



universität
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DIPLOMARBEIT

Titel der Diplomarbeit

„Target Tissues of Melatonin and PDF in *Platynereis*“

Verfasser

Stefan Hammer

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Wien, 2012

Studienkennzahl lt. Studienblatt:

A 441

Studienrichtung lt. Studienblatt:

Diplomstudium Genetik - Mikrobiologie (Stzw) UniStG

Betreuer:

Dr. Florian Raible

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1. Abstract

Platynereis dumerilii (Annelida) has become a successful model organism mainly used in evolutionary research, comparative genomics and neurobiology (D. Arendt et al. 2004; Raible et al. 2005; Jekely et al. 2008). The marine ragworm, like various other invertebrate marine species, ensures the success of external fertilisation through synchronous mass spawning (Fox 1924; Korrynga 1947). This phenomenon is driven by an endogenous circalunar clock, where the synchrony of the cycle is entrained only by a nocturnal light stimulus (Hauenschild 1960). Additionally *Platynereis* also comprises a circadian clock to maintain a proper day-night activity rhythm (D. Arendt et al. 2004).

Another reason for studying lophotrochozoan species and in particular *Platynereis* are their ancestral-type characteristics in gene structure, development and anatomy. They are less derived than other frequently used model systems and are therefore important for our understanding of animal evolution (Tessmar-Raible and Arendt 2003). These ancestral-type features also extend to components of the hormone-system, for example to steroid receptors (Eick and Thornton 2011).

Recently we identified several important key players of the aforementioned clock systems, among which are melatonin and the pigment dispersing factor, PDF (unpublished data). These two hormones also exhibit ancestral-type features and from other species it is known that they are regulated by light, either directly or indirectly through a clock. Melatonin was found in almost all organisms but is best studied in vertebrates. There it represents the major output factor of the circadian clock (Bell-Pedersen et al. 2005; Hardeland and Poeggeler 2003). PDF was first identified in crustaceans and was then investigated in detail in *Drosophila melanogaster* where it is discussed to play multiple roles in the circadian clock (Helfrich-Förster 2009).

The aim of this study was to identify and investigate the target receptors of these hormones, as a detailed understanding about the properties of hormone-receptors already helped to reveal the function of the binding hormone in the past (Lincoln et al. 2006). After identifying clear ortholog sequences of the melatonin and PDF receptor in *Platynereis*, I successfully cloned them. With in situ hybridisation data from embryonal stages I revealed that the Melatonin Receptor is constantly expressed in nervous tissues, predominantly in distinct areas of the brain. Comparing these staining patterns with those of Aanat and Hiomt led me to assume that melatonin is produced in the area posterior of the neuropil and then transported through neurons into the sensing cells anterior of the neuropil. I was also able to demonstrate a proper binding of melatonin to this receptor using the Cell Impedance Assay.

The PDF receptor sequence shows high similarities with ortholog genes and its expression is constant and exclusively found in nervous tissue. This includes cells in the ventral nerve plate for very young stages and dorsal and dorsolateral regions in the head. This expression pattern in the head is to a certain degree consistent with observations in *Drosophila* (Lear et al. 2005). Due to the fact that I was not able to clone the full length of the receptor, the obligatory verification of this receptor candidate binding its ligand, PDF, is still missing.

Using Immunohistochemistry and Whole Mount in Situ Hybridisation, I analysed the local and temporal patterns of the RNA and protein expression of PDF in larvae and adult worms. With this data it was possible to gain first insights into the temporal and spatial location of PDF and its receptor.

Zusammenfassung

Platynereis dumerilii (Annelida) ist zu einem erfolgreichen Modellorganismus geworden, der vor allem in Evolutionsforschung, vergleichender Genomik und Neurobiologie Verwendung findet (D. Arendt et al. 2004; Raible et al. 2005; Jekely et al. 2008). Die synchrone Massenbefruchtung, die zum Erfolg der externen Befruchtung führt, ist eine weitere Besonderheit von *Platynereis* und zahlreichen anderen marinen Invertebraten (Fox 1924; Korringa 1947). Dieses Phänomen wird durch eine endogene lunare Uhr gesteuert, wobei die Synchronität des Zyklus nur durch einen nächtlichen Lichtstimulus bewahrt wird (Hauenschild 1960). Zusätzlich verfügt *Platynereis* auch über eine circadiane Uhr, um einen geregelten Tag-Nacht-Rhythmus aufrecht zu erhalten (D. Arendt et al. 2004).

Ein weiterer Grund für die Erforschung von Lophotrochozoa und im Besonderen von *Platynereis* sind ihre Genstruktur, ihre Entwicklung und ihre Anatomie, die urzeitliche und evolutionär ursprüngliche Charakteristika aufweisen. Sie sind weniger stark evolviert als andere häufig verwendete Modellsysteme und daher wichtig für unser Verständnis der tierischen Evolution (Tessmar-Raible and Arendt 2003). Diese ursprünglichen Charakteristika betreffen auch die Komponenten des Hormonsystems, zum Beispiel Steroid-Rezeptoren (Eick and Thornton 2011).

Kürzlich identifizierten wir mehrere wichtige Komponenten der zuvor genannten Systeme der inneren Uhren, unter anderem Melatonin und den pigment dispersing factor, PDF (unpublished data). Auch diese zwei Hormone weisen ursprüngliche Charakteristika auf und anhand anderer Spezies ist erwiesen, dass sie durch Licht reguliert werden, entweder direkt oder indirekt durch eine endogene Uhr. Melatonin wurde in fast allen Organismen vorgefunden, kann jedoch am besten anhand von Vertebraten studiert werden. Hier stellt es den bedeutendsten Output-Faktor der circadianen Uhr dar (Bell-Pedersen et al. 2005; Hardeland and Poeggeler 2003). PDF wurde zuerst in Krustazeen vorgefunden und daraufhin im Detail in *Drosophila melanogaster* erforscht, wo es vielfältige Rollen für die circadiane Uhr spielt (Helfrich-Förster 2009).

Da ein detailliertes Verständnis der Eigenschaften von Hormonrezeptoren bereits in der Vergangenheit dazu beigetragen hat, die Funktionen des bindenden Hormons ans Licht zu bringen (Lincoln et al. 2006), war es das Ziel dieser Studie, die Zielrezeptoren der vorhin genannten Hormone zu identifizieren und zu erforschen. Nachdem ich eindeutige orthologe Sequenzen der Melatonin- und PDF-Rezeptoren in *Platynereis* identifiziert hatte, konnte ich sie erfolgreich klonieren. Anhand von durch In Situ Hybridisation erlangten Daten embryonaler Entwicklungsstadien konnte ich eruieren, dass der Melatoninrezeptor konstant im Nervengewebe, vorrangig in bestimmten Hirnregionen, exprimiert wird. Durch einen Vergleich

dieser Färbungsmuster mit jenen von Aanat und Hiomt gelangte ich zu der Vermutung, dass Melatonin im Gebiet posterior zum Neuropil produziert wird und dann durch Neuronen in die Melatonin wahrnehmenden Zellen anterior zum Neuropil transportiert wird. Ich konnte außerdem mit einem Cell Impedance Assay zeigen, dass dieser Rezeptor fähig ist, Melatonin zu binden.

Die PDF Rezeptor-Sequenz zeigt große Ähnlichkeit mit orthologen Genen, und ihre Expression findet konstant und ausschließlich im Nervengewebe statt. Dies schließt bei sehr jungen Entwicklungsstadien Zellen in der ventralen Nervenplatte, sowie allgemein dorsale und dorsolaterale Regionen im Kopf ein. Dieses Expressionsmuster im Kopf ist zu einem gewissen Grad konsistent mit Beobachtungen, die an *Drosophila* gemacht wurden (Lear et al. 2005). Aufgrund der Tatsache, dass ich nicht in der Lage war, die volle Länge des Rezeptors zu klonieren, fehlt die notwendige Verifikation für die Bindung dieses Rezeptor-Kandidaten an sein Bindungsprotein PDF.

Mit Immunohistochemie und Whole Mount in Situ Hybridisation analysierte ich die örtlichen und zeitlichen Muster der RNA- und Proteinexpression von PDF in Larven und adulten Würmern. Durch diese Daten war es möglich, eine erste Einsichten in die zeitliche und räumliche Lokalisation von PDF und seines Rezeptors zu erlangen.

2. Introduction

2.1 The Model Organism *Platynereis dumerilii*

2.1.1 Phylogenetics

Bilateral animals are recently categorized into Deuterostomia and Protostomia, whereas the latter are again subdivided into Ecdysozoa and Lophotrochozoa (Adoutte et al. 2000; Aguinaldo et al. 1997). This leads to three big ancient clades visualized in Figure 1, using the dataset of the Tree of Life web-project (Maddison, Schulz, and Maddison 2007).

In modern biosciences model organisms are studied to investigate particular biological phenomena and in specific cases it can be assumed that the observed knowledge is true for other organisms, too. Most of the molecular model organisms used nowadays belong either to the Deuterostomia such as *Mus musculus* (Mouse, Chordata), *Danio rerio* (Zebra fish, Chordata) and *Xenopus laevis* (African clawed frog, Chordata) or to the Ecdysozoa, like *Drosophila melanogaster* (Fruit fly, Arthropoda) and *Caenorhabditis elegans* (Nematoda).

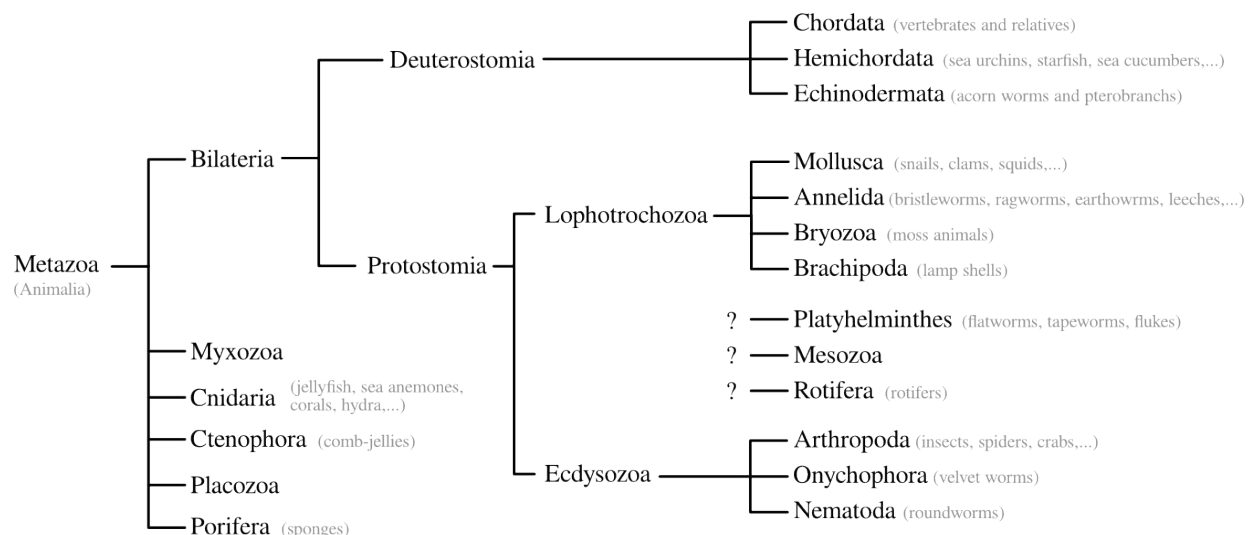


Figure 1: Overview of the phylogenetic relationships within the group of *Metazoa*. Drawn with information from the "Tree of Life webproject", www.tolweb.org; To keep the scheme simple, not all clades are included.

Although the Lophotrochozoans contain the largest number of animal phyla compared to other metazoan clades (Giribet 2008) and represent a huge and independent group of the metazoans, they have been understudied for a long time. Just recently, several lophotrochozoan species emerged as suitable model systems, like *Lottia gigantea* (Sea snail, Mollusca), *Helobdella robusta* (Leech, Annelida) or *Capitella teleta* (Polychaete worm, Annelida).

Also *Platynereis dumerilii* (marine ragworm, Annelida), the organism studied in this work, is getting increasingly attractive for molecular-biological research (Tessmar-Raible and Arendt 2003). Within the Annelids, *Platynereis* belongs to the Errantia because of its errant lifestyle and is further categorized into the group of Phyllodocida and Nereididae. This position is also supported by phylogenomic analyses (Struck et al. 2011).

2.1.2 Model Organism

During the last few years *Platynereis dumerilii* became a successful model organism mainly used in evolutionary research, comparative genomics and neurobiology (D. Arendt et al. 2004; Raible et al. 2005; Jékely et al. 2008). Recently, *Platynereis* was also more frequently used for studies in developmental biology. Topics are for example toxicology linked with growth and reproductive outputs, evolution of segmentation and molecular key players in segment formation (García-Alonso et al. 2011; Steinmetz et al. 2011; Dray et al. 2010). This animal has various features which are essential for the usage as a popular model organism. First, *Platynereis* can be easily cultured in boxes containing artificial sea water, which is rather atypical for marine species. Both, the larvae and the atoke worms are transparent, which is very important for applying spectroscopic methods. *Platynereis* also comprises amazing regeneration capabilities as it is characteristic for all Nereididae, which, for example, makes transplantation experiments very easy.

We maintain a culture with an consistent genomic background since 1953, originally set up by C. Hauenschild (Albrecht Fischer and Dorresteijn 2004). It is easy to perform inbreeding with hundreds of offspring and the generation time of about 6-7 months in average (at least 3 months) (Antje Fischer, Henrich, and Arendt 2010) is a moderate value.

Further, a set of standard techniques is available and well established for this organism. Whole mount in situ hybridisations and even double fluorescence in situ hybridisations (Tessmar-Raible et al. 2005) can be performed with visualization on the cellular expression level using confocal detection of the NBT/BCIP and fluorescence staining (Jékely and Arendt 2007). Immunohistochemistry can be used to visualize specific proteins in the embryos as well as in the whole atoke worm. Methods like the injection of DNA constructs for transgenesis, RNA interference or mutagenesis using Zinc-finger nucleases are currently developed and under heavy investigation (personal communication).

Additionally, there is lots of literature available which provides mentionable amounts of data on the anatomy, regeneration and behavioural biology of *Platynereis dumerilii* (Hauenschild 1953; Hauenschild 1954; Hauenschild 1955; Hauenschild 1956a; Hauenschild 1956b; Hauenschild 1960; Hofmann 1976; Heuer and Loesel 2007; Heuer et al. 2010). Sequence information is also accessible through a library of assembled expressed sequence tags (ESTs) and BAC libraries. With Next Generation Sequencing it was also already possible to derive a partially assembled Pdu genome (personal communication).

2.1.3 Life Cycle

The life cycle of *Platynereis dumerilii* starts with the external fertilization of up to three thousand eggs in open sea water. Two hours post fertilization (hpf) the Zygote (Figure 2 A) starts to cleave with a typical pattern (B). This happens in a protective jelly mass formed earlier by the Zygotes. The embryos rotate slowly driven by their ciliated prototroch cells until they hatch in the early trochophore stage. They soon start to show phototactic behaviour when swimming around (C).

Lipid oil droplets serve as energy resource during these early developmental stages. Three days post fertilization (dpf) all the important structures are already visible and the trunk starts to elongate rapidly with the development of additional segments (E). At this stage the larvae change to a benthic life style, they sometimes use their chaetae (bristles) to crawl¹. In the late nectochaetae stage (5 to 7dpf) embryos start to eat algae and plankton, although the stomodeum is still under development and the jaws just started to form. After three to four weeks the first pair of parapodia is transformed into a pair of tentacular cirri. Herewith the cephalic metamorphosis is finished and the worm only develops new segments at the posterior growth zone (F).

The worms live in characteristic tubes on the seabed built by themselves which they rarely leave although they are able to swim short distances. At the earliest at the age of three months (average 6-7 months) the worms undergo a sexual metamorphosis where they change their lifestyle to a pelagic one. Food intake stops and the morphology changes dramatically (G). The eyes increase in size, the parapodia change for better swimming capabilities and the colour gets yellow for female worms (full with oocytes) and white/red for male worms (anterior part filled with sperm cells, posterior: accumulation of blood capillaries). The reproduction is synchronized

between the animals with the lunar light cycle to ensure proper external fertilization of the gametes. With the highest peak being during new moon, the animals leave their tubes and perform a nuptial dance with subsequent mass spawning. *Platynereis* die after they have successfully mated (Antje Fischer, Henrich, and Arendt 2010).

The synchronous mass spawning is a highly interesting feature of *Platynereis dumerilii*, making this organism a excellent model for chronobiological studies.

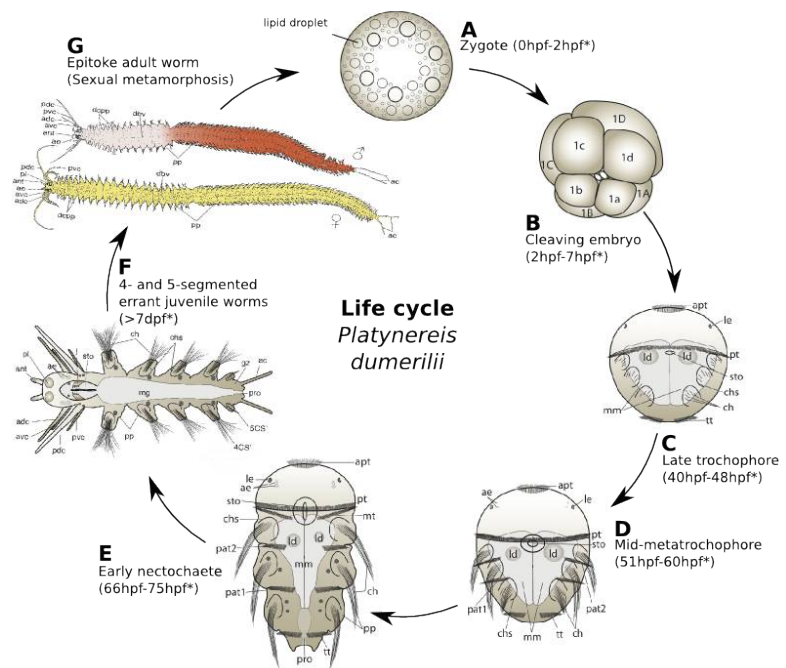


Figure 2: Life cycle of the rag-worm *Platynereis dumerilii*. Abbreviations: apt: apical tuft, le: larval eye, pt: prototroch, sto: stomodeum, chs: chaetal sac, tt: telotroch, mm: macromere, ld: lipid droplet, ae: adult eyes, pat1: first paratroch, pat2: second paratroch, ch: chaetae, pp: parapodium, pro: proctodeum, mg: midgut, pdc: posterior dorsal cirrus, h/dpf (hour/days post fertilization). Picture modified from Hauenschild and Fischer (1969). Drawings of the developmental stages are taken from Fischer et al. (2010). Drawn by Fiorella Schischlik.

1 <http://www.youtube.com/watch?v=g8auUQRIwCI>

2.2 Chronobiology in *Platynereis*

Concurrent mass spawning to ensure the success of the external fertilisation is not only important in *Platynereis*, but is also known to be essential for various other invertebrate marine species (Fox 1924; Korringa 1947). It is driven by an endogenous circalunar clock, where the synchrony of the cycle is entrained only with the nocturnal light stimuli (Hauenschild 1960). In his early studies, Hauenschild (1960) already included free-running experiments proving the former and showed that only lunar light is the zeitgeber for this circalunar clock.

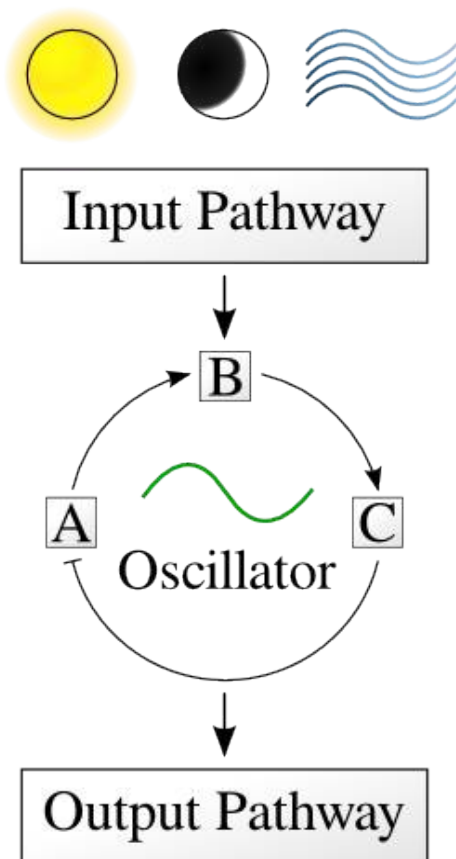


Figure 3: General concept of a biological clock: Environmental stimuli (e.g. solar, lunar light, tidal phases) serve as zeitgeber to entrain the clock. The signal is sensed through the input pathway and passed on to the oscillator, which usually consists of a small amount of genes connected with transcriptional and translational feedback loops. All pathways and genes directly underlying the control of the oscillator are defined as part of the output pathway.

Additionally *Platynereis* also comprises a circadian clock to maintain a proper day-night activity rhythm (D. Arendt et al. 2004). This daily rhythm must be in some way connected to the monthly lunar rhythm. This was shown with the observation of the reproduction being restricted to a certain circadian time window (Milton Fingerman 1957). This makes *Platynereis dumerilii* a very interesting model organism for studying chronobiology and its biological rhythms.

The general concept of a biological clock is already well known (Eskin 1979). It consists of an input stage which receives an endogenous stimulus from the environment – also called zeitgeber – for proper synchronization and entrainment. This signal is then passed to the oscillator, the main component of the clock. It is built of negative transcriptional and translational feedback loops controlled by regulatory genes (Roenneberg and Merrow 2005). This oscillator resonates with the period of the input stimulus and generates the internal sense of time. In a third step, the oscillating signal is then transferred to the output pathways where it affects physiology, metabolism and behaviour (Figure 3).

As already mentioned, the main function of the input stage is the synchronization of the endogenous clock with the environment. Using a specific environmental cue the clock is entrained, which means the phase is reset. This can be triggered by several recurring biological phenomena, such as tidal phases, the solar or even the lunar light (Tessmar-Raible, Raible, and Arboleda 2011). Usually in the input stage of circadian clocks, photoreceptors with their ability to sense light are used.

The oscillator consists of a rather small amount of genes, which build a regulatory network that swings in the right period. To ensure this on the biochemical level, there are four general

requirements necessary: “...*negative feedback, time delay, sufficient 'nonlinearity' of the reaction kinetics and proper balancing of the timescales of opposing chemical reactions*” (Novák and Tyson 2008).

The output stage is not defined as exactly as the others and varies from system to system. In general it includes all pathways and genes directly underlying the signal of the oscillator. This leads to a clock-controlled transcriptional and translational regulation of the output genes.

In *Platynereis dumerilii* possible molecular components of the circadian and the circalunar clock and their molecular mechanisms are studied eagerly (personal communication). It provides a better chronobiological model than other frequently studied organisms because they only represent a specialized and rather small subset of species. The slowly evolving marine rag-worm comprises many ancestral-type features (see Chapter 2.2). It still lives in the ancient marine environment and therefore does not have to cope with drastic habitat changes (Tessmar-Raible, Raible, and Arboleda 2011). Therefore, overall fundamental elements of the existing clocks could be identified this way.

Further, it would be highly interesting to observe how the different types of rhythms are connected and to which extent the clocks share specific molecular components. But not only insights into the crosstalk between clocks or between clock and physiological events would be possible. Another stunning aspect further downstream is that the regeneration capabilities of Nereididae often anti-correlate with the reproduction cycle. With the onset of gamete production the worms are not able to regenerate huge parts of their body any more (Hofmann 1976; Lawrence and Soame 2009). A functional and evolutionary understanding of these phenomena could lead to important observations also in terrestrial models (Tessmar-Raible, Raible, and Arboleda 2011).

2.3 Ancestral-type Features

Another reason to study lophotrochozoan species and in particular *Platynereis* are their already mentioned ancestral-type characteristics in gene structure, development and anatomy. They are less derived than other frequently used model systems and are therefore important for our understanding of animal evolution (Tessmar-Raible and Arendt 2003). *Platynereis dumerilii* exhibits several of these features which are atypical for ecdysozoans and rather similar to the deuterostomata.

The trochophore larva with its evolutionary conserved foregut and mouth region is, for example, considered to be an ancestral-type characteristic of the Urbilateria (D. Arendt, Technau, and Wittbrodt 2001). Further, unlike in insects and vertebrates two photoreceptor cell types could be found in the marine rag-worm. The eye comprises rhabdomeric photoreceptor cells and the medial brain ciliary photoreceptor cells (D. Arendt et al. 2004). Another example are distinct brain cell types with dual sensory-neurosecretory properties which were initially thought to be restricted to deuterostomes, but were then found in *Platynereis* and therefore considered to be involved in the origin of neurosecretory brain centres in Bilateria (Tessmar-Raible et al. 2007).

Further evidence for a common origin of a centralized nervous system in Bilateria is the architecture of the *Platynereis* nervous system patterning. Several domains match corresponding areas in the brain of deuterostomes (Denes et al. 2007). This was also found for the vertebrate pallium and the annelid mushroom bodies, which show deep homology (Tomer et al. 2010).

A genomic comparison of the exon/intron organisation of thirty genes led to the conclusion that the intron rich gene structure of *Platynereis* is highly similar to the one of vertebrates and that this structure was lost in other invertebrate species (Raible et al. 2005). A similar conclusion was found when investigating the ParaHox gene cluster. Here, Ecdysozoan models suffer from gene loss and cluster break-up. *Platynereis dumerilii* as a less-derived protostome animal enabled the reconstruction of the ParaHox cluster in the bilaterian ancestor (Hui et al. 2009).

These ancestral-type features also extend to the hormone-system. For example, steroid receptors were thought to be restricted to vertebrates only because of their absence in arthropods, nematodes and urochordates. As unambiguous homologs were found in annelids and molluscs it was clear that the family already derived from the protostome-deuterostome ancestor. These protostome receptors are very similar in sequence to the vertebrate estrogen receptors, show a high phylogenetic relationship and contain the same functional DNA binding domains as those of vertebrates (Eick and Thornton 2011).

Recently more such hormones with ancestral-type features were identified (Raible F, personal communication), among which are melatonin and the pigment dispersing factor, PDF. These two hormones are both known to be regulated by light, either directly or indirectly through a clock. Melatonin was found in almost all organisms but is best studied in vertebrates. There it represents the major output factor of the circadian clock (Bell-Pedersen et al. 2005; Hardeland and Poeggeler 2003). PDF was first identified in crustaceans and was then investigated in detail in *Drosophila melanogaster* where it is discussed to play multiple roles in the circadian clock (Helfrich-Förster 2009). We have evidence that both hormones are also important factors of the circadian and/or circalunar clock in *Platynereis dumerilii* (unpublished data).

2.4 Melatonin

During the last 15 years a lot of data was produced about melatonin in vertebrates, but until now still very little details are known concerning invertebrates. Therefore, I will summarize the functionality of melatonin in vertebrates first and then introduce the current knowledge of invertebrate species.

Melatonin is the main secreted hormone in the pineal gland of vertebrates. Its production and secretion is regulated by the suprachiasmatic nucleus (SCN) of the hypothalamus, the organ comprising the master circadian clock of mammals (Norman and Litwack 1997, 485ff). The SCN itself is entrained by the daily light-dark cycle through a direct retinal signalling pathway. This circadian signal is then sent to the pineal gland, where it controls the rhythmic melatonin production (Reppert and Weaver 1995). It was shown that melatonin-secretion is continuously higher during the night whereas the duration of this signal is adjusted to the night length

(Simonneaux and Ribelayga 2003).

While in vertebrate species as well as in many invertebrates the eyes comprise the photoreceptors to sense light as an input for the circadian oscillators in the optic lobe of the brain, there is evidence that in some animals both the receptors and the clocks are located in the brain. It could also be shown in lower vertebrates that photoreceptor cells in the retina and the pineal organ are responsible for melatonin secretion (Vivien-Roels and Pévet 1993).

The direct precursor of melatonin is serotonin, a monoamine neurotransmitter biochemically derived from the amino acid tryptophan. Serotonin is processed with two rate-limiting enzymes, Aanat (arylalkylamine N-acetyltransferase) and Hiomt (hydroxyindole-O-methyltransferase) which leads to the formation of NAS (N-acetylserotonin) and then melatonin (N-acetyl-5-methoxytryptamine) (Norman and Litwack 1997, 488ff). Whereas the dominant pathway is certainly the one shown before, there is indication from calculations and measurements that an alternative synthesis is also possible. Serotonin may be converted to 5-MT (5-Methoxytryptamine) and further processed to melatonin (Hardeland and Poeggeler 2003).

Besides, melatonin is also present in numerous non-vertebrate species. In fact, it could be detected almost everywhere in case appropriate methods for extraction and detection were applied (Hardeland and Poeggeler 2003). This includes bacteria, protozoans, macroalgae, fungi and vesicular plants, whereas information about melatonin is still missing in other organisms like archaea, ferns, mosses, gymnosperms, sponges, chelicerates, echinoderms and annelids (ibid.).

In vertebrates, the rhythmic circadian cycling of melatonin with the highest peak typically being at night is directly caused by transcriptional regulation of the Aanat gene (Reppert and Weaver 1995; Bell-Pedersen et al. 2005). Ancient homologs of this enzyme were published to be present in fungi and bacteria, but could not be detected in other eukaryotes than vertebrates yet. In unicellular green algae and amphioxus these ortholog sequences lack important functional parts found in vertebrate Aanats and appear intronless. This led to the hypothesis that a horizontal gene transfer from bacteria to green algae, fungi and chordates has happened (Coon and Klein 2006). As the expression pattern in vertebrates is also quite different from that in *Branchiostoma lanceolatum*, it was suggested that Aanat gained its known function at the onset of vertebrate evolution. In bacteria the enzyme may rather be used for detoxification or serves as a biogenic amine inactivator (Pavlicek et al. 2010).

2.4.1 Effects of Melatonin

There are different hypotheses from studies in various organisms about what the functions and effects of melatonin could be.

Reverse Ageing

In the early beginnings of research on melatonin, some papers were claiming that this hormone is capable of reverse ageing. It was shown how through the treatment with melatonin the life span of specific mice strains was significantly increased (Pierpaoli and Regelson 1994). However, in

another study by the same authors, treatment with similar amounts of melatonin induced tract tumours in these mice strains and therefore shortened their survival. Therefore, evidence of melatonin induced reverse ageing is very inconsistent (Reppert and Weaver 1995).

Protection from Radicals

Melatonin was also shown to be a more effective scavenger of hydroxyl and peroxy radicals than glutathione or vitamin E, two important antioxidants in the brain. Administered in vivo and in vitro it protected the tissue from oxidative stress and free radicals (Reiter 1995). Therefore it was assumed that this is not only an important function in vertebrates but that it came up much earlier in evolution (Hardeland et al. 1995). This theory was also criticized, because melatonin was applied in high pharmacological doses to gain the antioxidant potential. Concentrations about one million fold greater than under usual physiological conditions were thought to be of importance only for some therapeutic applications (Reppert and Weaver 1995). But with an experiment done in dinoflagellates, Hardeland Poegger showed once more that it was possible with physiological levels of melatonin to rescue cells from oxidative stress by hydrogen peroxide. They claim that quite high concentrations of melatonin can be found in some lower organisms and that the protective function is highly suggestive in plants, whereas published evidence is still rare (Hardeland and Poeggeler 2003).

Circadian Output

Rhythmic melatonin levels are known to regulate sleep-wake cycles and together with further neuronal and humoral outputs they are thought to adjust peripheral oscillator function. In mammals the pineal melatonin biosynthesis is directly regulated by the SCN, whereas the signal of this pacemaker is then passed on to tissues expressing melatonin receptors. Special functionality is known for melatonin receptors expressed in the SCN. They inhibit night time SCN activity and therefore restrict the pineal melatonin secretion to night-time. The pineal gland further influences the central nervous system and other peripheral sites (Bell-Pedersen et al. 2005).

Studies with administered exogenous melatonin revealed that melatonin can phase-shift circadian rhythms in humans and rodents. In some cases even entrainment was possible. Furthermore, observations like lower body temperature and induced transient sleepiness could be made (J. Arendt 1998).

In vertebrates and invertebrates, melatonin and its precursors of synthesizing enzymes were found in various organs involved in circadian pacemaking or photo-receptive processes, further supporting the function as a circadian output molecule. But due to huge variations of the data received from melatonin measurements in different animals it was hard to interpret the results. Sometimes nightly peaks were detected, the presence of a seasonal pattern was inconsistent and in some cases no cycling at all was observed. Further the oscillations were found to be on different levels in various species. In some studies transcriptional or translational regulation of the enzymes was observed, in other cases only melatonin secretion was found to be controlled

and in rare examples both was the case (Vivien-Roels and Pévet 1993).

In non-vertebrates, studies show that melatonin is not necessarily circadian clock-regulated. And if it is clock controlled, melatonin is not necessarily peaking at night, although it was frequently found. Several species even exhibit diurnal peaks or a lack of rhythmicity. But circadian rhythms with nocturnal maxima as in vertebrates is also frequently found in non-vertebrates (Hardeland and Poeggeler 2003).

Conveying Seasonal Adaptation

An important role of melatonin is also the regulation of seasonal adaptation (B. D. Goldman and Darrow 1983). The changing length of the day in temperate regions, also called photoperiod, is the main synchronising cue for seasonal animals. This signal is conveyed through the pineal gland by secreting melatonin rhythmically depending on the night length, which leads to adaptive changes in physiology, anatomy and endocrinology (Morgan and Hazlerigg 2008). These photoperiodic responses include adaptations of the body weight and the coat colour as well as changes of the behaviour and the reproductive status (J. Arendt 1998).

The relation between the two mentioned timing systems, the photoperiodic calendar and the circadian clock, is not exactly clear yet. Three major concepts are therefore possible: The two systems could operate functionally independent and physically completely separate from each other. Further, it would be plausible that both systems cooperate, but still exist as two separate machineries. In the third theory the daily and the seasonal rhythmicity are both controlled by one and the same molecular mechanism which just acts with two outputs (Vladimír 2011).

2.4.2 Melatonin in *Platynereis dumerilii*

However, melatonin with its rhythmic synthesis is apparently a common feature of all living organisms (Vivien-Roels and Pévet 1993). Still, as mentioned before, the two precursor enzymes Aanat and Hiomt could not be identified in any other metazoans than the chordates yet, leading to the suggestion that they have been inserted into the gene of this evolutionary lineage through horizontal gene transfer from prokaryotes. Evidence for this theory comes from the Aanat gene in *Amphioxus*, a unicellular green algae, which appears to be intronless (Iyer et al. 2004; Coon and Klein 2006).

