
	pGEX3'	CCGGGAGCTGCATGTGTCAGAGG

Quantitative RT-PCR primers:

ID#	name	Sequence
1	pducde5_L1	CCTATTGACATGGACGAAGATG
2	pducde5_R1	TTCCCTGTGTGTTTCGCAAG
593	Pdu_aanat L1	TGCATCCATTTCAGTGTGTGT
594	Pdu_aanat R1	GAAATGTTTCAACAGATTTAAAGCAA
599	Pdu_hiomt L2	CCCAATCATTTATGCTCCAAA
600	Pdu_hiomt R2	CCATCCTTTTAAGTACCAAGGAGA
902	RPS9_L1	CGCCAGAGAGTTGCTGACT
903	RPS9_R1	ACTCCAATACGGACCAGACG
2123	isotig12505_qPCR_L	CCATGAGGCCTTCTACAGATGC
2124	isotig12505_qPCR_R	GCTATGGCGTTACCAGAGTCA
2125	isotig22423_qPCR_L	CTACCAAGTCGTTGGTCTGCT
2126	isotig22423_qPCR_R	GCAAGAGTCTGTTTCATGACGA
2131	isotig08891_TN_Hormone_qPCR_L	AACGAGTACTACGCCATCGTG
2132	isotig08891_TN_Hormone_qPCR_R	GCCTCCTAGATAAGCCGACTG
2133	FV9XCA101BDK13_qPCR_L	CCGAAGATCTGAAGGACGAC
2134	FV9XCA101BDK13_qPCR_R	TTCTCCAAGAACGCTGCTC
2135	isotig25816_qPCR_L	CGGGATACTAAACGCCTCAC
2136	isotig25816_qPCR_R	GGGGACGATATGACGACCT
2137	isotig10193_qPCR_L	TGGACATCAGCTGAAACTAGGA
2138	isotig10193_qPCR_R	CATCAGCTGTGATGCTTACCA
2167	Pdu_FVMamide_qPCR_L1	GGGAAAGAGAAACCCAAAAGA
2168	Pdu_FVMamide_qPCR_R1	TCTGGTAATCTGGCTTTTGCTC
2208	Top1_qPCR_L1	CACAACCTGAGCAGCCTAATC
2209	Top1_qPCR_R1	AAAGGCCGCAATTTGTATCAC

7. APPENDIX

Script for Matlab regression:

```
>> mingfun.m

%to be used with lsqcurvefit, for example:
%x1 = lsqcurvefit(@mingfun, [0.1 0.1 0.1 0.1], data(:,1)', data(:,2)', [-1000
%-100 0.01 -1000], [1000 100 1000 1000])

function [F]=mingfun(x, xdata)
A=x(1);
B=x(2);
C=x(3);
D=x(4);
F=(A-D)./(1+(xdata/C).^B)+D;
End

>> evaluate_mingfun.m

function evaluate_mingfun
data = input('data:');
[x resnorm, residual]= lsqcurvefit(@mingfun, [0.1 0.1 0.1
0.1], data(:,1)', data(:,2)', ...
    [-1000 -100 0.01 -1000], [1000 100 1000 1000]);
A=x(1)
B=x(2)
C=x(3)
D=x(4)
R_squared=1-resnorm/sum((data(:,2)-mean(data(:,2))).^2)
xinterval=0:0.01:20;
figure(1);
hold on;
plot(xinterval, (A-D)./(1+(xinterval/C).^B)+D, 'r');
plot(data(:,1), data(:,2), 'bo');
end
```

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10. CURRICULUM VITAE

EDUCATION

- Since 02.2010 **PhD studies on molecular biology** in Max F. Perutz Laboratories, University of Vienna, Austria
- Thesis: Insights into the molecular output of the circalunar clock in *Platynereis dumerilii*
- Supervisor: Dr. Florian Raible
- 09.2007 – 07.2009 **Master of Engineering on biochemical engineering** at Department of Chemical Engineering, Tsinghua University, Beijing, China
- Thesis: Catalytic behavior of polymer-modified lipase in hydrophobic organic solvent
- Supervisor: Prof. Zheng Liu, Advisor: Dr. Diannan Lu
- Degree conferred: 07.2009
- 09.2004 – 06.2006 **Diplôme d'Ingénieur**, Ecole Centrale Paris, France
- Double-degree program with Tsinghua University
- Degree conferred: 10.2009
- 09.2002 – 06.2004 **Bachelor of Engineering on Polymer engineering** at Department of Chemical Engineering, Tsinghua University, Beijing, China
- Degree conferred: 07.2006

SCHOLARSHIPS AND AWARDS

- 2014 Award for best student oral presentation in MFPL Retreat 2014
- 2013 Travel award from the University of Vienna
- 2010 – 2012 Erasmus Mundus External Cooperation Window (EMECW) scholarship
- 2008 Friend of Tsinghua- Guanghua Scholarship
- 2004 – 2006 Eiffel excellence scholarship from the French Ministry of Foreign Affairs
- 2003, 2004 Friend of Tsinghua-Tangshi Scholarship for outstanding students

PUBLICATIONS AND CONFERENCE PRESENTATIONS

Design and synthesis of lipase nanogel with interpenetrating polymer networks for enhanced catalysis molecular simulation and experimental validation.

Mingliu Du, Diannan Lu, Zheng Liu.

Journal of Molecular Catalysis. B, Enzymatic, 88(2013) pp. 60-68.

Characterization of a protostome melatonin biosynthesis pathway.

Mingliu Du, Juliane Zantke, Agne Valinciute, Kristin Tessmar-Raible, Florian Raible.

13th International Congress on Invertebrate Reproduction and Development. Detroit USA, 14 – 19 July, 2013. Poster presentation.

Award for Outstanding Student Application.

Insights into the molecular output of the circalunar clock in *Platynereis dumerilii*.

Mingliu Du, Alessandra Polo, Olga Antonova, Juliane Zantke, Tobias Neumann, Arndt von Haeseler, Paola Oliveri, Kristin Tessmar-Raible, Florian Raible.

CIG Symposium 2014 "Rhythms in Biology". Lausanne Switzerland, 12-13 June, 2014. Abstract selected for oral presentation.

Via est.