universität
wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Tool Development and Behavioural Analysis to Investigate the Influence of Light on Platynereis dumerilii Behaviour"

verfasst von

Heinrich Oberlerchner

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Wien, 2013

Studienkennzahl lt. Studienblatt: A 441

Studienrichtung lt. Studienblatt: Diplomstudium Mikrobiologie –Genetik

Betreuerin: Dr. Kristin Teßmar-Raible

INHALT

1. INTRODUCTION

1.1. PLATYNEREIS DUMERILII AS MODEL ORGANISM

P. dumerilii is a marine bristleworm (Polychaeta, Annelida) and belongs to the Lophotrochozoan branch of Protostomia. Protostomia and Deuterostomia together form Bilateria. The traditional model organisms in Biology mostly derive from Deuterostomia (Chordates and vertebrates) or from the Ecdysozoan branch (*Caenorhabditis elegans, Drosophila melanogaster*) of Protostomia. Because *Platynereis* has many ancestral features in its lifestyle, anatomy and development, it is a very useful model organism to study the features of the last common ancestor of bilaterians, the so called Urbilateria, and to understand bilaterian evolution and relationships [1];[2];[56]. Also it has a relatively short life cycle of three to four months and can be easily cultured in plastic boxes with natural or artificial seawater. One of the most interesting biological characteristics of Platynereis is the photoperiodic spawning behaviour synchronised to the lunar cycle and the great regeneration capability.
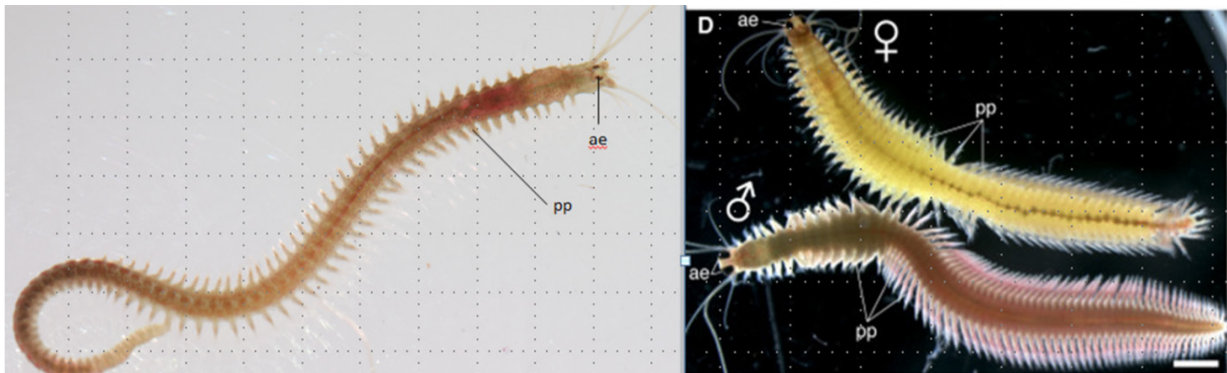
Fig.1.1: Dorsal view of the atoke worm (left) and the epitoke heteronereis (right, adapted from [3]); ae: adult eyes; pp: parapodia.

At the end of its life a female *Platynereis dumerilii* releases lots of eggs which are fertilized by a nearby spawning male. After fertilization the oozytes follow a typical spiral cleavage and develop in planktonic living trochophore larvae. At this stage the larva can swim actively with help of a ciliary band and it is phototactic. After 72 hours post fertilization (hpf) the juvenile animal is formed, it has three parapodial segments bearing chaetae. It changes its lifestyle from planktonic to benthic and it further elongates by forming new parapodial segments. In three to four week-old individuals cephalization occurs, meaning that the anterior parapodia are transformed into tentacular cirri. It continues the benthic lifestyle and elongation until maturing metamorphosis sets in and ends the atoke phase of the animal. During sexual maturation muscle cells and parapodia are decreased and blood vessels are added. This

stage is called the epitoke heteronereis. Males and female can easily be distinguished by the colour of their gametes. Female become yellow because they are packed with yellow oocytes. Male in contrast get a more reddish tail, but their anterior part is white or crème (Fig.1). From fertilization to a mature and spawning worm it takes about three months.[3, 31]
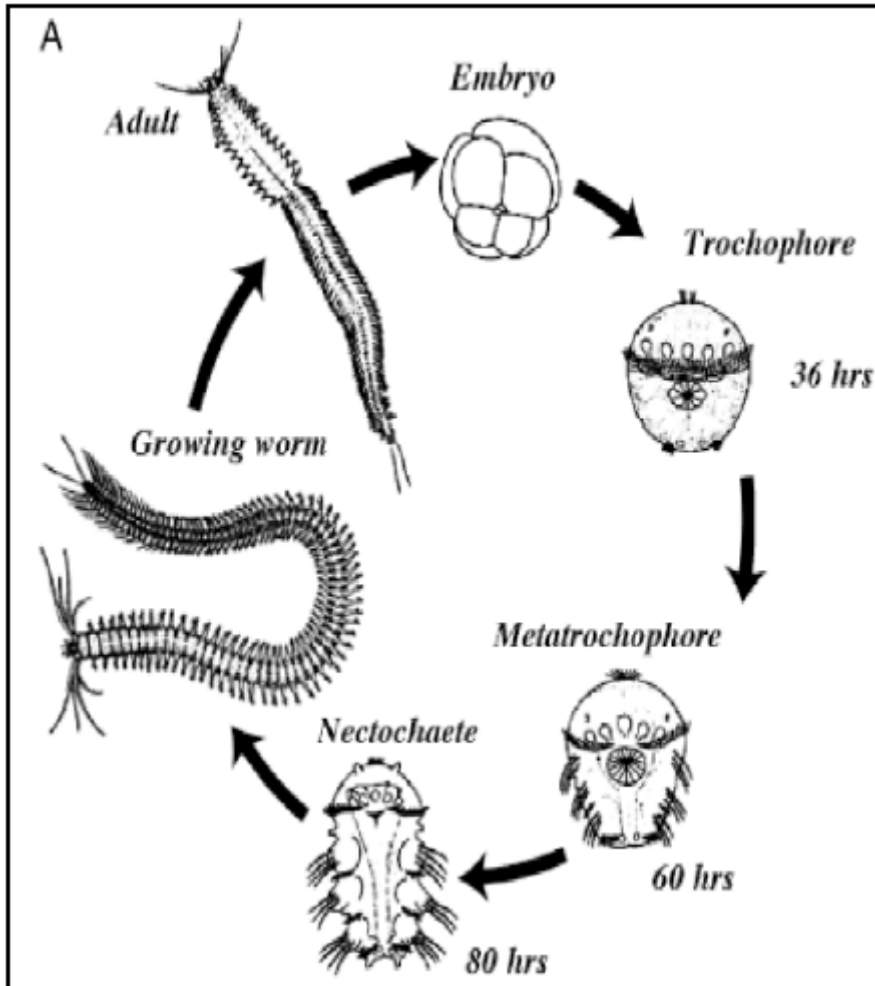
Fig.1.2: Illustration of the life cycle of *Platynereis dumerlii* (taken from [31])

1.3. PHOTORECEPTION

Besides the hole signal transduction cascade many photoreceptor cells (PRCs) contain two molecules needed for light sensing and which form the photoreceptor, retinaldehyde (or its derivates), a pigment which can absorb photons and this leads to conformation changes (photoisomerisation), and opsin, a protein detecting this changes, but not all PRCs are opsin based e.g. chryptochrome. Because opsins are membrane bound molecules, PRCs enlarge their surface to increase photosensitivity. In rhabdomeric PRCs the apical surface is folded or consists of microvilli. One by folding modified cilium is characteristic for ciliary type PRCs. The opsins employed in the different PRC types also differ and can be categorized in rhabdomeric opsin (*r-opsin*) and ciliary opsin (*c-opsin*), although both types are seven-membrane GPCRs. The c-terminal amino acid sequence is dissimilar, which results in

2

coupling to different types of GTP-binding protein. *C-opsin* binds to Gαi type (transducin) G-protein and r-opsin to Gαq type G-protein.
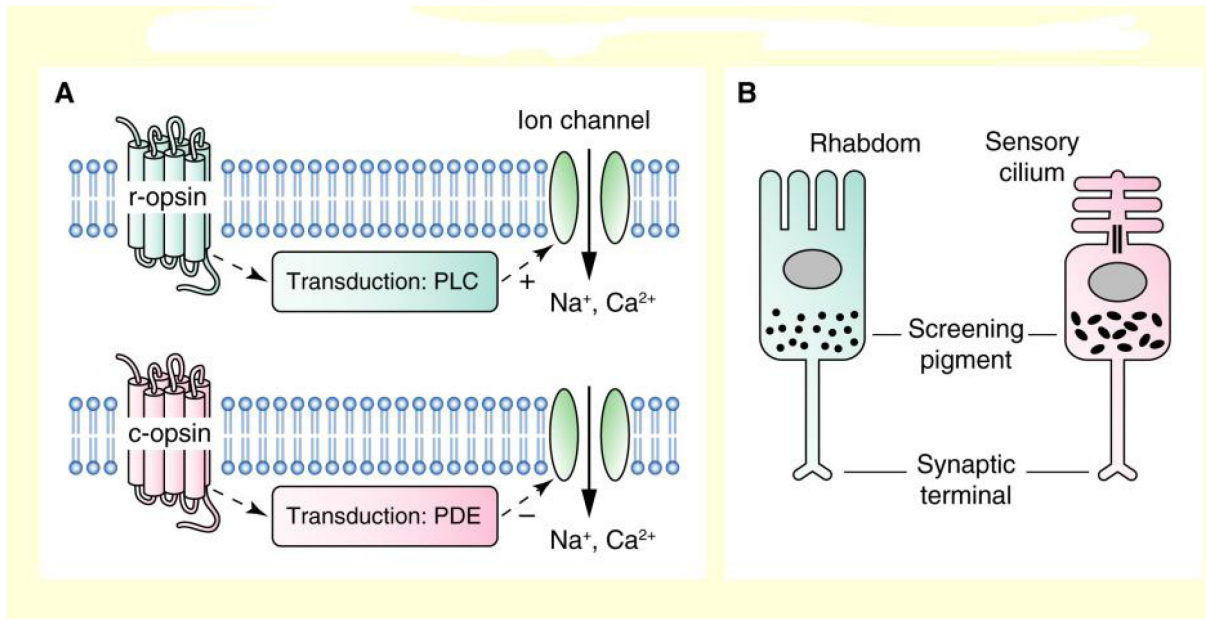
Fig.1.3: Phototransduction motifs (A) and cell morphology (B) (adopted from [43])
  r-opsin mediated light-reception results in depolarisation via PLC-pathway
  c-opsin mediated light-reception results in hyperpolarisation via PDE-pathway

Light activated *c-opsin* activates coupled G-protein by GDP-GTP exchange. Thereby Gαi gets separated and bound to phosphodiesterase (PDE), leading to its activation and hydrolysing cGMP. The decrease of cGMP level results in closure of cyclic nucleotide gated channels, which are open in darkness, and hyperpolarization of the cell membrane. Usually membrane potential lies at -30mV, because the CNG channels let cations inside the cell, this is needed for constant glutamate release. When they get closed the dark membrane potential drops and synaptic transmission gets reduced or even stopped.[4]

Phototransduction of *r-opsins* is still not completely understood and best studied in *Drosophila.* Light activation of *r-opsin* also leads to GDP/GTP exchange at G-protein and dissotiation of the α-subuntit. But here Phospholipase C (PLC) is activated which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositoltriphosphate (IP_3) and diacylglycerol (DAG). Further steps are still unclear and what exact role these three molecules play in further transduction. What is known is the target at the end of the cascade, the two transient-receptor-potential channels (TRP and TRPL). They are unselective cation-channels, but have a preference for Ca^{2+} and are opened under light, thus leading to membrane depolarization and transmitter release.[4]

1.4. PHOTORECEPTORS IN *PLATYNEREIS DUMERILII*

1.4.1. RHABDOMERIC PHOTORECEPTORS

Platynereis dumerilii has both types of photoreceptor cells. The rhabdomeric PRCs are adjacent to pigment cells, that are necessary for sensing the direction of light, and so make up the cerebral eye, as it is common amongst invertebrates [5]. The ciliary PRCs have no adjacent pigment cell and are so thought be luminance detectors [1]. For directional vision the pigment cells are as important as the PRCs and light sensory cells without a shading pigment are not considered as an eye, because directional vision is its purpose. In *Platynereis* backward radiation of the PRCs is prevented by a cup like structure consisting of pigment cells, leaving open one side where light can enter. Therefore this type of eye is named pigment cup eye. This organism's eyes are studied for understanding the evolution from a simple structured organ to a very complex one. It is assumed that the "proto-eye", the simplest form of an eye, only consists of on PRC and one pigment cell. The larval eye of *Platynereis* has exactly these features. One pair of larval eyes comes up at lateral position of the head 22 hpf. During on-going development they shift to ventral and end up near the ventral palps. [6] They control the phototaxis of the trochophore larva by directly stimulating ciliated cells used for swimming. [7] The function of the persisting larval eyes in adults is to be clarified.

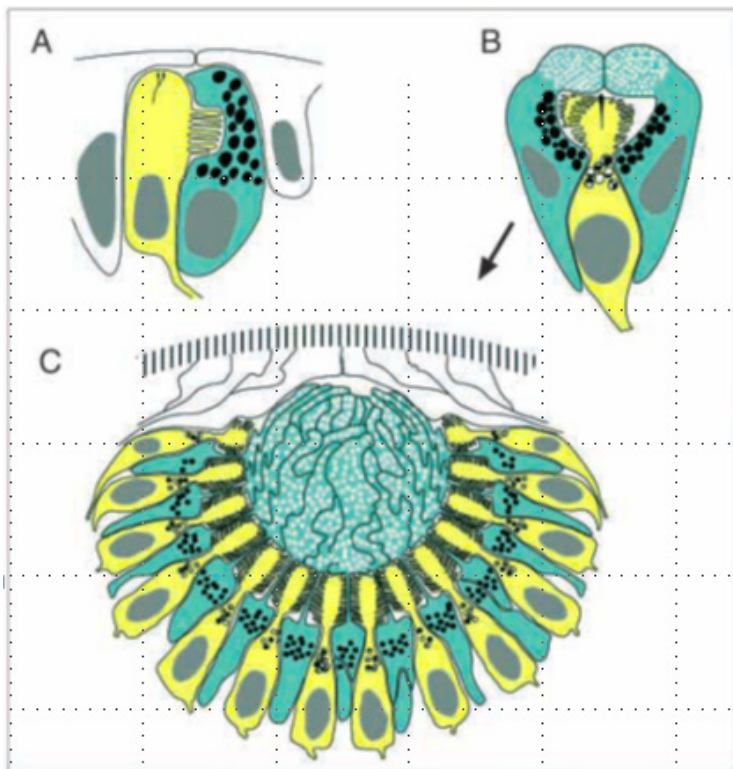**Fig.1.4:** Ultrastructure of PCRs (yellow) showing the inverse type (A), represented in larval eyes, and the everse type (B); C sows how PRCs, pigment cells (green) and support cells are combined to the adult eye. Taken from [8]

At 53hpf adult eyes appear [8] next to the larval eyes then migrate to the dorsal side of the head and split in two pairs till 72hpf. Consisting of pigment-, support- and photoreceptor cells

the eyes grow by mitotic division of the individual cell types. During maturation metamorphosis they are enlarged by cell growth. The adult eyes of *Platynereis* have multicellular layout and are not a derivate of the larval eyes. Because their photosensitive side faces away from pigment cells, they are called everse. In contrast to the inverse larval eyes where the photoreceptors look towards the pigment cells which form an invagination. But it is believed that the adult eye changes its layout during ontogeny. [9] Support cells could work as a lens, as they make up the vitreous body or inner mass, but for proof its refractory index still has to be measured. *Platynereis* is also able to change the size of the eyecup-opening, the aperture, and adapt to brightness. [10] Recently non-visual *Pdu-r-opsin* expressing cells apart from the eye [12] have been found.