Recent unpublished work from Tessmar and Raible labs not only uncovers both enzymes in the annelid *Platynereis dumerilii*, but also led to the identification of two Aanat and one Hiomt ortholog in *Capitella* and another ortholog of Aanat in *Lottia*. When analysing their gene structure, high similarity could be found. This supports the hypothesis that melatonin and its biosynthesis pathway are evolutionarily conserved at least in animals. Therefore an independent horizontal gene transfer seems very unlikely (Zantke J. et al., unpublished data). Furthermore, there is good evidence that in *Platynereis dumerilii* melatonin is not only regulated through a daily rhythm but shows significant fluctuation depending on the lunar phase. Free running experiments indicate that even the synthesis enzymes are regulated by the lunar clock (ibid.).

2.5 PDF

The pigment dispersing hormone (PDH) originally got its name as it was found to regulate the retinal pigmentation in several crustacean species. Later studies show that it is also strongly involved in the circadian clock of insects and crustaceans where it influences the behavioural output, the daily rhythmicity and is an important factor in intercellular communication (Helfrich-Förster 2009; Yoshii et al. 2009).

The PDHs, first isolated from the eyestalk of crustaceans (Rao and Riehm 1993), consist of at least two families, the alpha PDH and beta PDH, whereas the latter was identified to represent the more ancestral-type peptide (Rao 2001). In crustaceans the hormone causes distal migration of screening pigment in the retina of the eyes to protect the photoreceptor cells from bright light during the day (Fernlund 1974). Another hormone, the red pigment-concentrating hormone, provokes the exact reverse reaction. In insects there is no evidence that PDH controls pigment migrations, but when the insect hormone is applied to the eyestalk of a crab it leads to the same reaction as with crustacean PDH. Therefore the insect homologue of the pigment dispersing hormone was called PDF, pigment dispersing factor (Rao and Riehm 1989). Early studies in insects already indicated that PDF is controlled through a clock. It is produced in a daily rhythm, which also continues under constant, free running conditions. Additional injections of PDF cause advances or delays of this rhythmicity (Aréchiga and Rodríguez-Sosa 1998). So, in the nervous system of insects, PDF was supposed to operate as a neurotransmitter or neuromodulator (Rao 2001).

Recent experiments done in crabs and lobsters indicate that PDH neurones located close to the medulla show high similarities to the PDF neurons of insects. This could be explained with different functionality of the two to three isoforms expressed in several crustaceans (Hsu et al. 2008; Harzsch, Dirksen, and Beltz 2008). Also, the cricket *Gryllus bimaculatus* maintains two isoforms of PDH, one located in PDF cells of the distal optic lobe and the other expressed in neurons close to the medulla (Honda et al. 2006).

In *Drosophila melanogaster* another system emerged to gain different forms of PDF. In neurons, projecting into the optic lobes, only PDF with a C-terminally amidation is produced, whereas projections into the central brain contain the non-amidated hormone (Park et al. 2008; Park and Taghert 2009). Amidated PDF exerts a strong synchronizing ability between the neurons, whereas the non-amidated form transfers rhythmic signals to neurons downstream and provokes rhythmic locomotor activity. The latter may also feedback onto dorsal neurons of the molecular clock to adjust their period and synchronize their oscillations (Helfrich-Förster 2009).

2.5.1 Role of PDF

PDF neurons certainly play a crucial role in the circadian clock of insects. PDF is not only coexpressed with other proteins of the clock, but also partially located in clock neurons (Helfrich-Förster 2009). Helfrich-Förster (2009) reviewed the exact role of PDF in the circadian clock and suggested the following possibilities:

Output Factor

There is reliable evidence that PDF is at least an important output neurotransmitter of the clock. In the fruit fly PDF is released rhythmically under the control of the clock. Mutant flies with the clock protein PER missing have short periods (per^s) or lack this rhythm completely (per^0). A similar study with cockroaches was done to support this hypothesis. PDF fibres to the central brain were severed. This led to an arrhythmic locomotor activity which was restored after the fibres regrew. Also in the cricket it was shown that PDF oscillates significantly in the the central brain and the optic lobes under both light-dark and dark-dark (free running) conditions (References in Helfrich-Förster 2009).

Coupling Factor of the Clock

Still, some observations can hardly be explained with PDF just being active in the output pathway of the circadian clock. Flies with artificially altered PDF levels for example exhibit strong period changes and pdf^{01} mutants still show rhythms with short periods. Therefore PDF has to feed back somehow onto the clock to be able to alter its pace. This could be achieved with PDF being a coupling factor between the various oscillating clock neurons. Further evidence for this synchronizing function of PDF comes again from cockroaches. There, the oscillating phase between different cell assemblies is locked and synchronized by PDF (ibid.).

Modulator of the Clock Speed

As already mentioned, PDF is able to affect the molecular oscillation of the clock and alter its pace. This is supported by the fact that PDF receptors are expressed in clock neurons themselves. Therefore the molecular conditions are fulfilled for these neurons to be able to sense PDF. Further evidence comes from neural mutants in *Drosophila*. They show internal desynchronisation into parts showing shorter and longer periods. This is driven by PDF from misrouted l-LNv fibres (ibid.).

Light Input Factor

It is shown for certain neurons in the brain to increase the night activity of flies when hyperexcited artificially. The same observation can be made with exposing the flies to nightly dim light. In pdf^0 mutants this effect cannot be induced, therefore it is clearly mediated through PDF. In a cricket it is also shown that injection of PDF increases the spontaneous electrical brain-activity during the day, but not in the night. Nevertheless, to further prove that PDF is also involved in the input pathway of the circadian clock more data would be needed (ibid.).

2.5.2 PDF in *Platynereis dumerilii*

A lot is already known about PDF in insects and crustaceans and some recent studies already described homologous genes outside the arthropods, in the nematode worm *Caenorhabditis elegans* (Janssen et al. 2009). Still, very little is known about this important factor of the clock in lophotrochozoans. Phylogenetic analyses done by our group indicate that homologous genes of

PDF exist in several lophotrochozoan species (Zantke J. et al., personal communication) and that PDF is therefore evolutionarily conserved throughout the whole protostomian lineage, as already suggested by Janssen et al. (2009).

As the lophotrochozoan PDF genes built a separate cluster outside of the insect and crustacean ones, it is not clear which clade they are more related to (Keplinger S., personal communication). Therefore it is hard to make statements about the function of PDF in *Platynereis dumerilii*. Recent studies done show that the presence of a circadian fluctuation and even lunar rhythmicity may occur, but both have to be investigated more closely. PDF might also regulate the pigment in the marine rag-worm in a similar way to the crustacean homologue. This is also part of ongoing experiments (Raible F, personal communication).

2.6 Receptors

The effects or hormones are principally mediated through specific hormone-receptors on the surfaces of target cells. Therefore a detailed knowledge about these receptors, their localisation and expression is crucial to identifying the target tissues. Further, this knowledge helps a lot in getting a detailed understanding of the function of a certain hormone. For identifying the function of melatonin in mammals, studies about the receptor localisations already gave the decisive hint (Lincoln et al. 2006). For PDF in *Platynereis* the localisation of the receptor also might give the essential clue about whether it behaves like the insect brain hormone or the crustacean pigment dispersing hormone.

2.6.1 The Melatonin Receptor

Hormonal effects of melatonin are known to be mediated through specific receptors, although this very lipid-soluble molecule is able to leave and enter cells by free diffusion. Therefore the receptors may not be the only targets (Norman and Litwack 1997), but for sure the most prominent ones. The very first cDNA of a high affinity melatonin receptor (MelR) was identified through expression cloning from *Xenopus* dermal melanophores (Ebisawa et al. 1994) followed by receptors in a variety of vertebrates. These procedures were possible through the ability of the vertebrate receptor to bind the radioactive agonist 2-[¹²⁵I]iodomelatonin (Dubocovich 1995).

The melatonin receptors were identified as members of the superfamily of G protein (guanine nucleotide-binding protein) coupled receptors (Reppert, Weaver, and Ebisawa 1994). Compared to other receptors of this superfamily, they exhibit a few unique features. Vertebrate melatonin receptor sequences include a NRY motif downstream of the third transmembrane domain rather than DRY/ERY motif and a C(C/Y)ICHS motif directly downstream of the previously mentioned one. Further, the highly conserved sequence of G protein-coupled receptors, NPXXY, is exchanged to NAXXY in the transmembrane domain seven of melatonin receptors (Reppert and Weaver 1995).

Biochemical analysis of the hormone receptor lead to the identification of several specifically binding agonists and antagonists. Melatonin, 2-Iodomelatonin and 6-Chloromelatonin mediate a

positive signal to the receptor as agonists, while the two antagonists 4-P-PDOT and Luzindole just block the binding site as shown on the human 1A and 1B receptor (Audinot et al. 2003).

Different Subtypes can be distinguished

Investigating the phylogeny of various melatonin receptors it was observed that several subtypes can be distinguished. In mammals the MelR 1A was expressed in the mammalian SCN, while the MelR 1B with 60% identity on amino acid level could be found in the human retina. This led to the assumption that the 1A subtype mediates the circadian and also reproductive actions of melatonin, whereas the 1B receptor is responsible for actions on the mammalian retina (Reppert and Weaver 1995; Reppert, Godson, et al. 1995; Reppert, Weaver, and Ebisawa 1994).

However, the already mentioned receptor of *Xenopus* together with receptor genes in birds represent a third subtype which has similar functional and pharmacological properties as the mammalian subtypes. All three subtypes exhibit a very similar gene structure, further proofing their close relationship. Until now there is no evidence of a third melatonin receptor in vertebrate species (Reppert, Weaver, et al. 1995).

Expression Pattern led to the Function of the Ligand

Early experiments to identify the site of action of melatonin and to explain the observed photoperiodic effects already led to remarkable species-specific expression patterns in mammals (Morgan et al. 1994). These analyses, including in situ hybridisations and autoradiography using radio-labelled analogue of melatonin, revealed that the two possible target sites of melatonin are cells in the mediobasal hypothalamus or in the pars tuberalis. The pars tuberalis is located at the interface between the main pars distalis region of the anterior pituitary and the median eminence (Hazlerigg and Loudon 2008). Further, it was suggested that melatonin receptor 1A expressing cells provide an intermediate relay function in neighbouring areas (Dardente 2007). This expression pattern is also true for sheep, where it was discovered that the pars tuberalis is producing a melatonin modulated prolactin-releasing factor that further regulates the prolactin release by the lactotrophs in the pars distalis. It was therefore proposed that this communication within the pituitary gland plays a central role for the circannual prolactin rhythm and that the pars tuberalis cells may be the pacemaker for this system (Lincoln et al. 2006). Removal of the pineal and therefore removal of the dominant source of nocturnal produced melatonin prevents photoperiodic responsiveness (B. Goldman 2001). Therefore, the region of the anterior pituitary gland is thought to be the master controller of seasonal effects in mammals and birds (Hazlerigg and Loudon 2008).

In contrast to this very limited distribution in mammals, the melatonin receptor shows a more widespread expression in brains of birds and lower vertebrates (Cassone, Brooks, and Kelm 1995). These animal groups respond to seasonal photoperiod changes through extra-retinal photoreception. Direct effects of light through deep encephalic photoreceptors might be the main input pathway on the hypothalamic function leading to the seasonal response in reproduction. This was concluded because it was shown that melatonin is not required (Sharp 2005). In birds,

the pars tuberalis itself might be a possible location of deep-brain phototransduction (Hazlerigg and Loudon 2008).

Signalling targets downstream of melatonin are for example GnRH and other hypothalamic releasing hormones. GnRH inhibits FSH and LH secretion in presence of melatonin, as demonstrated with pituitary cells from young rats. This leads to the suggestion that melatonin also controls the reproductive state (Norman and Litwack 1997). Further evidence therefore comes from sheep and hamsters, where melatonin mediates control of seasonal changes in gonadotropin secretion, gonadal activity and controls prolactin secretion and its dependent biology (Lincoln et al. 2006).

2.6.2 The PDF-Receptor

As many other hormone receptors, the PDF receptor also belongs to the B1 subfamily of G-protein-coupled receptors (GPCRs). It consists of an extracellular N-terminus capable of binding peptide, seven transmembrane domains and the cytoplasmic G protein binding domain on the C-terminus (Mertens et al. 2005). With a cell-culture assay it was shown that the PDF receptor also regulates cAMP (cyclic adenosine monophosphate) as it is true for all other members of the B1 subfamily. This regulation is done through the coupling of the stimulatory G-protein to the adenylate cyclase (ibid.).

Although no homologue of the PDF peptide itself could be identified in vertebrates yet, there are genes closely related to the PDF receptor. It was shown that the mammalian VIP (vasoactive intestinal peptide) receptor and the calcitonin receptor are quite similar to the PDF receptor. Both are known to be expressed in the mammalian circadian clock, which suggests a certain conservation of the clock networks between arthropods and vertebrates (Helfrich-Förster 2005). Further, PDF was also able to slightly activate the vertebrate calcitonin receptor (Mertens et al. 2005). Comparing the functionality of VIP to PDF we can find strong similarities. It was also suggested for VIP to enhance and synchronize the oscillations of individual clocks. In the SCN (suprachiasmatic nucleus) it maintains the circadian rhythmicity in half of the neurons and controls the synchrony among intrinsically rhythmic neurons in the other 50% (Aton et al. 2005).

In *Drosophila melanogaster* the PDF receptor has already been studied very well, leading to a detailed knowledge about the localisation and the function. In situ hybridisations done with an antisense mRNA showed that the gene is expressed in the dorsal brain close to the dorsal clock neurons and the PDF terminals (Lear et al. 2005). Also two studies using Immunocytochemistry were done to identify the exact location of the protein itself. Cell bodies in the dorsal and lateral protocerebrum and the subesophageal ganglion showed specific staining. These areas have been previously described to harbour the relay between the master clock of the fly and the rhythmic behaviour (Mertens et al. 2005). Hyun et al. described that the receptor is present on a whole subset of the clock neurons and also on neurons not involved in the master clock (Hyun et al. 2005), leading to a broader expression pattern than identified by Mertens et al. This discrepancy can be discussed with the different antibodies used. Hyun et al. generated the antiserum against the more accessible extracellular part of the receptor (Helfrich-Förster 2005).

Further evidence that the PDF receptor is present on neurons involved in the circadian clock comes from an experiment done by Shafer et al. They show that almost all clock neurons respond to PDF with an increased level of cAMP. The only exception is one neuron that does not express the receptor gene (Shafer et al. 2008). Additionally, mutant flies lacking the PDF receptor show a phenotype identical to the one of Pdf⁰ mutants. This mutant phenotype could be easily rescued with expressing the PDF receptor in all neurons of the clock, showing that PDF signalling plays a crucial role for the function of clock neurons (Hyun et al. 2005).

In conclusion, the PDF receptor gene is not only expressed in clock neurons, but also outside the clock. Further, PDF is an important factor of the circadian clock, shown in insects and also in crustaceans, whereas the exact chronobiological role of this hormone still needs to be identified. PDF certainly not only functions in the output pathway, but also within the neural network of the circadian clock (Helfrich-Förster 2009; Mertens et al. 2005; Hyun et al. 2005; Lear et al. 2005).

2.7 Aim of the Study

The aim of this study was to identify the target tissues of melatonin and PDF, two hormones which are already known to be regulated by light, either directly or indirectly through a clock. We have evidence that in *Platynereis dumerilii* these hormones are also important factors of the circadian and/or circalunar clock (unpublished data), which further raises the interest in these hormones. A detailed understanding about the properties of hormone-receptors already helped to reveal the function of the binding hormone in the past (Lincoln et al. 2006).

The first step therefore was to identify true ortholog receptors of both hormones in *Platynereis dumerilii* using bioinformatic approaches. After cloning them, it was possible to further investigate their sequence properties, phylogeny and ligand-binding abilities. Subsequent experiments should determine the mRNA expression levels in adult animals and the localisation of the transcript. Immunohistochemistry using an antibody against the pigment dispersing factor itself should additionally allow detailed insights into the spatiotemporal expression patterns of PDF. Ideally, with this data it will be possible to gain a temporal and spacial idea of PDF generation, transportation, and recognition through its receptor.

Gaining extensive knowledge about the characteristics of both receptors is an important step in understanding the effects of melatonin and PDF in *Platynereis dumerilii* and in enlightening the downstream signalling pathway of these hormones.

3. Materials and Methods

3.1 *Platynereis* Culture

Platynereis dumerilii were held in approximately 30x40cm transparent plastic boxes stored in a shelving system. The boxes were filled with a 1:1 mixture of natural sea water and artificial salt water to a level of about 5 to 10 cm. For food, the animals received spinach leaves and *Platymonas* algae two times a week. Air conditioning provided a constant water temperature of 18°C.

Light conditions for a daily rhythm were 16h of artificial light followed by 8h of complete darkness. To imitate an environment during full moon a 1-10 lux lamp was turned on constantly for 7 days followed by 21 days of normal rhythm (Hauenschild 1960). In a separate outphase room the lunar rhythmicity was shifted 14 days compared to the inphase room. This helped to decrease waiting times while sampling. Some boxes were kept in an “inverted shelf” providing an inverted day-night rhythmicity.

3.2 Homology Search and Phylogenetic Analysis

3.2.1 Blast Search Programs

Except for the later mentioned organisms, blast search was performed using the web-interface on NCBI (Johnson et al. 2008). With the default properties I searched in the non-redundant databases of the respective animal using a protein sequence query. For searching *Lottia gigantea*, *Helobdella robusta* and *Capitella teleta* sequences I performed blast searches in the “all model transcripts” database from the Joint Genome Institute². *Platynereis* sequences were extracted from our local EST library after the identification using the blast2 package by National Centre for Biotechnology Information. For searching in nucleotide databases as the EST database and transcripts I performed a tblastn (Altschul et al. 1997), for searching in a protein database like on NCBI and JGI a blastp. All identified sequences can be found in the Appendix, Table 3.

3.2.2 Alignment, Phylogenetic Tools and TMHMM Prediction

For fast previews of alignments the CLC Bio standard alignment tool (v6.0) with a gap open cost of 10.0 and a gap extension cost of 1.0 led to good results. Proper alignments for the calculation of the phylogenetic tree was either done with ClustalW or ClustalX (Chenna et al. 2003). The latter was also convenient for calculating previews of trees with the Bootstrap Neighbour Joining algorithm. Therefore I always excluded positions with gaps and corrected for multiple substitutions. For converting between different file formats, extracting sequences and inverting them I used several Emboss programs (Rice, Longden, and Bleasby 2000). To calculate sequence similarities and identities between two amino acid sequences with the waterman-smith algorithm, I used the program “water” from the aforementioned package.

² <http://genome.jgi-psf.org/>

The phylogenetic trees shown in the results part were all done with the Maximum Likelihood method and the iqpnii program (Vinh and von Haeseler 2004). I usually calculated 100 trees with the following options: Blossum62 model, gamma rate type, num_reate set to 4; model of rate heterogeneity was gamma distributed, number of gamma rate categories was 4 and with a minimum of 200 iterations. The output trees of iqpnii had to be reconstructed into one representative tree. This was done with Tree-Puzzle 5.2 (Schmidt et al. 2002) with computing of clocklike branch lengths and the most exact parameter estimation. Presentation of the trees was either rendered with iTOL (Letunic and Bork 2006) or Figtree 1.3.1.

The 7-transmembrane domains were predicted with the CBS TMHMM server v2.0³ (Sonnhammer, von Heijne, and Krogh 1998; Krogh et al. 2001) as recommended by Möller et al. (Möller, Croning, and Apweiler 2001).

3.3 Cloning and Subcloning

3.3.1 RNA Extraction, cDNA Synthesis

RNA was extracted from larvae or *Platynereis* tissue using the QIAGEN RNeasy Mini Kit following the protocol “Purification of Total RNA from Animal Tissues” from the handbook.

For cDNA generation we used the QUAGEN QuantiTect Reverse Transcription Kit obeying the “Reverse Transcription with Elimination of Genomic DNA for Quantitative, Real-Time PCR” protocol. Both steps were done by Benjamin Backfisch (cloning the Pdu melatonin receptor) and Fiorella Schischlik (cloning the PDF receptor).

3.3.2 Specific PCR

For specific PCR I used several polymerases with their according buffers and protocols for different purposes. The concentration of dNTPs in H₂O was always 10mM each, from specific primer it was 10mM. The calculation of primer melting temperatures was done with OligoCalc (Kibbe 2007).

Pdu MelR candidate cloning: PCR ID#1 (Table 1, page 70); Phusion[®] High-Fidelity DNA Polymerase (Thermo Scientific), 5x Phusion HF Buffer; Primer #912 and #913 (Table 2, page 71); Template was 48hpf Pdu cDNA.

Mm MtnR1A cloning: PCR ID#2 (Table 1); Polymerase and Buffer as in Pdu MelR candidate cloning; Primer #1406 and #1254 (Table 2); Template: Mouse brain cDNA from Astriad Hagelkruys (Seiser Lab). For the nested PCR reaction I used the same set-up, only changed the primers and the template: #1251 and #1252 with T_m= 59.2°C (pre cycle) and 64.2°C (main cycle); Template was previous PCR reaction.

Subcloning of Pdu MelR candidate in mammalian expression vector pcDNA3.1: PCR ID#3 (Table 1); Polymerase and Buffer as in Pdu MelR candidate cloning; Primer #1249 and #1250 (Table 2); Template was plasmid #786 “Pdu_MelR (Melatonin Receptor)” 1:3000 diluted.

³ <http://www.cbs.dtu.dk/services/TMHMM/>

3.3.3 Degenerated PCR

For designing degenerated primers I created a protein alignment with sequences from related species and used BOXSHADE⁴ to display and spot conserved regions. Primers were then designed by hand in such regions. The most conserved AS sequence was converted into a degenerated nucleotide sequence considering AS exchanges between different species. I designed two lower and two upper primers for running various combinations of nested reactions.

With Oligo 6.8 for Mac (Molecular Biology Insights) I calculated the primer melting temperatures and estimated the length of the PCR product using the *Capitella* sequence. Following Primer with a concentration of 100mM were needed: up1 #971 ($T_M=57.6^\circ\text{C}$), lo1 #973 ($T_M=58.6^\circ\text{C}$), up2 #972 ($T_M=58.6^\circ\text{C}$), lo2 #974 ($T_M=60.2^\circ\text{C}$) (Table 2, page 71)

PCR Mix and Program: PCR ID#5 (Table 1, page 70); I did PCRs with four different combinations of primer using a T_M temperature gradient; HotStarTaq DNA Polymerase (QIAGEN) with according 10x PCR Buffer (QIAGEN); Template was Pdu cDNA from 2-8dpf old larvae.

Nested PCR: ID#6 (Table 1); I took the same polymerase and buffer as before, trying three different primer combinations; Template: 1 μl previous PCR reaction (up1lo1, up1lo2, up2lo1).

3.3.4 Gel Electrophoresis and Gel Extraction

To separate DNA by size I loaded it onto an 1-2% TAE agarose gel and ran it at a constant voltage of 100V for at least 1 hour. To get it faster I occasionally ran it on 120V for 35 minutes. For sharper DNA bands on important gels I mixed MetaPhor agarose (Lonza) 1:1 with regular agarose. As size marker I used the 2-log DNA Ladder (New England Biolabs). Gels were stained after the run for 20-60min in an ethidium bromide bath (1:10000 dilution in 1xTAE). Pictures were done using UV Light.

Gels for cutting out bands were stained during preparation with SybrSafe (Invitrogen). I cut out the band of interest with visualisation under blue LED light (Safe Imager transilluminator).

For DNA extraction from the gel I used the QIAGEN QIAquick Gel Extraction Kit with the appropriate protocol: "QIAquick Gel Extraction Kit Protocol: using a microcentrifuge". I just changed the last step and eluted the DNA with 30 μl of ddH₂O instead of elution buffer.

3.3.5 Ligation and Transformation

Cloning of PCR products with an Adenine overlap was done with the pGEM[®]-T Easy Vector Systems Kit (Promega). Therefore I followed the "Protocol for Ligations Using the pGEM[®]-T and pGEM[®]-T Easy Vectors and the 2X Rapid Ligation Buffer" from the pGEM-T and pGEM-T easy Vector Systems Technical Manual TM042. For blunt-end cloning I used the CloneJET[™] PCR Cloning Kit #K1231 (Fermentas). From the procedure recommended in the "Blunt-End Cloning Protocol" I halved the overall reaction volume to 10 μl .

⁴ <http://sourceforge.net/projects/boxshade/>

Transformation protocol: For each sample I thawed up 50µl of competent *E. coli* cells prepared by Claudia Lohs and Katharina Schipany on ice and added 5µl of ligation mix. After snipping the tube it was incubated on ice for 30 minutes. I performed the heat shock at 42°C for 30 seconds and placed the cells immediately on ice again for another 2 minutes. After adding 200µl of SOC Medium to each sample I shook the cells for 30 minutes to 1.5 hours (700rpm). For streaking them out on Petri dishes with selective agar medium I divided the sample into two different amounts (50µl and 200µl) because transformation efficiency was unknown and I wanted to get a proper colony density.

3.4 Analysis of Clones

3.4.1 Colony PCR

To check for the presence of a certain DNA insert in a vector I used colony PCR with either one specific primer for the insert and one from vector backbone or with only two specific primers, in case vector primers were not available. Therefore I prepared two 96 well plates, one with 20µl ddH₂O and another with PCR master-mix (PCR ID#4: Table 1, page 70). FirePol[®] polymerase (Solis Biotec) was used with its according 10x buffer. After picking one colony from the agar plate I swirled the tip in water and afterwards in the PCR mix (20µl per well). Annealing temperatures and elongation times were depended on the primer and the length of the expected amplicon. Bacteria dissolved in water were used to grow miniprep cultures afterwards.

3.4.2 Restriction Digests

For digesting DNA, FastDigest[®] restriction enzymes (Fermentas) as well as New England Biolabs (NEB) ones were available in the lab. The Fermentas enzymes, which I mostly used for double digests, were delivered with a universal 10x FastDigest buffer whereas for the NEB enzymes I determined the right NEB 10x buffer according to the protocol. A standard restriction digest with a total volume of 10µl was consisting of 6.5µl ddH₂O, 1µl 10x buffer, 2µl plasmid (~1µg), 0.5µl enzyme A. For double digesting I added 0.5µl enzyme B instead of 0.5µl water. For some NEB enzymes BSA was required. Therefore I included 1µl BSA (NEB 100x diluted 1:10) and subtracted the same amount from water.

To check for a positive insertion into the pGEM-T easy plasmid after cloning and for discrimination between different insert fragments I performed an EcoRI digest which cuts on both sides of the insert and EcoRI + HinfI double digest (polycutter).

3.4.3 Sequencing

Sequencing was done by sending plasmids dissolved in water (100ng/µl) to GATC Biotech⁵ or LGC Genomics⁶. DNA concentrations were measured with a Nanodrop Spectrophotometer.

⁵ www.gatc-biotech.com

⁶ www.agowa.de

3.5 Mini-/Midi-/Maxiprep

For a standard Miniprep with the alkaline lysis method I adopted our common lab protocol (see Appendix: 7.2 Miniprep). To perform bigger preps I used the QIAGEN Plasmid Maxi Kit (Cat. no. 12163) together with QIAfilter Maxi Cartridges (cat. no. 19763) for Maxiprep and the QIAGEN Plasmid Midi Kit (Cat. no. 12143) together with QIAfilter Midi Cartridges (Cat. no. 19743) for Minipreps. For both purposes I followed the protocol “Protocol: Plasmid or Cosmid DNA Purification Using QIAfilter Plasmid Midi and Maxi Kits” from the QIAfilter Plasmid Purification Handbook.

To receive endotoxin free DNA plasmids for transfection in cell culture assays I proceeded according to the protocol “Protocol: Plasmid or Cosmid DNA Purification Using the EndoFree Plasmid Maxi Kit” from the EndoFree Plasmid Purification Handbook using the EndoFree Plasmid Buffer Set (Cat. no. 19048).

3.6 Smart RACE (Rapid Amplification of cDNA Ends)

RACE-cDNA synthesis was carried out by Enrique Arboleda following the user manual “V. Generating RACE-Ready cDNA” from the SMARTer™ RACE cDNA Amplification Kit (Clontech). Previous RNA extraction from *Platynereis* tissue is mentioned in Chapter 3.3.1 (page 21).

I performed the RACE-PCR according to the protocol “VI. Rapid Amplification of cDNA Ends (RACE)” pipetting following PCR-Mix: PCR ID#7 (Table 1, page 70); fwd. primer: GSP1 #1151 (Table 2, page 71) with 5' RACE ready cDNA as template or GSP2 #1149 with 3' RACE ready cDNA; rev. primer: UPM (Universal Primer Mix, short and long primer); Polymerase: HotStarTaq® DNA Polymerase (QIAGEN) with according 10x PCR Buffer (QIAGEN).

For the nested reaction I used the same protocol and exchanged the fwd. primer to Nested Gene Specific Primer: 5' RACE, NGSP1 #1152 ($T_M = 71.5^\circ\text{C}$); 3' RACE, NGSP2 #1150 ($T_M = 70.7^\circ\text{C}$). A temperature gradient was necessary for the primer annealing step. In the pre cycle I therefore used a five degrees lower temperature. The previous PCR served as template.

After loading an aliquot of all PCR products onto an agarose gel I did a Southern Blot followed by radioactive DNA hybridisation. From another gel I cut out positive bands of interest and ligated the gel-extracted DNA into the pGEM-T easy cloning vector. To identify positive colonies a filter lift was done followed by hybridising these filters again. I picked these colonies, did an analytical restriction digest and sent promising samples for sequencing.

3.7 Southern Blot and Colony Lift

Blotting procedure: I separated the DNA on an agarose gel which I stained with ethidium bromide afterwards. To document it I photographed the gel with a ruler as visible size marker. The southern blot was built with a nylon membrane (PALL), two times Whatman blotting paper (3mm) and a staple of paper towels (5cm height). I added some weight on top of it for better capillary transfer. After blotting over night the DNA is bound to the nylon membrane. For

covalently binding it to the membrane I performed UV crosslinking (UV Stratalinker, STRATAGENE) using the auto-crosslink function. Finally the membrane was dried and stored in the dark for later DNA hybridisation.

Filter Lift procedure: Bacteria were grown over night at 37°C on selective medium. The Colonies were then partially transferred to a circular shaped membrane (Whatman BA-S 85 Optitran) where I marked the orientation cutting out edges with a scalpel. Afterwards I incubated the membranes with colonies upwards on three different blotting papers sucked with denaturing solution, neutralisation solution (5 minutes each) and SSC (2x2 minutes). Finally the membranes were air-dried and UV- crosslinked. I put the agar-plates on 37°C for another 2 hours to regrow the colonies.

DNA hybridisation: I amplified the probe-template for blotting using PCR ID#8 (Table 1, page 70): FirePol Polymerase (Solis Biodyne) with according 10x Buffer; primer #1153 + #1151 (Table 2, page 71) for 3' probe, #1145 and #1149 for 5' Probe; Template: pGEM-T easy + Pdu-pdfr-candidate-fragment 1:2000 diluted).

With the RadPrime labelling kit (Invitrogen) we generated a specific radioactive probe containing ^{32}P labelled dCTP. This probe was then purified (ProbeQuant G-50 Micro Columns, GE Healthcare) and hybridised to the DNA on the blotting membrane (Rapid-hyb Buffer, GE Healthcare) to visualize positive DNA fragments containing the sequence of interest. The latter three steps were performed either by Florian Raible, Enrique Arboleda, Benjamin Backfisch or Fiorella Schischlik using the standard kit protocols.

3.8 Whole Mount In Situ Hybridisation

3.8.1 Single Detection using a anti DIG-Antibody and NBT/BCIP

I did the *Platynereis* WMISH mainly following the common lab protocol version of Florian Raible from 2006 (See Appendix: 7.3 *Platynereis* Whole-mount in Situ Hybridisation, page 73).

Preparation of antisense RNA Probe: For optimising the transcription mix I pipetted different volumes compared to the protocol. I used up to 10µl of linearised template and counterbalanced the difference with ddH₂O (RNase free). Afterwards I added 2µl 100mM DTT, 2µl NTP/DIG-UTP-MIX, 0.5µl RNase inhibitor, 2µl 10x Transcription buffer and 1µl RNA-polymerase. The total reaction volume was 20µl. Incubation at 37°C was always done for 4 hours.

Furthermore I varied the Proteinase K digestion times (2dpf: 45", 3dpf: 1', 4d: 1'30" 5dpf: 1'45", 14d: 2'45, Adults: 3') and performed the pre-hybridisation always for 2 hours or even longer. For over night hybridisation a higher RNA Probe concentration, as mentioned in the protocol, led to better results. Thus I used up to 20µl RNA Probe in Hyb-Mix with 200µl Hyb-Mix per tube. I accomplished the PTW washing steps after Antibody detection with ascending washing times. The first time I shortly rinsed the animals in PTW following by 1x 5min, 2x 10 minutes, 1x 15min and 1x 20min washing while shaking. If crystals appeared during the staining procedure I washed them away with 75% Ethanol after stopping the reaction with PTW. Afterwards rinsing

2x with PTW was necessary to get rid of the Ethanol.

3.8.2 Double Detection using a Fluorescent and a Non-Fluorescent Detection Method

The procedure for double detection is equal in the first part for the non-fluorescent detection with chapter 3.8.1. Including a Fluo labelled RNA probe at the hybridisation step on 65°C over night is the only difference. For generating this RNA probe I varied the transcription mix mentioned in the original WHISH protocol (Appendix, chapter 7.3) and took 2µl Fluorescein RNA Labeling Mix (Roche) instead of NTP/DIG-UTP-MIX.

Later steps for the fluorescence detection were done as described the “fluorescent/nonfluorescent detection method” from Kristin Tessmar-Raible (Tessmar-Raible et al. 2005): After stopping the NBT/BCIP staining reaction with 2x washing with PTW I transferred the embryos in TNT buffer. This was done with 2 rinsing steps. Blocking for 2h at room temperature was performed using 1 ml 2% DIG-block (Roche) TNT. Subsequently the AB detection for 1h at RT was done with a 1:50 anti-fluo-POD fab fragments antibody in 1% Dig-Block/TNT. Afterwards I washed the animals 6 times for 5 minutes in TNT.

Equilibrating in Perkin 1x Amplification Diluent (100µl per tube) and following incubation with an 1:25 dilution of Cyanin fluorescein Fluorophore in Amplification-Diluent (25µl per tube) for 2,5h led to a fluorescence staining. After checking the staining quality under the microscope I stopped the reaction with washing one time in TNT and three times in PTW buffer.

3.9 Antibody Staining

3.9.1 Generation of the Antibody

Polyclonal antibodies against the amidated C-terminus of PDF conjugated to the carrier protein Ovalbumin were developed by the PRIMM company⁷. Therefore this peptide sequence was used: nh2-NPGTLDAVLD MPDLMSL-coNH (Amidated C-terminus). They performed immunization in two different rabbits, did an immunoaffinity purification of the serum and tested the antibodies with ELISA. After initially testing both purified antibodies with our immunostaining procedure, I chose the rabbit 1 purified antibody for further studies.