1.4.2 CILIARY PHOTORECEPTORS

With antibody staining of acetylated tubulin a paired ciliated structure in dorsal *Platynereis* brain becomes visible. A positive *in situ* hybridisation with probes for *Pdu-c-opsin* shows that this region is photoreceptive. In the adjacent area of those PRCs are cells which express several clock genes and they also contain an oscillator, which leads to the assumption that they function at the input stage of a clock [1], although it stays unclear whether it is the circadian clock or the circalunar clock. These characteristics can be compared to the vertebrate sub-mammalian pinealocytes which also have an oscillator entrained directly by *c-opsin* sensed light changes. It should be mentioned that recent literature focuses more on the evolution of ciliary PRCs from a simple non visual light sensor to the complex vertebrate eye. Most likely the two types of opsins evolved in parallel after gene duplication in a common ancestor and r-opsin lost its function and is rarely present in vertebrates [11].

1.5. BIOLOGICAL RHYTHM

Biological rhythms are biological or physiological changes that come in cyclic patterns and this is studied in the research field of chronobiology. Biological rhythms can be found in many different species from specialised eukaryotes, with changes in intervals of seconds in extreme cases, down to prokaryotes, which despite their multiple divisions per day can still measure a twenty-four hour cycle [13]. The rhythms are defined by their periods, not by their frequency, and are categorized by their length. If the period lasts longer than 24 hours, it is called an "infradial" rhythm, such as "circannular" rhythm that shows yearly periods, or as the "circalunar" rhythm that has a period of 29,5 days and resembles the moon cycle, or the by tidal changes entrained 14,8 day lasting "circatidal" rhythm. An "ultradial" rhythm is shorter than one day and can reflect short sleeping periods or the defecation behaviour of *C.elegans* lasting only 45 seconds [14] or even shorter like the approximately one second long human cardiac cycle. The most studied and prominent is the circadian rhythm with a period length of about one day. That this rhythm is regulated endogenously is indicated by the fact that it is

not exactly 24 hours long. Is it in phase with the meteorological day, it is called "diurnal", in contrast to the inverted "nocturnal" rhythm. Another feature used for describing biological rhythm is the amplitude, it should be high enough to give good output and not dampen during oscillation. Amplitudes of different cycles can be compared to determine which one is the more dominant rhythm and overdrives the other [15]. The third feature of a rhythm, the phase, is determined by an external cue called "zeitgeber" (environmental changes like e.g. temperature shifts). The internal biological cycles are compared to their zeitgeber-cycle to see if they are synchronous or shifted in their oscillation. If the zeitgeber signal is missing, but oscillation is still running with the same period, it is called a "free running" rhythm. "Entrainment" is the process which leads to the synchronisation of environmental cycle with the endogenous biological clock.

So period, phase and amplitude only describe an oscillation, but reveal nothing about its causes. The underlying mechanism which connects the free-running internal rhythms of an organism with the rhythms of the environment is called "biological clock". The very frequent biological rhythms which do not connect to an environmental rhythm (e.g. cell cycle) are not considered as clock. The most prominent zeitgeber for the circadian rhythm is photoperiodicity and is marked by light and dark periods, labelled as "LD" and with the ratio of light to dark (e.g. 16:8). Constant conditions are termed "LL" for permanent light and "DD" for permanent darkness.
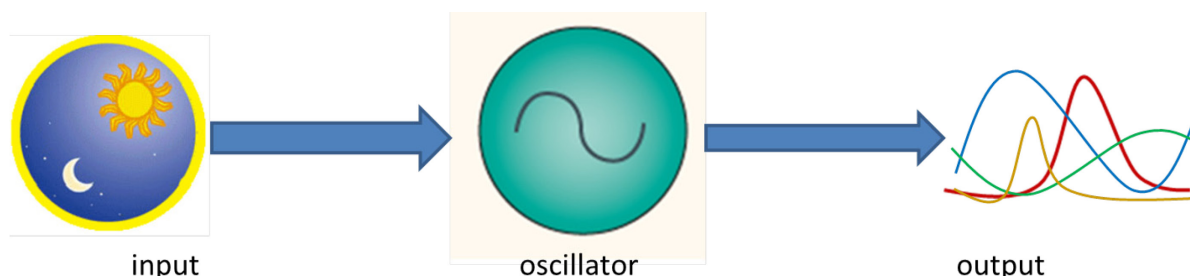
Fig.1.5: The three elements of a biological clock. Signal transduction happens from left to right. Input is given by a zeitgeber (sun, moon) to the oscillator where the signal is modulated to an instructive signal for the output pathway (curves indicating gene expression patterns).

The biological clock consists of three elements [16]. At the input stage receptors recognize stimuli from the environment and pass this information on to the oscillator stage. The oscillator is synchronised with the zeitgeber cycle and regulates the output pathways of the last stage, which lead to certain behavioural, metabolic and physiological changes. Manipulations at different stages of the clock give different answers, for instance if the first or second stage is interfered, one gets information about how the clock works. But interfering at the output level leads to insights of its biological function. The adjustment to periodic

environmental events by their expectancy of an organism is allowed by the biological clock through comparison of external cues to an internal mechanism.

1.5.1. THE INPUT STAGE

Due to some factors (relocation; seasonal changes) resetting of the endogenous clock can become necessary, this is performed by the input stage, which recognises those changes and is linked to the oscillator to keep it in phase with the zeitgeber. Without the ability to adapt to changes in the zeitgeber rhythm the clock would be a permanently free running system. There are a lot recurrent stimuli in the environment especially in a geophysical manner (lunar, tidal, solar…), which can lead to a strong and robust endogenous oscillation (=entrainment). For entraining an oscillator the stimulus has to be sensed and translated to an instructive signal by a receptor in a way that it can cause a suitable physiological reaction of the cell and so trigger the input pathway. As pointed out before one of the strongest periodic environmental stimuli is the daily light cycle and by being recognised by photoreceptors it entrains the most prominent clock, the circadian clock. Because of second messengers in the signal transduction cascade, weak light signals get enhanced and therefore give a strong enough stimulus to the oscillator [17, 18].

In mammals light cycles are recepted by retinal ganglion cells, a small cell population in the retina which expresses melanopsin (an r-opsin) as chromophor [19], although their loss can be compensated by rods, a ciliary type PRC in the retina. The exact pathway is unknown, but it is thought that they use the phospholipase C pathway like rhabdomeric cells [4], to release glutamate and PACAP [20] upon neurons of the suprachiasmatic nucleus (SCN). Those neurons get excitatory by depolarization and trigger the downstream pathways, mostly chromatin remodelling mechanisms, thus making them to the cellular substrate of the pacemaker.

In insects light entrainment is mostly received by the compound eyes, but there also photoreceptors apart from the eye, like the ocelli, chryptochrome or Hofbauer-Buchner eyelet of *Drosophila*. [21] Decapitated flies still can be entrained to the circadian rhythm [22]. So do mutants of the phospholipase C encoding gene *norpA* which can be considered blind. Only when all receptors are eliminated (*glass mutation*) and cry is mutated, Drosophila cannot entrain to a light-dark cycle. [23]

It should be mentioned that there is a gateway mechanism, so an input cannot always re-entrain an oscillator, mostly depending on its phase in which this stimulus is given.

1.5.2. THE OSCILLATOR STAGE

An oscillator runs independent from an input in order to create a self-sustained rhythmicity. Only if an internal rhythm correlates with an external one, fitness is increased [24]. If there is more than one oscillator active, the dominant one is called "pacemaker" or "master clock". The *Goodwin oscillator* is a model often used to explain the function of a basal oscillator [25]. Time delay, negative feedback, a balance of the time scales and non-linear kinetics of the involved reactions are the four elements needed to establish rhythmicity instead of homeostasis [26]. Time delay ensures an oscillating pattern by preventing steady-state equilibrium of a reaction. Negative feedback resets signals to their start or initial state. Non-linearity of reactions is needed for curve fitting in order to get a better oscillation. Balance of time scales stabilizes the other elements. All these features are present in the circadian clock. Its oscillation is characterized as amplified negative feedback and delayed negative feedback. The element X3 inhibits X1 giving the negative feedback. On the other hand X1 enhances X3 via induction of X2; this is why it is called amplified. The time delay is a result of protein synthesis and transport. Non-linearity emerges from enzyme activities (kinases, phosphatases) and oligomerisation of the components. The balance of time/speed relies on every step happening at the right velocity [26].
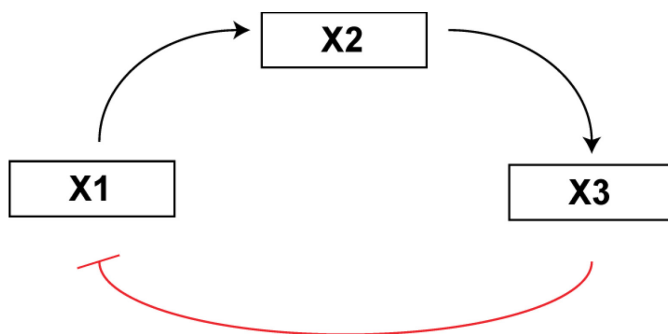
Fig.1.6: Schematic view of the dynamics of a *Goodwin oscillator*. X1 induces X2 which induces X3 and that inhibits X1.

When experimental data about circadian clocks of any species are reduced to their basic elements, the *Goodwin oscillator* gives good explanation of how they work. So called "positive elements" are represented by X1 and X2 and X3 is regarded as "negative element". Those elements underlie the regulation of the input stage, which can induce (mammals) or destabilize (Drosophila) them (in both cases negative elements are affected). The transcriptional or posttranslational regulated "clock genes" encode the physical representatives of the elements (X1-3).

1.5.3. THE OUTPUT STAGE

This stage also must have a periodic manner, although an exact definition does not exist. So called "clock controlled genes" are closely linked to the oscillator by for instance sharing promoter motives. Alternatively it also can be regulated by slave oscillators or gene-induction by intermediates (D-element binding proteins). Genes of the output stage are

expressed in a cyclic manner ad lead to behavioural (sleep, locomotion…) and physiological (blood pressure) changes. Among the thousands of genes appearing in rhythms [27, 28] (about 10% of mammalian m-RNAs show circadian rhythmicity [29]), the most prominent ones are PDF and melatonin. This vast number of output genes leads to the connection of chronobiology to many other disciplines in biology.

1.6. BEHAVIOURAL ANALYSIS AS A TOOL TO STUDY BIOLOGICAL RHYTHMS

Analysis of an organism's behaviour gives insights of how it reacts to a certain stimulus. This stimulus can be used for orientation, like light, gravity or even hydrostatic pressure. Also settlement only takes place, when conditions are as preferred. A suitable substrate for an organism can be determined by many characteristics, chemical ones seem to be the most important for some species though. Physical features (e.g. sand grain size) of a substrate are also criteria for settling down at a certain location. The mentioned examples only describe the reaction of an animal to a stimulus. If however this reaction comes in a periodic manner and also in absence of the stimulus, then this behaviour is controlled by an endogenous clock [revised in 30]. Determination of the period length gives hint about the type of clock employed (e.g. circadian clock with a period of about 24hrs). To understand more about the mechanisms working, other stages of the clock have to be manipulated. For instance to determine the entrainment factor by changing the zeitgeber itself (different light conditions, e.g. LL or DD) or eliminating the receptors, or manipulate clock genes (mutate, knock down) at the oscillator stage. As mentioned before output can be very variant, hence there is a huge number of possible investigations. The most prominent and therefore most studied rhythmic behaviours are sleeping, feeding and locomotion. If manipulation of one clock factor affects the rhythm, they give easily detectable changes in behaviour. Locomotion is studied in many different species, like the wheel-running behaviour of mice and wing-movement (flying respectively) in *Drosophila*. Because behaviour in Polychaetes is not as complex as in mammals and other vertebrates, they should make particularly useful subjects to study comparative behaviour [30]. In *Platynereis* one behaviour is already well investigated: the spawning behaviour. To make it easier to find a mating partner, they synchronize metamorphosis to the heteronereis by adapting to the moon cycle. The beginning of metamorphosis is linked to full moon, but by what exactly it is triggered is still unclear, and one or two weeks later the majority of the population spawns [30, 31]. The circadian behaviour of the atoke worm has also been investigated and termed nocturnal, because most of locomotion and feeding takes place at night, while during the day the worm mostly rests within its tube [31]. Summarizing one can say that behavioural studies give information about the output of a clock and the effect of its manipulation, but not really about the

underlying mechanism. In the early stages behaviour studies were done by simply watching the animal for a period and taking notes about their activity. This cannot be done for a very long time, so filming the animals soon was discovered as a helpful tool. When using an infrared sensitive camera, individuals can also be filmed in complete darkness, if they are not able to recognize infrared light. Films can last for several days or even weeks, but during analysis they are watched at higher velocity. Also software exists for scoring the activity of an individual, especially for *Drosophila*, allowing to analyse the behaviour for weeks. The results are visualized in so called actograms (fig.7), showing only one type of behaviour (feeding vs. not feeding; movement vs. no movement). Said simplified the period can be measured as the distance between two peaks of the actogram. It is calculated with programs using statistical approaches (Fourier; Lomb-Scargle), meaning the longer the observation the more robust the result gets, because more oscillations get included. These two features give enough results to interpret the effects of clock-gene manipulation on the behaviour (e.g. maximum of activity is not at the same time point anymore; or the period length is altered).
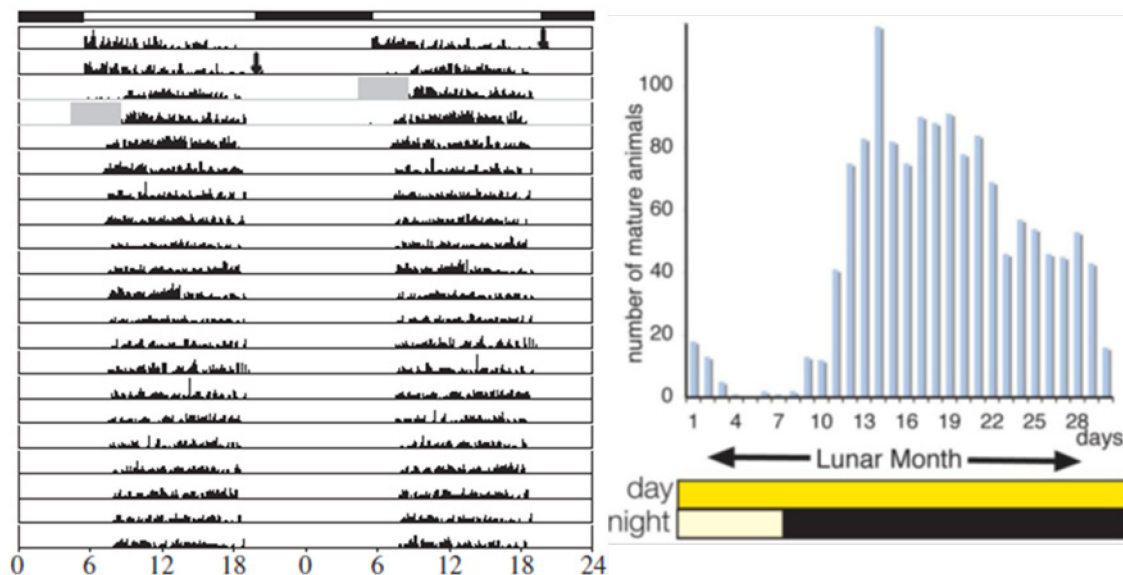
Fig.1.7: Example for an actogram of several days, each line represents a day, time is marked at the bottom, the black boxes at the top indicate dark periods (left, adopted from [44]). Spawning pattern of *Platynereis* (right): only few animals spawn when nocturnal light is given (full moon), maximum of spawning happens 9-14 days afterwards (from Zantke J., unpublished)

10

1.7. TALENS AS A TOOL TO INDUCE MUTATIONS

For engineering genes and chromosomes of higher eukaryotes site specific cleavage is a very useful method. Provocation of repair of the targeted locus can lead to genetic changes by gene disruption or gene editing. Transcription activator-like effector (TALE) proteins of the plant pathogen *Xanthomonas ssp.* naturally have the function of modulating host gene expression by entering the nucleus and bind to promoters and thus induce transcription [32]. The binding specificity is provided by 30-35 amino acid long tandem repeats followed by a 20 amino-acid long single truncated repeat and naturally there are 12-27 full repeats [33]. Within the tandem repeats there are two adjacent amino acids, called "repeat variable di-residue" (RVD), which specify the target by having different affinity to different nucleotides [34, 35]. As each RVD targets its base independently, TALEs can be redesigned to target a predicted sequence [34]. This modularity makes TALEs a very interesting tool for gene editing, because it allows simple assembly of new RVD combinations. They can be linked to the functional domain of *Fok1* restriction enzyme and used as site-specific endonucleases [36, 37]. Sequence of each repeat is cloned into a plasmid and via Golden Gate cloning assembled in one vector. This method uses Type IIS restriction enzymes, which create a 4bp overhang apart from the actual recognition site. In the same reaction mixture also ligation takes place, because it eliminates the original recognition site [38]. The design of TALEN sequences can be done with software freely available on the internet [39].

Fig.1.8: Structure of a naturally occurring Tal effector (a) and TALEN (b). Two monomeric TALENs are needed to bind at a target site and allow Fok1 to cleave. Taken from [39]

1.8. TRANSGENESIS AS A TOOL TO STUDY GENE FUNCTION

Transgenesis means a foreign gene is brought into an organism to provide it with a new property or to find out the function and/or location of either that gene or a co-introduced host gene. If the latter is the case the introduced gene is mostly a chromophor or a fluorophor (GFP, m-cherry) and used for locating host gene expression on protein level, it is then termed reporter-gene. One advantage of transgenesis is that it is passed on from generation to generation, thus creating a strain and once gene-introduction is successful it is no longer necessary. One of simpler methods to introduce two or more tagged genes into a eukaryotic organism is via the bacterial artificial chromosome (BAC). The BAC can, if not integrated into a host chromosome, exist as an independent molecule within the cell. But more often used is introduction of genes with plasmids as vector. Here the exogenous gene has to be integrated in the host genome. This can be facilitated by simply co-introducing a meganuclease (Sce1) or a transposase (Tol2, Mos1), when the vector also carries the target sequence. Another common use of transgenesis is to create inducible gene expression, meaning that genes can be switched on and/or off as desired. One prominent example for this is beta-galactosidase, which only works with the presence of IPTG that binds and releases the repressor of the promoter [40]. On other approaches inducible cell death is used to investigate gene function by killing specific cell-populations (ablation). This happens via introduction of a "suicide-gene", which does not lead to immediate cell death. *Nitroreductase* (NTR) for example is permanently expressed in a host cell, but only in presence of a chemical like metronidazole (MTZ) it becomes cytotoxic, hence it cleaves MTZ to its poisonous components [41]. Combined with a reporter like EGFP *nitroreductase* mediated ablation [42] makes a very good tool to inquire the function of a certain cell type (e.g. rhabdomeric PRCs). Cell death can be easily controlled by lack of EGFP-expression.

Amongst all the techniques (viral vectors, electroporation…) to transport foreign material into a cell, microinjection is the most popular, because of its high efficiency in terms of cell damage, cell waste, cell viability, specificity or effectiveness of delivering molecules. Hereby a liquid containing for instance plasmids is injected into a cell by using a very thin glass needle.

1.9. AIMS OF STUDY

The goal of my work was to understand if and how the two different photoreceptors contribute to entrainment of the circadian and/or circalunar clock of *Platynereis dumerilii.* Therefore I tried to create a transgenic reporter line for c-opsin1 to locate ciliary type PRCs and to study their function. C-opsin1 expression was found near clock gene expressing cells hinting an involvement in entraining a biological clock. Further I tried to create c-opsin1 mutants to help establishing a protocol for TALEN induced mutagenesis in *Platynereis* and

12

later on, if the permanent loss of this photoreceptor affects the circadian or circalunar rhythmicity of the organism. The second big part of this thesis is to use and optimize a newly established behavioural assay to record and visualize the output stage of the circadian rhythm of *Platynereis* and then study the effects of various light treatments (LL,DD) during different moon phases (NM, FM, FR-FM) and the effects of r-opsin ablation on the circadian behaviour.

2. Materials and methods

2.0.1 Equipment
APPLIED BIOSYSTEMS Step-One-Plus cycler

VWR Quattro cycler

LEICA LMD 6500

ZEISS Stemi 2000

ZEISS Axioplan 2

ZEISS LSM-510 Meta

HANNA INSTRUMENTS pH210 Microprocessor pH meter

HERAEUS PICO 17 centrifuge

INVITROGEN Safe Imager blue-light transilluminator

BIORAD Mini PROTEAN Tetra cell

Chameleon™ USB 2.0 digital video camera

ROSCHWEGE GmbH infrared light LED array (990 nm)

2.0.2 Software
CLC Main Workbench 6.6.2, CLC Bio

Axiovision 40 V4.8.1.0, ZEISS

Graph Pad Prism 6

Image J 1.46 and ActogramJ as plug in

Excel 2010, Microsoft

2.0.3 Kits
AMBION mMessage mMachine SP6 transcription kit

PROMEGA pGEM-T Easy Vector System

PROMEGA TnT **Sp6** Quick Coupled Transcription/Translation System

QIAGEN QIAquick PCR Purification Kit

QIAGEN Plasmid Maxi Kit

QIAGEN QIAfilter Plasmid Maxi Kit

QIAGEN MinElute Gel extraction kit

QIAGEN RNeasy Mini kit

THERMO Super Signal West Pico Chemiluminescent Substrate (Prod# 34080)

2.0.4 MARKERS
2log DNA ladder (NEB)

PageRuler Prestained Protein ladder (THERMO Scientific)

2.0.5 MEDIA
SOC: 10mM NaCl; 20g Trypton; 5g Yeast extract; 2,5mM KCl; 10mM $MgCl_2$; 10mM $MgSO_4$; 20mM Glucose

LB: 20g NaCL; 20g Trypton; 10g Yeast extract; fill up to 2L with ddH_2O, autoclave

LB+AMP: LB + ampicillin (50mg/ml)

LB+Spec: LB+spectinomycin (

LB agar: 7,5g agar powder to 500ml LB, autoclave

2.0.6 PRIMERS

Primers	Sequence	Target gene
cOps1_exon1_TnT_fw	GTTCCAGAAAATGCCAGTTGAGGGCTTATTGGCA	c-opsin1
cOps1_exon1_TnT_rev	GTGACAACCTGCCCCTGTGACAACCAGCCCCGGT	c-opsin1
HRM-copsin1-exon1-fw	CAAGACCTACCTCCCAAATAAGT	c-opsin1
HRM-copsin1-exon1-rev	CTTGCCCTTGGTGATCTCTTAG	c-opsin1
c-opsin1-Genomic-up1	ATAGTGATCCAATGACACTGCGTGGG	c-opsin1+EGFP
c-opsin1-Genomic-lo1	ATGAGATATTGCCACAAGGAGGCTC	c-opsin1+EGFP
C1-exon1-TT1-fw	GGTGGTCCAATCGAAGACACTGTACTG	c-opsin1
C1-exon1-TT1-rev	GGAGCACCTGCCTGCCACTTTATG	c-opsin1
C1-exon1-TT2-fw	CCGTCGTGTGGACTCTCCCCTATG	c-opsin1
C1-exon1-TT2-rev	GACAACCTGCCCCGAAAGTGTGAC	c-opsin1
cOps2_exon1_TnT_fw	GCCTATTCGCCAGTGTTGTGATTCTATTAGCAT	c-opsin2
cOps2_exon1_TnT_rev	CTGTTGATTTTCTTTCCACATTATCTATATTTC	c-opsin2
PCR8-F1	TTGATGCCTGGCAGTTCCCT	pFus vector
PCR8-R1	CGAACCGAACAGGCTTATGT	pFus vector
TAL-F1	TTGGCGTCGGCAAACAGTGG	pTALvector
TAL-R1	GGCGACGAGGTGGTCGTTGG	pTALvector
EGFP-fw	GGTGAGCAAGGGCGAGGAGC	EGFP
EGFP-rev	CTCGTCCATGCCGAGAGTGATCC	EGFP

2.0.7 VECTORS AND BACTERIA
One Shot Top10 cells (INVITROGEN)

pGEM-T easy (PROMEGA)

2.0.8 ANIMAL CULTURE

Worms are kept in translucent plastic boxes at 18°C. The usual light regime is 16hrs artificial light and 8hrs of complete darkness. To mimic full moon a dim light source (1-10 lux) is switched on during the usual dark period for a week, following three weeks of normal light dark cycle. For practical reasons two culture rooms are established with a 14 day difference between their full moon periods (termed inphase, because corresponds to actual moon cycle, and outphase). Animals are fed twice a week with spinach leaves and algae (platymonas). (Established by Hauenschild [35, 53, 54, 55]) During my experiments I did not distinguish between the two rooms, but I tried to use mostly the B321-strain (also called VIO, PIN or ORA).

2.1. LABELLING C-OPSIN1 EXPRESSING CELLS

2.1.1 ANTIBODY STAINING
Used chemicals:
2x Laemmli buffer: 4% SDS; 20% glycerol;10% 2-mercaptoethanol; 0.004% bromphenol blue; 0.125 M Tris HCl

RIPA buffer: 20 mM Tris-HCl (pH 7.5); 150 mM NaCl; 1 mM ; Na_2EDTA; 1 mM EGTA; 1% NP-40; 1% sodium deoxycholate; 2.5 mM sodium pyrophosphate; 1 mM b-glycerophosphate; 1 mM Na_3VO_4; 1 µg/ml leupeptin

SDS PAGE running buffer: 250mM Tris; 1,9M Glycine; 35mM SDS

Western blot transfer buffer: 0,19M Glycine; 25mM Tris; 10% Methanol

10x PBS: 70g NaCl, 62,4g $Na_2HPO_4.2H_2O$, 3,4g KH_2PO_4, pH7.4

1x PTW: 1xPBS; 0.1% Tween

Bouin's fixative: picric acid: 35%-formaldehyde: acetic acid = 15:5:1

NSW: natural sea water (from North Sea)

Fixation
3-10 day old larvae (or from stage desired) are collected with a nylon-net and washed with NSW. Then they are transferred to a 2ml Eppendorf tube by rinsing them out of the filter with the fixative.

Bouin's fixative fixation: let embryos sink for one minute and remove supernatant. 1-2ml Bouin's solution are added and the tube is shaken (Belly Dancer) for 30 min. The supernatant is removed and larvae are washed 5 times with PTW (1-2-5-10-20min). PTW is replaced again and larvae can be stored at 4°C

PFA fixation: After transfer embryos are incubated in 4% PFA for 90min (or o/n at 4°C) shaking. Afterwards supernatant is removed and larvae are washed as described above and stored in PTW at 4°C.

Proteinase digestion and blocking

All steps are done at room temperature. Fixed larvae are transferred to a nylon filter and submerged into 40ml of PTW in an old tip-box. They are washed by shaking two times for five minutes. Then the nets are transferred to a Proteinase K solution (160µl of stock in 40ml PTW) and incubated without shaking. Time of incubation depends on stage of embryos (see table). Rinse the embryos 2 times for 1 min in freshly prepared 20mg/ml glycine/PTW and then wash them 5 times (1-2-5-10-20min) with PTW shaking (put boxes on thermoblock). Larvae are transferred to Eppendorf tubes, supernatant is removed and replaced with 5% sheep serum in PTW and incubated for 1 hour gently shaking (400 rpm).