3.9.2 Antibody Staining Procedure

For this approach I adapted our students course protocol “Platynereis dumerilii Immunostaining Protocol” (Appendix, chapter 7.4). In contrast to the protocol I performed this method with PFA fixed animals stored in Methanol. Therefore I added two rehydration steps before rinsing with 2x 5min in 1x PTW in the beginning. After transferring the embryos into nets submerged in methanol I rinsed them with 66% and 33% Methanol in 1xPTW. Furthermore, the Proteinase K digestion time of adults (2 months and older) was 3 minutes.

⁷ www.primm.it

The anti-PDF antibody was concentrated 1:500 in 2.5% sheep serum and served as first primary antibody. The second primary antibody was anti-acetylated tubulin as described in the protocol. I changed the dilution of PIS to 1:500. For mounting the samples Glycerol with DABCO (250mg dissolved in 10ml PBS, added 90ml Glycerol, pH 8.6) was taken.

3.10 Quantitative Real-time PCR

I received frozen RNA for my approaches from Enrique Arboleda and Mingliu Du extracted with the QIAGEN RNeasy Mini Kit following the Protocol “Purification of Total RNA from Animal Tissues”. cDNA was then generated directly before the qPCR run.

3.10.1 Primer Design and Testing

For designing suitable primer the Roche “Universal ProbeLibrary Assay Design” ProbeFinder Version 2.45⁸ was used. Therefore I did alignments with known melatonin receptors and PDF receptors of various species, included the *Platynereis* receptors and tried to annotate conserved exon-intron borders. With this approach I placed all primers on conserved borders to avoid genomic amplification. I ordered 3 primer pairs per gene for further testing.

For testing the primers I performed a quantitative real-time PCR including four different concentrations of cDNA (1, 1:10, 1:100, 1:1000) done by serial dilution, a -RT control (including water instead of reverse transcriptase) and a water control (no cDNA). As positive control I used primer amplifying the *rps9* or *cdc5* gene.

3.10.2 General qPCR Protocol

For transcribing RNA into cDNA we used the QIAGEN QuantiTect Reverse Transcription Kit following the protocol “Reverse Transcription with Elimination of Genomic DNA for Quantitative, Real-Time PCR”. Therefore I always took exactly 0.4 µg RNA as input measured by Nanodrop.

I implemented some changes to this protocol suggested by Enrique: In step three I incubated for 5 minutes at 42°C instead of two and in step number six I changed the incubation time to 20 minutes at 42°C. The RNAs, water and the gDNA wipeout buffer were pipetted one by one. For the remaining steps I worked with a mastermix. Finally the completed cDNA was diluted with 40 µl of water. For setting up the plate I first pipetted 20 µl of mastermix in each well consisted of 1 µl forward and 1 µl reverse primer (0.5 pMol/µl final concentration), 10 µl Power Sybr Green PCR Master Mix (Applied Biosystems) and 3 µl water. Afterwards 5 µl of cDNA template were added. The reaction was performed by an Applied Biosystems Step-One-Plus Cyclet using the StepOne Software 2.1. I always ran a 2h complete run including the standard PCR protocol and the melting curve analysis.

8 https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_030000

3.10.3 Analysis

Normalisation of the Ct values was done with the house keeping gene *cdc5*. Using OpenOffice 3.2 Calc I computed the standard error and the standard deviation. The Student's T-Test (Student 1908) for calculating statistical significance was also done with Calc.

3.11 Receptor-Ligand Binding Assay

3.11.1 Mammalian Cell Culture

The assay was performed with COS-7 cells. I obtained a frozen aliquot of passage 3 from Andrea Varga (Baccarini Lab), thawed it up and kept the cells in culture for 14 days, splitting them always 1:8. As cell culture medium I used High Glucose DMEM with L-Glutamine (PAA Cell Culture Company, Cat.No. E15-810) with 10% FCS and Penicillin/Streptomycin (1000x).

Thawing up: After warming the cells to 37°C in a water bath I added 1ml culture medium, re-suspended the pellet and plated the cells on a 10cm culture treated Petri dish containing 9ml more medium.

Splitting: Therefore I sucked off the growth medium and washed the cells with roughly 2 ml PBS. The following incubation with 1ml Trypsin (PAA Cell Culture Company, Cat.No. L11-003) for about 5 minutes at 37°C detached the cells from the dish. After isolating them from each other by pipetting I plated 1/8 of the volume on a new dish and added medium to a volume of 10ml.

Freezing: To freeze the cells they obtained the same initial treatment as with splitting. Instead of plating them, the liquid cell suspension was transferred into a falcon, filled up with medium and spun down (1100rpm, 5min, 37°C). Afterwards, I sucked off the supernatant and dissolved the cell pellet in freezing medium (90%FCS, 10%DMSO). I transferred 1ml of this suspension into each freezing tube and cooled them down in the -80°C fridge. For permanent storage I used liquid nitrogen.

3.11.2 Transfection of Mammalian Cells

COS-7 cells were transfected after growing them to 90% confluence. Therefore I used the Transit-LT1 Transfection Reagent (Mirus) in OptiMEM Medium (Invitrogen). I mixed 0.2µl/0.3µl of transfection reagent and 10µl OptiMEM per well, depending on the ratio 2:1 or 3:1. After incubating the solution for 5 to 20 minutes I added 0.1µg Plasmid DNA per well and incubated the transfection mixture for another 15-30 minutes. Afterwards a total volume of 10µl TraffoMIX was transferred to each well.

3.11.3 Impedance Assay

The measurement and also the analysis were done with the xCELLigence System (Roche) and RTCA Software version 1.2.1.1002 (ACEA Biosciences Inc.). I started the procedure with seeding passage 9 COS-7 cells in the suitable 96well plate. The standard cell number was 32000

cells/well in 100µl culture medium, but I also tested different cell numbers (10000 cells/100µl, 20000 cells/100µl) and different transfection-reagent to DNA ratios (2:1 and 3:1). After 24h I transfected the cells with Pdu MelR candidate in pcDNA3.1, Mouse Mm_MelR_1A (NP_032665.1) in pcDNA3.1, and oxytocin receptor pEGFP-N3, EGFP fused to C-terminus.

69h after seeding the cells the total culture medium was changed to 160µl/well starvation medium (0.5% FCS in DMEM + Pen/Strep) and incubated for another 3 hours. Then I added 20µl of the receptor-antagonists dissolved in DMSO (0.2µl) + Starvation Medium to each well. The final concentration thereof was 100µM. Furthermore I added only DMSO + Medium to wells without Antagonist treatment and excluded the oxytocin receptor + oxytocin wells. The two used Antagonists were Luzindole (TOCRIS Bioscience) and 4-P-PDOT (TOCRIS Bioscience).

After an incubation of 15 minutes on room temperature 20µl melatonin (0.004µl melatonin dissolved in DMSO + Starvation Medium) was added to all wells. In addition 40µl oxytocin (0.4µl oxytocin dissolved in PBS + Medium) were added to OxytocinR + oxytocin wells. Wells which did not get ligand treatment were filled up with starvation medium to a total volume of 200µl. The final concentration of melatonin (Bachem, 5g No. Q1300) was 100nM, from oxytocin (Bachem, 5mg No. H-2510) 200nM. After the addition of the antagonists the measuring interval of the impedance values was set to the minimum of 15 seconds to get a high resolution curve. During this time the reaction was run on room temperature.

3.12 Microscopy

3.12.1 Confocal Laser Microscopy

I used the Zeiss LSM-510 Meta Confocal Microscope with a 10x dry or a 40X oil objective together with the Zeiss LSM software (version 4.2) to detect fluorescence antibody staining and MBT/BICP staining of whole mount in situ hybridisations. The latter was observed with the confocal reflection microscopy technique (Jékely and Arendt 2007), where the in situ staining is exposed to a specific laser light and reflects it at the same wavelength. Therefore the embryos or adult worms were mounted in 87% glycerol plus DABCO. To avoid squeezing of the sample I used several layers (2-4) of tape or square cover-slips for thicker adult animals at the borders as spacing material between slide and cover slip.

To obtain better results I performed Deconvolution using the Huygens Software. 3D reconstruction was either also done with this software or with ImageJ (version 1.44I). Analysis, Z-Stacks projections and smaller image processing steps like brightness/contrast were only done with ImageJ. I also used the latter for measuring fluorescent pixels in stacks of confocal scans. Therefore I set a threshold of 77-255 and calculated the particles bigger than 10 pixels with all circular shapes.

3.12.2 Fluorescence Microscopy and Bright Light Microscopy

A Zeiss Axioplan 2 microscope was used for differential interference contrast (DIC) microscopy. Images therefore were done with either 63x, 40x oil objectives or 20x dry objective using the Axiovision (AxioVS40 V4.8.1.0, Carl Zeiss Imaging Solutions) software. For quickly checking the stainings and several other small tasks the Zeiss STEMI 2000 binocular was more than sufficient.

4. Results

4.1 Melatonin Receptor

4.1.1 Identification of Melatonin-Receptor candidates

To obtain a novel gene in *Platynereis* there are two principal approaches available. Cloning with a specific primer is only possible if the sequence is known – at least on both sides, to be able to design the primers. For cloning with a degenerated primer only some sequences of the gene of interest from related species are necessary. Therefore we tried to identify potential melatonin-receptor candidates in our *Platynereis* sequence pool. We started blasting the *Mus musculus* annotated MelR1A against our local EST database and found a candidate consisting of two EST reads (internal IDs: IB0AAA39CB08EM1, IB0AAA39CB08FM1) covering the 3' and 5' end of the receptor sequence, but lagging several nucleotides in the middle. The hits showed outstanding e-values and score values and the alignment with the query showed high conservation, too. This and several other blast searches against our EST databases gave no indication for a second melatonin-receptor-candidate.

To get further evidence for the real homology of the discovered *Platynereis* melatonin receptor candidate, we calculated phylogenetic trees consisting of melatonin receptors from related species. Therefore we collected annotated and predicted melatonin receptor genes, as well as yet unknown receptor candidates using the same blast search procedure as described before. Here, the following database sources were used: NCBI non-redundant protein database, several JGI protein databases (*Lottia*, *Capitella*,...) and our local EST database. As outgroups for the tree I decided to take adrenergic receptor and dopamine receptor because they showed most similarity with the mouse MtnR1A when performing the blast searches. For vertebrates several annotated genes could be found besides predicted receptors. In lophotrochozoan species such as *Lottia*, *Apis* or *Capitella* I just obtained untouched sequence reads and therefore marked them as candidates, only discovered through blast search analysis (Figure 6).

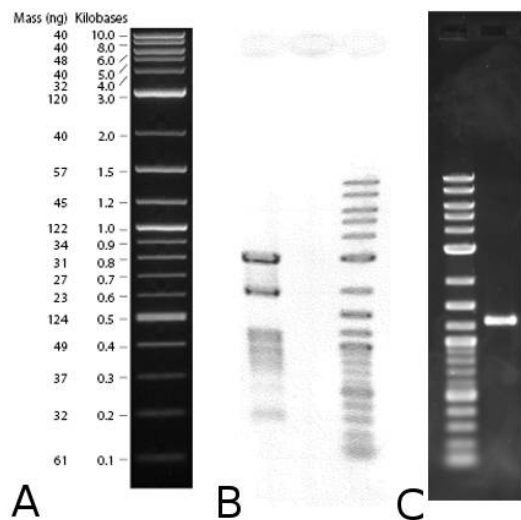


Figure 4: A) 2-Log DNA Ladder (0.1–10.0 kb, Picture from www.neb.com); B) Cloning of the pdu-MelR-candidate with specific PCR-Primer resulted in two major bands with 1.7kb and 3kb size, whereas only the smaller one was specific. Lane 2: Water control of PCR, Lane 3: 2-Log Ladder; C) Cloning of Mm-MtnR1A led to a single band at the expected size of ~1050bp. Lane 2: 2-Log Ladder;

4.1.2 Cloning of Pdu-MelR-candidate

Cloning from larval cDNA using a PCR program with specific Primer (see chapter 3.3.2, ID#1) resulted in two major bands at ~1.7kb and ~3kb (Figure 4 B). Sequencing showed that the larger

3kb piece was amplified with just one primer (MelR-up) on both ends. Blast search of this fragment against the mouse non-redundant database did not provide any usable, long results, either.

The sequence of the smaller band with 1.894kb was identical to the database EST reads. Predicting an open reading frame resulted in an ORF with 1.284kb size that starts at position 278. Its translated protein is 428 amino acids long. Furthermore, analysis for potential transmembrane domains with the TMHMM method (as described in chapter 3.2.2) gave a high probability for seven transmembrane domains. The N-terminus is predicted to be outside of the cell, whereas the C-terminus appears to be inside (Figure 5). The existence of these criteria verify that the obtained sequence, as well as the melatonin receptor, belongs to the family of G-Protein Coupled Receptors, also known as 7-transmembrane receptors.

The complete nucleotide sequence, the predicted transmembrane domains and also the translated amino acid sequence can be found in the Appendix, chapter 7.5.

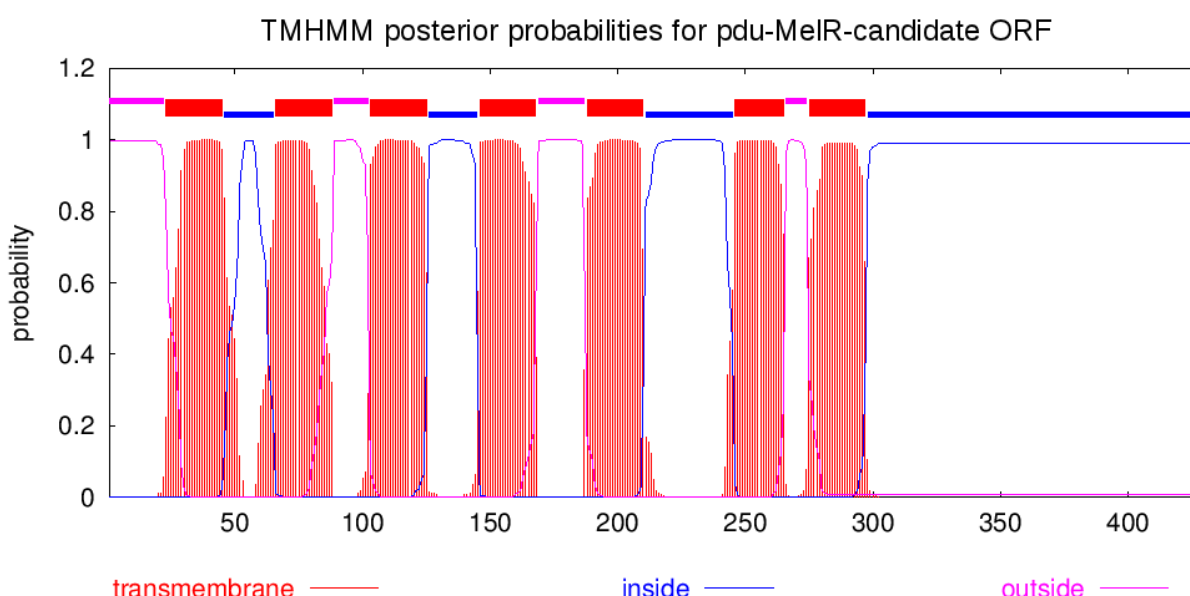


Figure 5: TMHMM probabilities for the Pdu-MelR-candidate calculated with the translated nucleotide sequence of the predicted open reading frame. The x-axis shows the position on the amino acid sequence (N to C-terminus) and the y-axis the probability for a transmembrane domain or an inner/outer domain.

4.1.3 Cloning of *Mm-MtnR1A*

As positive control for the later described Ligand-Receptor Binding Assay (Chapter 4.1.7) we needed a physical clone of the *Mus musculus* MtnR1A. Therefore we amplified it from mouse brain cDNA using specific primer as described in chapter 3.3.2. This approach resulted in a single band at the expected size of 1062bp (Figure 4C). Furthermore, during this cloning step we extended the sequence with two restriction enzyme sites, EcoRI and XhoI, and a Kozak sequence through primer engineering. Sequencing verified the integrity of the obtained gene and that all modifications were present.

For the receptor to be expressed in mammalian cell culture we needed to cut and ligate it into the mammalian expression vector pcDNA3.1. This step was confirmed through several analytical

restriction digests. In the following, an endotoxin free maxi prep (see chapter 3.5) was performed to get enough DNA for the transfection into mammalian cells.

4.1.4 Phylogenetic Analysis

Phylogenetic trees help to calculate and visualize the relationship of related homologous and orthologous sequences. I computed the following tree two times: At first without including the novel *Platynereis* MelR candidate to check the phylogeny of all other melatonin and adrenergic receptors, especially from the ones just predicted and from candidate genes. During a second run, I added the new melatonin receptor candidate from Pdu. This gene can cluster to the most related group with the biggest sequence similarity (Figure 6).

The required alignments and the phylogenetic trees were calculated as described in chapter 3.2.2 using the programs ClustalX, iqpnmi and tree-puzzle. As outgroup for the final tree (Figure 6) I chose adrenergic receptors because of their closest relationship to the melatonin receptor.

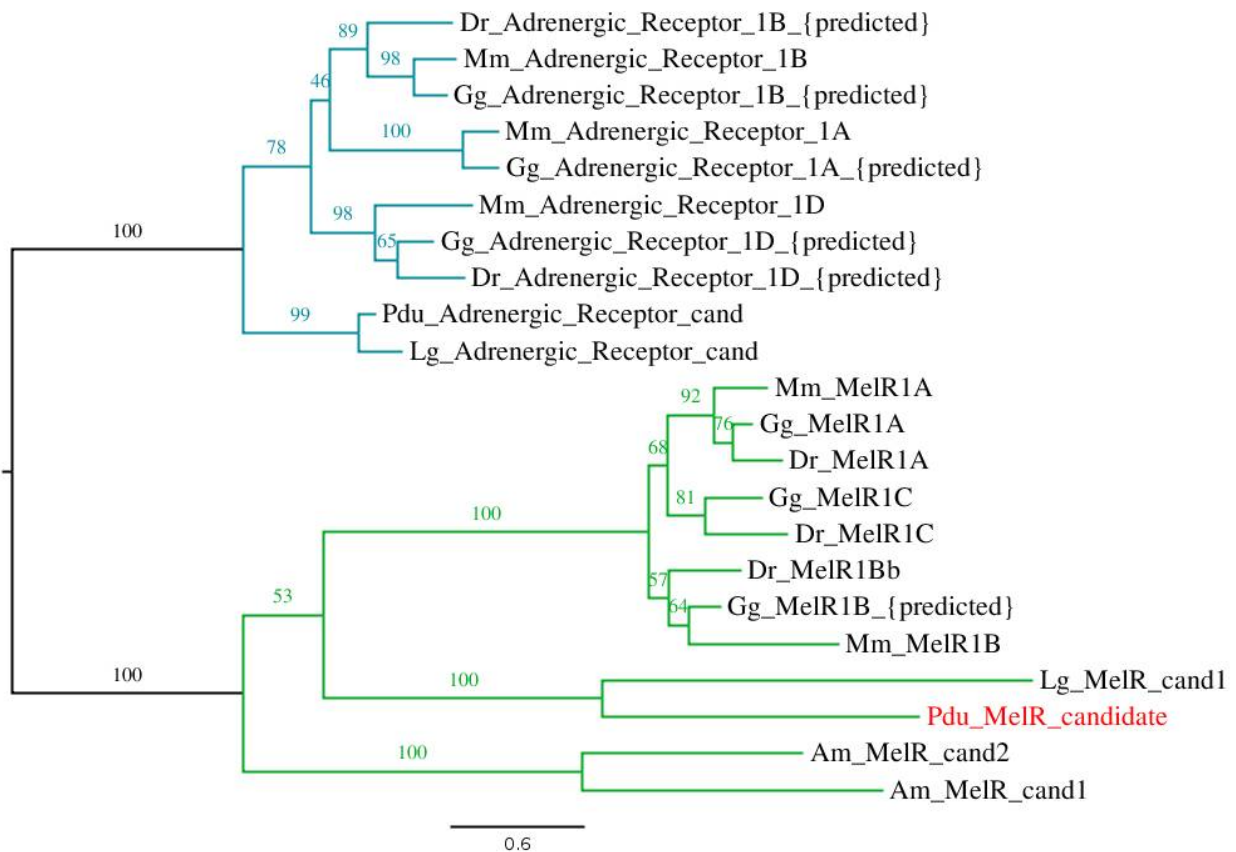


Figure 6: Maximum Likelihood tree of Melatonin receptors and adrenergic receptors. Tree consists of annotated proteins, predicted ones (marked as predicted) and candidates discovered by blast search analysis ("cand" for candidate). The cloned Pdu-MelR-candidate is highlighted in red. Numbers are Bootstrap values of 100 calculated trees. Abbreviations: Pdu - *Platynereis dumerilii* (polychaete worm), Mm - *Mus musculus* (mouse), Dr - *Danio rerio* (zebrafish), Lg - *Lottia gigantea* (giant owl limpet, a mollusc), Am - *Apis mellifera* (honey bee), Gg - *Gallus gallus* (chicken)

The Pdu MelR candidate clusters very well together with the predicted *Lottia* candidate. Outside of these Lophotrochozoa genes is one cluster of all Vertebrate sequences and another of two MelR candidates from *Apis mellifera*, the honey bee, which cluster outside of the receptors from Deuterostomia and Lophotrochozoa. Inside the Vertebrates, the genes group very well according

to their subgroup A to C. This is also visible in the outgroup of adrenergic receptors, where the sequences also cluster according to their subfamilies. The lophotrochozoan candidates from *Platynereis* and *Lottia* form a separate cluster outside of the vertebrate sequences, as expected.

4.1.5 Expression Localisation in Embryos

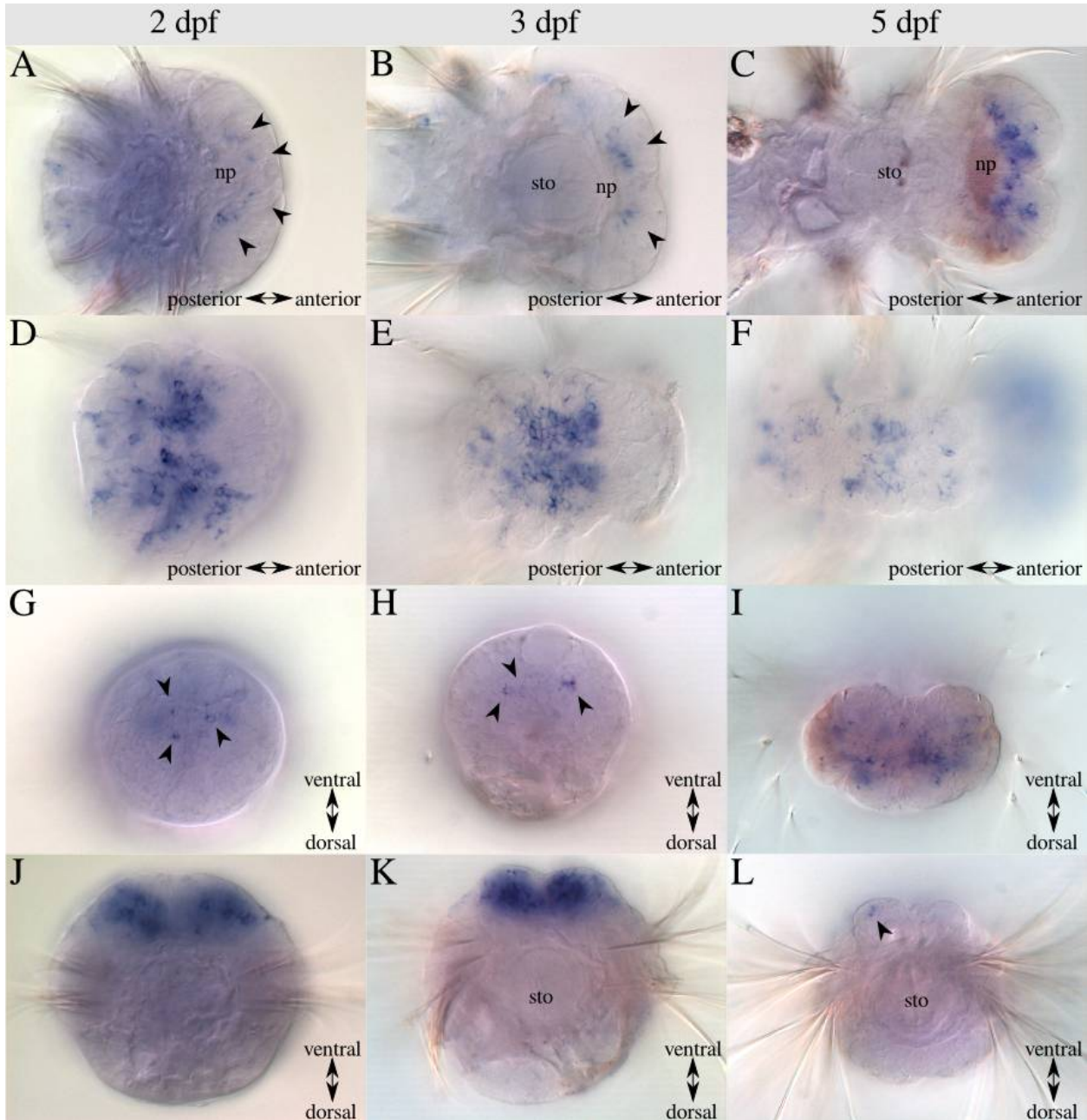


Figure 7: WMISH with an RNA antisense probe of Pdu-MelR-candidate. Animals were fixed at an age of 2, 3 and 5 days post fertilisation. Arrows indicate stained cells. A-F: Ventral view, anterior is right; G-L: Apical view, ventral on top; A-C and G-I: The stained cells in the brain are in focus; D-F and J-L: Ventral nerve plate is in focus; Abbreviations: sto - stomodeum, np - neuropil

To prove whether or not our newly identified melatonin receptor candidate is expressed and to localize such cells, I performed a whole mount in situ hybridisation as described in chapter 3.8.1. Bright field microscopy and also Confocal laser microscopy were used to visualize these results (chapter 3.12.1). The RNA antisense probe of the Pdu-MelR-candidate gave a blue NBT/BCIP

staining after developing for at least 3 days in the dark (Figure 7). Unfortunately, I could not get any cellular staining for other stages tested, including 7dpf and 14dpf animals. Two days after fertilisation the embryos showed a massive, symmetric staining in the ventral nerve plate (7D,J) and some single cells in the head (7A,G). The latter are arranged in a semicircular shape anterior to the neuropil (np) in the medial region of the brain (indicated through black arrows).

Platynereis with an age of 3dpf still have the dominant staining in the ventral nerve plate (7E,K), but in the medial brain region more cells are expressing the MelR candidate (7B,H). At this stage the stomodeum (sto) becomes visible very well because of strong cells proliferation (Antje Fischer, Henrich, and Arendt 2010). Posterior to the stomodeum two symmetrical cells could be detected in the Confocal Microscopy scans (Figure 8 C).

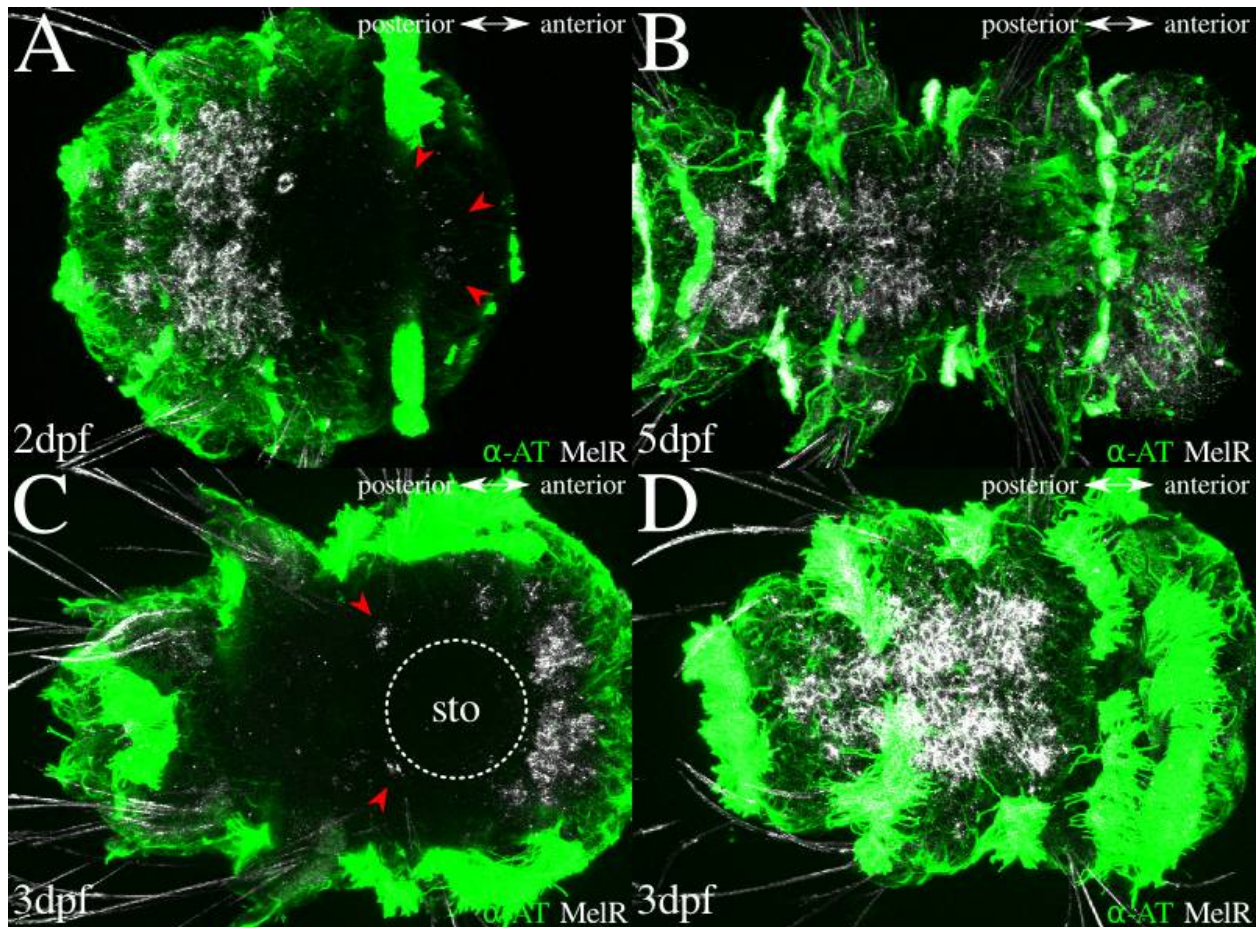


Figure 8: Confocal Microscopy images as Z-Stack. White: In Situ Hybridisation Staining, Green: anti-acetylated tubulin antibody staining; A) 2dpf larvae B) 5dpf larvae C+D) 3dpf embryo, whereas C are dorsal layers and D ventral layers.

At the stage of 5 days post fertilisation the ventral nerve chord staining decreased enormously. It shows expression in the range of just some single cells to randomly distributed to small weakly stained cell-clusters (7F,L). This fluctuation does not depend on the timepoint of the day, tested with embryos fixed at 3am, 9am and 3pm, but maybe rather on the developmental stage of the individuals. Further, we see a broad gene expression in the head which is still in the medial, apical brain region (7C,I), but sometimes appears to be distributed more dispersedly (Figure 8 B).

Figure 8 indicates the location of MelR expressing cells in relation to the neurons, coloured with antibody staining using a monoclonal antibody against acetylated tubulin. The proto-, meta-, para-, and telotroch also appear coloured very strongly, as they contain acetylated tubulin for stabilisation. Unfortunately the staining did not work that well and the antibody could just detect α AT at the very surface, but not inside the embryo, especially at the 3dpf stage (8C,D).

4.1.6 Expression Quantification in Immature Adults

For Adults, the gene expression of the MelR candidate was measured with quantitative real-time PCR. Therefore I designed several primer pairs and tested them for proper functionality (for details see chapter 3.10.1). To avoid amplification of genomic DNA, the primers were located on conserved exon-exon borders, predicted using an alignment of genomic sequences of several known and well annotated melatonin receptors and the new *Platynereis* candidate messenger RNA.

Primers were tested with an RNA mix from Mingliu Du, extracted out of Pdu head, body and larval tissue. The primer pair number one gave an R^2 of 0.9965 when doing linear regression on the plot of cycle count to cDNA dilutions. Further, the results of the water control (no RNA included) and the -RT control (no reverse transcriptase included) were fine, no amplification could be detected for this primer pair. Therefore I used this one for further analysis.

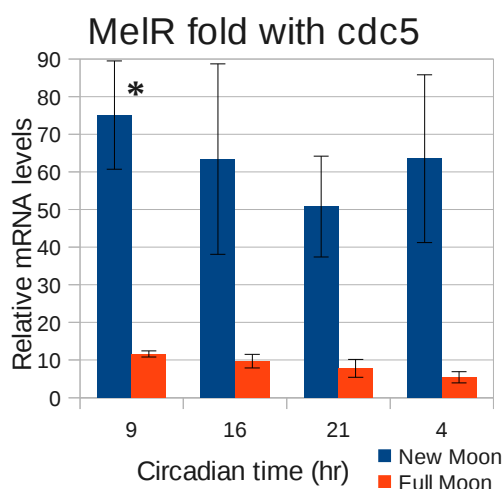


Figure 9: qPCR Results from Enrique RNA; Stars represent T-Test p-values: * < 0.05, ** < 0.01, *** < 0.005; Error bars show Standard Error;

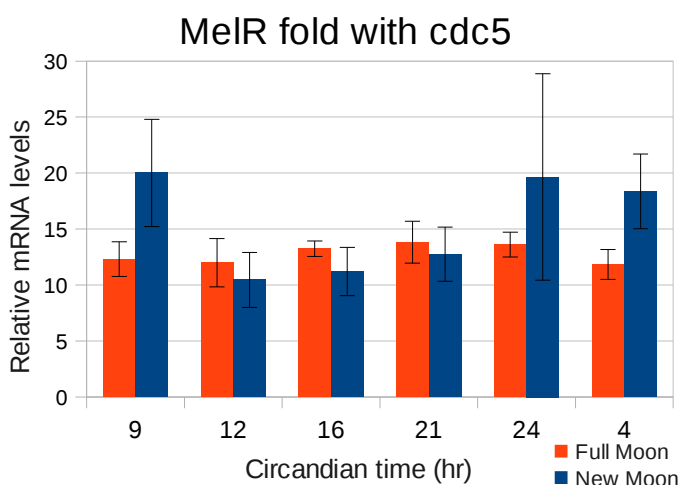


Figure 10: qPCR Results from Mingliu RNA; Stars represent T-Test p-values: * < 0.05, ** < 0.01, *** < 0.005; Error bars show Standard Error; mRNA levels are relative to the cdc5 housekeeping gene

To quantify the Pdu MelR expression I did two test runs with several timepoints during 24 hours for full moon (FM) and new moon (NM) conditions. For the first run I obtained RNA from heads sampled and extracted by Enrique Arboleda, for the second run the RNA was done by Mingliu Du. I always analysed 3 biological replicates containing 5 worm heads each (see also chapter 3.10.2). Only for the 9h, 16h and 21h timepoint of Enrique's full moon RNA, there were just two biological replicates available. I performed each run with two technical replicates and calculated the mean value for further processing. To assess if the data showed statistically significant

differences I did the Student T-Test, as described in chapter 3.10.3.