Stage	Time of Proteinase K digestion
16h-72h	45sec-1min
72h-5d	2min
1 week to 6 weeks	3min

Staining with antibodies

Provided antibody solutions (anti c-opsin1 rat serum) are diluted as desired with 2.5%. Animals are suspended and divided into different tubes (depending on number of antibodies or dilutions), let sink and supernatant is removed. 200µl of 1st antibody solution is added and incubated for an hour at RT (or overnight at 4°C). Antibody solution is removed (can be re-used up to 3 times) and immediately replaced with PTW. Larvae are washed 5 times with PTW for 10 minutes. During last wash 2nd AB-solution (Alexa488-α-rat, 1:200) is prepared and covered with tinfoil. PTW is fully removed from the larvae and 200µl of 2nd AB-solution added and incubated for 1h at RT or o/n at 4°C. After removal of 2nd AB-solution embryos are washed again 5 times 10min covered with tinfoil and then transferred to a six well dish. The water should be removed completely and DABCO-glycerol is poured over the larvae. Now dishes can be stored at 4°C wrapped up in tinfoil.

2.1.2 WESTERN BLOT
Protein extraction

A batch is collected and washed with NSW in a nylon-net and then transferred to a tube and water is removed completely. It now can be shock-frozen at -80°C or protein extraction is started immediately. One tablet of Complete Mini proteinase inhibitor is dissolved in 7ml of RIPA buffer. 150µl of this solution is added to the batch, which is then homogenized with a

syringe (1ml, ø 0.4mm). Samples are centrifuged at 4000xg for 10 min and supernatant is transferred to a fresh tube and mixed with same amount of 2x Laemmli buffer. This is then boiled at 95°C for 5-10min and then samples can be stored at -80°C.

SDS-PAGE and Western Blot

10% Acrylamide gel is casted (total volume of 10ml, sufficient for 2-3 gels):

Resolving gel:
3.3ml   30% Acrylamide
2.5ml   1,5M Tris/HCl p.H:8.8
4ml   water
100µl   10% SDS
100µl   10% APS
4µl   TEMED

Stacking gel:
660µl   30% Acrylamide
500µl   1M Tris/HCl p.H.6.8
2.8ml   water
40µl   10% SDS
40µl   10% APS
4µl   TEMED

Gels are poured between two glass plates and are 1mm thick. 15µl of sample (preheated at 95°C) and 5µl of PAGE-Ruler marker is loaded on the gel. Gels are put in running chamber and run at 200V/0.12A until blue marker lane almost reaches bottom. Then gels are 15min washed in transfer buffer. PUDF membrane is activated in Methanol for 5min, shortly rinsed in water and also washed 15min in transfer buffer. Gel is placed on membrane between Whatman-paper that is soaked up with transfer buffer. When there are no more air bubbles in between the layers, the blotting machine is turned on and run for 45-60min at 12V/0.4A. When the marker is clearly visible on membrane the transfer is stopped and the membrane is blocked for 2 hours in 5% milk-powder in PTW. Antibodies (rat serum) are diluted as wished in 2.5% milk/PTW and incubated on the membrane overnight at 4°C. The next day the blot is washed with PTW for 1 hour, PTW is exchanged several times. Membrane is incubated for 1.5 hours at room temperature (or O/N at 4°C) in 2nd antibody solution (anti-rat-HRP; 1:10000) and washed again in PTW for at least 1 hour.

For imaging the membrane is put in the peroxide/enhancer solution (THERMO Super Signal West Pico Chemiluminescent Substrate kit) and incubated for about 90sec. Imaging itself happens in complete darkness and is done on a photosensitive film.

2.1.3 CREATION OF A C-OPSIN1-EGFP-NTR TRANSGENIC ANIMALS
Vectors:

pMosSce{Nvi_c-opsin1::EGFP::NTR}

pTol2Sce{Nvi_c-opsin1::EGFP::NTR}

Microinjection:

Plasmid carrying c-opsin1 tagged with EGFP are injected along with the corresponding transposase mRNA into zygotes, as described in TALEN chapter (2.2.4).

Injection mix:

3µg	plasmid DNA
3µg	transposase mRNA
3µl	TRITC-dextran
To15µl	dH_2O

Zygotes are checked under the Lumar for red TRITC-signal, to verify successful injection. Two or three day old larvae can be checked for GFP signal. pMos injected animals are not very likely to show GFP expression in F_0. Worms are raised in culture and when 2-3 months old, they can be tail clipped and screened for GFP in their genome via PCR.

Reaction mix:		PCR-program:		
1µl	gDNA	95°C	5min	
1µl	dNTPs (10mM)	95°C	20sec	
2.5µl	primer EGFP-fw (5µM)	62°C	30sec	30cycles
2.5µl	primer EGFP-rev (5µM)	72°C	1min	
2.5µl	HotStar buffer (10x)	72°C	10min	
0.25µl	HotStar Taq	10°C	hold	
To 25µl	dH_2O			

Alternatively the same PCR was done with c-opsin1-genomic-up1/lo1 primers which bind in the c-opsin1 sequence adjacent to the EGFP-region.

When injected animals mature, they are mated with a partner of any strain (preferred B321). 3-10 day old larvae of the batch are screened under the microscope for GFP expression.

Mounting and microscopy of larvae:

To provide a suitable space between microscope slide and coverslip two or three layers of scotch tape are attached in a way that they form a chamber in the middle. Larvae are incubated in a 1:1 mix of 0.7M $MgCl_2$ solution and NSW. This paralysing solution inhibits neuromuscular transmission and thus movement of the animals. But long exposure or high concentrations of paralysing solution may lead to death of worms. Larvae have been mounted with their dorsal side up and checked at 20x magnification for EGFP signal. The

exposure time to the light beam was as short as possible. After screening the larvae have been released into the cup again.

2.2. CREATION OF TALEN INDUCED C-OPSIN1 MUTANTS

2.2.0 USED CHEMICALS

50xTAE: 242g Tris base; 57,1ml acetic acid; 100ml 0.5M EDTA, pH 8.5

Agarose gel: if not stated differently 1% (m/v) agarose in 1xTAE

NSW: natural sea water (from North Sea)

Mini-prep:

P1: 10mM Glucose; 25mM Tris (pH 8.0); 10mM EDTA (pH 8.0); 100mg RNase A in 1l buffer

P2: 0.2M NaOH; 1% SDS

P3: 3M Potassium acetate; 11.5% glacial acetic acid; in water

2.2.1 TALEN CONSTRUCTION

TALEN sites are predicted with the online software: http://boglabx.plp.iastate.edu/TALENT/ and found regions are checked for SNPs in them. Preferred features for selection are no SNPs in target site, whole region is in exon, and site is within a sequence encoding a crucial protein domain or is near the 5' end of gene. For later analysis and screening for mutants *in vivo* it is recommended to have a unique restriction site in the spacer region between the binding sites of a TALEN pair. Reaction lists can be created using the *Golden Gate TAL assembly tool* found on https://tale-nt.cac.cornell.edu/protocols.

TALEN name	Target sequence	RVD sequence
c-opsin1 TALENS		
c1-TAL-L1	TCTTCTTCTCTGAGTA	HD NG NG HD NG NG HD NG HD NG NN NI NN NG NI
c1-TAL-R1	GATGATAAACTTGAAA	NG NG HD NI NI NN NG NG NG NI NG HD NI NG HD
c1-TAL-L2	GATGATAAACTTGAAA	NG HD HD NI NI NI NN NI HD NN NN HD NG NI HD
c1-TAL-R2	GTCTTTGATGAAAAGA	HD HD NG NN NG NI HD NN HG HD NI NN NG HD NG
c1-TAL-L3	TTCCAAAGACGGCTAC	NI HD NI NN NN NI NI NI NG NG NI NG NG NI NN
c1-TAL-R3	AGACTGACGTACAGGA	HD NG NG NG NG HD NI NG HD NI NI NI NN NI HD
c1-TAL-ZL1	TAAAAAATTAATCCTCCTT GC	NI NI NI NI NI NI NG NG NI NI NG HD HD NG HD HD NG NG NN HD
c1-TAL-ZR1	AAATGCTGAATGGCCA	NN NN HD HD NI NG NG HD NI NN HD NN NG NG NG
c1-TAL-ZL2	TTTGCGGTCACACTGC	NG NG NN HD NN NN NG HD NI HD NI HD NG NN HD
c1-TAL-ZR2	AGCCTCGTCCGCCACA	NN NG NN NN HD NN NN NI HD NN NI NN NN HD NG
c-opsin2 TALENs		
c2-TAL-L1	AGCCTCGTCCGCCACA	NN NN NN HD HD NG NI HD NI NG HD HD NG NI NG
c2-TAL-R1	ACCTGTGTATAGTGGG	HD HD HD NI HD NG NI NG NI HD NI HD NI NN NN NG
c2-TAL-R2	ATAGTGGGAGTGATTGGG	HD HD HD NI NI NG HD NI HD NG HD HD HD NI HD NG

		NI NG
c2-TAL-R3	ATAGTGGGAGTGATTGGG AC	NN NG HD HD HD NI NI NG HD NI HD NG HD HD HD NI HD NG NI NG
c2-TAL-R4	ATAGTGGGAGTGATTGGG ACACT	NI NN NG NN NG HD HD HD NI NI NG HD NI HD NG HD HD HD NI HD NG NI NG

All reagents are thawed and pipetted together on ice as directed (see fig.2.1), but a master mix of all enzymes and buffers is made and added last.

☐ **Reaction A**

☐	4 µl	Water
☐	1 µl	pFUS-A
☐	1 µl	Bsal
☐	1 µl	BSA 2mg/ml
☐	1 µl	Quick Ligase
☐	2 µl	10x Ligase Buffer
☐	1 µl	NG 1
☐	1 µl	NG 2
☐	1 µl	NN 3
☐	1 µl	HD 4
☐	1 µl	NN 5
☐	1 µl	NN 6
☐	1 µl	NG 7
☐	1 µl	HD 8
☐	1 µl	NI 9
☐	1 µl	HD 10
	20 µl	

☐ **Reaction B**

☐	10 µl	Water
☐	1 µl	pFus-B - 4
☐	1 µl	Bsal
☐	1 µl	BSA 2mg/ml
☐	1 µl	Quick Ligase
☐	2 µl	10X Ligase Buffer
☐	1 µl	NI 1
☐	1 µl	HD 2
☐	1 µl	NG 3
☐	1 µl	NN 4
☐	0 µl	
☐	0 µl	
☐	0 µl	
☐	0 µl	
☐	0 µl	
☐	0 µl	
	20 µl	

Fig.2.1: example for a reaction mix for a 15-RVD long TALEN. If the RVD sequence is longer that 20 pFus-A vector is replaced with pFus-A30A and pFus-A30B, resulting in three reactions. Concentration of pFus vectors is 75ng/µl and of RVD plasmids it is 150ng/µl. T4-ligase was used instead of Quick Ligase.

Reactions are incubated in the PCR amchine using following program:

37°C 5min ⎤
16°C 10min ⎦ 10 cycles
50°C   5min (in 2nd ligation cycle this step is changed to 37°C for 15min)
80°C   5min
4°C   hold

Then digest with 1µl of Plasmid Safe Nuclease and 1µl of ATP solution for 1 hour at 37°C to get rid of linear DNA. 5µl of ligation reaction are transformed into One Shot Top 10 competent cells: cell aliquots are thawed on ice; the ligation mix is added and let on ice for 30 minutes. Cells are heatshocked for 30 seconds at 42°C and then immediately put back on

ice for some minutes. 250µl of SOC medium is added and cells are incubated for 1 hour at 37°C shaking. 50-100µl of bacteria are plated out on LB+Spectinomycin+X-Gal+IPTG plates (blue-with screen) and grown overnight at 37°C. Pick 5-10 colonies, dissolve them in 20µl water and perform colony PCR:

Reaction mix:	PCR-program:	
1.0µl dissolved bacteria	94°C 2min	
0.5µl FIRE Pol	94°C 30sec	
2.5µl 10X Buffer	55°C 30sec	35 cycles
1.0µl dNTPs(10mM)	72°C 1min45sec	
1.0µl pCR8_F1(5µM)	72°C 10min	
1.0µl pCR8_R1(5µM)	10°C hold	
1.5µl MgCl$_2$ (50mM)		
16.5µl Water		
25.0µl TOTAL		

PCR-products are run on a 1% agarose gel (~100V for ~40min). The expected band size is about 100bp per RVD. Positive colonies are inoculated in 3ml of LB+Spec medium and grown overnight at 37°C shaking for mini preps.

Mini prep:

2ml of overnight culture are transferred to a 2ml Eppendorf tube and spun down for one minute. Supernatant is removed completely and cells are re-suspended in 200µl P1 buffer (resuspension buffer). 200µl of buffer P2 (lysis buffer) are added and mixed well by inverting the tube. 200µl of buffer P3 (neutralisation buffer) are added and mixed by inverting the tube. Tubes are centrifuged for 10min at full speed (13000rpm) and supernatant is transferred to new 1.5ml Eppendorf tube. 500µl of Isopropanol are added and tubes are centrifuged again for 10min ant full speed. Supernatant is removed and discarded, while pellet is washed with 1ml of 70% Ethanol. Pellet is air-dried for several minutes and then dissolved in 30-50µl of dH$_2$O.

For confirmation of right insert size sequencing of plasmids or a test restriction can be done:

```
1µl          XbaI
1µl          AflII (BstBI)
1µl          Orange Fermentas Buffer
1-2 µg       of plasmid DNA
up to 10µl   water
```

Second cycle reaction is set up and incubated in the PCR machine:

1ul   pCS2TAL3-DD **or** pCS2TAL3-RR pDNA (75ng/µl)

22

1ul   last-repeat plasmid (150ng/µl)
1ul   of **each** pFUS plasmid (150ng/ul)
1ul   T4 ligase
2ul   T4 ligase buffer (10x)
1ul   Esp3I enzyme
to 20ul water

Ligation mixes are transformed in Top10 cells like described above and plated on LB+Ampicillin+X-Gal+IPTG plates and grown overnight at 37°C. 5-10 white colonies are picked and screened with colony-PCR.

Reaction mix:	PCR-program:
1.0µl dissolved bacteria	94°C 2min
0.5µl FIRE Pol	94°C 30sec ⎤
2.5µl 10X Buffer	55°C 30sec ⎬ 35 cycles
1.0µl dNTPs(10mM)	72°C 3min ⎦
1.0µl TAL_F1(5µM)	72°C 10min
1.0µl TAL_R1(5µM)	10°C hold
1.5µl MgCl$_2$ (50mM)	
16.5µl Water	
25.0µl TOTAL	

Positive colonies are inoculated in 3ml LB+Amp medium and grown overnight for mini preps (as above). The remaining culture is used for making a glycerol stock (spin down culture and re-suspend in 200µl fresh LB-medium; add 800µl of glycerol and mix by pipetting; freeze and store at -80°C). Plasmids are sequenced and checked with restriction.

1µg   pDNA
1µl   NEB4 buffer (10x)
1µl   StuI enzyme (NEB)
1µl   EcoRI-HF enzyme (NEB)
To 10µl   water

Restriction mix is incubated for at least 1hour at 37°C and run product on 1% agarose gel. To check sequences: generate plasmid sequence with the tool provided at https://tale-nt.cac.cornell.edu/protocols and compare with sequencing result of own plasmids.

2.2.2 TnT ASSAY- CHECKING THE FUNCTION OF TALENs *IN VITRO*

A DNA target for the TALENs is made by PCR (about 1kb long; cleavage site should not be in exact middle of DNA fragment). Three reactions are set up and run on a 1.5% agarose gel and eluted on the same column of QIAGEN MinElute gel extraction kit. Concentration is measured on *nanodrop* and estimated by band intensity on gel.

The PROMEGA TnT **Sp6** Quick Coupled Transcription/Translation System kit is used for this experiment. When DNA target is ready TnT reactions can be mixed together in 1.5ml Eppendorf tubes. Master-mix is added last.

1µg	left TALEN (1-2µl)
1µg	right TALEN (1-2µl)
225ng	target DNA (1-5µl)
To 9µl	dH$_2$O
1µl	Methionine (1mM)
40µl	Master mix

Reactions are mixed briefly by vortexing, shortly spun down and then incubated for 3 hours at 30°C.

For DNA clean up a Phenol-Chloroform-Precipitation is performed. Therefore TnT reactions are diluted with 150µl of nuclease-free or RNase-free water to a final volume of 200µl. For facilitation of phase separation transfer reactions to Phase Lock tubes (Eppendorf). 200µl of Phenol:Chloroform:Isoamyl alcohol (25:24:1) are added and mixed well with vortex. Reactions are centrifuged for 5min at 16000xg. Aqueous phase is transferred to new tube and this step is repeated with an equal amount of Phenol chloroform mix. Aqueous phase is transferred to a 1.5ml tube and 300µl of 100% Ethanol and 10µl of 3M Sodium-acetate (NaAc) are added. This mix is kept at -80°C for at least 30min or overnight at -20°C and then centrifuged for 15min at 16000xg in 4°C centrifuge. Supernatant is removed and pellet is washed with 500µl ice cold 70% Ethanol. The pellet is air dried for 10-15min and resuspended in 8µl NF-water. 2µl of RNase A/H solution (RNaseA/H from QUIAGEN Midi kit is diluted 1:1000 in P1) are added and left for 5min at room temperature. RNaseA/H is inactivated at 70°C for 5min. 2µl 6xDNA loading dye are added, reactions are loaded on a 1-2% agarose gel and run at 100V/0.18A for 1-1.5 hours. Gel is stained with Ethidium-bromide.
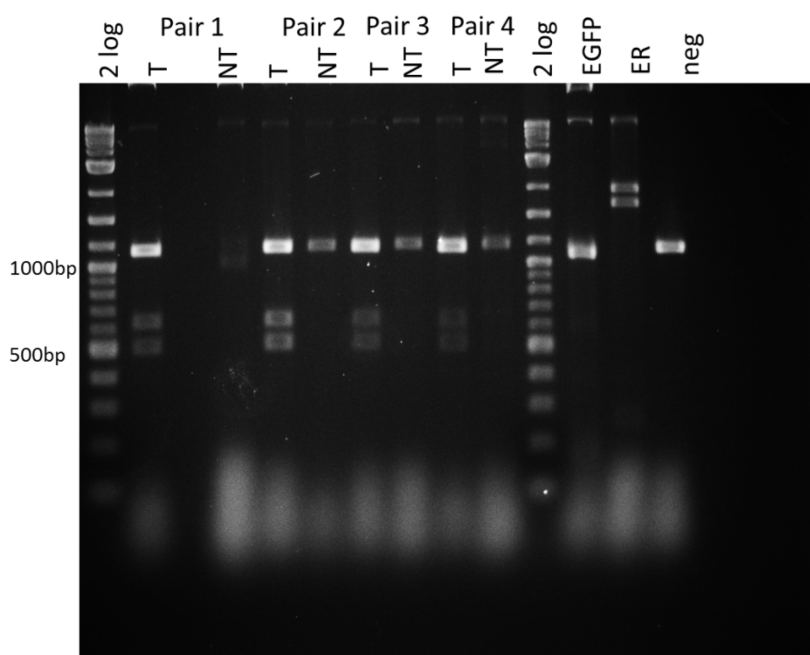

Fig.2.2: TnT assay of c-opsin2 TALENs. All four pairs cleave the target fragment (T) and specifity is proven by a non-target control (NT; c-opsin1). Target size: 1.1kb. Size of cleaved fragments: 600bp+500bp). Positive controls: EGFP and ER (estrogen receptor). Neg: negative control (target treated with Tnt master mix)

2.2.3 *IN VITRO* TRANSCRIPTION OF TALENS

Before the *in vitro* transcription the plasmid DNA has to be linearized.

2-5µg	pDNA
1µl	KpnI (NEB)
2µl	NEB buffer2 (10x)
To 20µl	water

Note: KpnI is used for pCS2-TAL3 vectors, for pCS2TAL3-DD (or -RR) vectors NotI is used.

Incubate for 1-1.5 hours at 37°C then load on 1%agarose gel and run at 120V for 40min.

TALextract DNA from gel with QUIAGEN kit and elute in 30µl water. For transcription mMessage mMachine SP6 kit is used.

6µl	linear plasmid DNA
2µl	10X Reaction buffer
10µl	2X NTP/CAP mix
2µl	Enzyme Mix

This mix is incubated for 3 hours at 37°C. Then 1µl of Turbo DNase is added and incubated for further 15min. RNA-purification is done with RNeasy kit from QUIAGEN. To get better concentrations several reactions per TALEN are made and cleaned up on the same column.

RNA samples should be aliquoted to a smaller volume (ideally for one injection) and diluted to desired injection concentration (e.g. 40ng/µl) and stored at -80°C.

2.2.4 MICROINJECTION OF TALENS

A fresh batch is set up by mating a couple of well and very actively swimming worms. They are put together in a cup and left there for several minutes so they can spawn. The male can be squeezed to induce egg-release of female. The batch is kept for 40-45min at 18°C. During that period make Injection mix in 1.5ml Eppendorf tube:

Xµl	mRNA of left TALEN (concentration at will)
Xµl	mRNA of right TALEN (concentration at will)
2-3µl	TRITC-dextran solution
To 10µl	water

The amount of mRNA from left TALEN and right TALEN should be the same. To get rid of larger particles of TRITC dye which may block the needle two things can be done. Either spin injection mix 5min at 10000rpm in 4°C centrifuge and try not to take liquid from the bottom of the tube, or put mix on Microspin-filter column and spin for 5min at full speed. Injection mix should always be kept on ice.

The batch is checked if it is fertilized and the jelly is removed. Eggs are transferred to a nylon filter and NSW is poured onto them 5-6 times to create current, which washes away the jelly, oocytes always have to be in water. Then eggs are digested in a ProteinaseK solution (75µl ProtK in 30ml NSW) for 30-35 sec. and washed 5-6 times with a cup of NSW. The so treated

oocytes are transferred to a six-well dish with a Pasteur-pipette. 200-300 eggs are picked up with a P10 pipette under binocular (only very healthy looking zygotes are chosen) and mounted on an already prepared injection-stage (fig.2.3) and injection-mix (~5µl) is transferred to needle (Eppendorf Femtotips II). As little liquid as possible is injected in the zygote, just as much that the droplet becomes visible. Successful injection is checked under the Lumar for red TRITC signal and glowing zygotes are separated in different well and kept at 18°C wrapped up in tinfoil for growing.
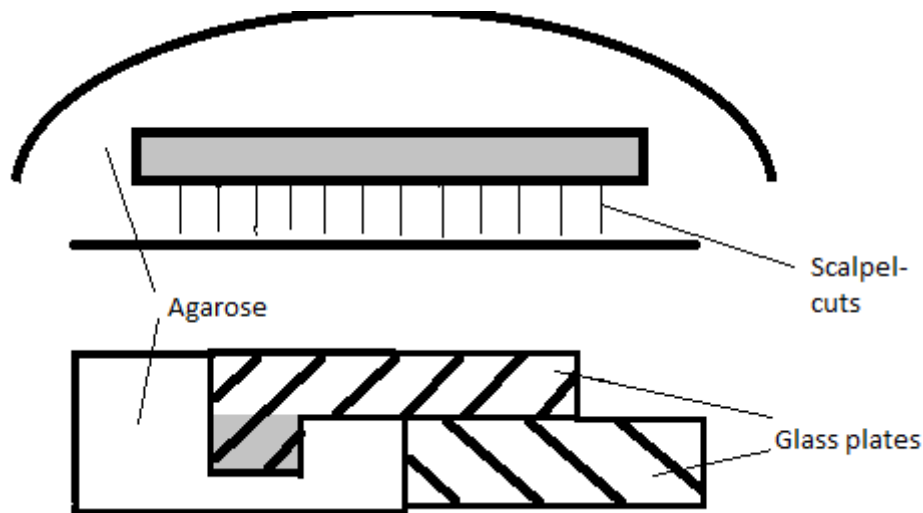

Fig.2.3: The Injection stage from above (top) and from side (bottom). A Plexiglas plate with a rim is laid upon a microscope slide in a way that they form a trough (grey area) in which the zygotes are laced. 1.5% agarose melted in NSW is poured around them at let dry. The glass plates are removed and scalpel cuts are made in the agarose, so that later on zygotes can be removed from the needle. This stage is placed on a small petri-dish and covered in NSW.
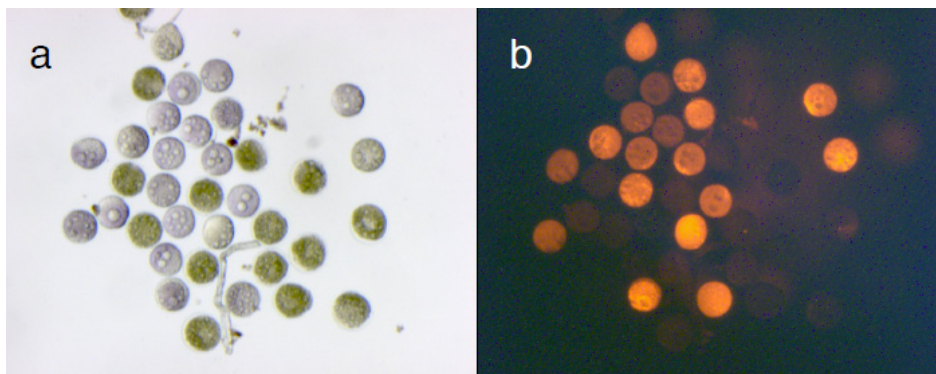

Fig.2.4: Injected embryos 2hpf under bright light (a) and under fluorescent light (b)

2.2.5 SCREENING INJECTED *PLATYNEREIS* FOR MUTATIONS

There are two possibilities to screen for mutations. Either let worms grow 2-3 months, tail-clip (last 15-20 segments are cut off) them, extract DNA and make restriction enzyme test, or take 1-2 day old embryos, digest them with ProtK and make restriction enzyme test.

For the first option:

App. 2cm long worms are taken and tail is cut off with a scalpel under the binocular and transferred to 1,5ml Eppendorf tube. Genomic DNA is extracted from tails with kit from MN. 180µl buffer T1 and 25µl Proteinase K are put onto tails and incubated for at least 3hrs at 56°C. Lysis is done by adding 200µl of preheated B3 buffer and incubation for 10min at 70°C. 210µl of abs. Ethanol are added mixed and whole sample is loaded on the spin-column. In between the next steps there is always a 1 minute centrifugation step at 11000xg. First wash column with 500µl BW buffer then with 650µl of B5 buffer. Silica membrane is dried by spinning 1 min without buffer. 50µl of preheated (70°C) elution buffer BE are dropped directly on membrane and let incubate 1 minute. At last column is put in a fresh 1.5ml tube and spun another minute and genomic DNA sample is ready.

Second option:

1-2dpf larvae are picked in 8µl of sea water and place in 0,2ml tube. Sea water is removed and replaced by 10µl digestion buffer (1µl ProtK+ 1µl 10x HotStar buffer+ 9µl water). This mix is incubated at 55°C for 2.5 hours the ProtK is inactivated for 10min at 95°C. If amount of injected/noninjected embryos is high, they can be pooled but volume of digestion buffer should be adjusted (10µl/embryo). 1µl of that lysate is used to set up PCR-reaction.

From now steps are the same for both options. Control samples are taken from un-injected embryos/worms, ideally from same batch. A PCR reaction is set up. Primers are chosen in a way that the product has the TAL-binding site not exactly in the middle or even better a second restriction site of the used enzyme is within the fragment, for controlling if enzyme did cut at all.

Reaction mix:		PCR-program:		
1µl	embryo lysate/ gDNA from tails	95°C	5min	
1.5µl	$MgCl_2$ (25mM)	95°C	20sec	
2.5µl	primer c1-exon1-TT1 fw (5µM)	63°C	1min	35 cycles
2.5µl	primer c1-exon1-TT1 rev (5µM)	72°C	1.5min	
2,5µl	10x Hot Star buffer	72°C	10min	
1µl	dNTPs (10mM)	10°C	hold	
0.25µl	HotStar Taq (QUIAGEN)			
To 25µl	water			

This PCR product is then digested with the restriction enzyme that cuts in the spacer region of the injected TALEN pair (see table).

5µl	PCR product
1µl	of appropriate enzyme buffer
1µl	enzyme
3µl	water

Digestions are incubated for 2 hours at 37°C and then heat deactivation (10min; 65°C) is done. The digested and undigested PCR-products are run on a 2-3% agarose gel. Gel is checked for uncut bands (in digested lanes) and band shifts (in undigested lanes) compared to the control samples.

As a second method for screening for mutations High Resolution Melt Analysis (HRM) can be performed, but due to many polymorphisms in the *Pdu* genome results were often not conclusive and so I am not going further into detail.