The first run with RNA from Enrique (Figure 9) shows a big difference of the relative mRNA levels between full moon and new moon samples. During new moon the animals show a relative expression of 50% to 75% *cdc5*, while during full moon the gene expression is just 5% to 10% of *cdc5*. These results would exhibit an almost 10 times higher expression of the melatonin receptor candidate during new moon, but the Students T-Test only showed a weak significant difference for the 9am timepoint with a p-value of 0.04. Furthermore, a big Standard Error was calculated which is shown in the graph as error bars. I also couldn't detect any significant daily variation, which would indicate no circadian rhythmicity.

For the second analysis with the RNA from Mingliu, samples from FM and NM next to each other were available, with just two weeks in between. In contrast, the RNA I got from Enrique was sampled during two completely independent moon phases.

With the data from the second run (Figure 10) I could not totally reproduce the previous values. The relative mRNA expression still showed no significant difference between the two moon conditions, and also no significant alteration during the day. But the expression levels I obtained are in the range of 12% to 20% of *cdc5*.

4.1.7 Receptor-Ligand Binding Assay

To test the melatonin receptor candidate's ability of properly binding his ligand, melatonin, we performed a Cell Impedance Assay (Yu et al. 2005; Roche Applied Science). This assay was designed in three steps: On the first day, the mammalian COS-7 cells were seeded onto a special 96 well plate and then transfected on the second day with our receptors of interest. On the third day the Ligand and also two known Antagonists of the mouse melatonin receptor were added while measuring the change of the impedance values (Materials and Methods, chapter 3.11). For each condition eight technical replicates were used and obvious outliers were taken out when calculating the mean value for further processing.

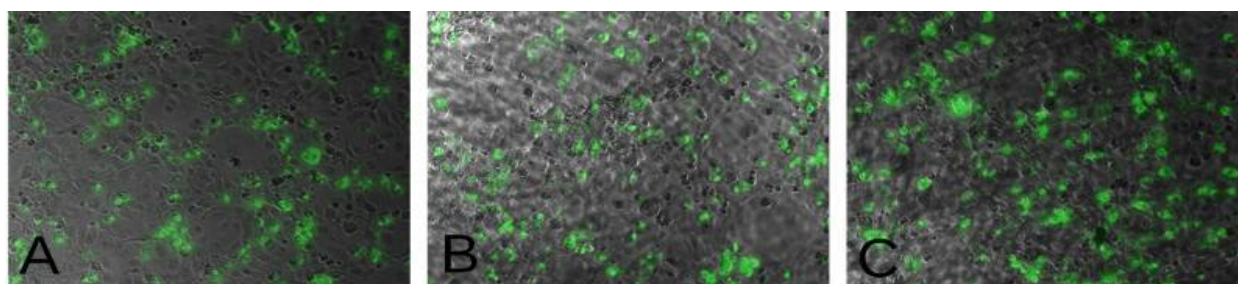


Figure 11: Fluorescence Microscopy Picture showing the transfection efficiency of Cos-7 cells with EGFP tagged oxytocin receptor. Cells are visible through phase contrast microscopy, fluorescence signal as a screen overlay. A) 10 000 cells B) 20 000 cells C) 32 000 cells, All images with 3:1 ratio of transfection reagent and Plasmid-DNA.

To establish this assay, we needed to examine different conditions to obtain optimal results. Therefore different ratios of transfection reagent to Plasmid-DNA and different cell numbers were tested. The transfection efficiency showed best results for wells with 32 000 cells seeded at day 1, as demonstrated for the EGFP tagged oxytocin receptor (Figure 11) using fluorescence microscopy. These wells reached 90% confluence on the second day when the transfection was

performed. Unfortunately, we don't have any data for the transfection efficiency of the Pdu_MelR or the Mm_MtnR1A, but we assume from the data of the oxytocin receptor that these genes also show the best efficiency at 90% confluence. Furthermore, the 3:1 ratio of Transit-LT1 to Plasmid-DNA exhibited a better transfection efficiency too and also higher Impedance peaks than the 2:1 ratio, so all results are only shown with these data.

The impedance values were measured during the whole experiment to check for a proper adhesion of the cells to the surface of the 96 well plate and to observe the proliferation during this time. The gain of the impedance and therefore of the cell proliferation slowed down and then stopped in all wells during the second day. The addition of the transfection mixture could also be detected on the plots through a small positive peak. Changing to starvation medium at the third day also affected the impedance in all wells, but during the following three hours of incubation time the impedance recovered again to a stable value.

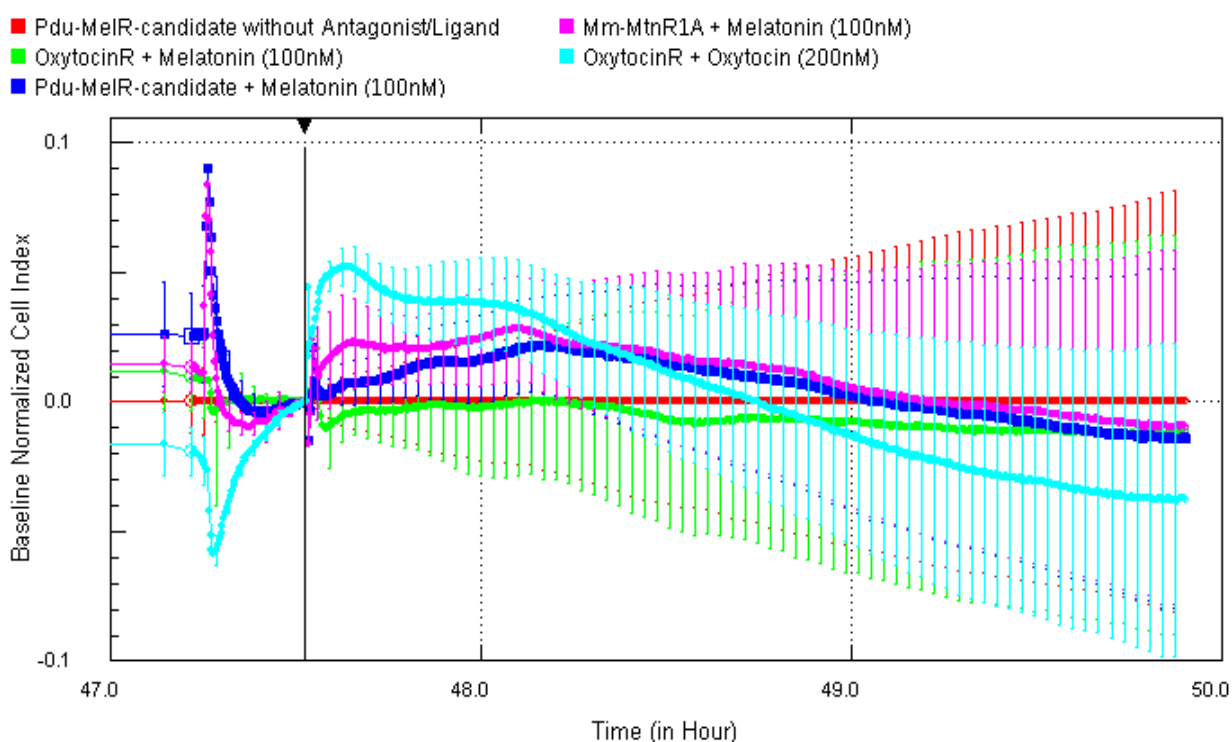


Figure 12: Impedance measurement: Black arrow in diagrams indicates selected timepoint for normalising the data. There the ligands were added to the cells. Error bars indicate the standard deviation. All data are normalised to the negative control shown in red.

Adding the receptor-antagonists and all further steps including the incubation phases were performed on room temperature. This had a strong influence on the impedance of the cells. The values were rising and produced a big positive slope independent of the added substances. To be able to spot real ligand-receptor interaction peaks I had to post-process the data and normalise all the curves to my negative control, COS-7 cells transfected with Pdu-MelR-candidate and no compounds added. Hence, this negative control always appears as a zero base line (Figure 12, 13 and 14). Further, this phenomenon is the reason for the big error bars, which are additionally increasing more and more during the measurement. The black arrow in the three diagrams indicates the selected timepoint for normalising the data. At this timepoint the ligands melatonin or oxytocin were added to the cells. The peak visible 15 minutes earlier is the change of

impedance through the addition of Antagonist or solvent.

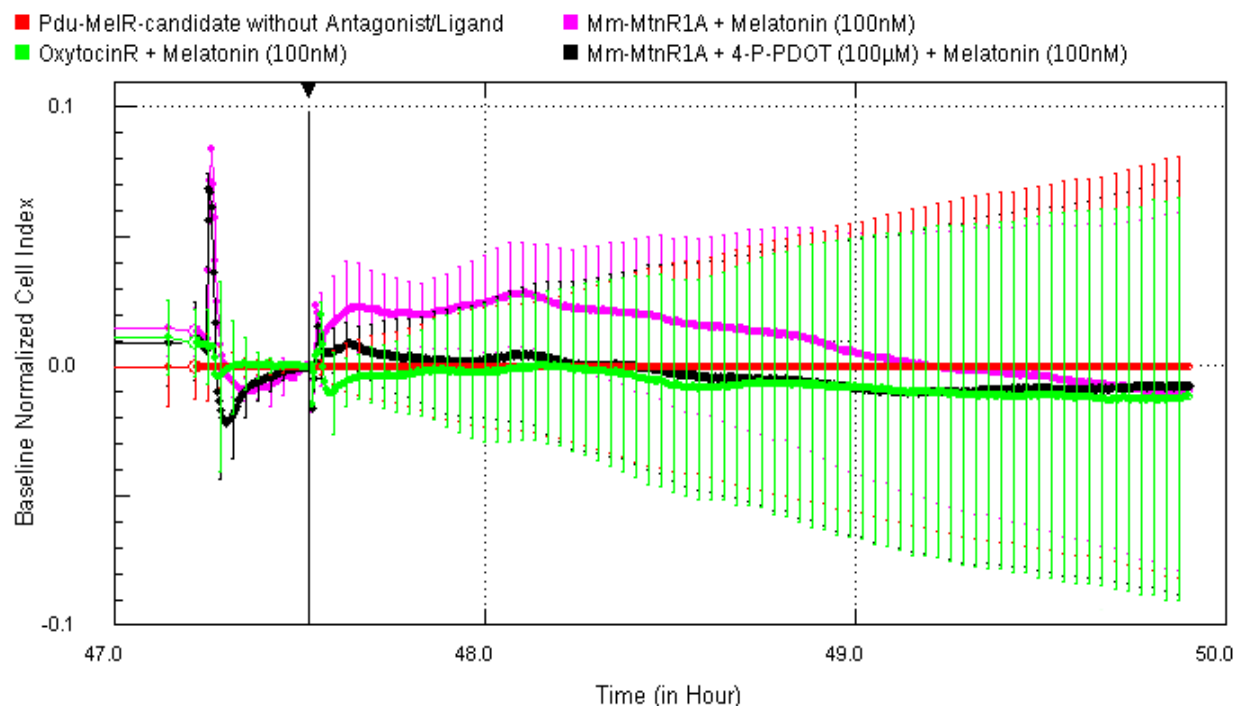


Figure 13: Impedance measurement of cells expressing Mm-MtnR1A treated with the antagonist 4-P-PDOT: Black arrow in diagrams indicates selected timepoint for normalising the data. There the ligands were added to the cells. Error bars indicate the standard deviation. All data are normalised to the negative control shown in red.

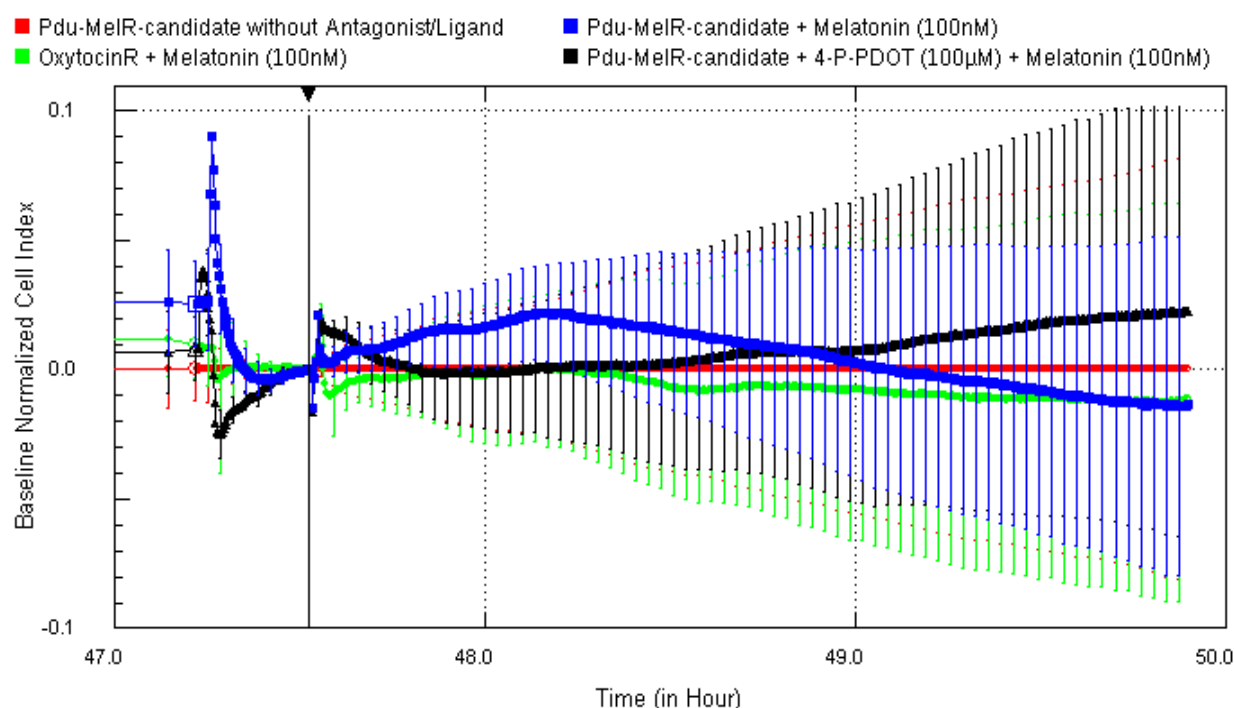


Figure 14: Impedance measurement of cells expressing Pdu-MelR-candidate treated with the antagonist 4-P-PDOT: Black arrow in diagrams indicates selected timepoint for normalising the data. There the ligands were added to the cells. Error bars indicate the standard deviation. All data are normalised to the negative control shown in red.

As seen in Figure 12, cells transfected with the oxytocin receptor showed the best response to oxytocin exposure, whereas our positive control, Mouse MtnR1A with addition of melatonin, also reacts quite strongly with a CI increase of ~ 0.3 . Cells expressing the *Platynereis* MelR

candidate respond to melatonin with a clearly visible peak similarly shaped as the curve of the mouse receptor. All three GPCR react almost immediately and the CI reaches a normal level again after about 1.5 hours. Another negative control, cells with a transfected oxytocin receptor and treatment with melatonin, also showed no change of impedance. This tests the natural response of COS-7 cells to melatonin exposure. Only a very small negative alteration is visible, probably due to the addition of solvent.

Further, I tested the effect of two Antagonists of the mouse melatonin receptor: Luzindole and 4-P-PDOT. Both are known to efficiently inhibit the binding of melatonin to the mouse MtnR1A and 1B.

In our assay, addition of only Luzindole already showed an enormous negative peak on the plots. This dramatic change of the CI is probably due to a strong reaction of the cells to Luzindole. Because of this huge discrepancy, it was not possible to include these curves in the normalisation procedure. Further, it would be impossible to detect a proper Ligand-binding peak after the addition of melatonin because CI values were still changing rapidly from the Luzindole reaction.

The cells did not react that strongly with the addition of 4-P-PDOT and only a small negative peak was observed in this case. Also, the cell impedance values stabilized during the following 15 minutes incubation time. MtnR1A (Figure 13) as well as the Pdu-MelR-candidate (Figure 14) show a clearly reduced response to melatonin with a prior treatment with 4-P-PDOT (black curves). Only the shape of the reduced signal was different in both receptors. A clearly reduced peak during the whole ligand response was measured for the mouse receptor with antagonist treatment, whereas the Pdu receptor first showed a higher peak than without 4-P-PDOT, which disappears shortly afterwards.

4.1.8 Search in EMBL Databases Resulted in Another Pdu MelR candidate

At the end of my research I did several blast searches as described in chapter 4.1.1 against a new sequence database provided by the Arendt Group at the EMBL Institute (unpublished data). They led to the observation of a genomic fragment very similar to the already cloned melatonin receptor candidate and also of a second candidate assembled from a contig of genomic DNA and a transcript.

This new resource provided an EST library and even genomic transcripts. Blasting against both databases resulted in two hits with great scores and p-values. MelR candidate number three was retrieved from an assembled sequences of the genomic database (internal IDs: scaffold202018, scaffold229599) and had an identical amino acid sequence from position 85 on compared to my already studied Pdu MelR candidate. Except for some single nucleotide polymorphisms or sequencing errors there are also no notable differences between the nucleotide sequence of candidate 1 and 3. Only at the 3' end the cand3 sequence shows huge changes and it is a little bit longer at 5'.

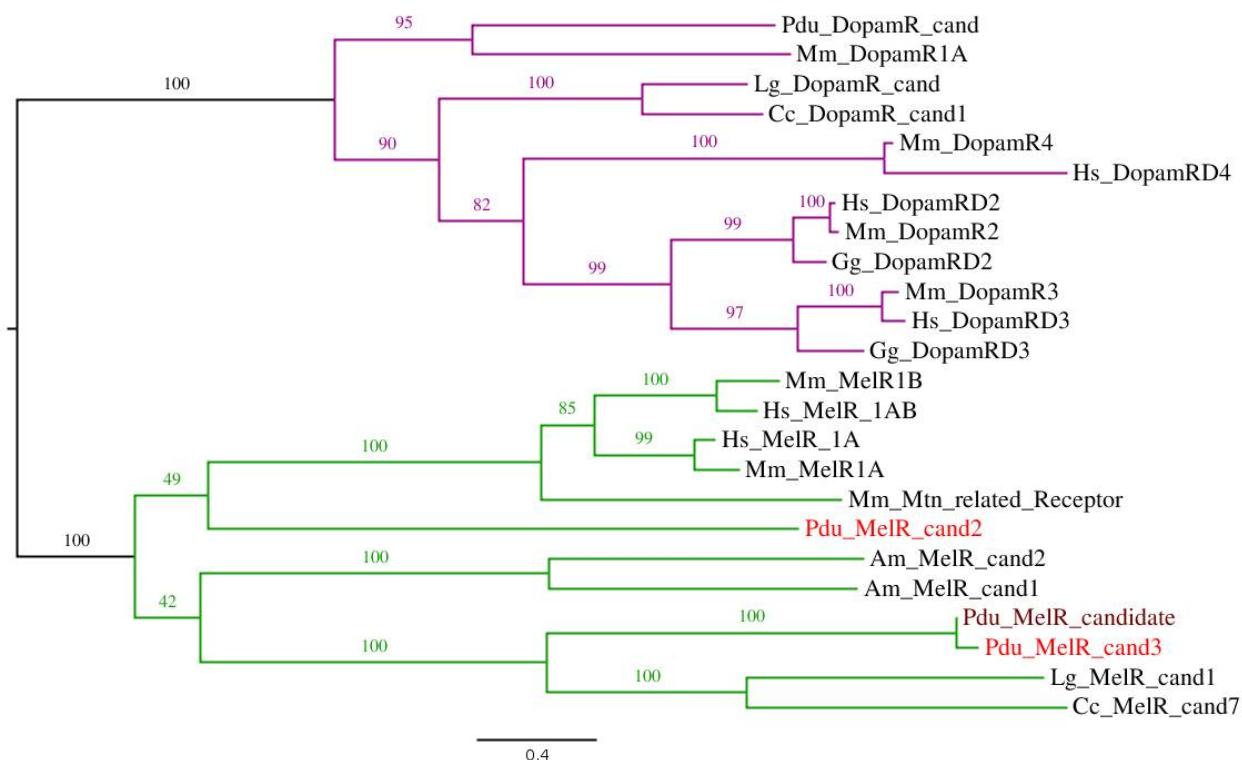


Figure 15: Maximum Likelihood tree of melatonin receptors and dopamine receptors. The newly discovered melatonin receptor candidates are highlighted in red. Tree consists of annotated proteins, predicted ones (marked as predicted) and candidates discovered by blast search analysis ("cand" for candidate after names). Numbers are Bootstrap values of 100 calculated trees. Abbreviations: Pdu - *Platynereis dumerilii* (polychaete worm), Hs - *Homo sapiens* (human), Mm - *Mus musculus* (mouse), Cc - *Capitella capitata* (a polychaete worm), Lg - *Lottia gigantea* (giant owl limpet, a mollusc), Gg - *Gallus gallus* (chicken)

To obtain the full length of the second hit, sequence assembling of a small piece of genomic DNA to the EST transcript was necessary (internal IDs: scaffold952079, KN-1127-B-93_O24_SP6.abi_Sanger_Jekely_KN-1127). Blasting back this candidate against the *Mus musculus* non-redundant NCBI database gave a strong similarity to MtnR1B. Building a maximum likelihood tree (Figure 15) shows that this new *Platynereis* MelR candidate 2 clusters even more closely to the annotated vertebrate melatonin receptors than our previous candidate. It also consists of seven transmembrane domains predicted by TMHMM, whereas the N-terminus is calculated to be outside and the C-terminus inside. The sequence is 1377 nucleotides and the predicted open reading frame 459 amino acids long. The Pdu MelR candidate 3 also has seven predicted transmembrane domains, whereas on the C-terminus there is a huge area (amino acid 350 to end) with probabilities of about 0.5. Further, I was able to identify a *Platynereis* dopamine receptor candidate in the EMBL sequences (internal ID: scaffold1027908) and included it with other annotated dopamine receptors as an outgroup for the phylogenetic tree (Figure 15). The whole sequences and the annotations can be found in the Appendix (chapter 7.5).

4.2 PDF Receptor

4.2.1 Identification of Pdf-Receptor candidates

As already described for the melatonin receptor (chapter 4.1.1), two approaches are available to clone a novel gene in *Platynereis*. If the sequence is at least known on both ends of the mRNA, cloning with specific primer is possible, whereas completely unknown *Platynereis* genes have to be cloned using degenerated primers. So, we tried to identify potential PDF receptor candidates in our *Platynereis* sequence pool. We blasted the annotated and well described sequence of the PdfR from *Drosophila melanogaster* against our local EST database, but could not find any related sequences.

With the assumption that the PdfR mRNA is missing in our sequence database, we decided to perform a degenerated PCR. Therefore a good alignment of the gene of interest from different related species is needed to be able to design the primers. Using blast I searched for PDF receptors from other species and found many candidates, but most of them were just predicted or not yet annotated at all. Among these were genes from insects, such as *Tribolium* and *Apis*, obtained from the NCBI non-redundant database and also lophotrochozoan candidates, like *Lottia* and *Capitella*, derived from the JGI protein database.

4.2.2 Cloning of Pdu-PdfR-candidate

Degenerated PCR was used to clone a *Platynereis* PDF receptor candidate from larval cDNA. For designing the primer, I built an alignment of PdfR genes from related species and calculated a phylogenetic tree. The latter was used to verify the observed candidates as PdfR. After computing a precise alignment, conserved amino acid regions were highlighted and translated into a degenerated nucleotide code, with respect to amino acid exchanges between the different species. I designed two primers in each direction, leading to four possible combinations. With them I performed PCR reactions from larval cDNA (for details see chapter 3.3.3) and also nested reactions, where a previous PCR product, amplified with more exterior primers, is used as template.

With a nested reaction I was able to clone a 225bp long, specific fragment using the primers Pdu_PdfR_up2 and lo2. Using a radioactive probe against this newly identified sequence, we performed a Southern Blot of these PCR products. This led to an even longer fragment amplified with the primers up2 and lo1. The fragment is 709 base pairs long and has a predicted open reading frame with a length of 236 amino acids. Doing a blast search against the *Drosophila* non-redundant NCBI database gave PdfR as the first hit and I also observed a high sequence similarity of 68.6% between the Pdu fragment and the *Drosophila* amino acid sequence of the same length.

Further, checking for transmembrane domains with the calculation of TMHMM probabilities showed that six out of seven transmembrane domains were already on the known sequence (Figure 16). Aligning this fragment to other known PDF receptors indicated that only the

transmembrane domain number one (tm1) was missing. The C-terminus was predicted on the inside of the cell, as it is expected for a GPCR. There were still long parts of the sequence missing up and down-stream. Therefore I declared it as a fragment of the Pdu PdfR candidate.

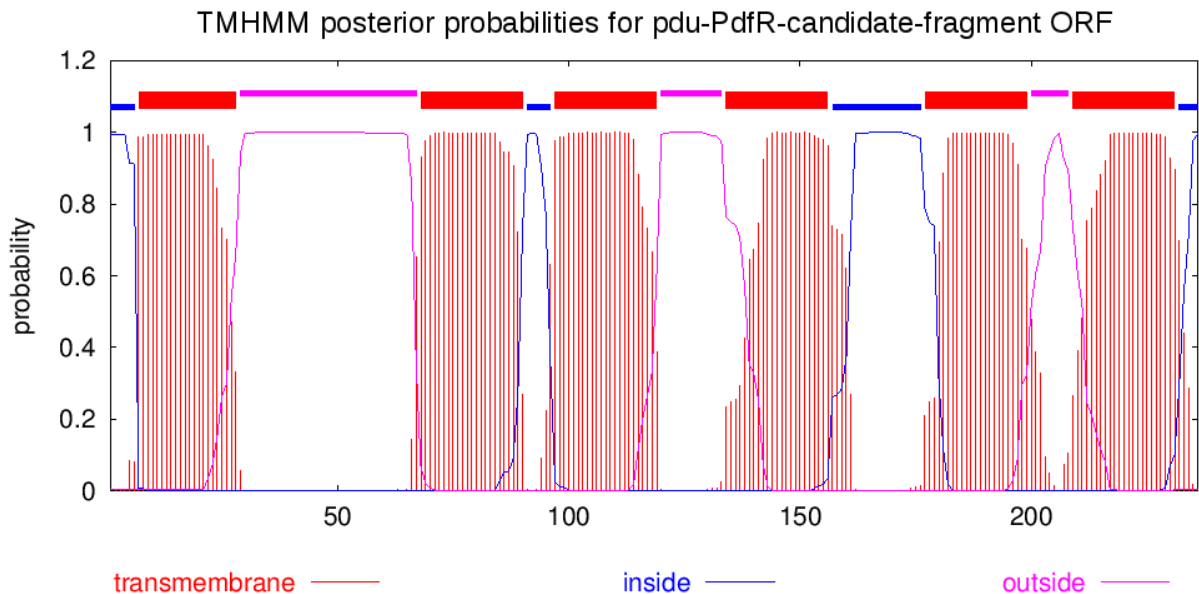


Figure 16: TMHMM probabilities for the Pdu-PdfR-candidate-fragment calculated with the translated nucleotide sequence of the predicted open reading frame. The x-axis shows the position on the amino acid sequence (N to C-terminus) and the y-axis the probability of a transmembrane domain or an inner/outer domain. First visible transmembrane domain from N-terminus is tm2.

4.2.3 Phylogenetic Analysis

The calculation of a phylogenetic tree is a tool to further prove the genuineness of the novel receptor fragment. I included several PdfR candidates from different species and also calcitonin and corticotropin receptors as two out-groups (Figure 17).

The tree was first computed without the novel PdfR fragment and then with it to investigate whether the new sequence clusters rather with other PDF receptors than with closely related genes, the outgroups. Figure 17 shows that the *Platynereis* PdfR candidate (highlighted in red) groups with the PdfR candidates from other lophotrochozoan species, such as *Capitella* and *Lottia*. The Insect receptors from *Drosophila*, *Apis* and *Tribolium* cluster together on a branch next to them (dark red). The Bootstrap values to the root are always 100 out of 100 trees, indicating a clear differentiation to the outgroups.

The internal phylogenetic relationship of the corticotropin receptor branch shows a similar structure. The *Capitella* and *Lottia* genes cluster together with the mouse corticotropin receptor, whereas the insect genes build a branch next to this group. The *Drosophila* receptors of both outgroups are annotated as Diuretic Hormone Receptors. Within the calcitonin receptors, the lophotrochozoan genes also group together, including *Helobdella*, *Capitella* and two observed candidates from *Lottia*. Other sequences from *Capitella* and *Lottia* build an extra branch more closely related to the insect PdfRs. I was also able to detect a mouse sequence of a calcitonin receptor isoform. These results further indicate that the observed fragment of the Pdu PdfR candidate could be a real PDF receptor.

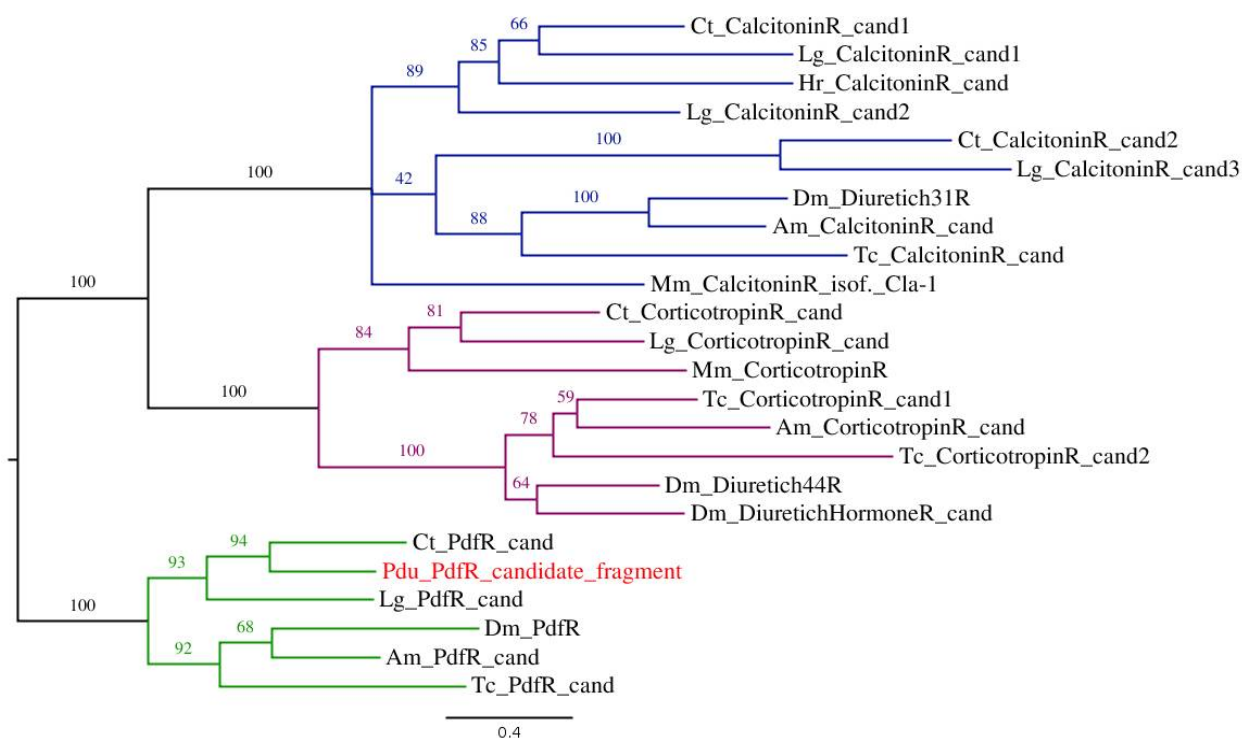


Figure 17: Maximum Likelihood tree of PDF receptors, calcitonin receptors and corticotropin receptors. The Pdu PDF receptor candidate is highlighted in red. Tree consists of annotated proteins, predicted ones (marked as predicted) and candidates discovered by blast search analysis ("cand" for candidate after names). Numbers are Bootstrap values of 100 calculated trees. Abbreviations: Pdu - *Platynereis dumerilii* (polychaete worm), Mm - *Mus musculus* (mouse), Ct - *Capitella teleta* (a polychaete worm), Lg - *Lottia gigantea* (giant owl limpet, a mollusc), Hr - *Helobdella robusta* (a leech), Am - *Apis mellifera* (honey bee), Tc - *Tribolium castaneum* (red flour beetle), Dm - *Drosophila melanogaster* (fruit fly)

4.2.4 Elongation of the Cloned PdfR Fragment

To elongate the previously obtained Pdu PDF receptor fragment in both directions, 3' and 5', we performed a smart RACE (Rapid Amplification of cDNA Ends) as described in chapter 3.6. The used primers, the cDNA generation procedure and also the PCR protocol are described in Materials and Methods. After running an agarose gel of the PCR products (Figure 19) to separate them by size, a

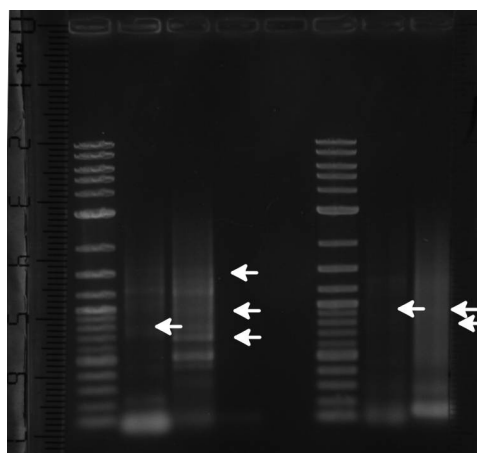


Figure 19: Agarose gel of RACE products; Lanes: 2-log Ladder, 3' reaction, N3' (nested), 2 empty lanes, Ladder, 5', N5'; Arrows indicate positive bands seen on the hybridisation blot (Figure 18), which were cut out and cloned.

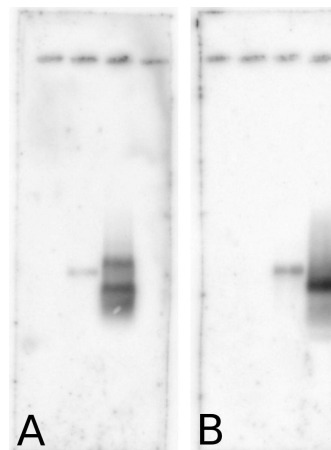


Figure 18: Southern Blot of the agarose gel in Figure 19 hybridized with pdu-pdfR probe. A) 3' RACE, B) 5' RACE; Both include a nested PCR in 2nd lane.

Southern Blot was done, using a radioactive probe against part of the already known fragment, to label specific PCR products. The detailed Protocol for it, the sequence of the probes and the used kits can be found in chapter 3.7. To visualise the radioactivity we scanned the blot with a phosphorscreen imager (Figure 18). We could detect some highly specific bands marked with a

white arrow in Figure 19. These were then cut out, eluted from the gel, cloned into the pGEM-T easy vector and transformed into *E. coli* bacteria, as described in chapter 3.3.4 and 3.3.5.