2.3. BEHAVIOUR STUDIES OF *PLATYNEREIS DUMERILII*

2.3.1 GENERAL EXPERIMENTAL SET UP

Prior to filming (1-2 days before start) the box (20x20cm) has to be cleaned (algae are removed) so that worms are clearly visible, or worms were transferred to a new box (10x10cm or 20x20cm). The number of animals in one box correlates with the size of the box. In 10x10cm boxes there are maximal ten worms, in 20x20cm boxes there are up to 25 animals. Because *Platynereis* tend to settle near edges, microscope-slides can be put in the box to provide more edges. Otherwise many worms settle in the corners of the box, which brings a higher density (nearby animals can influence each other's activity) and worms cannot be seen clearly. One day before the experiment starts worms are fed with spinach leaves, to prevent behaviour changes due to hunger or feeding (worms are much more active during day when (fresh) food is within the box). The box is placed on the infrared light array and so illuminated from beneath, to control light and temperature changes a HOBO device is laid next to it, and filmed from above. The camera (Chameleon™ USB 2.0 digital video camera) set up is placed in a black box, so worms can be put under completely independent light regimes (LL, DD, inverted…). The recording starts at late as possible in the evening. Analysis of the behaviour starts when light is switched on again the next day (7:00 a.m.). Up to 96hours (4 days) are analysed, 24 hours are enough to see, if activity pattern is changed, but to see changes in period length at least two days have to be analysed.
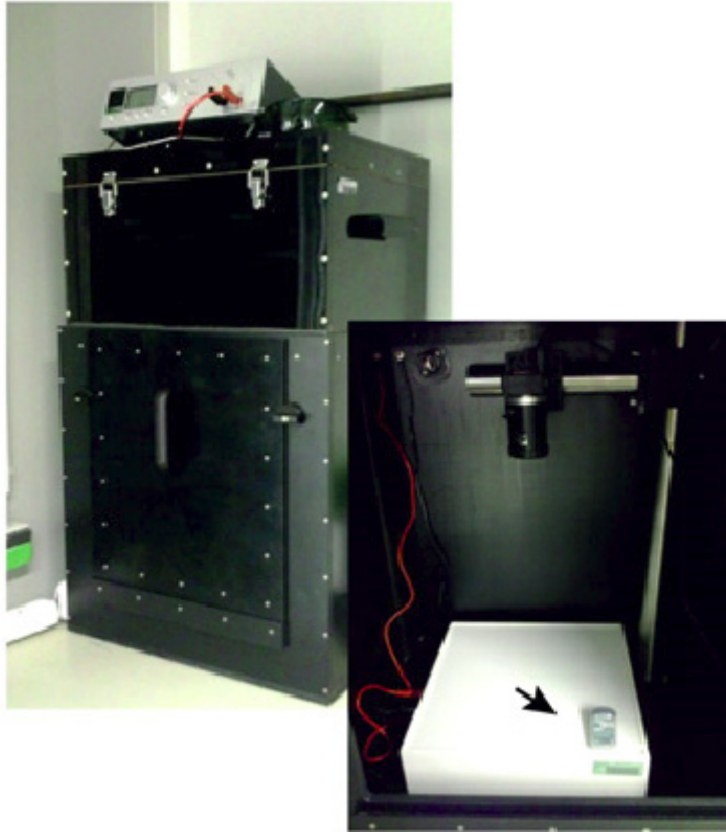
Fig.2.5: The camera set up. Black box closed on the left picture and open on the right one. Camera is fixed above the infrared array (white box). A light and temperature detecting device (HOBO) is placed in the box for control (arrow).

2.3.2 BEHAVIOURAL ANALYSIS

For *Platynereis* six different behaviours have been defined and categorized in active (scored as 1) and inactive (scored as 0). The first ten minutes of each hour have been analysed, meaning that the type of behaviour was reported at every full minute during this period, resulting in ten time points per hour.

Category	Behaviour	Explanation
active	Searching	Anterior end is outside tube searching movements are made to locate nearby food
	Interaction	Animals have contact to each other and/or fight over food or tubes
	Outside tubes	Worms are crawling or swimming outside their tubes
inactive	Inactive	Complete inactivity within/half outside/outside the tube
	Ventilation	Ventilation of the tube to produce respiratory currents by muscular movements of the body
	Others	Minor movements of head or tail or movement within the tube

A seventh type of behaviour called turning (animals turn within the tube) was used in the beginning, but then removed because it seems to occur rarely and thus is difficult to score.

The obtained data was imported to ActogramJ software (University of Würzburg) for circadian analysis. The periodograms were calculated with Lomb-Scargle analysis and confirmed with Fourier transform analysis (FFT) and Chi-square analysis. This was done for each individual and the average was calculated. The rhythmicity was categorized in non-significant (peaks are below p-value), weakly significant (peaks are slightly higher than p-value) and significant. Periods below 4 hours were regarded as arrhythmic.

2.3.4 BEHAVIOUR OF R-OPSIN ABLATED WORMS

Ablation: Worms are treated with 10mM Metronidazole in NSW for 48 hours (solution is exchanged after 24 hours) in complete darkness, because the chemical is light-sensitive. If GFP was still visible, drug treatment was prolonged for another day.

As a control a film was made before ablation. The animals were given a three day long recovery phase after drug-treatment. Two types of worms were used: pMos-copsin1::gfp::ntr strain [12] (called transgenic) and wild type worms (siblings of transgenic worms, but without GFP-expression) and treated the same way. The recordings took place under normal light dark conditions (16:8).

2.3.5 BEHAVIOUR OF B321 STRAIN DURING DIFFERENT MOON PHASES

Worms undergo a normal light dark entrainment with circadian and circalunar light regimes for at least two months. Behaviour analysis starts on full moon day or on new moon day and is done for 3 days. Two experiments have been done during full moon phase.

1. Worms undergo a normal lunar cycle (with nocturnal light) and are filmed under constant light conditions.
2. Worms are moved to another room, so that they do not receive nocturnal light, and are then filmed under light dark conditions.

3. RESULTS

3.1. LABELLING C-OPSIN1 EXPRESSING CELLS

Due to the fact that ciliated PRCs are located in the so-called unpigmented ciliary ocelli, which are extensively ciliated structures located in *Platynereis dumerilii* dorsal brain and not adjacent to pigment-cells, targeting and identification is rather difficult. The existence of ciliary type PRCs has been proven by Arendt, Tessmar-Raible et al. (2004) [1] in 3-day old larvae by Whole mount *in situ* hybridisation (WMISH). This method detects gene expression on mRNA level by binding of labelled RNA probes to the mRNA of interest. At older stages staining of c-opsin expressing cells is problematic, because the transcription rate and/or RNA stability seems to be very low. Labelling of this cell type on protein level might be more promising to succeed. Especially creating a transgenic reporter line would be very useful. Therefore *Platynereis* zygotes have been injected with c-opsin1::EGFP constructs with two different transposase recognition sites (mariner/Mos; Tol2). GFP signal can be observed at 2dpf, but to ensure proper development of embryos, the screening for GFP signal was performed earliest at 3dpf. But still with all precautions only very few normally developed animals with a positive signal were found. When injecting the Tol2 construct in total 4 transient larvae were observed. In two cases a GFP expressing cell was found in the dorsal part of the head in between the anterior eye-pair with axon-projections into the brain (Fig.3.1 A). In this region antibody staining of acetylated α- tubulin, a protein found in stabilized microtubules of axons and cilia, indicates a paired ciliary structure (Fig.3.1. D). In other animals GFP signal was located near the adult eye and in one case with a projection into the anterior dorsal cirrus (Fig.3.1 B). Although GFP expression was found in the approximate region were the ciliary structures should be, which hinted the existence of ciliary PRCs in the first place, the number of observed animals is too low to claim this as the right signal. Immunostaining with antibodies against Pdu-c-opsin1 would bring more clarification in this topic, but the antibodies tested during my work could not be proven functional. This was done by western blotting, after immunohistochemistry staining was unsuccessful. Therefore a membrane with protein extract was incubated with the different rat sera first with different concentrations, then with one concentration. At the second step the rat sera were pre-incubated overnight with the antigen-peptide, with which the animals were immunized, provided by the producing company (PRIMM). This should inhibit the antibodies to bind to membrane bound proteins and thus lead to disappearance of specific bands (Fig.3.2.). This can be easily seen by putting blocked and unblocked solutions next to each other, but it could not be observed in this case.
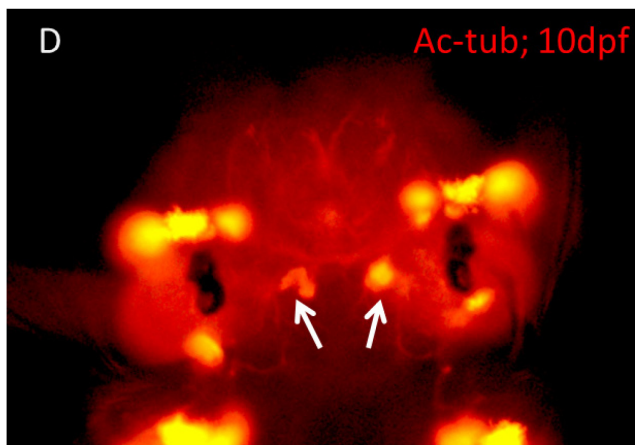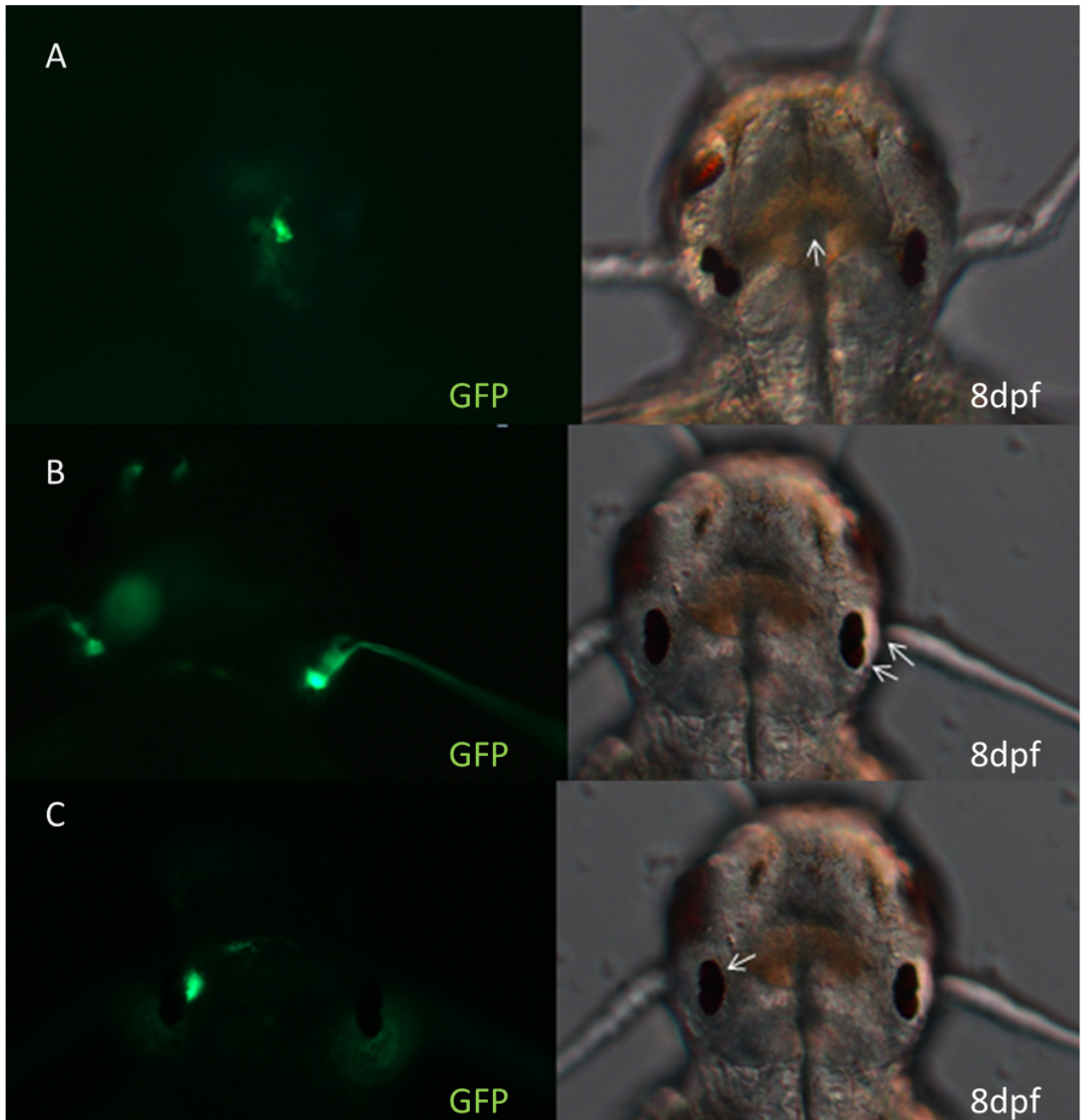
Fig.3.1: Dorsal view of 8 day old larvae expressing EGFP after being injected with pTol2-c-opsin1::EGFP-NTR constructs (A-C); white arrows in bright light pictures indicate location of GFP-signal. Dorsal view of 10day old larvae (D) stained with acetic-tubulin antibody; white arrows indicate paired ciliary structures in the brain.
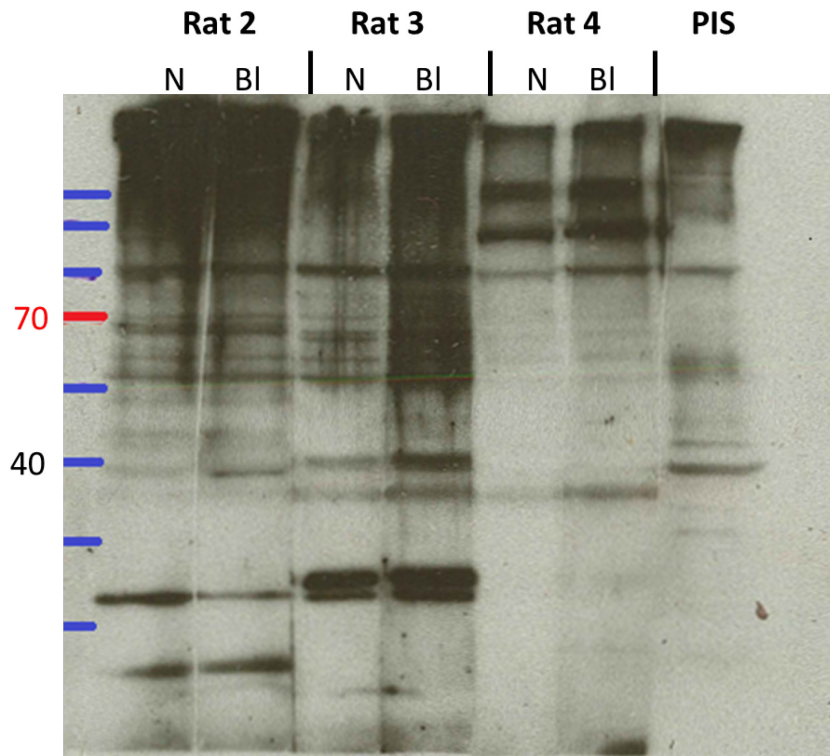
Fig.3.2: Western blot with different rat sera diluted 1:500 in 2.5% milk/PTW. The expected size of c-opsin1 is around 39kDa. Serum of rat 1 was excluded, because it did not even stain the antigen-peptide. N=unblocked; Bl=blocked; PIS=pre-immune serum (pooled).

Unfortunately, I was also not able to create a stable transgenic reporter strain. Although GFP expression was observed in F_0 animals injected with Tol2 constructs, the signal gets lost in F_1. Using mariner based constructs has been shown to eliminate this problem [12], even if there is no GFP signal visible in F_0 animals. From the creation of other transgenic reporter lines we know that about 1 in 20 animals has GFP positive offspring. Neither in batches derived from pMOs-c-opsin1::EGFP injected worms, nor in genomic DNA samples from said worms a GFP signal could be observed, despite of increasing the needed number of raised worms by threefold (see table below).

	Number of larvae/worms
Set out for growing	~60
Reached adult stage	47
Matured	36
Mated	31
Bad batches	25 from 13 animals
Screened batches	32 from 18 animals (all neg.)
gDNA samples of immature worms screened for GFP (PCR)	11 (all neg.)

3.2. TALEN CONSTRUCTION AND TESTING

To determine and study the function of a gene the loss of function approach is the most used tool. This is mostly done by simply creating mutants of the gene of interest. The advantage of mutations is that once a mal-functioning or non-functioning mutant has been created it stays in the genome and can be inherited (if the mutation is not lethal) to other generations. A stable line of animals without a functional gene of interest can be generated by breeding. In other species TALENs have been proven to successfully induce mutations by causing double strand breaks. In *Platynereis dumerilii* no protocol to induce or detect mutations by TALENs is established so far. Because of their simple construction TALENs would be a powerful tool to generate mutants and creation of a c-opsin1 mutant will shed light on its function. Five TALEN pairs targeting different loci in exon1 of c-opsin1 were constructed and proven to work *in vitro*. To test the function of TALENs in vivo the so called "single embryo restriction assay" was used. Therefore 1-2 day old larvae are digested with proteinase K and this lysate is used as a genomic DNA template. In the spacer region between the two TALEN binding sites of the two monomers is a specific restriction site. By amplifying this region with PCR and digest it with the appropriate enzyme one can test, if this sequence was cut by TALENs and a miss-repair took place (restriction enzyme does not cut at this locus anymore). During my work only pair 3 was tested in vivo with BceA1 restriction enzyme (NEB; Fig.3.3). In some cases a larger band occurs, but in undigested control also a large band occurs together with the expected one (960bp). Sequencing of that fragment showed that there is an intronic polymorphism in this region having an approximately 200bp insert upstream of the exon. Also 4 TALEN pairs targeting c-opsin2-exon1 were assembled and tested *in vitro* (fig.2.2).
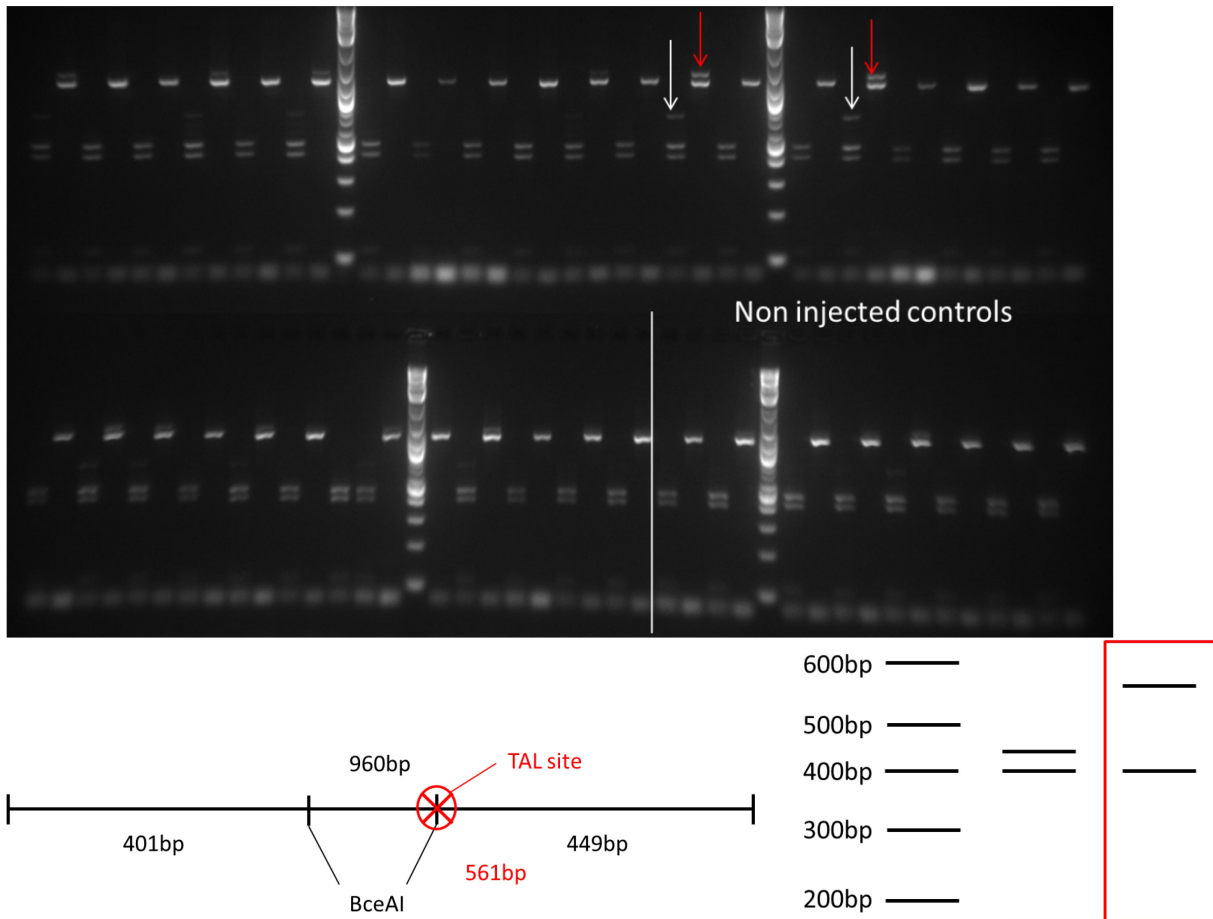
Fig.3.3: At the bottom is a map of the amplified PCR product with the restriction sites of BceA1(left); and how the bands on the gel look like when digested with BceA1 (right) when both sites are functional or one is deleted by a TALEN (red box). At top: gel of 32 injected embryos loaded alternately digested and undigested PCR product. Bands of longer fragments (white arrows) come always together with a double band in undigested control (red arrows).Non injected controls are from same batch and 5 animals are pooled in one sample.

3.3. BEHAVIOUR ANALYSES OF *PLATYNEREIS DUMERILII*

3.3.1 LOCOMOTOR ACTIVITY BEFORE AND AFTER LOSS OF R-OPSIN EXPRESSING CELLS

The behaviour of Platynereis was analysed before and after chemical ablation of r-opsin expressing cells. In F_1 of the newly established r-opsin::EGFP::NTR line [12] in our culture are also animals without GFP expression amongst their transgenic siblings (termed "Transgenic" or "TG"). Those were used as the control group (termed "Wild Type" or "WT") to minimize differing results by genomic diversity. At first an analysis was done over 24 hours at three time points: before, 1 hour after and 72 hours after ablation (when drug is removed). As 24 hours are too short to get a reliable period length, this analysis relies on peak shifts. *Platynereis dumerilii* is a nocturnal organism, meaning its locomotor activity is higher at night than during the day, when it rests in the tube [31]. This is the case before ablation in both

Wild Type and Transgenic group (n=4) the activity is restricted to the night period. The average activity per hour (Fig.3.4 last image), which is calculated by dividing the sum of active behaviours during a given period by the time (e.g.: 16h light period), raises from 0.8 during the day to 2.1 during night in Wild Type animals and from 0.4 to 2.3 in Transgenic animals. Shortly after drug treatment both groups are affected as both groups are more active during day and less during the night compared to before treatment. Regarding the activities per hour there is almost no difference between darkness and light (WT: 1.3 light, 1.5 dark; TG: 1.1 light, 0.9 dark). After three days of recovery Wild Type animals show again nocturnal behaviour, their activity during day is again decreased and much lower than during night (0.7 light, 3.6 dark). The Transgenic group fails to do so and their activity during light period is still elevated and only slightly lower than during the night (0.8 light, 1.0 dark).
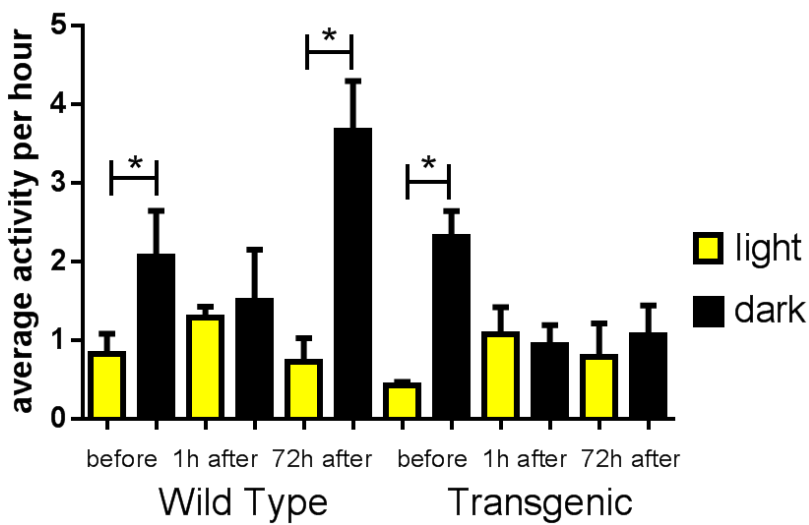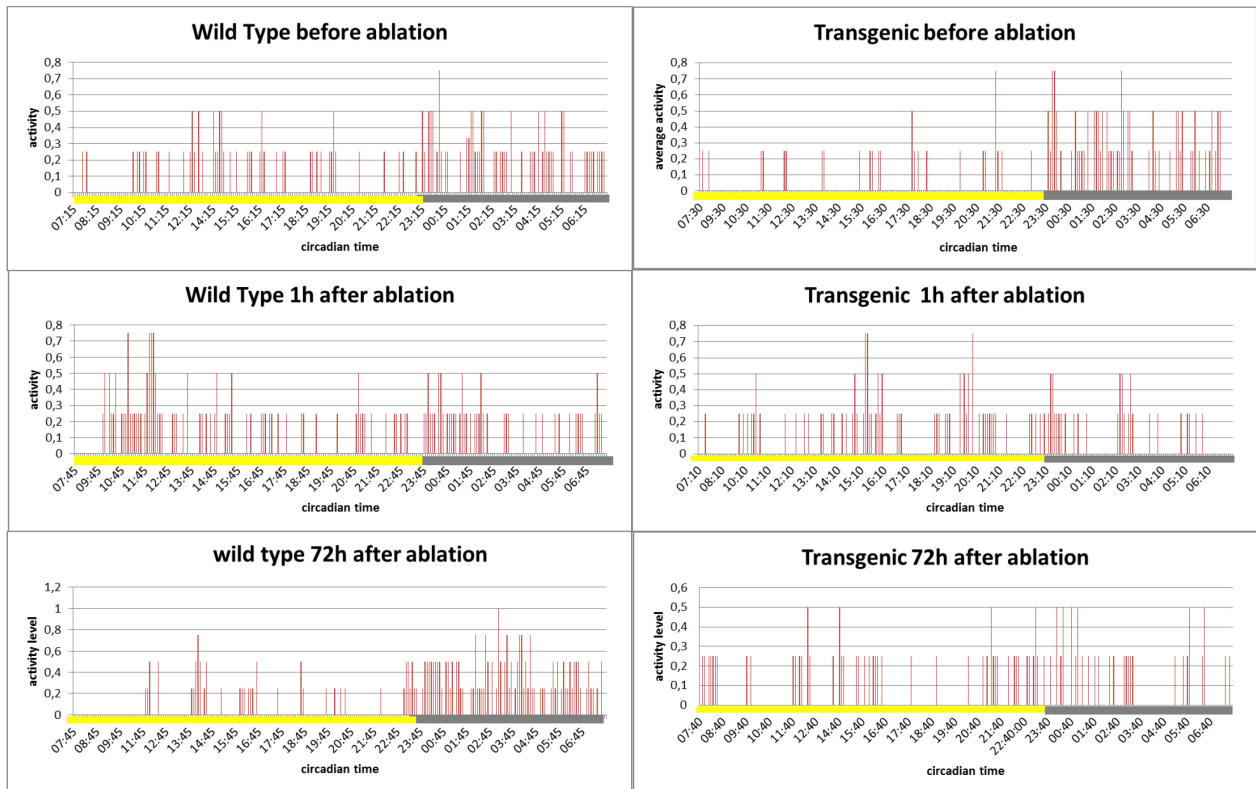
Fig.3.4: Above: Activity plots of Wild Type and Transgenic groups; average activity of 4 animals is plotted against time; yellow bar indicates light period and grey bar dark period. Left: Average activity per hour is calculated for light period and dark period; asterisks indicate statistical significant difference between light and darkness. Error bars represent SEM.
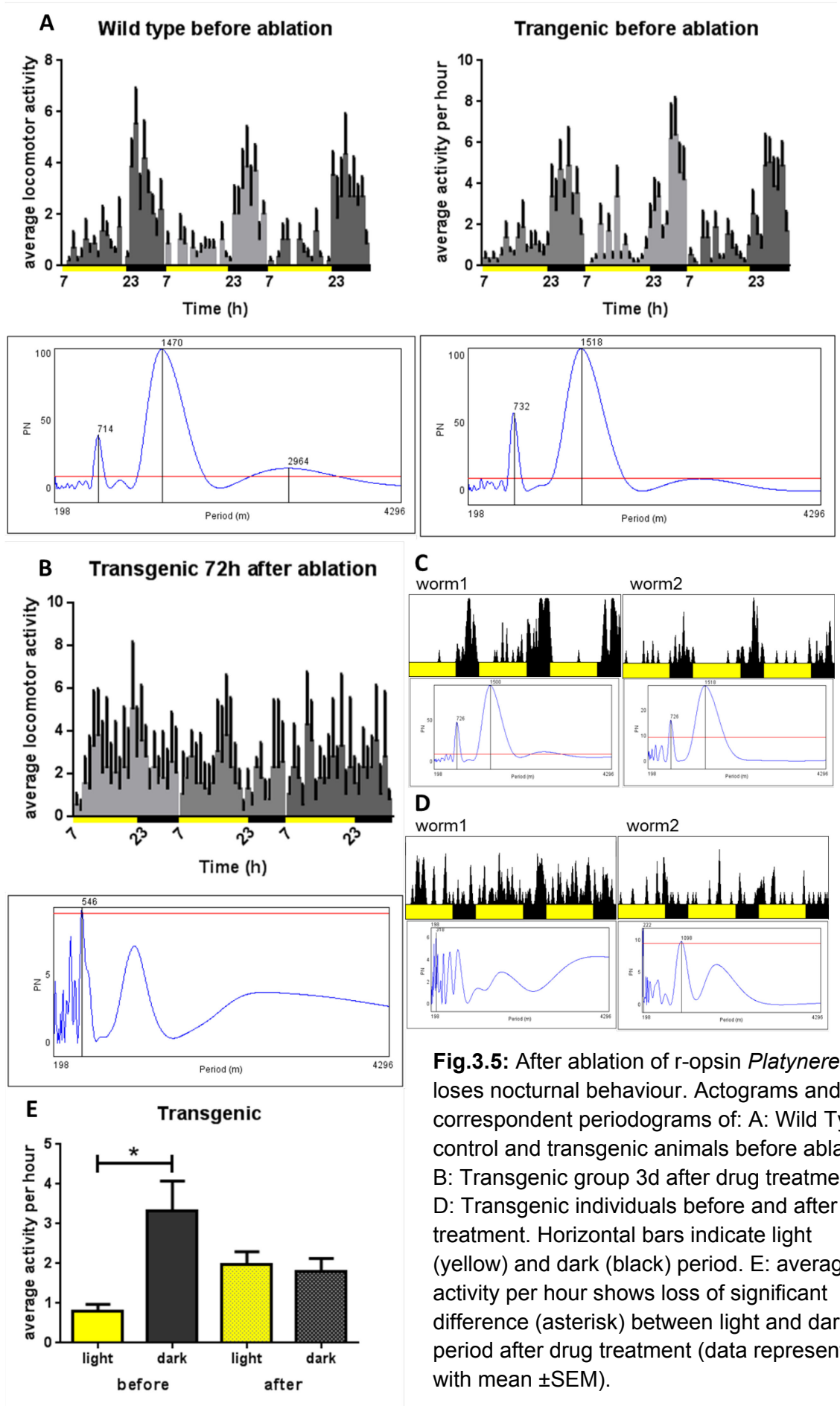
Fig.3.5: After ablation of r-opsin *Platynereis* loses nocturnal behaviour. Actograms and correspondent periodograms of: A: Wild Type control and transgenic animals before ablation; B: Transgenic group 3d after drug treatment; C, D: Transgenic individuals before and after drug treatment. Horizontal bars indicate light (yellow) and dark (black) period. E: average activity per hour shows loss of significant difference (asterisk) between light and dark period after drug treatment (data represented with mean ±SEM).