To select for colonies containing specific PdfR DNA we also blotted Filter lifts of the agar-agar plates and obtained many positive colonies. The clones were analysed through analytical restriction digests (chapter 3.4) and sent for sequencing. Unfortunately, all inspected clones showed either no elongation of the known fragment or no similarity at all. Some short (<400 base pairs) sequences contained the specific PdfR sequence but just the already known fragment. The longer fragments had no similarity to the Pdu PdfR candidate. As a control, blotting of some digested miniprep plasmids gave a positive signal for all small bands, but not for any large inserts.

So, even after several runs, I was not able to elongate the known fragment of 709 nucleotides.

4.2.5 Expression Localisation in Embryos

With performing a whole mount in situ hybridisation (for details see chapter 3.8) it is possible to answer the question whether our novel *Platynereis* PdfR candidate is expressed in some cells and where these cells are located. Therefore I fixed embryos at different embryonic stages and time points, including 2, 3, 4, 5, 7 and 14dpf, and used them for this approach. Bright field microscopy and also Confocal laser microscopy helped to visualize these results (chapter 3.12). For stages not shown, I could not get any staining probably due to technical issues.

At 3 days post fertilisation (9am) the receptor is already expressed, showing the blue NBT/BCIP staining in the ventral nerve plate as well as in the head (Figure 20). The staining in the ventral nerve plate can be described as two to four single cells which appear bilaterally symmetric if there are more than two cells expressing the PdfR (20 B). In the head there is a medial, very dorsally located patch out of four to six cells (20 A, C) which is located anterior and dorsal to the neuropil (np). Further, there are smaller cell clusters lateral and a bit posterior to these cells (20 A, D). They are probably located directly anterior to the eyes, but double stainings with marker genes which would prove this thesis are missing. Some embryos at this stage only showed four cells in the medial region and none lateral, or sometimes the lateral clusters also appear more medial, as shown here.

The cells in the ventral nerve plate are visible much better in the Confocal Laser Microscopy scan shown in Figure 21, picture B. Also the previously described six medial cells in the head can be seen in the confocal scans (21 A) and are marked with red arrows. With this technique it is possible to visualize the neurons, coloured with antibody staining using a monoclonal antibody against acetylated tubulin. Therefore, the location of PdfR expressing cells can be shown in relation to the neurons appearing green in the brain. The proto-, meta-, para-, and telotroch also contain acetylated tubulin and therefore also appear coloured very strongly. Unfortunately, the antibody did not detect the α AT inside the embryo, but just at the surface of the embryo due to problems with the antibody staining technique. There were even unspecific cell outlines appearing in fluorescent green, as it is visible in Figure 21 B.

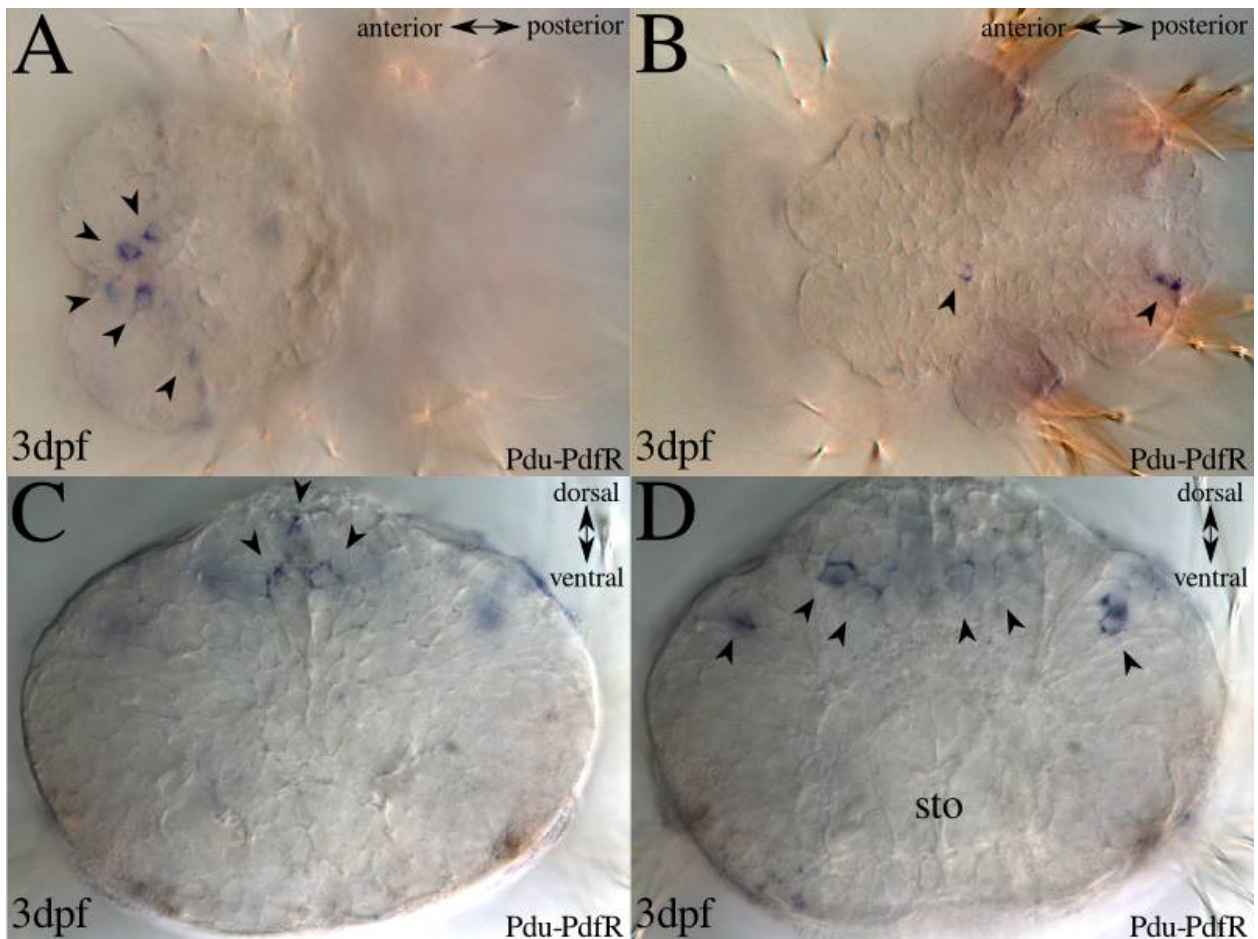


Figure 20: 3dpf 9am, Pdu PdfR In situ staining A+B) ventral view, anterior left. A) showing head staining B) focus on ventral nerve plate C+D) apical view, dorsal on top – focus in D is more posterior. Abbreviations: sto – stomodeum, np: neuropil

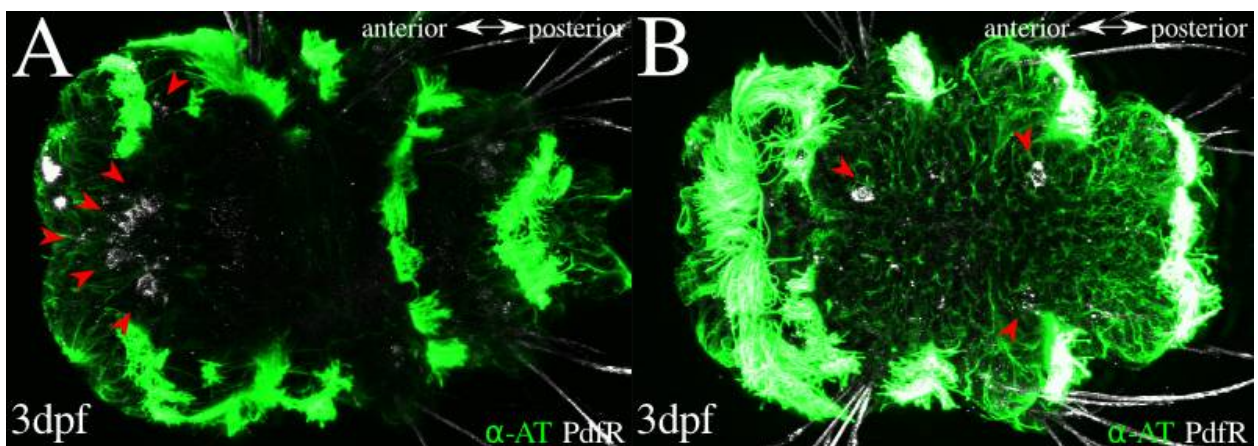


Figure 21: 3dpf 9am, Ventral view, z-stack projection of confocal image, green anti acetylated tubulin antibody staining, white: in situ hybridisation PdfR staining. A) ventral nerve plate B) medial and dorsal layers

In comparison to the 3dpf stage there are generally more cells expressing the PdfR candidate in five day old embryos (Figure 22). The medial patch in the brain spread all over the dorsal region of the head with more cells expressing PdfR (22 A, B). As seen in the lateral view (22 B), these cells are located on the outermost cell layer and consist of weak expressing cells and also a few cells with more gene expression. In this embryonic stage I could not find any staining in the ventral nerve plate any more. Also the four lateral domains seem to express the PdfR candidate more strongly at this age. They show two deeply coloured cell cluster on each side, marked with

black arrows. This can be seen in Figure 22 A from the dorsal view and in picture B from the lateral view.

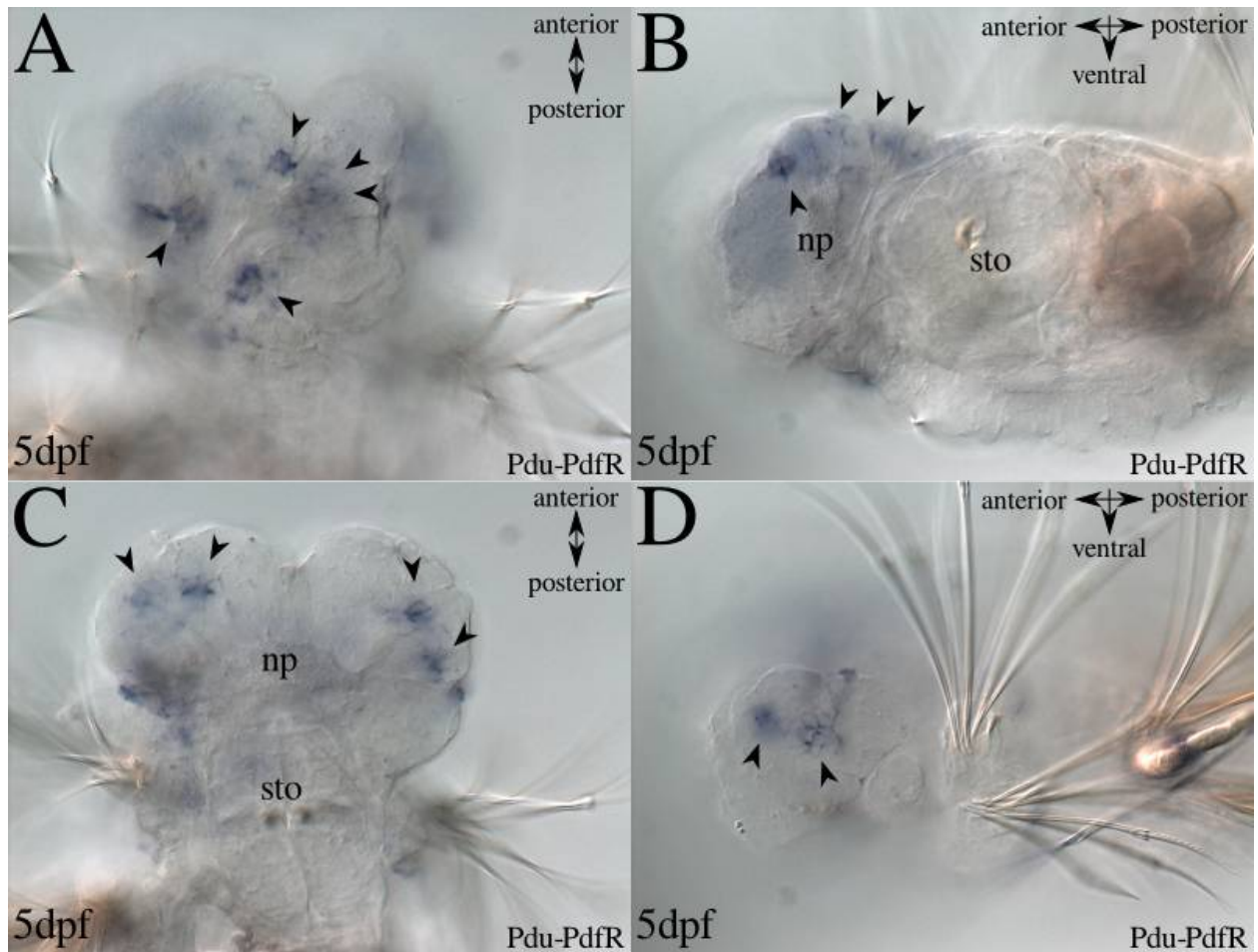


Figure 22: 5dpf embryo In situ hybridisation Pdu-PdfR-candidate A+C) ventral view, anterior top, whereas focus in C is more ventral B+D) lateral view, dorsal on top, focus in B is medial, in D more lateral. Abbreviations: sto - stomodeum, np - neuropil

4.2.6 Expression Quantification in Immature Adults

We were also interested in the expression levels of the PdfR candidate in adult worms and therefore did quantitative real-time PCR. As already described for the MelR (chapter 4.1.6) I also designed three primer pairs for the PdfR and tested them before doing the analysis.

The primer pair number one had the best R^2 of 0.9975 when doing linear regression on the plot of cycle count to cDNA dilutions. Further, the water control (no RNA included) and the -RT control (no reverse transcriptase included) showed no amplification. Only a very late amplification in one well from the water control, with 10 cycles later than the real amplification signal, could be detected. Further, I used the same time points, moon conditions and also the same RNA, obtained from Enrique Arboleda and Mingliu Du, as described for the MelR qPCR set-up.

Both runs showed almost the same results (Figure 23 and 24). The expression level of the PdfR candidate is constant during the day and does not show any clear differences between full moon (FM) and new moon (NM) conditions. But there is a difference between the two runs in the

amount of expression. Enrique RNA shows around 25% of cdc5 (Figure 23), while with Mingliu RNA a level of about 12% of cdc5 was measured (Figure 24).

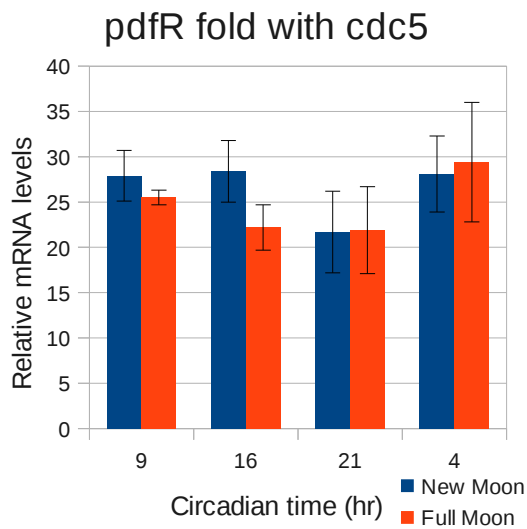


Figure 23: Enrique RNA; Error bars show Standard Error. Stars represent T-Test p-value: * < 0.05, ** < 0.01, *** < 0.005

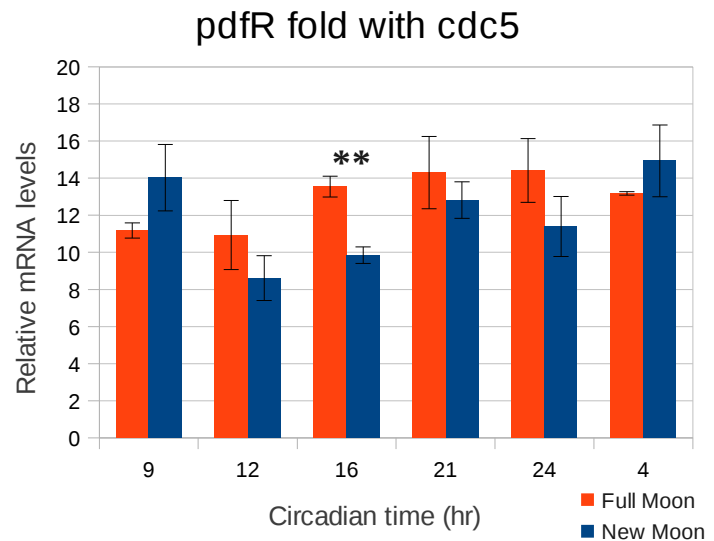


Figure 24: Mingliu RNA, Error bars show Standard Error; Stars represent T-Test p-value: * < 0.05, ** < 0.01, *** < 0.005 relative mRNA levels are relative to cdc5 housekeeping gene

With the Student T-Test I analysed if there is a statistically significant difference between the time points of the day or between lunar conditions of the same time of the day. For the latter I found that the 16 o'clock sample of the Mingliu RNA showed a T-Test p-value of 0.0063 between full moon and new moon and therefore there is a clearly significant difference.

4.3 Spatial and Temporal PDF Production, Secretion and Recognition

4.3.1 Localisation of PDF RNA and Protein in Embryonal Stages

To visualize different staining patterns of the pigment dispersing factor in developmental stages double fluorescence whole mount in situ hybridisations (ISH) as described in chapter 3.8.1 were performed. The ISH was done with an antisense probe against the mRNA of PDF. The blue NBT/BCIP staining indicates the location of the transcript and therefore the site of production on the cellular level. An antisense probe against r-opsin marked the location of the eyes with a fluorescent dye. The illustrations (Figure 25) were done with bright field microscopy as described in chapter 3.12.1 and arranged the way that one column represents one observed embryonal stage. Confocal laser microscopy was used to detect fluorescent stainings for analysis, but pictures are not shown for the embryonal stages. The exact timepoints of fixation were 3d 9am, 4d 10am, 5d 9am and 7d 9am. I decided to present ventral views with the anterior side being always on the right. In the first row focusing on the ventral nerve plate (vp) and going more dorsal from row to row, it is possible to get a three-dimensional idea. Cell cluster and cells belonging together are marked with arrows of the same colour.

In the ventral nerve plate 2 to 6 bilaterally symmetric cells are visible in different layers (Figure 25A-F) from very ventral close to the surface to two cells appearing ventral to the stomodeum, close to the first pair of chaetae (indicated in black). During development within the observed stages the amount of cells decreases to just 2 cells located ventral to the stomodeum (E, F) from 5dpf onwards. They show much weaker staining, meaning that the amount of transcripts is less in these cells. These cells seem to be located just next to pigment cells as shown by Fischer et al. (2010) in Figure 26. The mentioned pigment cells are not visible at 6dpf any more, which might be related to my observation of PDF expressions getting weaker and weaker at the older stages. The very ventral cells close to the surface could not be detected any more after 4dpf.

The green arrows indicate two PDF expressing cells located rather lateral and just posterior to the head in the 3dpf stage (G). They seem to move more medial during the development of the stomodeum and start to proliferate. At 7dpf these cells built a whole cell cluster of 4 to 8 symmetrically arranged cells (J).

A similar proliferation event can be observed in positive cells anterior to the anal cirri in 3dpf embryos (G, indicated in blue). These cells also move more medial and anterior towards the developing proctodeum (Figure 25O) and proliferate to at least three strongly PDF-expressing cells (P, M). These cells are in all stages close to the detected pigment by Fischer et al. (2010) in Figure 26.

The staining in the head can be categorized into three bilaterally symmetric domains: Four cells (red arrow) distributed just caudal along the palpus nerve (Antje Fischer, Henrich, and Arendt 2010) as seen in confocal laser scans (data not shown). Further, two big symmetric cell clusters are located just medial to the eyes (black circle). The eyes were marked with fluorescent ISH

against r-opsin, the most important light sensor in the eyes and therefore a good reference gene to indicate their position. Using this approach, no coexpression could be detected, although the cells appear very close to each other (confocal scans, data not shown). Some weakly expressing cells marked in yellow appear very dorsal in the head. They are dispersedly distributed or sometimes even appear in an almost circular arrangement (R-U).

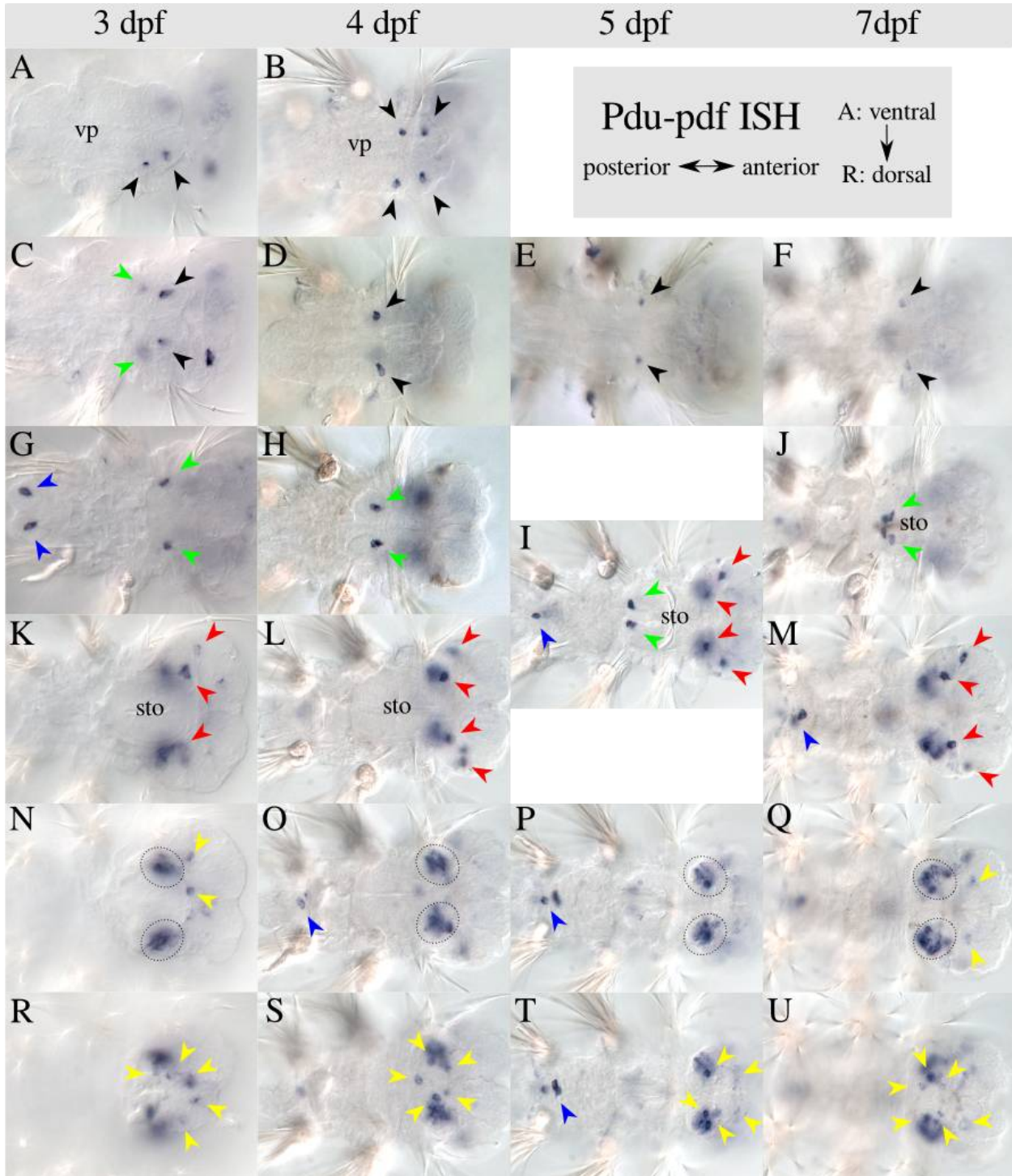


Figure 25: NBT/BCIP stainings of an antisense RNA probe against Pdu Pdf gained in a whole mount in situ hybridisation. In columns developmental stages are shown, rows indicate different focus levels in the embryo beginning from very ventral to dorsal. Ventral view, anterior is always right. Characteristic cells and cell clusters are marked with arrows and circles (described in the text!) Abbreviations: sto – stomodeum, vp – ventral nerve plate, dpf – days post fertilization

All three domains in the head do not show any clear alteration between the observed embryonal stages (3dpf to 7dpf). The staining of the cells close to the palpus nerve might not be complete at 3dpf (K).

To be able to locate the PDF peptide itself I used Immunohistochemistry (see chapter 3.9). The antibody stainings against the amidated form of PDF were done with the same batches of embryos as used for the ISH. Therefore the exact same stages and timepoints were examined to analyse the PDF protein during development. The negative control with pre-immune serum (PIS) instead of antibody does not show any strong fluorescence at all (data not shown). The procedure worked very well and it was possible to obtain specific staining in all tested stages. The PDF stainings showed some alteration within the same stages, but the overall pattern did not differ much from 3 to 7dpf. Therefore only the most representative stage (5dpf) is shown (Figure 26).

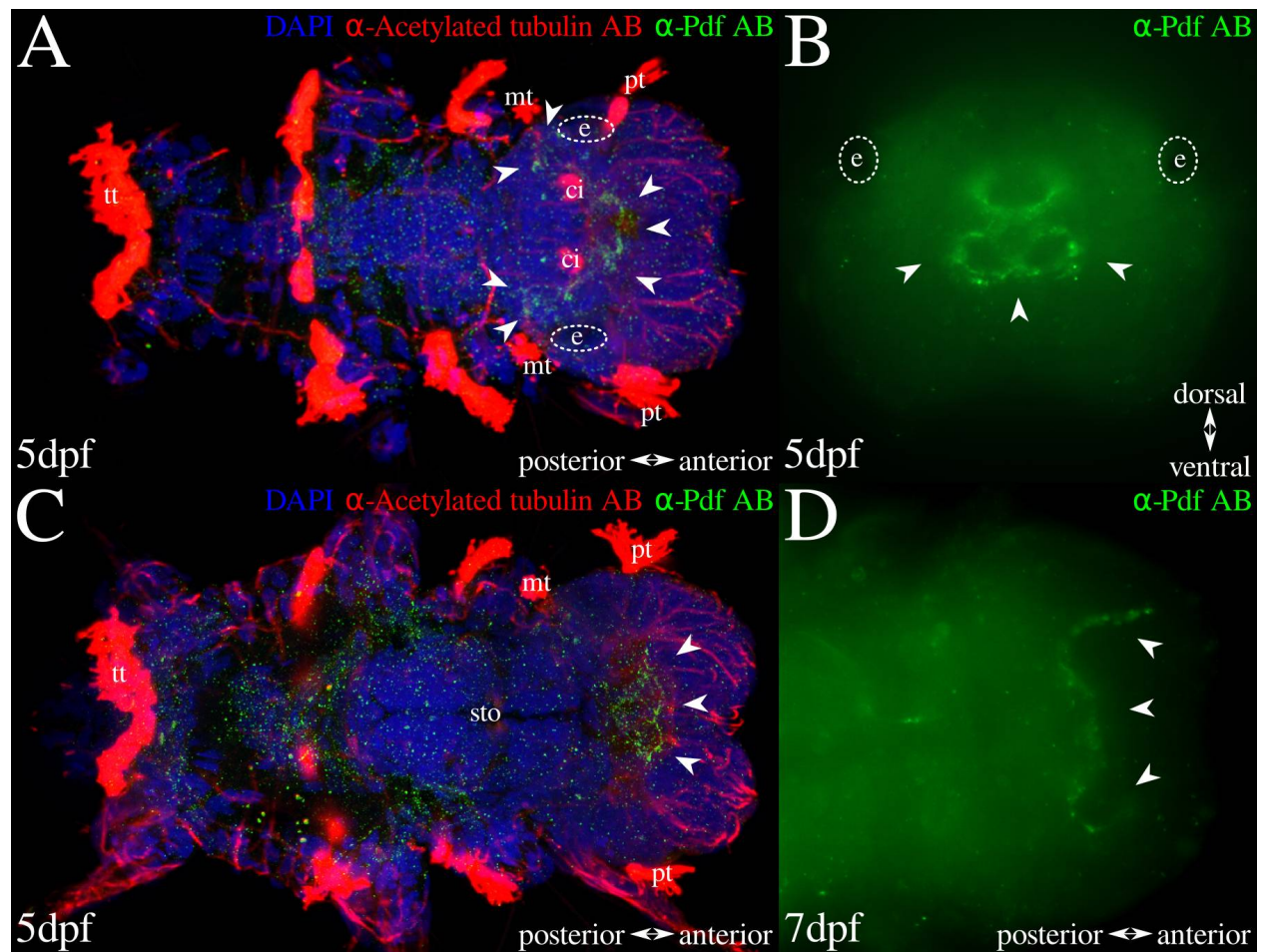


Figure 26: Antibody staining of 5dpf embryos. A: Confocal Laser microscope stack from very dorsal layers, eyes are marked with white circle. B: Layers more ventral with neuropil visible C: Apical view with Axioplan microscope; D: 7dpf embryo, dorsal view of antennal nerve; Abbreviations: sto – stomodeum, e – adult eyes, pt – paratroch, mt – metatroch, tt – telotroch, ci - cilia of the ciliary photoreceptor cells, dpf – days post fertilization

The most prominent staining could be detected in various nerve chords of the neuropil (Figure 26 A-C). This staining was also the only one already present in the youngest stage tested (3dpf). It consists of a whole network of PDF neurons which gets bigger during development. In the 7dpf stage PDF is additionally located in the palpus nerve (D). This can be best seen as co-localisation of acetylated tubulin and PDF using confocal microscopy (Figure 27K-N).

Completely stained cellular outlines can just be seen for some cells belonging to the huge cluster close to the eyes. Two to eight of them (A, indicated with white arrows) show moderate PDF expression with projections going medial and anterior to the neuropil.

The cells in the stomodeum previously described for the ISH could also be detected using Immunohistochemistry. They start to express the PDF protein at 5dpf, whereas very early just a small part of the whole cells outline is coloured. This staining differs much in intensity from one individual to another, but is usually clearly visible. Comparing the images of these cells from both detection methods, a proper co-localisation of mRNA and protein can be found.

However, I could not detect cells positive for amidated PDF in the ventral nerve plate or close to the proctodeum in the observed larvae, although mRNA transcripts were clearly present.

4.3.2 Circadian Changes and Localisation of PDF RNA and Protein in the 14dpf stage

In general, the staining patterns of PDF mRNA and protein in embryos that I described before could also be observed in the more developed stage, 14 days post fertilization. Additionally, I tested these embryos for circadian alterations of PDF by including four different timepoints during 24 hours: 9am, 3pm, 9pm, 3am. The used procedure was the same as already explained in chapter 4.3.1. A double fluorescence WMISH with antisense probes against PDF and r-opsin were used to detect mRNA transcripts and Immunohistochemistry with an α -PDF antibody was used to detect the peptide itself. The anti-acetylated tubulin antibody was used to indicate neurons as a localisation reference. Due to a technical issue there are no in situ hybridisation results for the 9pm timepoint. The negative control using preimmune serum instead of antibodies again led to no obvious staining (data not shown).

The very dorsal cells in the head, marked yellow, are also present in 14dpf (Figure 27A-C). They appear with a rather weak in situ staining and are disperse distributed. It was not possible to detect these cells using Immunohistochemistry. Therefore they are probably not producing the amidated form or any PDF peptide at all. When comparing the different timepoints not much difference can be detected for these cells. There might be less overall expression in cells at 3am, as observed for several individuals.

Further, the cluster of cells located between the cilia of the ciliary photoreceptor cells (ci) and the eyes is present during the whole day without any alterations (A-C). There are constantly 3 to 4 cells on each bilateral side expressing the PDF messenger RNA. This observation is consistent with the number of cell outlines seen when using Immunohistochemistry to colour the peptide (Figure 27D-G, indicated with an arrow). Although these cells seem to constantly produce PDF, the secretion is regulated by a circadian rhythm. The projections of this cluster going medial and anterior into the neuropil are not present or very weak at 9am. Same is true for the projection network in the neuropil and the palpus nerve (K-N). These nerve chords are filled with PDF during the day, but show almost no protein secretion at 3am (K).

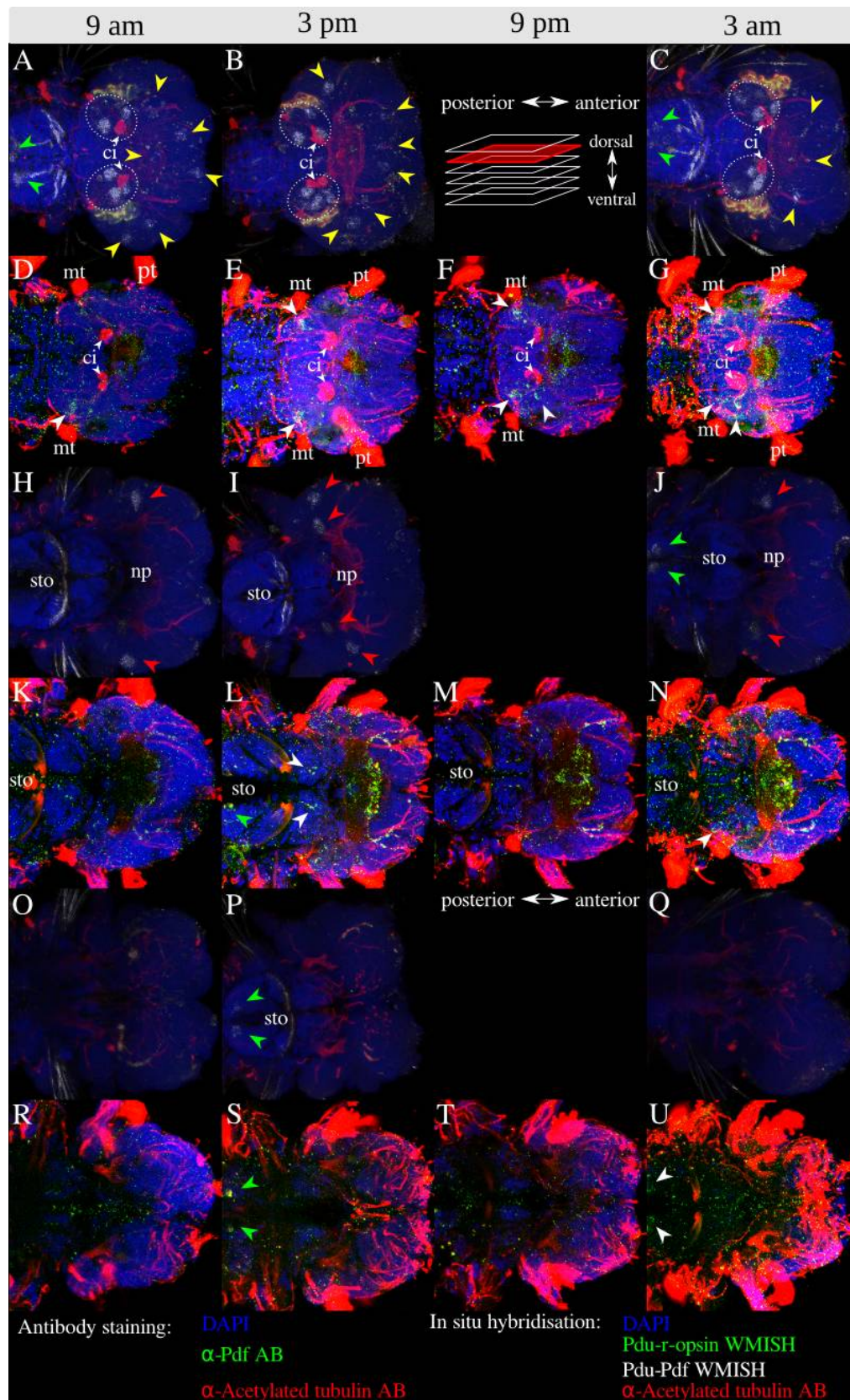


Figure 27: *Platynereis* larvae 14dpf stained with whole mount in situ hybridisation and Antibody staining to visualize Pdu PDF RNA and Protein localisation. α -acetylated tubulin Antibody staining and Pdu r-opsin WMISH staining served as reference. DAPI staining labels cell nuclei. Images were done with confocal laser scanning microscopy and visualized through a z-stack projection of several layers, starting with very dorsal ones (A-G) followed by medial (H-M) and ventral ones (O-U). Row 1, 3 and 5 are WMISH stainings while row 2, 4 and 6 are antibody stainings. Abbreviations: np – neuropil, sto – stomodeum, ci - cilia of the ciliary photoreceptor cells, mt – metatroch, pt - paratroch

However, for these observations outliers could be found showing enormous PDF secretion into the neuropil at 3am. Therefore, circadian cycling and variations could certainly be detected, but diversity within biological replicates makes it hard to describe a distinct pattern. The variations could of course also be triggered by other external influences than the daily rhythm.

With this huge cluster of cells expressing PDF next to the eyes the question arose whether or not there are some cells co-expressing the main light sensor r-opsin and the pigment dispersing factor. Both transcripts could be detected within the same animals using double fluorescence in situ hybridisation (chapter 3.8.2). As seen in Figure 27A-C no coexpression could be found, although the light sensing cells are located in close vicinity to the PDF cells, as already observed in the early stages.

The four cells posterior to the palpus nerve are again indicated with a red arrow. They show mRNA transcription of PDF at all observed timepoints in the 14dpf stage, whereas the staining is not always complete in all individuals (H-J). Furthermore, in contrast to earlier stages, these cells do also produce PDF protein 14 days after fertilization. Four weak fluorescent coloured cell outlines could be detected in most of the observed embryos. As it can also be seen in Figure 27, there are some noticeable variations in the amount of cells and the intensity of the stainings, but due to huge variations within biological replicates of the same batch it is unclear whether or not they are regulated in a circadian manner.

In the posterior part of the stomodeum two to four cells (indicated in green) were visible in all temporal samples in the in situ staining as well as in the antibody staining. Again, the intensity of the antibody staining differs between individuals and in rare cases the staining is completely absent.

As already observed for the embryonal stages, the PDF transcript is located in two to maximum four cells close to the proctodeum, but in contrast to the early stages could also amidated PDF be detected there. Further, there is no cellular staining in the ventral nerve chord any more, although in some cases projections in four nerves along the entire body could be found.

However, it seems that the mRNA is not much regulated at all. There is a rather constant expression between biological replicates and timepoints of the day. Some variations could be found for cells close to the palpus nerve (red) and for the very dorsal batch in the head (yellow). The PDF peptide, in contrast, is regulated very strongly. A lot of alterations were found when analysing the antibody stainings, although it is hard to make final statements by only studying these data.

4.3.3 PDF Localisation in pre-Adult Worms

As our interests were not only the developmental aspects of PDF, but rather the roles of this important factor in the circadian clock and probably also in the lunar clock, we also investigated the spatial distribution of the PDF peptide in pre-adult worms. Therefore I used the same protocol as described before, just on whole worms fixed with PFA at a certain timepoint of the day. To describe one worm in detail and to specify its PDF cell clusters and expression domains,

I used about 2 month old worms fixed at 4am during new moon. During that time, no nightly light exposure can interfere with the daily regulation of PDF, and therefore circadian changes should be seen best. I decided to use the 4am timepoint to describe the overall staining because of the data gained for 14 day old animals. There, the 3am timepoint showed the strongest anti-PDF staining.

An experiment done with preimmune serum (PIS) did again not exhibit any strong staining, just an equally distributed fluorescent background could be detected (data not shown).

Figure 28A-F shows the distribution of PDF in the head. The PDF protein detected with the polyclonal rabbit anti-PDF antibody (green) could best be seen in the ventral view. As location-reference I used anti-acetylated tubulin antibodies which stain the neuronal network and DAPI to indicate the cell nuclei. Another reference are the eyes (e) themselves, because of their auto-fluorescent pigment cells (Figure 28A). These cells and other pigment cells (marked as pi) appear rather yellowish, because of their detection in all three channels. Unfortunately, in order to get the neuronal outlines visualised in the very deep layers of this thick tissue, the red Cy3 staining had to be over-saturated when scanning with the confocal microscope.

PDF cells are either assembled to clusters with a white circular outline or single cells are indicated through a white arrow. Bad visible projections are marked with a smaller, green arrow, whereas very well visible ones are not further labelled for clarity. The brain and its hormone secreting tissues were previously very well described (Hofmann 1976; Heuer and Loesel 2007; Heuer et al. 2010). Therefore I use the terminology of these publications to describe the location of PDF cells and neurons.

The probably most dominant staining is a huge cell cluster between the posterior minor strand of connective tissue and the nuchal region (28A-D). Two cells within this cluster located just medial to the posterior eyes are indicated separately (B). They were found to be extremely strongly stained at all timepoints and show long neuronal projections going apical and medial to the later described projection network. Also visible even in very weakly stained animals were two symmetric medial cells close to the anterior impaired tubular strands of connective tissue (A and B). Both the huge cell cluster and the described cells were already described before using WMISH (Keplinger S., Steinkellner E., unpublished data).

Further, I found about three to four cells located very closely around the anterior eyes and at least one cell just next to the posterior eyes (B-E). These cells did not always show bilateral symmetry and their staining intensity showed huge variations. However, one to two cells anterior and medial to the anterior eyes seem to be constantly present. These cells could also be detected in whole mount in situ hybridisations (Keplinger S., Steinkellner E., unpublished data).

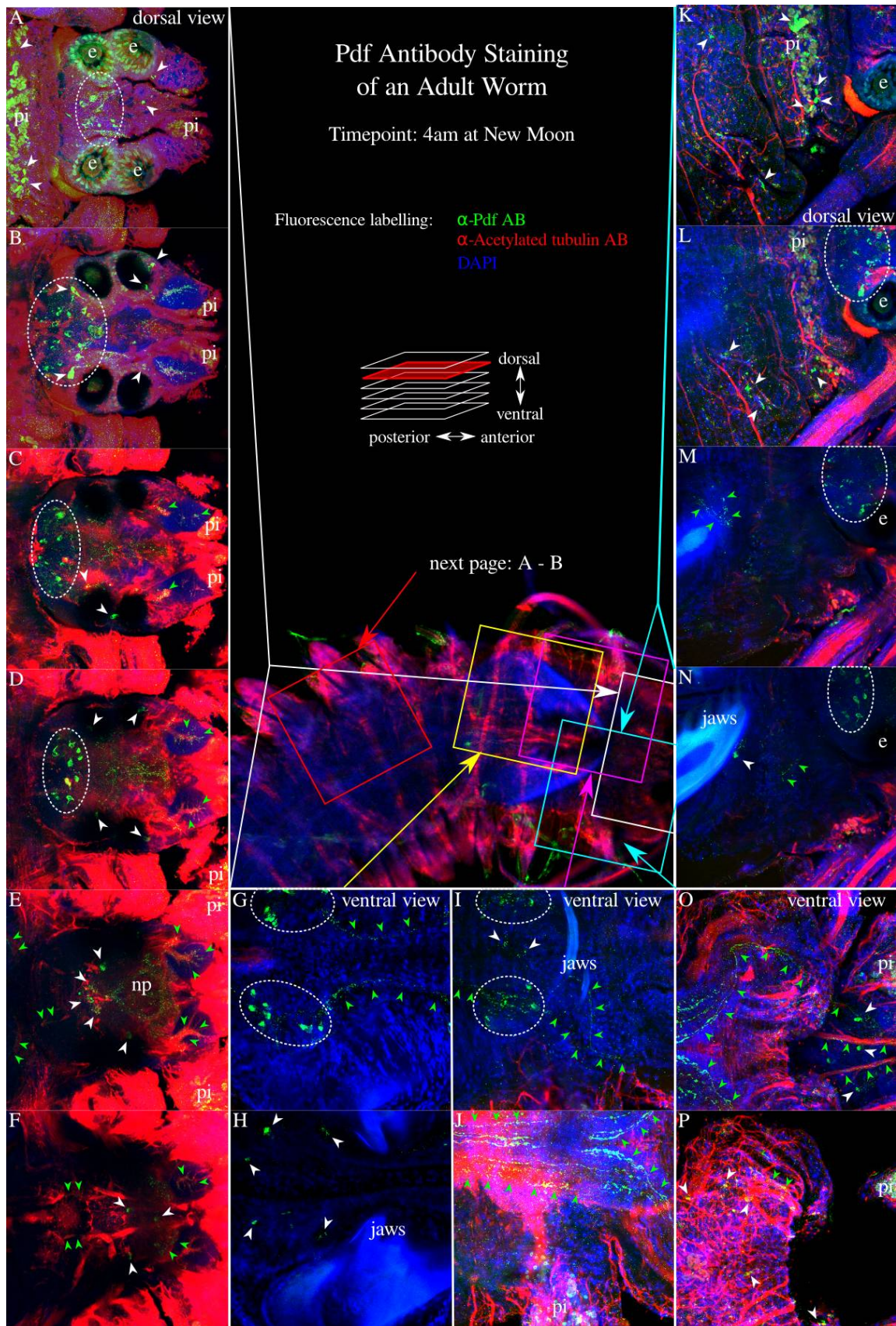


Figure 28: Antibody staining of adult worm: Confocal laser microscopy scan, stack displayed as z-stack projections of several frames. Images in one white boarder show a stack, dorsal pictures on top, going more and more ventral. Scans are done either from dorsal or from ventral indicated in the picture. The exact location of the stack is shown in the middle graphic. White arrows indicate PDF cells, smaller green arrows projections. Good visible projections in the brain are not marked due to clarity. Abbreviations: pi – pigment, e – adult eyes, np – neuropil, jaws

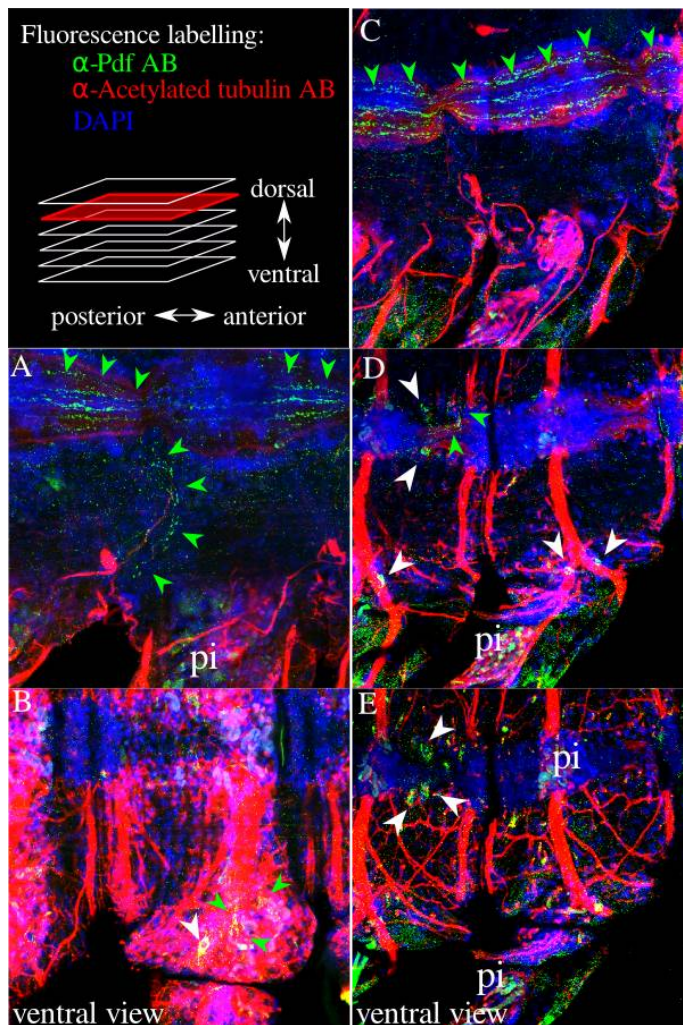


Figure 29: Antibody staining of adult worm: Confocal laser microscopy scan, stack partially displayd as z-stack projection. Images in white frame show one stack, dorsal pictures on top, going more and more ventral. Scans are done either from dorsal or from ventral indicated in the picture. The exact location of the stack A-B is shown in the middle graphic of Figure 28. Stack C-E shows two smaller segments more posterior of the worm.

same cells as in 28F.

The staining in the head including the cell cluster, the described single cells and the PDF projection network can be also seen in the video of a calculated 3D model (Attached CD). Unfortunately, due to the surface rendering algorithm, it is harder to distinguish pigment cells from PDF cells, although it is still possible because of the characteristic shape of their surface. Pigment cells look as if they were composed of small granule whereas PDF cells have a smoother surface.

However, the pigment dispersing factor occurrence is not restricted to the head and the brain. Two bilaterally symmetric cell clusters of about 3 cells each are located posterior of the jaws (28I). The relation of this clusters to the head and eyes can be seen in Figure 28M-N. Starting from these cells, projections go anterior and even bigger ones posterior (green arrows). The latter connect these cells to another two symmetric cell clusters of about 8 cells caudal of the first ones (G, H).

Four to six cells are located next to some massive neuronal tracks more ventral in the region of the infracerebral gland (28E). As they appear posterior to the neuronal network of PDF fibres in the neuropil (np), they might also be responsible for PDF secretion into this structure.

The aforementioned projection network in the neuropil appears shaped in a triangular way with two main PDF nerve chords going anterior into the palpa, one more ventral (B) and the other more dorsal (E). There the PDF fibres spread into the whole tissue.

In Figure 28E, F and O projections coming from the ventral nerve chord can be seen (green arrows). Unfortunately it is unclear whether they go to the medial cells mentioned before or to the very deep, ventral cells (about 4) under the projection network (F). When analysing the scan from the ventral side (O) it seems that rather the latter is the case, whereas the fibres in the neural tracks (red) are passing these cells and go more apical. For making it clear, the cells seen here are the

Furthermore, the aforementioned projections along the ventral nerve chord are present throughout the whole worm. Starting from cells in the brain (O), they are at first assembled to one big fibre and then divided into approximately 6 strands (J) going posterior through every segment (Figure 29A, C). As seen in Figure 29A, it seems that these PDF fibres are even projecting to lateral regions of the bigger segments.

In each segment at least four cells are present generating PDF. Two cells on each bilateral side could be detected close to the chaetae (29B, D). They appear strongly stained and are sometimes attached to short PDF fibres. Another two cells are visible just ventral to the ventral nerve chord projections (29D, E). This staining pattern is true for big segments as well as for smaller segments more caudal, whereas the two medial, ventral cells can unfortunately not be seen in the big segment shown here.

Cell outlines of PDF cells and small projections could frequently be detected close to the skin respectively in surface-near tissue (28P, K, L and 29E). These stainings appear randomly distributed with no obvious pattern. Some of these cells with a very strong expression also seem to be present between the pigment cells of 28K. Due to their shape and characteristic colouring, they can easily be distinguished from neighbouring pigment cells. Furthermore, in these surface-near tissues more staining in form of small dots is visible. It is unclear whether this is background-staining of unspecific bound antibodies or specific staining (28K, L).

Although I was able to also stain other timepoints of the day and worms fixed during full and new moon, it was hard to reliably describe circadian and/or lunar changes due to huge variations between biological replicates. These experiments are topic of current research.

5. Discussion

5.1 Target Tissues of Melatonin in *Platynereis*

To gain some knowledge about the downstream signalling pathway of melatonin and its harbouring tissues in *Platynereis*, I concentrated on the melatonin receptor as the primary melatonin sensing protein. During my research I have identified three candidate sequences. Also, after extensive searches with several queries in various accessible databases, there is currently no evidence for further homologue genes in *Platynereis*. I already extensively tested the first candidate for a proper ligand-binding, its expression localisation and transcript quantification. Now, I first want to discuss what is already known about the second and third candidate and then explain the results for cand1 in detail.

5.1.1 Sequence Analysis shows remarkable similarities of our Receptor Candidates to Vertebrate Receptors

The melatonin receptor candidate three is almost identical with the candidate one, just the 5' end until the middle of transmembrane domain number two is completely different. Additionally the sequence of this fragment is shorter at this end. As the candidate 3 is a genomic sequence read, it could be that this different part is an intron and that the coding exon lies further upstream. This hypothesis is supported by the fact that the identical part of the reads start with a CAG, a conserved pattern for intron/exon splicing sites. The upstream exon/intron boarder and the exon are unfortunately not present on the observed genomic sequence reads. Therefore a whole genomic melatonin receptor sequence is not available yet. However, at the 5' end we gained quite a long piece, probably of the non coding region downstream of the gene. This also explains why the TMHMM prediction could not calculate the probabilities for this region.

In contrast, the candidate number two exhibits some quite remarkable differences. Therefore, I analysed the sequences of candidate one and two in detail and compared them to the very well described vertebrate melatonin receptors. For both candidates a sequence similarity of 50% (identity: 30%) with the mouse melatonin receptor could be calculated, which is quite high compared to the phylogenetic distance. Further, seven transmembrane domains could be detected for both candidates using TMHMM (Figure 5). They even appear on the same loci as in vertebrates, as seen in the alignment.

As already described in chapter 2.6.1, vertebrate melatonin receptors exhibit various very conserved regions. These are a NRY motif downstream of transmembrane domain number three directly followed by a CXXCH motif and a NAXXY pattern in the seventh transmembrane domain. The NRY motif is present in a slightly different form in all G protein coupled receptors. There the sequence normally consists of DRY or ERY. The same is true for the NAXXY motive, which is exchanged to NPXXY in all other members of this superfamily (Reppert and Weaver 1995).

These patterns could in general be found for the *Platynereis* melatonin receptor candidates, too. Candidate one also includes the NRY motif as vertebrate melatonin receptors, but in the CXXCH motif the first Cysteine is exchanged to a Valine and in the NAXXY the Alanine to a Phenylalanine (Figure 30). These small discrepancies could be due to the huge evolutionary distance between these clades and do not necessarily interfere with the hypothesis of candidate one being a functional receptor binding melatonin.

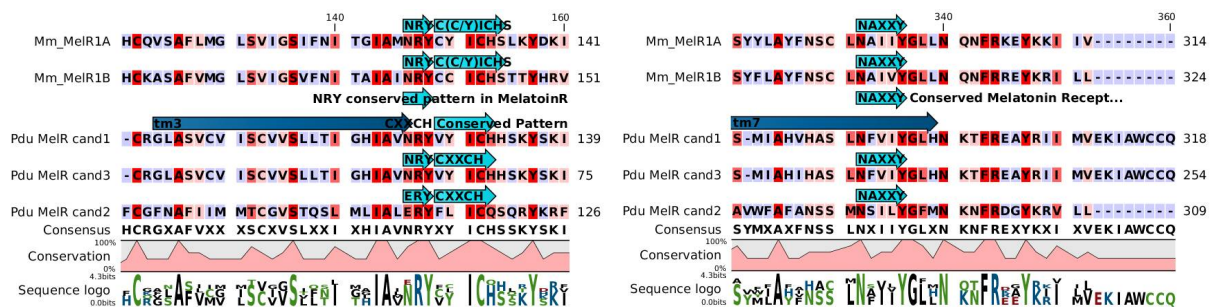


Figure 30: Alignment of *Mus musculus* MtnR1A and 1B with the three *Platynereis* MelR candidates. The conserved domains described in several studies as well as the transmembrane domains are indicated with arrows. The degree of conservation can be seen in the diagram on the bottom.

In candidate two an ERY motif could be detected, which was described before as being conserved within G protein coupled receptors, but is different for melatonin receptors. Further, the pattern CXXCH is not easily recognisable as it appears as FXXCQ. Similar to candidate one, the second amino acid of the NAXXY motif is also exchanged, but in this case to Serotonin. In my opinion, these results do not really prove the affiliation of candidate two to the group of melatonin receptors, although the main features of G protein-coupled receptors are present. However, the described conserved motives are not necessarily functional and therefore these differences can probably be ignored.

Other conserved amino acids in vertebrate sequences, which are in contrast important for a functional melatonin receptor, are a certain Histidine in tm5 and a Glycine in tm6. They form the binding pocket of the receptor to specifically detect melatonin (Barrett, Conway, and Morgan 2003). In both *Platynereis* sequence candidates the Histidine is substituted with Glycine and the Glycine with Leucine. Hence I assume that the binding pocket is formed differently in lophotrochozoan melatonin receptors.

The sequences of both MelR candidates exhibit a similarity of 50% (30% identity) to each other when calculating a pairwise alignment. Compared to the two mouse MtnR subtypes 1A and 1B, which show a similarity of 75% and an identity of 57%, these values are rather small. This is also visible through the different branch lengths of the tree (Figure 6). The protostomian genes are less similar to each other and therefore exhibit very long branches.

5.1.2 The Cell Impedance Assay indicates a proper Ligand-Binding

The probably most important step to verify a candidate of a gene is to test whether it fulfils the proposed function. Therefore I tested if the receptor cand1 is binding and responding to addition of melatonin in a Cell Impedance Assay. Although the graphs show a positive result, there are several issues which have to be eliminated. Until then, these results are at best first hints for a

proper melatonin binding of our candidate.

It was shown before (Yu et al. 2005; Roche Applied Science) that G protein-coupled receptors can react with an CI up to 0.8. The assay positive control, oxytocin receptor with its ligand, almost exhibits such a value, whereas neither the mouse melatonin receptor nor the *Platynereis* receptor show a value above 0.3. Reasons for that could be a bad transfection rate for the melatonin receptors. As just the oxytocin receptor was tagged with a fluorescent labelling, it was not easily possible to determine their transfection efficiency. Further, the sequence of the *Platynereis* candidate was not codon optimized for expression in a mammalian cell culture. This certainly also led to a smaller expression rate and probably explains the CI differences to the mouse melatonin receptor.

Another important change would be to perform the addition of ligands and the following incubation steps on 37°C to avoid the observed massive change of CI due to the falling temperature of the system. These circumstances were also the reason for the huge error bars which were even increasing the more the temperature fell.

To really prove a proper ligand-binding reaction with this assay it is necessary to test different ligand concentrations. Only then it is possible to calculate the proposed IC50 value (Roche Applied Science) which even indicates quantitatively how good a receptor is binding this ligand.

The same is true for the tested antagonists, 4-P-PDOT and Luzindole. Here also different concentrations would be necessary to be certain about the inhibiting effect of these reagents. However, for the antagonist 4-P-PDOT we already have a very good hint that it does not only inhibit the mouse melatonin receptor (Figure 13), but also the effect of melatonin on our Pdu candidate (Figure 14). The curves with the prior 4-P-PDOT treatment show an overall reduced CI value although these changes are very little, also compared to the huge error bars. Therefore some optimisation has to be done to confirm the obtained results. In contrast, Luzindole can probably not be tested using the COS-7 cells because they reacted quite strongly after adding this substance. It might be possible that other cell lineages do not show the same behaviour and can be used for this kind of assay.

If the antagonists were used to treat living worms, this would open the doors for various experiments. The animals with the blocked receptor would behave as if they had the receptor or enzymes of the melatonin production pathway knocked out. Therefore it would be possible to test the effects of a missing melatonin signalling under various environmental circumstances. However, these first results are quite good indicators that the *Platynereis* MelR candidate one binds melatonin and is inhibited by 4-P-PDOT, but more experiments are necessary to confirm this hypothesis.

5.1.3 Melatonin Receptor is constantly expressed in Nervous Tissue

With whole mount in situ hybridisation it was possible to locate cells expressing the receptor candidate one in embryonal stages. In very early stages they are mainly present in the ventral nerve plate, whereas from five days post fertilisation on, this previously massive staining is

strongly reduced. In contrast, the head-expression is detected anterior to the neuropil increasing from a few single cells to a broad expression throughout the whole area during these developmental stages (Figure 7, 8). A widespread expression pattern for melatonin receptors was already described before for birds and lower vertebrates (Cassone, Brooks, and Kelm 1995). Therefore it is possible that the broad staining in the head is maintained in adult worms and acts as the main melatonin sensing tissue. Whole mount in situ hybridisation stainings in adult worms are technically very hard to obtain. Hence, we do not have any data about the expression localisation in adults to verify this assumption.

The melatonin receptor expression in the whole ventral nerve plate could play a developmental role in the very early stages. Thus, we even tested if *Platynereis* larvae change in behaviour, anatomy or development when adding big amounts of melatonin into their seawater. But we could not observe any change, leading to the hypothesis that the receptors might not be functional at all during these stages.

Another step necessary to gain knowledge about the localisation of melatonin production and detection is to compare and analyse expression patterns found for Aanat and Hiomt, the two enzymes responsible for melatonin production, with the patterns found for their receptor. Aanat and Hiomt were described to be located in a circular structure just posterior of the neuropil in 60hpf and 14dpf animals (S. Keplinger, unpublished data). Therefore I assume that melatonin is produced in the area posterior of the neuropil and then transported through neurons into the sensing cells anterior of the neuropil. Again, this fact needs to be confirmed for adult animals, as we currently just have data from embryonal stages.

When testing the MelR expression quantity in adults, the main results probably are that the receptor is expressed at all and that the expression is constant during the day and during the lunar month. In total I quantified the transcript in *Platynereis* heads at 4 different days, including two new moon and two full moon nights. The relative expression in these four measurements varied between 8% and 75% of *cdc5*, a very discontinuous result almost without any statistical significance (Figure 9, 10). The RNA obtained from Enrique Arboleda was almost one year old with many frosting-defrosting cycles. It could be that this RNA was already partially degraded and the data therefore not reliable. This is supported by the fact that the biological replicates showed very different expression levels, leading to huge error bars (Figure 9). The data achieved for the positive control, *bmal*, speak against this theory. They showed expected values in all timepoints and biological replicates.

Huge differences between the replicates could also be obtained due to different cutting of the worm heads. When assumed that the melatonin receptor is present in the cut region you will get different expression levels in the biological replicates when cutting more anterior or posterior. Further, I thought of seasonal variations because the full moon samples from Enrique were taken in December, whereas the new moon samples and all samples from Mingliu were obtained in May and early June. As the culture room is completely isolated from the outside we should not have any seasonal variations at all. Unfortunately, we already measured slightly higher temperatures in summer than in winter, which could be sensed by the worms and might led to the

observed changes. Although the second qPCR run provided more stable values with smaller errors, at least one more experiment of this kind would be necessary to confirm them.

As the receptor is constantly expressed, we can assume that changes of the melatonin level are directly sensed in the same proportion. Therefore, in future experiments specifically measuring melatonin levels it is not necessary to include the measurement of the receptor quantity.

In conclusion, it was possible to gain some ideas about the targets of melatonin. I could not only identify and clone sequences of the *Platynereis* orthologs, but also achieved first hints for the expression localisation and the transcript quantity of the candidate number one. Further, I was able to demonstrate a proper binding of melatonin to the receptor and confirmed the negative effect of the antagonist 4-P-PDOT using the Cell Impedance Assay. However, even with these findings it is not yet possible to determine the precise function of melatonin in *Platynereis*. There are still many open questions, the signalling targets downstream of the melatonin receptor need to be identified and the melatonin-regulating mechanisms detected.

5.2 Target Tissues of PDF in *Platynereis*

The neurotransmitter PDF can only be sensed by its receptor. Therefore, it is necessary to study this protein extensively to gain some knowledge about the target tissues of the pigment dispersing factor. This included studying the phylogeny and sequence characteristics of possible receptor candidate genes as well as experiments to identify the expression levels and localisation.

5.2.1 The uncovered PDF Receptor Candidates exhibits highly conserved Sequence Patterns

Searches in our *Platynereis* sequence databases did at no time give any specific results when querying with a PDF receptor sequence. Not even in the genomic database of the EMBL institute it was possible to achieve a PdfR sequence read. The reason for this might be a very low gene expression which was also previously observed for the orthologous gene in *Drosophila*. Nevertheless it was possible to clone the *Platynereis* PDF receptor using degenerated PCR primers.

The obtained fragment of the gene has a high sequence similarity with other PDF receptors. When comparing the Pdu receptor fragment with its *Drosophila* ortholog, I detected a similarity of 69% (identity: 50%) between these two genes. Further, the alignment exhibits many highly conserved regions and sequence motifs, especially in and also downstream of transmembrane domains. These conserved regions are an HXNFL motif at the beginning of tm2, a TPXXCE pattern between tm2 and tm3, the pattern FXWMFIEGXXLHN in tm3, WIXEXPR in tm5, FLXNII/VRVLI/VXKL downstream of tm5, KAVXAAXLLPL in tm6 and the motif YCLXNGEV downstream of tm7 (Figure 31). The seven transmembrane domains predicted with TMHMM are also indicated in the alignment with blue arrows. Notice that the predicted transmembrane regions of *Platynereis* and *Drosophila* overlap quite well and that the discovered highly conserved regions are often within transmembrane domains. Therefore, some

evolutionary pressure might lie on maintaining the sequence of the transmembrane domains. Probably some of the conserved motives are important for recognising the PDF peptide.

The calculated phylogenetic tree (Figure 17) also supports the hypothesis that our novel sequence is a true PDF receptor. The fragment clusters very well to the other lophotrochozoan genes. However, I want to point out that the tree was only calculated with gap-free loci of the alignment. Thus only the highly conserved part between the 2nd and the 7th transmembrane domain were considered. The C- and N-termini which show much more variation were not included. Therefore, the tree will probably exhibit longer branch length when calculated with a full sequence of the *Platynereis* PDF receptor.

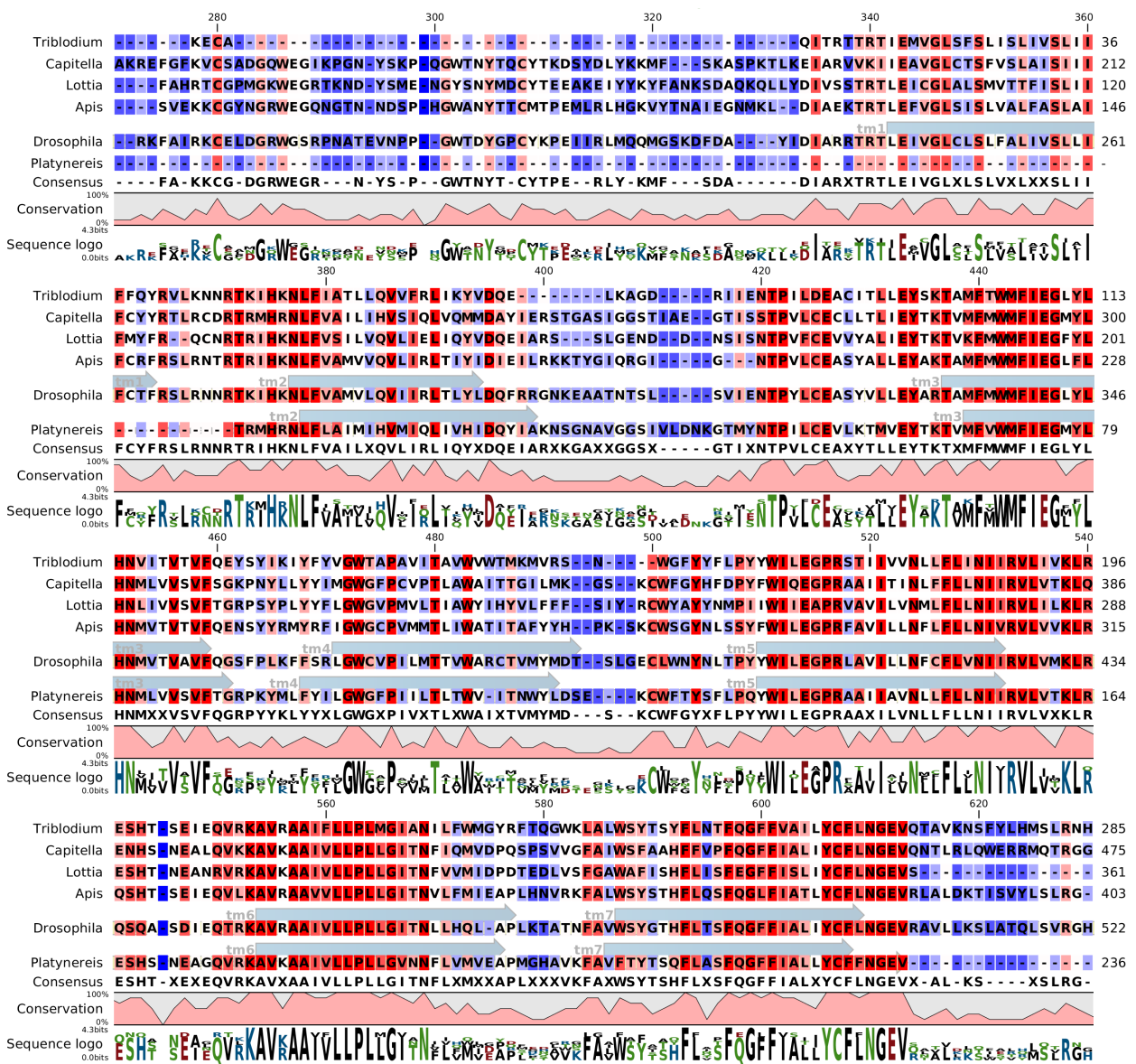


Figure 31: Alignment of PDF receptor genes including the Pdu PdfR fragment. Red indicates a high similarity between the ortholog sequences, blue a very low one. Transmembrane domains are marked with blue arrows above the sequences.

5.2.2 Obtaining a full-length Clone of the PDF Receptor Candidate failed

The elongation of the obtained fragment with the RACE (rapid amplification of cDNA ends) technique unfortunately failed (see Chapter 4.2.4). I got colonies which were positive when hybridising, but the sequence was either unspecific or not elongated. The issue of the wrong positive cells must be in some way related to technical issues in the the hybridising step. When searching for an explanation for the negative elongation, I came up with several possibilities: The cDNA probably was degraded on the ends or otherwise not able to bind the universal primers, the used specific primers might have bound to unspecific regions or the PCR conditions were chosen wrongly. However, the cDNA did work for other elongations done in our lab and ordering new primers binding to different sites of the fragment also did not solve the problem. I also tested different PCR conditions, following the protocol of the RACE kit as well as performing the steps previously established in our lab. When analysing the PdfR fragment in detail, I found some sequence similarity with the RACE short universal primer. This might have led to an amplification of the known fragment with two universal primers and without the specific primer. Under these circumstances we would also expect to see no elongation, but positive blotting signals. Trying a different method would probably help to obtain the full length PDF receptor of *Platynereis*. A BAC-screening might therefore be the right approach.

5.2.3 PDF Receptor is steadily expressed in distinct Cells of the Nervous Tissue

To analyse the expression quantity of the PDF receptor transcript in adult heads, I performed two runs of qPCR with RNA extracted at timepoints of four different days. I observed a constant expression during the day without any significant cycling. The relative expression levels were 25% of *cdc5* for one qPCR run and about 12% for the second run (Figure 23, 24). These differences are much smaller than the ones observed for the melatonin receptor, but still, as already discussed, it is hard to explain these differences. The reason might again be the different cutting of the heads, degraded RNA, temperature changes or other technical issues. Thus, I would suggest to perform another experiment to verify one or the other value.

When analysing the changes between the two lunar conditions new moon and full moon using the Student's T-Test, one timepoint showed a quite significant alteration. At 4 pm, the PDF receptor expression at new moon is smaller than at full moon. Thus, a hint for a lunar influence on this gene expression could be detected, whereas it is still unclear if the nocturnal light or the lunar clock triggers this change. A free-running experiment would provide the answer for this. However, this incident could only be observed in one of the two performed qPCR experiments, containing only 3 biological replicates, and therefore has to be verified with more data.

The whole mount in situ hybridisation revealed that the *Platynereis* PDF receptor is only expressed in nervous tissue. This includes cells in the ventral nerve plate for very young stages and dorsal and dorsolateral regions in the head. The expression pattern in the head is to a certain degree consistent with observations in *Drosophila*, where the PDF receptor is restricted to certain

neurons in the dorsal brain close to clock neurons and PDF terminals (Lear et al. 2005). The role of the PDF receptor in some ventral nerve plate cells at 3dpf is completely unclear. It might be involved in developmental processes. When comparing the staining of the 3dpf to the 5dpf stage, I observed an expansion of the expression domain in the very dorsal region of the head. Further, the cells in the dorsolateral cluster are increasing in number during these developmental stages. Unfortunately, we do not have any data about the first occurrence of the PDF receptor expression in development yet. In situ hybridisations in earlier stages would answer this question. Additional interesting, but still missing, experiments would be expression analyses of 14dpf and adult animals to gain a more accurate knowledge of the PDF sensing tissues.

In conclusion, all performed experiments support the hypothesis of our PDF receptor candidate being expressed as a functional receptor able to sense the pigment dispersing factor. The obtained sequence shows extraordinary conservations with ortholog genes, the calculated phylogenetic tree exhibits clustering of all included PDF receptors. In the expression localisation experiment, data consistent with literature from *Drosophila* could be gained. Further, the candidate gene is also expressed in the head of immature adult animals and belongs to the family of G protein coupled receptors, shown with transmembrane prediction. Due to the fact that I was not able to clone the full length receptor, the obligatory verification of this receptor candidate binding its ligand, PDF, is missing.

5.2.4 Localisation of PDF

We are of course not only interested in the target tissue of PDF, but in its complete functionality. Therefore, much information can be gained by knowing where this important neurotransmitter is produced, secreted and detected. Production takes place in cells where mRNA is present. These cells can be detected using WMISH. The peptide itself is located in secreting tissues as well as in neuronal projections, where it is transported. It can be visualised with Immunohistochemistry. We visualised the PdfR with an RNA antisense probe against its mRNA. As the receptor protein does not leave the cell, we can assume that the mRNA and the protein have the same location. Neurons expressing the receptor will however not be visible with this technique. Therefore Immunohistochemistry with an antibody against the receptor would be necessary. In the following, I want to summarize the described cell cluster and expression domains, compare them to literature of *Drosophila* and discuss their possible function.

Embryonal Localisation

At the beginning, I want to point out that I could in general verify the data of previous in situ hybridisations using PDF antisense probes on 14dpf young worms (Stefan Keplinger, unpublished data). The only difference is that I observed more cells in the cell clusters medial to the eyes. Data from Immunohistochemistry experiments show that the PDF peptide is already present three days post fertilisation. It might already be expressed earlier in development, but unfortunately I have not tested younger stages.

The amidated form of the PDF peptide could also be detected in many cells where the mRNA

transcript was present. Exceptions thereof are the described cells in the ventral nerve plate which are visible only in three to four day old animals (Figure 25A-B). I therefore assume that these cells do not produce PDF or the non-amidated form of the peptide. Further the four cells posterior to the the palpus nerve and the cells close to the proctodeum are not positive in the antibody stainings in stages up to 7dpf. But in contrast to the young stages, the PDF production is switched on in 14dpf old larvae, leading to weakly stained but very well visible cell outlines.

In the 3dpf and 5dpf stage, I managed to get the PDF receptor ISH staining working. Thus, it is possible to predict not only the localisation of the production, but also the target tissue. PdfR cells were found to be located in a medial, dorsal patch of the head as well as more lateral, anterior to the neuropil (Figure 20, 22). The peptide detected with antibody staining was found in a network of neurons in the neuropil (Figure 26). In 5dpf embryos it even seems that the cells containing the PdfR transcript are located close to the palpus nerve, a structure also positive for PDF protein. Therefore, one can argue that PDF is secreted by cell cluster medial to the eyes, transported in neurons to the neuropil and detected anterior to the neuropil and the palpus nerve by the PdfR. This hypothesis is supported by literature about the fruit fly. There, amidated PDF was described to exert a strong synchronizing ability between neurons of the clock (Helfrich-Förster 2009).

As already mentioned, the PdfR is also present in cells very dorsal of the head. In exactly this area cells also showed a positive staining when trying to sense the PDF transcript (Figure 25R-U, yellow arrows). This leads to the assumption that a feedback-system is present there. However, it was not possible to detect the amidated form of PDF in these cells. In *Drosophila*, non-amidated PDF was found to feed back onto dorsal neurons of the molecular clock, adjusting their period and synchronizing their oscillations (Helfrich-Förster 2009). These clock neurons express the PDF receptors and are able to sense PDF for that reason. Therefore, the results obtained in *Platynereis* are somehow consistent with previous findings. Unfortunately, there were no antibody stainings done with an antibody against the PdfR. Thus, it is not possible to locate the protein in the neurons itself, which would further verify this theory.

Functions of PDF discussed for *Drosophila* are that it might act as an output factor of the circadian clock, as a modulator of the clock or even as a light input factor (Helfrich-Förster 2009). In *Platynereis* I found a huge cell cluster producing PDF next to the eyes which were indicated with colouring the light receptor r-opsin. This is certainly a good hint that PDF plays similar roles in the rag-worm and is also controlled through light, either directly or indirectly. It is of course difficult to argue whether or not these findings are related, but I am sure this is a great starting point for further studies.

Furthermore, I tried to analyse circadian fluctuations of the PDF mRNA and the protein and came to the conclusion that the gene transcription is not much regulated. In contrast, a lot of alterations were found when analysing the antibody stainings, although it is hard to analyse the stainings in detail. There were huge differences between the biological replicates, the staining itself was not always complete, and due to the three-dimensionality enormous amounts of data were produced, making it hard to manually compare them. I suggest to use different techniques

to answer this question. It would for example be possible to cut specific tissues and to quantify the overall amount of PDF in there using Western Blot or ELISA. The disadvantage of these methods is of course the loss of information about the exact location of the cells. But as a first description of circadian changes, these might be the right approaches.

Adult Localisation

With all these experiments we gained a lot of information about PDF and its receptor in embryonal stages, but certainly need to confirm this data in adult worms. Therefore, I tried to relate the already described embryonal PDF cell cluster to the stainings detected for adult worms.

Assuming that the very dorsal cells in the embryonal stages (Figure 25, yellow arrows) still do not produce amidated PDF in adults and are therefore not visible in the antibody staining, I conclude that the huge dorsomedial cell cluster in adults (Figure 28) is probably related to the embryonal clusters medial to the eyes. These bilaterally symmetric clusters might have spread massively and therefore fused in the middle. Furthermore, the cells located posterior to the neuropil (red arrows) in embryos might be the same cells as the ones located in the area of the infracerebral gland in adults (Figure 28E). Also the projection network in this area still looks the same. There are also PDF projections going apical in adults like the palpus nerve in 14dpf animals.

Not only in the young stages, but also in adults some cells are still located in closely to the eyes, further indicating that PDF's function is somehow connected to light. Also the cells expressing the pigment dispersing factor in the stomodeum are still present in adults, where I described them as two cell clusters posterior to the jaws connected with several projections. The other projections found in the adult body, along the ventral nerve chord, were also occasionally already visible in 14dpf animals. The only segmental cellular staining in the early stages were two to four cells close to the proctodeum (Figure 25). It is quite possible that this staining pattern led to the four PDF cells in adults which are repeated in every segment.

Although I was able to also stain other timepoints of the day and worms fixed during full and new moon, it was hard to reliably describe circadian and/or lunar changes due to huge variations between biological replicates. Juliane Zantke observed similar alterations when analysing the PDF in situ staining for adult worms (unpublished data). Reasons for this could be the selection of worms from different strains, sizes and ages. Within this study it was also not possible to measure the exact amount of light the worm was exposed to and also the differences in pigmentation. The grade of pigmentation could certainly be related to the occurrence of PDF. These subsequent studies are already in progress in our group.

I would propose to pick interesting timepoints showing maximal changes with data achieved from qPCR, Western Blot or ELISA and analyse and localise the differences for only these samples. It is not helpful to stain as many timepoints as possible due to the huge amount of data. One ends up with image-stacks comprising thousands of single photos, which furthermore contain lots of cells stained differently in the examined biological replicates. To be able to detect lunar periodicity, it might also be helpful to make a timecourse during the whole lunar month to

get a better resolution of the overall alterations. It could easily be that the biggest change is not between new moon and full moon, but rather between slightly shifted timepoints, due to probable long responses to upregulated expressions.

In adult animals, only the antibody staining of PDF has been described in detail so far. The next important step would be to compare these results to an extensive ISH staining analysis and to get a proper PdfR staining for adults. The latter could be either whole mount in situ hybridisations or also antibody stainings. The same is true for expression data of the PdfR in 14dpf worms. Thus, for these older stages not much information could be gained concerning target tissues.

However, with the observed data it is already possible to argue that PDF in *Platynereis dumerilii* might have a similar role as the PDF in *Drosophila* (Helfrich-Förster 2009; Helfrich-Förster 2005). It seems to be also somehow involved in the circadian clock. Observations leading to this hypothesis are that PDF cell clusters are located next to light sensing tissue, that projections are going into the neuropil and further into the region of the infracerebral gland. It would also be possible that non-amidated PDF feeds back onto cells in the dorsal region of the brain. However, the question came up if PDF does also act as a pigment regulating factor as observed in crustaceans. When analysing the adult antibody staining I discovered some particularities which indicate that this is the case. For example some PDF cells were located among many pigment cells in the nuchal region or in the segments close to the chaetae. Further, the observed surface-near cellular staining with small projections outside of any nervous tissues can be seen as an indicator for the pigment regulatory role. Certainly, there are still many experiments to be done to uncover the role of the pigment dispersing hormone bit by bit.

6. Acknowledgements

I want to thank my supervisor Dr. Florian Raible for giving me the opportunity to do research on this interesting topic and for his support throughout the whole thesis. Special thanks also to my lab colleagues and my classmates for the great time during my studies. For the great linguistic support and the extensive proof-reading, I particularly want to thank Isabel Heger.

7. Appendix

7.1 PCR Pipetting Schemes, Programs and Primers

ID#	1	2	3	4	5	6	7	8
PCR Mix	Volumes (μl)	Volumes (μl)	Volumes (μl)	Volumes (μl)	Volumes (μl)	Volumes (μl)	Volumes (μl)	Volumes (μl)
Total reaction	25	25	25	20	50	50	50	25
Fwd. Primer	1	1.25	1.25	0.5	1.5	1.5	1	1
Rev. Primer	1	1.25	1.25	0.5	1.5	1.5	5	1
Polymerase	0.25	0.25	0.25	0.3	0.2	0.2	1	0.5
Buffer	5	5	5	2.5	5	5	5	2.5
MgCl ₂	0	0	0	1.2	0	0	0	1.5
dNTPs	0.5	0.5	0.5	0.5	1.25	1.25	1	0.5
Template	5	1.25	1.25	0	1.5	1	2.5	1
ddH ₂ O	12.25	15.5	15.5	14.5	39.05	39.55	34.5	17
Program	Temp. (°C) / Time (min s)	Temp. (°C) / Time (min s)	Temp. (°C) / Time (min s)	Temp. (°C) / Time (min s)	Temp. (°C) / Time (min s)	Temp. (°C) / Time (min s)	Temp. (°C) / Time (min s)	Temp. (°C) / Time (min s)
Initial Denaturation	98° / 30"	98° / 2'	98° / 2'	94° / 5'	95° / 15'	95° / 15'	95° / 5'	94° / 2'
Denaturation		98° / 10"			94° / 1'	94° / 1'	94° / 1'	
Annealing		61.6° / 15"			T _M – 5° / 2'	T _M – 5° / 2'	66.5° / 2'	
Elongation		72° / 25"			72° / 4'	72° / 4'	72° / 3'	
# Of cycles		5			5	5	5	
Denaturation	98° / 15"	98° / 10"	98° / 10"	94° / 30"	94° / 1'	94° / 1'	94° / 1'	94° / 30"
Annealing	60° / 1'	66.6° / 15"	59.2° / 15"	T _M ° / 1'	T _M ° / 2'	T _M ° / 2'	71.5° / 2'	62.9-63.6° / 30"
Elongation	72° / 1' 30"	72° / 25"	72° / 45"	72° / >1'	72° / 4'	72° / 4'	72° / 3'	72° / 1'
# Of cycles	35	30	35	35	35	35	35	35
Final Elong.	72° / 5'	72° / 10'	72° / 10'	72° / 10'	72° / 10'	72° / 10'	72° / 10'	72° / 10'
Storage	4° / infinite	4° / infinite	4° / infinite	4° / infinite	4° / infinite	4° / infinite	4° / infinite	4° / infinite

Table 1: PCR pipetting schemes and program conditions

ID#	Name	Sequence (5' → 3')	length (bp)	Organsim
89	pGEM-T_easy_up	CCATGGCGGCCGCGGGAATTC	21	Platynereis
90	pGEM-T_easy_lo	CAGGCGGCCGCGGAATTCAGTGTG	24	Platynereis
140	pJETreverse	AAGAACATCGATTTTCCATGGCAG	24	pJETblunt vector
141	pJETforward	CGACTCACTATAGGGAGAGCGGC	23	pJETblunt vector
912	Pdu_MelR_up	GTGCACAAGGCACACCGTTATG	22	Platynereis
913	Pdu_MelR_lo	CAAAACAAACGGAATGGATGATC	23	Platynereis
924	Pdu_MelR seq	GGCATGCGATGACCTCTTG	18	Platynereis
971	Pdu_PdfR_up1	YTNTGYTGGCCISCIAC	17	Platynereis
972	Pdu_PdfR_up2	ACNMRNATICAYMRIAAAYTITT	23	Platynereis
973	Pdu_PdfR_lo1	ACYTCNCCRTTIARRAARCARTIA	25	Platynereis
974	Pdu_PdfR_lo2	TGNARRWANADICCYTCDATRAACATC	27	Platynereis
1049	pdu_pdfR_race_up1	CTCTATGAACATCCAACAACATAACAG	29	Platynereis
1050	pdu_pdfR_race_up2	CAGTCTTGGTGTATTCACCATTTGTC	26	Platynereis
1051	pdu_pdfR_race_up3_probe	CTTTAGTACTTCACATAAATTTGGAG	26	Platynereis
1052	pdu_pdfR_race_lo3_probe	CAACTCATTGTACACATCGACC	22	Platynereis
1053	pdu_pdfR_race_lo2	GCCATTATGATCCACGTCATGATTCAAC	28	Platynereis
1054	pdu_pdfR_race_lo1	GCGTATGCATCGGAATTTGTTTCTGGCC	28	Platynereis
1149	GSP2_3'RACE_pdfR_720bp	CGGATGCATCGGAATTTGTTTCTGGCC	28	Platynereis
1150	NGSP2_3'RACE_pdfR_720bp	GAATTCTGGGAATGCCGTAGGGGGC	25	Platynereis
1151	GSP1_5'RACE_pdfR_720bp	GCCGTTGAAGAAGCAGTAGAGCAGAGC	27	Platynereis
1152	NGSP1_5'RACE_pdfR_720bp	CTTGGAAGGAGGCTAGGAACGTGTAGG	27	Platynereis
1153	probe2_primer_3'RACE pdfR 720bp	ATGTGAAGTACTAAAGACAATGGTGG	26	Platynereis
1154	probe1_primer_5'RACE pdfR 720bp	GTAAGACAGCAAACTTGACAGCG	24	Platynereis
1171	pdu_melR_qPCR_4_forward	GTTGGTAATTGCAGCACTGG	20	Platynereis
1172	pdu_melR_qPCR_4_reverse	AAGAACAATAATTTCCAACCTCTC	26	Platynereis
1173	pdu_melR_qPCR_1_forward	TGATCTGGTTGTTACGGCTTT	21	Platynereis
1174	pdu_melR_qPCR_1_reverse	AATGATGTCCCCTTGACTGC	20	Platynereis
1175	pdu_melR_qPCR_3_forward	CCACATTGCTGTCAATCGTT	20	Platynereis
1176	pdu_melR_qPCR_3_reverse	CATCTTCCTTTACTGTAGATCTTGGAG	27	Platynereis
1177	pdu_pdfR_qPCR_1_forward	TGCTGGTCACCAAGTTAAGAGA	22	Platynereis
1178	pdu_pdfR_qPCR_1_reverse	CGCCAAGTAACGGAAGAAGA	20	Platynereis
1179	pdu_pdfR_qPCR_2_forward	GTAGGGGGCTCCATTGTTCT	20	Platynereis
1180	pdu_pdfR_qPCR_2_reverse	TCCAAACAACATAACAGTCTTGG	24	Platynereis
1181	pdu_pdfR_qPCR_3_forward	TCCTTTTTACCTCAGTACTGGATACTC	27	Platynereis
1182	pdu_pdfR_qPCR_3_reverse	CCCGTATGATGTTAAGAAGAAAAAGT	26	Platynereis
1249	Pdu_melR_fwd_TMT03	TCAGAAATTCGCCGCCACCATGGAATACCAATTTGTGCTTAATG	43	Platynereis
1250	Pdu_melR_rev_TMT03	TCTAGACTCGAGCTAGATGGAGGATGTGTCCATC	34	Platynereis
1251	Mm_MtnR1A_fwd_TMT03	TCAGAAATTCGCCGCCACCATGAAGGGCAATGTCAGCGAGC	40	mouse
1252	Mm_MtnR1A_rev_TMT03	TCTAGACTCGAGTTAAACAGAGTCCACCTTTATTAAGTTATTATTG	46	mouse
1253	Mm_MtnR1A_fwd_outer	AAGTGGCGGAGAGGGCTCGAG	22	mouse
1254	Mm_MtnR1A_rev_outer	AAGGAGAGCAGTAAGACCCCAACC	25	mouse
1406	Mm_MtnR1A_fwd_outer2	TGGCTGTGCTGCGGTGAGGC	21	mouse

Table 2: Database IDs, names and sequences of all primers used during the thesis

7.2 Miniprep

- spin 2ml of culture for 8 minutes at 5000rpm
- add 200 ul buffer P1 and resuspend
- add 200ul buffer P2, mix by inverting the tube 4-6x
- add 200ul buffer P3, mix by inverting the tube 4-6x
- spin 10 minutes at 14000rpm
- take supernatant to another tube
- add 450ul 2-Propanol, mix by inverting the tube
- spin for 10 minutes at 14000rpm
- wash pellet with 1ml 70% EtOH
- spin 5 minutes at 14000 rpm
- air dry and resuspend in 50ul H₂O

(P1, P2,P3 ready -to-use lab stocks):

Resuspension buffer P1 (steril filter and store at 4°C) 500 ml

- | | |
|------------------------|---------|
| - 10 mM Glucose | 4,5 g |
| - 25mM Tris (pH 8.0) | 12,5 ml |
| - 10 mM EDTA (pH 8.0) | 10 ml |

Alkaline Lysis buffer P2 (mix fresh of both components, use no longer than 1 week old)

- 0,2 M NaOH
- 1% SDS

Neutralization buffer P3 (store at RT) 500 ml

- | | |
|-----------------------|----------|
| -5M Potassium acetate | 300 ml |
| - Glacial acetic acid | 57,5 ml |
| - H ₂ O | 142,5 ml |

7.3 *Platynereis* Whole-mount in Situ Hybridisation

Version Florian Raible 2006, based on Kristin's, Detlev's and Jochen's protocol

Fixation and storage of embryos

Reagents:

PBS: Tablets (Sigma No. P4417, 10x Stock: 5 tablets in 100ml water) **PTW (2xPTW):** 1x PBS (2xPBS), pH 7.5, add Tween20 to 0.1% and sterile filter (0.2 µm, nitrocellulose).

16% PFA (common stock): dissolve 16% paraformaldehyde in PBS by stirring and heating to 65°C, add dropwise 1 M NaOH until the solution gets clear (check pH ≈ 7.5), cool to room temperature and store at 4°C (note from Medaka protocol: store for up to 4 weeks; if Medaka embryos do not stay clear but turn "milky" in MeOH prepare fresh 16% PFA stock).

4% PFA (working stock): Mix with 3 volumes of 2xPTW just prior to use.

PFA Fixation (*Platynereis*): Pour embryos through filter, wash with sea water (keeping the embryos wet), transfer them to a small filter, invert it and rinse with 4%PFA into 2ml tube. **Note:** Use 2x PTW + PFA for the fixation!

Put on a shaker for 2hrs, RT

Methanol transfer:

- wash larvae 3 x 5 min in 1ml 2x PTW (**optional, skipped for *Platynereis***)
- wash 3 x 5 min at room temperature in 100% MeOH
- replace MeOH and store embryos at least over night at -20°C

Comments: MeOH treatment enhances probe penetration, a longer storage of embryos at -20°C in MeOH is advantageous.

RNA probe preparation

Reagents:

NTP-Mix: ATP, CTP, GTP 15.4 mM each, UTP 10.0 mM (all Roche)

Digoxigenin-11-UTP 10 mM (Roche)

-> NTP/DIG-UTP-Mix: 1.3 vol NTP-Mix, 0.7 vol Dig-11-UTP

Fluorescein-12-UTP 10 mM (Roche)

RNasin 20-40 U/µl (Promega, Pharmacia)

T7-/SP6-/T3-RNA-Polymerase 20 U/µl (Roche)

5xTranscriptionbuffer (Stratagene)

DNaseI RNase-free 10U/µl (Roche)

STE: 100 mM NaCl/20 mM TrisCl, pH 7.5/10 mM EDTA

NucTrap™ push-columns (Stratagene) or **RNeasy** (Qiagen)

Hybridisation Mix-H₂O:

	stock	HYB-H ₂ O
Formamide	100 %	5 ml
SSC	20 x	2.5 ml
Heparin	50 mg/ml	10 µl
Torula-RNA (Sigma)	solid	50 mg
Tween20	10 %	10 µl

Nylon-membrane (HybondN, Amersham)

PI: 100 mM TrisCl, pH 7.5/150 mM NaCl

PII: PBS/0.1% TritonX-100

PIII: 100 mM TrisCl, pH 9.5/100 mM NaCl/50 mM MgCl₂

Anti-Digoxigenin-Fab fragments/Anti-Fluoresceine-Fab fragments (Roche)

BCIP (Roche): 50 mg/ml in 100% DMF

NBT (Roche): 75 mg/ml in 70% DMF/H₂O

- linearize 10 µg of template with a suitable enzyme allowing as transcription (blunt or 5-prime overhang should be preferred to avoid snap back effects)
- purify template from enzyme and digestion buffer (QiaQuick nucleotide removal kit, Qiagen / **or use gel purification, followed by Qiaquick purification kit**)

-> elute in 30 µl of H₂O

- control for a complete digest on an agarose gel
- add – **in the following order** – to a total volume of 20 µl:

linearized template	1 µg (max 12.5 µl)
100 mM DTT	2 µl
NTP/ DIG-UTP -Mix	2 µl
RNase inhibitor	0.5 µl
10xTranscriptionbuffer	2 µl
H ₂ O	ad 19 µl
RNA-Polymerase	1 µl

Final NTP conc. will be 1mM, with 35% of UTP being DIG-11-UTP

- incubate for 2-4 hrs at 37°C
- add 1 µl DNaseI and incubate for another 15 min at 37°C
- purify RNA using the Qiagen RNeasy kit (faster, less expensive; 50 µl H₂O final; elute 2x (re-apply eluate to column, spin 1x again))
- take an aliquot of 2 µl, mix with 4 µl RNA loading dye, heat to 80°C for 1', snap cool, run on a TAE agarose gel
- dilute the remaining probe by adding 75 µl Hyb-buffer and store at -20°C

Comments:

As a rule of the thumb 4 µl of the probe in 100 µl of Hyb-mix will give a good staining.

Probes from 600bp- 2.5 kb were used successfully with this protocol, short probes below 0.5 kb will generate only faint staining although this varies with the specificity of each probe and may be worth being tried.

Hydrolysis of the probe might be required in a few exceptional cases.

Proteinase digestion and postfixation

Reagents:

ProteinaseK: prepare a stock solution of 20 mg/ml **in PBS** and store frozen aliquots at -20°C, dilute 1:200 just prior to use to a final concentration of 100 µg/ml in PTW

Glycine stock (100x): 200mg/ml in PBS (50g/250ml)

4% PFA see above

all steps are performed at room temperature, **in hand-made nets submerged into 40ml volumes (lids of pipet boxes)**

If you put the embryos onto a shaker, don't put more than 32ml (embryos could fall out of the nets)

- transfer embryos of different stages **into nets submerged in 100% MeOH**
- rehydrate 1-5 min in 75% MeOH/PTW (**30ml MeOH + 10ml PTW**)
- rehydrate 1-5 min in 50% MeOH/PTW (**20ml MeOH + 20ml PTW**)
- rehydrate 1-5 min in 25% MeOH/PTW (**10ml MeOH + 30ml PTW**)
- rinse 2 x 5 min each in PTW
- digest with ProteinaseK (100 µg/ml in PTW) without shaking for several minutes depending on the stage of the embryos (**see below**)
- rinse 2 x shortly in freshly prepared 2 mg/ml glycine/PTW (also in boxes)
- fix in 4% PFA/PTW for 20 min (**24ml PTW + 8ml 16%PFA**)
- wash 5 x 5 min in PTW

Comments: the ProteinaseK-digestion is a critical step of this protocol and will substantially influence the quality of the whole mount in situ, thus, times of digestion have to be optimized. The following list of digestion times worked well in our hands and can be used as guideline:

stage	time of ProteinaseK digestion
1h-15h	use 'early WMISH-protocol'!
16h-72h	45" - 1 min
72h-5d	2 min
1week to 6weeks	3 min

Hybridisation

Reagents:

Heparin: make a stock of 50 mg/ml in H₂O, store at -20°C

Hybridisation Mix: 50% formamide (Fluka, ultra pure), 5xSSC, 50 µg/ml heparin, 0.1%Tween20, 5 mg/ml torula RNA, store at -20°C,

for 50 ml of Hyb-Mix:

	<u>stock</u>	<u>Hyb-mix</u>
Formamide	100 %	25 ml
SSC	20 x	12.5 ml
Heparin	50 mg/ml	150 µl
Torula-RNA (Sigma)solid		250 mg
Tween20	10 %	500 µl
H ₂ O		ad 50 ml

all steps are performed in a water bath preheated to 65°C

- transfer embryos to 2 ml Eppendorf tubes
- prehybridize 1-2 hrs in 1 ml Hyb-Mix at 65°C

- denature probe (4-10 µl/200µl Hyb-Mix, as a rule of the thumb 4 µl of the probe will give a good staining) in 200 µl of Hyb-Mix for 10 min at 80°C
- remove prehybridisation solution leaving embryos slightly covered to avoid their dessication, the embryos are very sensitive at 65°C
- quickly add hybridisation probe, mix gently and hybridize at 65°C overnight

Washes

Reagents:

SSCT solutions: dilute from 20xSSC, and add Tween20 to 0.1% (= 1:200)

Note that (in contrast to common zebrafish practice) 50%formamide / 2xSSCT means that the final SSCT concentration should be 2x (not just a 1:1 mix of 2x SSCT with formamide)

all steps are performed in a water bath, all wash solutions are preheated to 65°C

- wash embryos 2 x **20** min in 1 ml 50% formamide/2xSSCT at 65°C
- wash embryos 15 min in 1 ml 2xSSCT at 65°C
- wash embryos 2 x each **20** min in 1 ml 0.2xSSCT at 65°C

Detection

Reagents:

BCIP (Boehringer): 50 mg/ml in 100% DMF

NBT (Boehringer): 75 mg/ml in 70% DMF/H₂O

SB: 100 mM TrisCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 0.1%Tween20

for 50 ml of SB add:

	<u>stock</u>	<u>1xSB</u>	<u>1xSB</u>
TrisCl, pH 9.5 2 M	2.5 ml	5 ml	
NaCl 5 M	1.0 ml	2 ml	
MgCl ₂ 1 M	2.5 ml	5 ml	
Tween20	20 %	250 µl	0.5 ml
H ₂ O	ad 50 ml	100 ml	

SB+NBT+BCIP: to be added per 1 ml SB (= per each sample)

NBT (75mg/ml; final: 337.5 µg/ml) 4.5µl

BCIP (50mg/ml; final: 175 µg/ml) 3.5µl

BLOCK: either 5% sheep serum/PTW or 2% NEN Blocking Reagent/TNT buffer

ANTIBODY: dilute in BLOCK, using the following dilutions:

Anti-DIG-AP, F_{ab} fragments 1:2000

Anti-fluo-AP, F_{ab} fragments 1:1000

Anti-acetylated tubulin, mAB (optional) 1:200

Original recommendation: preabsorb anti-Dig-AP F_{ab} fragments

Primary staining

- block embryos 1(-2) hrs with 1ml of **BLOCK** at room temperature

- incubate embryos in 200 μ l of **ANTIBODY**
either, for 1(-2) hrs at RT; alternatively, overnight at 4°C
- **in the tubes, wash** 5 x 10 min in PTW at RT
(original recommendation: one wash can be performed at 4°C overnight)
- transfer embryos to a 6-well dish and equilibrate 2 x 5 min in Staining buffer (SB)
 - add **1ml SB+NBT+BCIP** to the embryos
 - stain in the dark without shaking for up to 48 hrs
 - if necessary, replace solution with fresh staining solution
 - wash 3 x 5 min in PTW
- store at least overnight in PTW/4%PFA

Mounting

- transfer embryos to 87% glycerol
- leave in 87% glycerol at least overnight for complete equilibration
- mount in 87% glycerol in viewing chamber and take pictures

Comments: Glue two or three stripes of tape on microscope slide, add droplet of glycerol with embryos and cover with coverslip to prepare a viewing chamber.

Additional hints

I usually rock the anti-Dig antibody in 2.5% sheep serum in PTW for the time I pre-absorb the embryos in 5% sheep serum in PTW. It might serve as a kind of pre-absorption.

7.4 *Platynereis dumerilii* Immunostaining Protocol

Fixation of larvae

Reagents:

2xPTW: dilute 10x PBS to 2x PBS, add Tween20 to 0.1%.

16% PFA (common stock!): dissolve 16% paraformaldehyde in water by stirring and heating to 65°C, add dropwise 1 M NaOH until the solution gets clear (check pH $\approx$ 7.5), cool to room temperature and store at 4°C.

4% PFA/PTW (to prepare): For 10ml, mix 2,5ml 16%PFA with 7,5ml 2xPTW.

Procedure:

PFA Fixation:

- Pour larvae batch through big filter.
- Wash with sea water (keeping the embryos wet - in cup with NSW!!).
- With a pipette, transfer them to a small filter, invert it upon an Eppi and rinse with 4%PFA/PTW into 2ml tube.

Note: Use 2x PTW + PFA for the fixation!

- Put on shaker for 2hrs at room temperature.
- Wash 5 x 5 min at room temperature, on shaker.
- Store at 4°C.

Note: The storage of fixed larvae for immunostaining should not be longer than 3 days.

Proteinase digestion

Reagents:

ProteinaseK: 20 mg/ml Stock

Reagents you have to prepare before you start:

- **Proteinase K Solution:** 150µl ProtK in 40ml PTW
- **2mg/ml Glycine/PTW:** 80mg/40ml PTW

Procedure:

- All steps are performed at room temperature, in hand-made nets submerged into 40ml volumes (lids of pipette boxes).

Note: If you put the embryos onto a shaker, don't put more than 32ml (embryos could fall out of the nets).

- Transfer larvae of each different stage into nets submerged in fresh 1x PTW – rinse 2 x 5 min (transfer the nets between boxes).
- digest with ProteinaseK, 150µl in 40ml PTW, without shaking for several minutes depending on the stage of the embryos (**see below**).
- rinse 2 x shortly in freshly prepared 2 mg/ml glycine/PTW (also in boxes).
- wash 5 x 5 min in PTW – transfer nets in boxes (on shaker).

Comments: the ProteinaseK-digestion is a critical step of this protocol and will substantially influence the quality of the whole procedure, thus, times of digestion have to be optimized. The following list of digestion times worked well in our hands and can be used as guideline.

stage	time of ProteinaseK digestion
1h-15h	use 'early WMISH-protocol'!
16h-72h	45" - 1 min
72h-5d	1 min 30 sec
1week to 6weeks	2 min 30 sec

Blocking

Reagents:

Sheep serum

1xPTW

Procedure:

- Using a 1ml pipette, transfer the larvae in 1.5 or 2 ml Eppendorf tubes. Label the tubes, indicating the stage of the embryos. Let them sink (it takes maximum 5 minutes) and aspirate the PTW out.
- Immediately, add 1ml of sheep serum 5%. Leave them shaking at room temperature for 1 hour.
- Meanwhile, start preparing the tubes and antibody dilutions for the next step.

Primary Antibody incubation and wash

Reagents:

Pre-Immune serum (rabbit 1)

Primary antibody: anti c-Opisn2 (rabbit 1)

Primary antibody: anti acetylated tubulin

Sheep serum

1xPTW

Procedure:

Preparation of the antibody dilutions

You will incubate the larvae in:

- Pre-immune serum (PIS): dilution 1:50 in a total volume of 200µl containing 2.5% sheep serum.
- 2 primary antibody dilutions: anti-c-Opisn2 (dilution 1:50) and anti-acetylated tubulin (dilution 1:200), both in 200 µl containing 2.5% sheep serum.
- Label the tubes you will use during the procedure and after it, to keep the primary antibodies for further uses.
- Prepare the dilutions you will use.

After the blocking step: Antibody incubation

- With a pipette, mix the embryos in the same tubes where they are, so they are more or less

equally distributed in the 5% sheep serum.

- Immediately, distribute them among the empty labelled Eppis you will use. Try to add similar amounts of embryos in the different tubes.
- Let them sink. Aspirate as much as you can the remaining sheep serum. Immediately, add the corresponding AB or PIS dilution from the Eppi you already prepared.
- Let them incubate during 1 hour at room temperature, shaking. (Alternatively: overnight at 4°C, shaking).
- Let them sink. With a pipette, aspirate as much liquid as you can. (NOTE: The antibody solutions can be re-used, so KEEP the liquid you remove in the empty tubes you already labelled. Store them at 4°C)
- Immediately, add 1ml of PTW in each Eppi. Do 5 x 10 min washes. On shaker.
- Meanwhile, start preparing the secondary antibodies dilutions for the next step.

Secondary Antibody incubation and wash

Reagents:

Secondary antibody: Alexa Fluor 488 goat anti-rabbit

Secondary antibody: Cy3 goat anti-mouse

DAPI (4',6-diamidino-2-phenylindole)

2.5% sheep serum

1xPTW

Procedure:

You will incubate the larvae in 2 secondary antibody dilutions: Alexa Fluor 488 goat anti-rabbit (dilution 1:200) and Cy3 goat anti-mouse (dilution 1:200), both in a volume of 200µl containing 2.5% sheep serum. It will also contain DAPI (300 ng/ml).

Mix everything and wrap the tube in aluminium paper.

- After the last wash of the previous step, add 200µl of your secondary antibodies solution in each tube.
- Incubate during 1 hour at room temperature, shaking. (Alternatively: overnight at 4°C, shaking). Keep your samples covered (darkness).
- Let them sink, and aspirate as much liquid as you can. (You don't have to keep this solution).
- Immediately, add 1ml of PTW in each Eppi. Do 5 x 10 min washes. On shaker, covered.

Samples mount

Reagents:

Glycerol

ProLong Gold antifade reagent

Procedure:

- Mounting in glycerol, in 6-wells plates: add your larvae in PTW in a well, aspirate the PTW, cover the larvae with glycerol. Keep wrapped at 4°C. Label the plates!
- The day before doing the observation under the confocal microscope, mount in antifade solution:

- Prepare cover slides taped with 2-3 stripes of tape at the edges. (For fixed embryos, 2 tapes for animals up to 50hpf and 3 tapes for animals 50hpf to 5dpf usually work well).
- With a pipette, pick one animal from the ones you mounted in glycerol and place it on the middle of a slide.

Note: When choosing the animals, you can check under the fluorescence microscope which larvae have a better signal. Those will be the ones you will check under the confocal microscope.

- Fill up space with some drops of antifade reagent, so that the embryos are spatially fixed.
- Put one clean cover slip on top.
- Embryos can be moved into different position for viewing by moving cover slip (under binocular). Under the slide, mark where is the animal located, so it will be easier to find.
- Keep them covered (darkness) at 4°C.

7.5 Sequences

Sequence Abbreviation	ID Type	Sequence ID
Pdu_Adrenergic_Receptor_cand	Appendix	Pdu-AdrenergicR-candidate
Pdu_DopamR_cand	Appendix	Pdu-DopamineR-candidate
Pdu_MelR_cand2	Appendix	Pdu-MelR-candidate2
Pdu_MelR_cand3	Appendix	Pdu-MelR-candidate3
Pdu_MelR_candidate	Appendix	Pdu-MelR-candidate
Pdu_pdfR_candidate_fragment	Appendix	Pdu-PdfR-candidate-fragment
Gg_DopamRD3	GenBank	ACR48171.1
Hs_DopamRD2	GenBank	AAC78779.1
Hs_DopamRD3	GenBank	AAH95510.1
Hs_DopamRD4	GenBank	AAL58637.1
Mm_CalcitoninR_isof._Cla-1	GenBank	AAK56132.1
Mm_CorticotropinR_cand	GenBank	AAC52243.1
Mm_DopamR1A	GenBank	EDL41132.1
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Tc_CorticotropinR_cand1	GenBank	EFA02803.1
Tc_CorticotropinR_cand2	GenBank	EFA10791.1
Cc_DopamR_cand1	JGI protein ID	Capca1 19210
Cc_MelR_cand7	JGI protein ID	Capca1 83540
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Ct_CalcitoninR_cand2	JGI protein ID	Capca1 187285
Ct_CorticotropinR_cand1	JGI protein ID	Capca1 187232
Ct_PdfR_cand	JGI protein ID	Capca1 219483
Hr_CalcitoninR_cand	JGI protein ID	Helro1 90909
Lg_Adrenergic_Receptor_cand	JGI protein ID	Lotgi1 14230
Lg_CalcitoninR_cand1	JGI protein ID	Lotgi1 172709
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Lg_CalcitoninR_cand3	JGI protein ID	Lotgi1 109332
Lg_CorticotropinR_cand	JGI protein ID	Lotgi1 141716
Lg_DopamR_cand	JGI protein ID	Lotgi1 92859
Lg_MelR_cand1	JGI protein ID	Lotgi1 154252
Lg_PdfR_cand	JGI protein ID	Lotgi1 134519
Am_CalcitoninR_cand	NCBI Reference Sequence	XP_396046.4
Am_CorticotropinR_cand	NCBI Reference Sequence	XP_397268.3
Am_MelR_cand1	NCBI Reference Sequence	XP_392683.1
Am_MelR_cand2	NCBI Reference Sequence	XP_001120910.2
Am_PdfR_cand	NCBI Reference Sequence	XP_395896.4
Dm_Diuretic31R	NCBI Reference Sequence	NP_725278.1
Dm_Diuretic44R	NCBI Reference Sequence	NP_610960.1
Dm_DiureticHormoneR_cand	NCBI Reference Sequence	NP_725175.3
Dm_PdfR	NCBI Reference Sequence	NP_570007.2
Dr_Adrenergic_Receptor_1B_{predicted}	NCBI Reference Sequence	XP_001922013.2
Dr_Adrenergic_Receptor_1D_{predicted}	NCBI Reference Sequence	XP_697043.2
Dr_MelR_1A	NCBI Reference Sequence	NP_571468.1
Dr_MelR_1Bb	NCBI Reference Sequence	NP_571469.1
Dr_MelR_1C	NCBI Reference Sequence	NP_001154956.1
Gg_Adrenergic_Receptor_1A_{predicted}	NCBI Reference Sequence	XP_425762.2
Gg_Adrenergic_Receptor_1B_{predicted}	NCBI Reference Sequence	XP_414483.2
Gg_Adrenergic_Receptor_1D_{predicted}	NCBI Reference Sequence	XP_420871.2
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Mm_Adrenergic_Receptor_1D	NCBI Reference Sequence	NP_038488.4
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Mm_DopamR3	NCBI Reference Sequence	NP_031903.1
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Mm_MelR_1B	NCBI Reference Sequence	NP_663758.2
Mm_Mtn_related_Receptor	NCBI Reference Sequence	NP_034470.1
Tc_PdfR_cand	NCBI Reference Sequence	XP_971738.1

Table 3: Sequence used for Alignments and phylogenetic trees

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ORIGIN

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  181 FDRKFYAYT AFFCIVGIML PLVVIIIFYL KIFIHMSAAK IKLDKYAHLG THKIFFKTLR
  241 QARMMFVTFV AFSFCWSPYV FVLLLDKDDT YPLEAHL LTS MIAHVHASLN FVIYGLHNKT
  301 FREAYRIIMV EKIAWCCQNC KPEVKENPGD ATSISTCCDD VTSPIFEDQH FHEGLCHCFQ
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  901 CGTCAACCTC GCAGTGGCAG ACATCATCGT CTCCTGCTAC ATCATGCCAA TTGGATTAGC
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 1981 GGAAGACTGT GAGACCAAAA ATCACCCTCG TCCTCCCGGC TCAGTTGCTA CTCCGGAAAC
 2041 CAGACCTCG AGTTGTGCT CACGTGTGAC AACTATCTTC AATGAAAAGG AGGAGGCGAC
 2101 CGAGACACAG TTATGAAGTG CATCCAATTG GAAAA

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LOCUS      Pdu-MelR-candidate2 459 aa linear UNA
DEFINITION Pdu Melatonin Receptor candidate 2
FEATURES   Location/Qualifiers
    CDS     1..459

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Region 32..293
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/note="E-value: 6.5E-46"
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/note="Predicted from database: 100 most common domains"
/note="Predicted from a full length model"
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/label=7tm_1
Region 55..74
/label=tm2
Region 89..111
/label=tm3
Conserved Seq 112..114
/note="NRY motif in Melatonin Receptors"
/label="D/ERY motif in most GPCR"
Region 131..153
/label=tm4
Region 180..202
/label=tm5
Region 241..263
/label=tm6
Region 273..295
/label=tm7
Conserved Seq 289..293
/note="NPXXY in other GPCR"
/label="NAXXY Conserved MelatoninR Pattern"

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ORIGIN

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1 MANTTLNLDE HRHLPVGWV TFTLLVIIGT LGNLLIIGAV AVSHRLRVKG HAFTVNLAVA
61 DIIVSCYIMP IGLATSRYS NPFGEFGCGF NAFIIMMTCG VSTQSLMLIA LERYFLICQS
121 QRYKRFFSKP LIAGYIAFTW IYSAIWTQCG WTGWTRYIYG LDIWLCLFDG TFSISYDVCL
181 VLFGLMLPMG ILCFCYTKIF LLVHKNNKIM KQHRQRPHTT QEQDNLTDQ KRSKKELRLT
241 VTLFVIVLVF SVCWTPASIV LLLSGVWTDN PKSFYTVAVW FAFANSSMNS ILYGFMNKNF
301 RDGYKRVLLT MFCKKKDDCN IAYHCPCTSS EEHSNSSRQH SNTAKTDTL SKRKNMIIRG
361 AKAVIPQRTN PNSVSDIKID PVHLWSPEEV DRSCYMQQL STDSQVIEDQ DPFEEDCETK
421 NHPAPPGSVA TPETRPSSCR SRVTTIFNEK EEATETQL*

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//

LOCUS Pdu-MelR-candidate3 1940 bp DNA linear UNA

DEFINITION Pdu Melatonin Receptor candidate 3

FEATURES Location/Qualifiers

```

CDS complement(131..1596)
/note="Length: 1092"
/note="Found at strand: negative"
/note="Start codon: CAG"
/label=ORF

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ORIGIN

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1 CAGAATAAAA AGACAAAAAT ATCAAGATCA TATGTTAGTA AGACCAATCC TCTTACATCT
61 TTGAATGAAA GAAATCAATG AAGACAAATA TGTTACAGAA TCTTTATTTG GTTTCATATT
121 GCCTTAAATG TTATTGAATT TTTTCAAAAA CAAACAGAAAT GGATAATCTT TCAAATATTA
181 CAACAAAATT ACAACACTGA AGAGCTTCCA TCGGTACGCA ATCCTTGGGT ACATCCAAAA
241 TATGACCAAT CCTTACATCT ATTGGTACAT AGGTACAATG GTACATGTGC ATTGAAATGA
301 CCTTATGGTA ATCATAATTT GAACAAAATA TCCAGCAACA ATTTTGAGTT TGTTCAAAAG
361 ACAGTGGATA GCAATCAGAA ATTTGAAACC GACAGAATAA ATAATCTTTA TGTTTCGATC
421 CTGTATAATA GGGTAGCAAA CTGTGACAGC TTAGATAATT GCAAAATATC AAGTGATGCC
481 ATAGAAATGA TCACAAATTG GGTGCTAGAT GGAGGATGTG TCCATCTCTT TAGCAGACTT
541 AGCAGTTAAT GGTTC TGCCA CAGGCAAGT TCTTGAGTA CTTTGGCAGT TGGCGAGAAA
601 CCCGTTGATA TGGTTTTGAG AATATAATGG ACTCAATGGT GTAAGAGTCT TTGTGTCGGT
661 AACAGCGTCA TCACACTTCT CTTGCAAGG GAGACTGCGG CTGTCACTTT GAAAGCAATG
721 ACATAATCCT TCATGAAAGT GTTGGTCCTC AAAAATAGGT GAAGTGACAT CATCACAAAC
781 AGTAGATATA GAAGTGGCAT CGCCAGGATT CTCCTTCAAT TCTGGCTTAC AATTTTGACA
841 ACACCAAGCA ATCTTTTCCA CCATGATAAT CCGGTAAGCT TCCTGAACG TCTTGTTGTG
901 GAGGCCATAA ATGACAAAAT TAAGGCTAGC ATGAATGTGA GCAATCATGG ACGTTAATAA
961 GTGAGCTTCA AGTGGATATG TGTCATCTTT GTCAAGCAGC AAAACAAACA CATAAGGACT
1021 CCAGCAAAAA CTAAAGGCTA CAAAGTCCAC AAACATCATG CGAGCCTGCC GCAGAGTTT
1081 GAAGAATATC TTATGTGTTT CAAGATGTGC ATATTTATCC AATTTTATCT TTGCAGCACT

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1141 CATGTGGATG AATATCTTTA GGTAAAAAAT TATTATCACA ACAAGAGGTA ACATTATCCC
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1261 AGATTTGTTG TCGAAACCAT GTTGGCTCCA CCCCAAATGG CTTGGCAAAGT CAATTGCCAC
1321 TCCAATGAGC CAAGAGGTCA TGCATGCCAC CACACATCTG CCTTTACTGT AGATCTTGGA
1381 GTATTTAGAG TGATGGCAGA TGTAACATA ACGATTGACA GCAATATGGC CAATAGTGAG
1441 AAGAGACACT ACGCAGCTTA TGACACAGAC AGAAGCCAGA CCTCGACAGA GGCCCAAATT
1501 ATCCCGGAAG AATGATGTCC CCTTGACTGC CCTGAAAAG AGAAAACAAA CTGTCACTAC
1561 AGATCTATAA GAGCTTAATA TATGAGATGG GGAAGTTAC ATTTTCATATT ATACCATCAT
1621 CAGAATTGAT AACAGGTAGC ATCATATCAA GGAAACAAG ATAAGCTTTC ACAAGGTACC
1681 ACAAGTTTGT CCTTAAGTCA ATTCAAGATT CCAATTTTGG CGAAGTACAA GCACAACACC
1741 CAACACTTCC AGCTAGAATG GGAATTGTGT GAACATGCAG TAGTCTGTGA AGTGTCTGAG
1801 ACCACCAAAA CTAGTTGGCA GCCATAAAAG CTAAGAGGAG GCCACAAAAG CAACTGCACT
1861 ACTGATTCCT GCAAATCAGG TGCCTCTATG GCGATCAATC AGAGGATGGA CCTTCCTACC
1921 TCGCAGATAC ATCGGGAGAG

//

LOCUS      Pdu-MelR-candidate3      363 aa                linear      UNA
DEFINITION Pdu Melatonin Receptor candidate 3
FEATURES             Location/Qualifiers
ORIGIN
    1 QSPSHILSSY RSVVTVCFLF SGAVKGSFF RDNLGLCRGL ASVCVISCVV SLLTIGHIAV
    61 NRVVYICHHS KYSKIYSKGR CVVACMTSWL IGVAIDLPSH LGWSQHGFND KSHKCIFDRK
   121 FKYAYTAFFC IVGIMLPLVV IIFYLKIFI HMSAAKIKLD KYAHLGTHKI FFKTLRQARM
   181 MFVTFVAFSF CWSPYFVLL LDKDDTYPLE AHLTSMIAH IHASLNFVIY GLHNKTFREA
   241 YRIMVEKIA WCCQNCKPEL KENPGDATSI STCCDDVTSP IFEDQHFHEG LCHCFQSDSR
   301 SLPCKEKCDD AVTDTKTLP LSPLYSQNHI NGFLANCQST PRTLPAEPL TAKSAKEMDT
   361 SSI*

//

LOCUS      Pdu-PdFR-candidate-fragment      709 bp      DNA                linear      UNA 06-Aug-2010
DEFINITION PDF Receptor fragment from deg. PCR (lo1up2_after_blot)
FEATURES             Location/Qualifiers
    CDS
        2..709
        /note="Length: 708"
        /note="Found at strand: positive"
        /note="Start codon: ACC"
        /label=ORF
ORIGIN
    1 TACCCGGATG CATCGGAATT TGTTCCTGGC CATTATGATC CACGTCATGA TTCAACTTAT
    61 TGTACACATC GACCAATATA TCGCAAAGAA TTCTGGGAAT GCCGTAGGGG GCTCCATTGT
   121 TCTTGACAAT AAAGGAACAA TGTACAATAC TCCAATATTA TGTGAAGTAC TAAAGACAAT
   181 GGTGGAATAC ACCAAGACTG TTATGTTTGT TTGGATGTTT ATCGAAGGAA TGTATTTACA
   241 CAACATGCTT GTCGTTTCGG TGTTACACGG ACGGCCTAAA TACATGCTTT TCTACATCCT
   301 TGGATGGGGC TTTCCCATCA TTTAACCTT AACCTGGGT ATTACCAACT GGTACCTTGA
   361 TTCAGAGAAG TGTTGGTTTA CTTATTCCTT TTTACCTCAG TACTGGATAC TCGAGGGGCC
   421 AAGAGCTGCC ATCATAGCTG TGAATTTACT TTTTCTTCTT AACATCATAC GGGTGCTGGT
   481 CACCAAGTTA AGAGAGAGTC ATTCCAATGA AGCTGGGCAA GTCAGGAAAG CGGTGAAGGC
   541 GGCCATTGTT CTTCTTCCGT TACTTGCGCT CAACAACCTT CTAGTGATGG TCGAGGCTCC
   601 GATGGGACAC GCTGTCAAGT TTGCTGTCTT TACATACACC TCACAGTTCC TAGCCTCCTT
   661 CCAAGGTTTC TTCATAGCTC TGCTCTACTG CTTCTTCAAC GGCGAAGTA

//

LOCUS      Pdu-PdFR-candidate-fragment      236 aa                linear      UNA
DEFINITION PDF Receptor fragment from deg. PCR (lo1up2_after_blot)
FEATURES             Location/Qualifiers
    CDS
        1..236
        /note="only fragment, 3' and 5' end missing"
        /label=Pdu-PdFR-candidate-fragment
    Region
        7..28
        /note="inside to outside"
        /label=tm2
    Region
        32..229
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        /note="Accession: PF00001"
        /note="Score: -149.7"
        /note="E-value: 0.25"
        /note="Domain: 7tm_1"
        /note="Predicted by CLC Main Workbench version 6.0.1"
        /note="Predicted from database: 100 most common domains"
        /note="Predicted from a full length model"
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target=_blank>Link to PFAM</a></html>"

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Region      97..119
            /note="inside to outside"
            /label=tm4
Region      134..156
            /note="outside to inside"
            /label=tm5
Region      177..199
            /note="inside to outside"
            /label=tm6
Region      209..231
            /note="outside to inside"
            /label=tm7

ORIGIN
    1 TRMHRNLFLLA IMIHVMIQLI VHIDQYIAKN SGNVAGGSIV LDNKGTMYNP PILCEVLKTM
   61 VEYTKTVMFV WMFIEGMYLH NMLVSVFTG RPKYMLFYIL GWGFPIILTL TWVITNWYLD
  121 SEKCWFYTSF LPQYWLEGP RAAIIAVNLL FLLNIIRVLV TKLRESHSNE AGQVRKAVKA
  181 AIVLLPLLGV NNFLVMVEAP MGHAVKFAVF TYTSQFLASF QGFFIALLYC FFNGEV
//

LOCUS       Pdu-DopamineR-candidate 3371 bp DNA linear UNA
DEFINITION  Pdu Dopamine Receptor candidate
FEATURES             Location/Qualifiers
     CDS             complement(1111..2421)
                     /note="Length: 1311"
                     /note="Found at strand: negative"
                     /note="Start codon: AGT"
                     /label=ORF

ORIGIN
    1 AGTTTGTGAT CACTGCTACG GTTGTATTCT AATTGGAATT TGTCATGTGA CTTTGAACA
   61 GTCTCCCACT AGTTTGTAT TTCTCAAAA TATGCAACTA CTACAAAAGA AAAAAATACT
  121 TTGCAACAAT ATATGAAAT AAGTGAGTTG CACAAGGTCA ACTATTGCAT TCAAAATGCAC
  181 CATATCTGTG TGTTATTTGC TAAATGGCTC TGCTCCTGCA GTCTCTAAAT TCGAATTTCA
  241 AACGCTTGTA TTGCCATATAG AGTATTATTA GCATTTCGTGC GCGTGTATTT GAGGCATGAT
  301 CGATGCAAAAT GGCTGCCCAT TTTCTTTGCT CTACGAACAG TTACGCATCG GTCAAAATCT
  361 GTCATAAGTA AGCGAACATC CATCCCAATC GAAATCGCCT TTAGAAGCAA TGGGATTTAT
  421 TTACTTATAT TCGATTTGGG CCATATGTAA CTCTTGGTGC AGCAAAAGAA TGTGGCAGTT
  481 ATCTTACCTC AACTATACAA CTTCATGAAT GTTGATTATC AATATAAAAG GAACCATACT
  541 TTACCTATTT ATATTGCAC TCAATAAGTT TAATTCCAGT GACGTCATAT ATTTCTGTCAG
  601 CAACATGTCA TATGCTATTT TCAATGGTAA CAATTTGTGA CATGACTAAA GTGTATAATA
  661 TTATCTTAAT AGTAATATAA CTCCAATTAA CATAATACAA TGGCTGCCAA TAAATTTTCT
  721 ATGCAAAACA ATAATTTGTA AATATGTGCA ACTTGGCAGG TCTTTGTAAA GATAAAACAG
  781 AAAAAACGCT TATCGGTAAT GAACTCCAGT GAAGTTTGAA ACAGTTTCAT GACATTCATG
  841 TATTGCACAT AGTGGAAATA CAAATAATCA ATCTTCAAAG ATATTGAGAC ATCTTTAAAT
  901 TCAAAATCTC ATACCAGGAT ATATAAATAA ATTAGGGTAG CACCAAACTA CTTATATTGT
  961 AAAAAATATT TAACAATACC ACTAGATCCA TTCACTTTGT GTGTATGTAA ACAGTTTGAC
 1021 CTTCAACCTT GAGTTAAGGT CATGAGTAAT TAAGAGGTCA AGGTCAGGTC AAGGTTAGGT
 1081 CAAGGTTGTT CTATAAATAG ATTATCACAG TCATACAGAA GTAACTGGC TCTGGTCGGG
 1141 GGCTATAGTC CGAGGAGGCT TTTGGGCCAC AGTACCGTAA ACATTGCCCT TGGATGGGAG
 1201 ATGCATGAAT CTGATTGGTC GCCTGAATTC TACGCTTTTG AGTTTGAAGC ACTGCAGGTA
 1261 TGAGAGGACA CGCTTAAAGG CGTCTCTGAA TTCACGGTTG AAGATGGAGT ATATAACAGG
 1321 ATTTAAGGTT GAGTTGAAAT AACCAGGACA AGTGAAAACA CTAAACAGGA CCTCAGGAAC
 1381 ACAGGACGGT CAGAAGGATG AGATAATGTT AATTGTGAAG AAGGGCGCCC AACATAATAA
 1441 GAACGTTCCC ATAATAACTC CTAACGTGAT GGCAGCCTTG TGGTCCGACA CCTTATGCTG
 1501 GTGCTGTTGG CGGGGAGAGG GGACGTCATA CGCAGGGACC CTGGTTGTCC GCTTAATACT
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 1621 TGGAGCGTAA AAAGTGATGA GGGATGAGAC CACGGCATAA ATGGGGTTGA GCTCCATTAA
 1681 GCAAATGTAA TGGCCGTGGT GGTCTGACTG TCCTTCCGGG TTGTAGGGGC CAGTCCGGTG
 1741 CCATCCCAGG TGGATTGGCA GGAAGGAAAT AAGAGCGGAT AATAACCAGA CTAGTGACAT
 1801 ACACAGACAT GTCCGCAAGG GAGTCACGAG TCTCTCGTAA TGCAATGGGT TCTTGATATG
 1861 AATGTACCTG TCCAGGCTGA TGGCACACAG GTTCAAAATG GACGCCGTAG AACACATGAT
 1921 ATCAAATGAG ATCCACACTG AAAAAAAGTG GTGGCCAAAT ATCCAGTAAC CAAGAATGTC
 1981 ATTGGCTACG GCAAAAGTCA TAACCATTAT TGCAGCCAGA GTATCCGCCA GAGCTAAAGA
 2041 GATGATAAAG TAATTGCTAG TTTTACGCAA GTGCTGTGTA GTCAAAATGG CCACCAACAC
 2101 GAGAATGTTT CCCGCAATTG AGAAGAAGCA GATGAGAGAT AATACGACGC CAATTACAAC
 2161 CGCTTCCGGG AGACTGTACT TGGCTGGGTA GTCACCGAGG GCACTGATCT CCACACTGTT
 2221 TCCGTTGAGG GGACCAAGTC AGTAGTTGAG GGAGGACGCG AAGCCTTCCC ATGATGCATT
 2281 GCTTTCGGGA GGTGCTCTCC AGGAGTGCTC ATCAGGGGGC GCTGCAAGTG TGCTTCTGTG
 2341 AACGACGTCT ATGGCAGCTA AGACAAGCCA GATGGCGCTG CGAGAGGTGC CCATATCACT
 2401 TCCAGTCTCT GACCTCTCAC TTTATTACTG CTGCCACGTA ATGATCATGC GTAATGATTC

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2461 TGCCAGCCAT TAATAATTCA CTGGGGGAAG CGGGCGAGAG ACTCACGGGG AGTCTAGTAC
2521 AGGCGCAGTT GGACGAATCA CAGCCTTAAA GTGCCTTAAG GGCATTTGGG GGCTTGGCAA
2581 ACCCTGTGTG TACTCATTGT GGGCATTTAA GGGTTCAGCG ACCCACGCGA TCCCTCTAAT
2641 TATTGACTCG CTATGGCTTT GCTGCTGGGT TTAAACTTCG TGTGAATGGC CAAATTGTCC
2701 TGTGCTGGTC TCCGCCTGCT GTGTGCTCAC TCAATGAGAA GCCTGTCAAT GCTGCCAATC
2761 GTGCCCTCTA TTTTCACCAT ATCCTCACTC ATTTCACTTT CCTTGCTCTCT CGTTTATTTT
2821 ACTTTGTTCT CACTCTTTTG TGTGATTTT TATTTCACTT CCATTTCCTT GTGTTCCGAA
2881 CAGTTCGTTA GTTCAGGAAG CCTTTGTAAT TGATTCTCCC TAGTGATTA TCAGGGTGCT
2941 TCAGTGGTTG GTAGTGTGTC AGGCGCTCAT AAAGACAGAC AGGGCCACAA TGCCTGCTTG
3001 TTATGGCGTG CTAATACACA TGTGGTGGCT CCTTCAATGG CTCAGAAATC CACAATTGCT
3061 CACAGAACTT CCGGAGTGCC TGGGAGCTAA TGAAGTGTCT TCTCGGAGAA CAAGAATCTT
3121 GGATGTTGCT GTCTGAGAAC TCCAGTAATT TGTCAACAGA AGATGTTACT TTTTACTCCT
3181 ACAGAAGTAC TGTTTCACAG ACGAAGACAC TATATCACAC GACATAATTG CCTTGCAGTC
3241 AAAAGCGACT TCTTGGAAAG CGAAGGTTTT CTAAAAAGT CCCTTGCAAG TTACAATCCA
3301 ATATGTTTCG AGATATCACA AATATTATGC AGTATTCTTG AGTATTGTG ACTGTCTTGG
3361 TGGTAATTAA A

//

LOCUS      Pdu-DopamineR-candidate      437 aa                linear      UNA
DEFINITION Pdu Dopamine Receptor candidate
FEATURES   Location/Qualifiers
ORIGIN
    1 SERSETGSDM GTSRSAIWLV LAAIDVVTGS TTAAPPDEHS WKQPPESNAS WEGFASSLNY
    61 STGPLNGNSV EISAPGDYPA KYSLPEAVVI GVVLSLICFF SIAGNILVVV AILTDRHLRK
   121 TSNYFIIISLA LADTLVAIMV MTFAVANDIL GYWIFGHQFC SVWISFDIMC STASILNLCA
   181 ISLDRIYHIK NPLHYERLVT PLRTCLCMSL VWLLSALISF LPIHLGWHRT GPYNPEGQYD
   241 HHGHYICLME LNPIYAVVSS LISFYAPCVV MLLIYLRFL FARRVQSIK RTTRVPAYDV
   301 PSRQQHQHK VSDHKAAITL GVIMGTFLLC WAPFFTINII SSFCPSCVPE VLFSVFTWLG
   361 YFNSTLNPVI YSIFNREFRD AFKRVLSYLQ CFKLKDVEFR RPIRFMHLPS KGNVYGTVAQ
   421 KPPRTIAPDQ SQVTSV*

//

LOCUS      Pdu-AdrenergicR-candidate      443 bp      DNA                linear      UNA
DEFINITION Pdu Adrenergic Receptor candidate
FEATURES   Location/Qualifiers
     CDS           1..441
                   /note="Length: 441"
                   /note="Found at strand: positive"
                   /note="Start codon: CGG"
                   /label=ORF
ORIGIN
    1 CGGCCGGGGG TGCAGGAGAA GATTAATATA TTAGGTCTAA TTGGGATGAC GTTATCAGGA
    61 GTGACCCTGG GTACTATAGT GTTGATGACG GTCTGCGGGA ATGTTTTAGT TTTATTAGCA
   121 GTTTTTGTAA ACTCACACTT GCGGAGCACA ACTAATTTCT TCATCGTCAA CTTAGCAATC
   181 GCTGATTTGC TCCTGGGCAC TTTAGTCTCT CCATTTTCTG CGAGCTTGGA GATCGTCAAC
   241 TATTGGGCAT TTGGGCAGCT GTTCTGCGAC ATTTGGGCAG CAGTAGATGT GTTATGCTGC
   301 ACTGCCTCCA TCATGAGTTT GTGTGTGATA AGTATAGACC GTTATATCGG AGTGACTAGA
   361 CCGTTGCAAC ACTCAACCAT AGTATCGGAG AAACGGGCTT TTTANACGAT CATCTACTGT
   421 TTGGTTAGCT ATCATCCGCT ATA

//

LOCUS      Pdu-AdrenergicR-candidate      147 aa                linear      UNA
DEFINITION Pdu Adrenergic Receptor candidate
FEATURES   Location/Qualifiers
ORIGIN
    1 RPGVQEKINI LGLIGMTLSG VTLGTIVLMT VCGNVLVLLA VEVNSHLRST TNFFIVNLAI
    61 ADLLLGLTLV PFSASLEIVN YWAFGQLFCD IWAADVLLCC TASIMSLCVI SIDRYIGVTR
   121 PLQHSTIVSE KRAFYTIYC LVSYPHL

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All sequences are attached in digital form on the enclosed CD.

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Digital, high resolution versions of all figures showing staining patterns are attached on the CD as scalable vector graphic files. The tables listed above are also included there.

8. References

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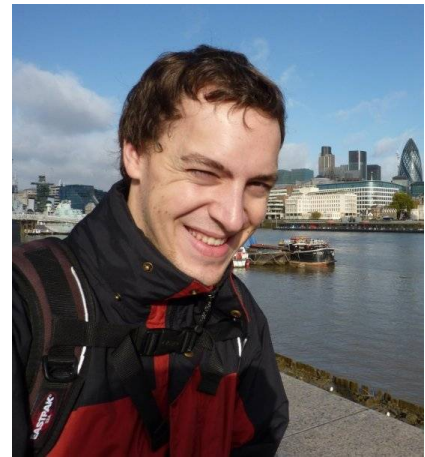
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Curriculum Vitae

Contact Information

E-Mail: s.hammer@univie.ac.at

Website: <http://homepage.univie.ac.at/s.hammer>



Personal Information

Name: Stefan Hammer

Date of Birth: March 2nd, 1988

Place of Birth: Waidhofen an der Thaya, Austria

Nationality: Austria

Education

September 1998 – June 2006:

Sciences orientated High School, Waidhofen an der Thaya;
graduated with honours on June 20th, 2006

October 2006 – March 2008:

Student at the University of Vienna, Diploma studies in Biology

April 2008 – February 2010:

Diploma studies in Genetics and Microbiology with specialisation in Developmental Biology, Bioinformatics and Immunology

Scientific career

since March 2010: Diploma thesis with the title "Target Tissues of Melatonin and PDF in Platynereis" in the group of Dr. Florian Raible at the University of Vienna / MFPL

Grants/Performance Scholarships

2009: Leistungsstipendium nach dem Studienförderungsgesetz der Universität Wien

2010: Leistungsstipendium nach dem Studienförderungsgesetz der Universität Wien

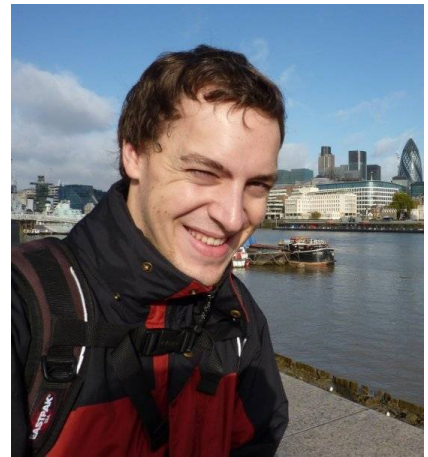
2010/2011: Leistungsstipendium der Johann Wilhelm Ritter von Mannagetta – Stiftung

Lebenslauf

Kontakt

E-Mail: s.hammer@univie.ac.at

Webseite: <http://homepage.univie.ac.at/s.hammer>



Persönliche Informationen

Name: Stefan Hammer

Geburtsdatum: 2. März 1988

Geburtsort: Waidhofen an der Thaya, Österreich

Nationalität: Österreich

Ausbildung

September 1998 – Juni 2006:

Naturwissenschaftliches Realgymnasium, Waidhofen an der Thaya;
am 20. Juni 2006 mit Ausgezeichnetem Erfolg abgeschlossen

Oktober 2006 – März 2008:

Student an der Universität Wien, Diplomstudium Biologie

April 2008 – Februar 2010:

Diplomstudium Genetik und Mikrobiologie mit Spezialisierung in Entwicklungsbiologie,
Bioinformatik und Immunologie

Wissenschaftlicher Werdegang

seit März 2010: Diplomarbeit mit dem Titel "Target Tissues of Melatonin and PDF in
Platynereis" in der Gruppe von Dr. Florian Raible an der Universität Wien / MFPL

Forschungsbeihilfen/Leistungsstipendien

2009: Leistungsstipendium nach dem Studienförderungsgesetz der Universität Wien

2010: Leistungsstipendium nach dem Studienförderungsgesetz der Universität Wien

2010/2011: Leistungsstipendium der Johann Wilhelm Ritter von Mannagetta – Stiftung