Repetition of this experiment and analysis of behaviour over three consecutive days reveals more details. Both Transgenic and Wild Type group show a significant nocturnal activity (Fig.3.5 A+E, n=6) before treatment with metronidazole and also a significant periodicity of about one day (WT: 24.5h±0.8h; TG: 25.3±1.5h). In all individuals of both groups (except one animal in Transgenic group) a significant, yet weaker, period can be observed around 12 hours (WT: 12.3h±0.6h; TG: 11.6h±0.6h). The average activity per hour of Transgenic is four times higher at night (3.3) than during the day (0.8), in Wild Type the difference is even higher (day: 0.56; night: 2.6; data not shown). Immediately after drug removal no experiment was performed. Animals could recover for 72h under normal light/dark regime before next analysis started. A mistake occurred during drug treatment of Wild Type group; therefore these results had to be excluded. Transgenic group (n=4) has elevated activity during day (1.96/h) and decreased activity at night (1.81/h) (Fig.3.5 B+E) after drug treatment. This population has a slightly significant period of 9.1h (power<10, before ablation: power>100). On individual level (Fig.3.5 D) 50% show no significant rhythmicity and 50% show weak rhythmicity, thus Transgenic group can be considered arrhythmic. Ablation of r-opsin leads to loss of rhythmicity and nocturnal behaviour.

3.3.2 COMPARISON OF LOCOMOTOR ACTIVITY AT NEW MOON AND FULL MOON

Spawning of *Platynereis dumerilii* stays rhythmic for more than three months after the zeitgeber (nocturnal light mimicking full moon) is removed. Expression of certain clock genes is altered during full moon phase compared to new moon. To see if these changes do lead to an altered locomotor activity, behaviour analysis was performed over three consecutive days during full moon (FM) period and (NM) new moon period (done by Juliane Zantke, a member of Tessmar-Raible group). During new moon animals are recorded under normal light conditions (LD; 16:8). For full moon two experiments have been done: 1. Animals undergo normal full moon period and recording starts on FM-day under LL-conditions. 2. Animals are set on free running conditions (last nocturnal light about 1 month before) and filmed under normal LD conditions. The second experiment is done to prove potential behaviour changes are clock driven and not caused by permanent light conditions (work done by me and Juliane Zantke). For this experiment B321-strain was used, because it was also used to obtain molecular data (gene expression patterns). At new moon worms behave clearly nocturnal (Fig.3.6.A+C) with an average activity per hour of 2.9 at night and 0.8 at day. A period length of 24.1h±0.3h has been observed and in 40% of the animals a second significant period of about 12h. Only 10% of the animals are arrhythmic, the rest is either rhythmic (60%) or weakly rhythmic (30%). At full moon nocturnal behaviour is lost, as the average activity per hour is almost the same during day hours (1.98) and night hours (2.0). In addition the period

is shortened to 16.3h±3.2h (Fig.3.6B,C,D). The power of the rhythmicity (height of the peak) is about 15.3±1.3 and thus reduced to the one at new moon which is 44±4.6. On individual level 10% (1 worm) are rhythmic, 60% (6 worms) are weakly rhythmic and 30% (3 worms) are arrhythmic.
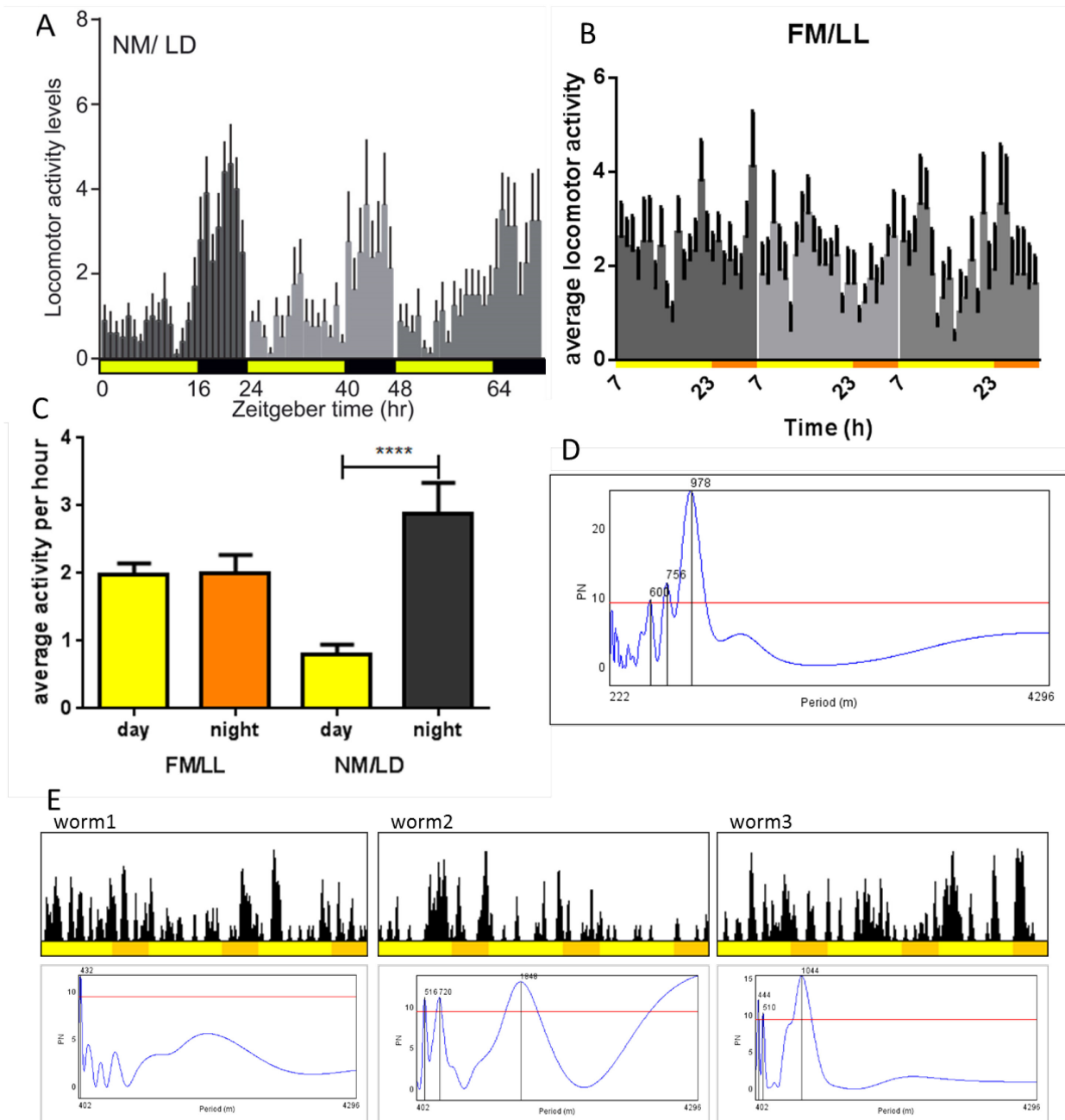

Fig.3.6: At full moon *Platynereis* has a shorter period. A, B: Activity of Platynereis at new moon (A, n=10) and full moon (B, n=10); C: comparison of activities per hour at FM and NM; D: average periodogram at full moon; E: actograms and corresponding periodograms of individuals at full moon. Horizontal bars below actograms indicate day/light period (yellow) and night hours (black=dark; orange=light)

Under free running full moon conditions Platynereis is still nocturnal, but activity during day hours is elevated (day: 1.7; night: 3.8activities/hour). The animals behave significantly less rhythmic (power=16.9±1.4) and have a shortened period of 16.7h±1.4h. 39% of investigated

40

worms are rhythmic, 52% are weakly rhythmic and 9% are arrhythmic (n=23). This gives good evidence that the circalunar clock affects circadian behaviour.

4. DISCUSSION

4.1. LOCATION OF CILIARY TYPE PRCS IN *PLATYNEREIS DUMERILII*

As outlined in the introduction *Platynereis* has two types of photoreceptor cells. The rhabdomeric type, which is located in the cerebral eyes, frontolateral eyelet and other non-cephalic locations, is used for directional vision as it has adjacent pigment-cells, at least in the cephalic locations. The rhabdomeric PRCs in the trunk do not have a visible pigment in their vicinity, but decapitated animals show a negative phototactic response, when illuminated after a period of darkness [12]. The ciliary type is settled in the dorsal part of the brain between the adult eyes and lacks nearby pigmented cells. It is thought to sense general light changes (in length or intensity) and entrain adjacent clock-gene expressing cells [1]. These PRCs express two types of sensory protein: c-opsin1 and c-opsin2. This study is focused on labelling c-opsin1 by creating c-opsin1-EGFP hybrid construct and injecting it into Platynereis. Doing this with a construct containing Tol2 transposase target sites led to transient animals having GFP signal at various cephalic locations. Although 50% (n=4) of the worms had the signal at the predicted site, near the ciliated structure visualized by acetylated-α-tubulin staining, the number of investigated animals is too low to call this the correct signal. In the other 50% of animals the signal is located very close to the adult eye and this high diversity of c-opsin1::EGFP signal-location makes it difficult to interpret. It is possible that c-opsin1 expressing cells are not restricted to a distinguished area and localization may differ. An alternative explanation is that the construct was integrated into the genome near a regulatory network of another gene and thus is controlled by it and expressed in cells that do not naturally express c-opsin1. What contradicts this option is that it is very incidental and this variance was not observed while creation of other reporter strains. To prove one or the other possibility wrong the number of animals with EGFP signal has to be increased and verified with different approaches. This can be done with two methods whole mount in situ hybridisation (WMISH) and immunostaining. WMISH as used to verify the existence of c-opsin in *Pdu* brain in three day old larvae, but despite of many attempts it could not be visualized at older stages. Therefore a functional antibody against c-opsin1 would be very helpful to find out if the location of ciliary PRCs does vary or not. Unfortunately none produced during this study. The advantages of a stable transgenic reporter line have been outlined in the introduction. Creation of a c-opsin1::EGFP::NTR strain was unsuccessful. Explanation for that can either be an unusual low integration rate (normally 1 in

20 animals gives transgenic offspring) or that the construct got integrated in a silenced genomic region and hence expression is impossible. A third and rather simple possibility is that the transient animals in the injected population died before maturation or screening for EGFP. Although many questions stay unanswered the first step of labelling and identifying c-opsin1 expressing cells was made.

4.2. SCREENING FOR TALEN INDUCED MUTATIONS

In several other species TALENs have been proven to induce mutations at a moderate to good rate. Their modularity facilitates assembly of TALENs with a new target sequence and using the Golden Gate assembly system makes it fast. Several pairs targeting *Pdu-c-opsins* had been constructed and were proven functional and site specific *in vitro*. To find mutations *in vivo* a protocol was established and optimized which allows screening in larvae as well as in adults. Therefore the region of interest is amplified via PCR and digested with a specific restriction enzyme which cuts at the TALEN-target site. If the region is altered by mutation the enzyme is not able to cut and this results in a band shift. This method is rather simple and fast and a high number of samples can be tested (established by Stephanie Bannister, member of Raible group). Several members of our lab were injecting TALENs targeting various genes and detected mutations with the method described above. Efficiency varies amongst TALEN pairs targeting the same gene and also from gene to gene. For example in the case of estrogen receptor (er) five out of thirteen samples were tested positive (samples were pooled with 4-5 larvae) with 4-18% of mutated genome copies, when injected with 200-300ng/µl of TALEN mRNA. After injection with two TALEN pairs deletions were found, but at a very low rate (less than 5% of genome copies). Furthermore mutants of diverse genes have been raised to adult stage (15 mutants of 40 animals for *lcry*, 1 of 8 animals for *vtn* and 3 of 44 animals for *er*). One reason why no c-opsin1 mutations were found might be that TALEN mRNA was injected at a lower concentration (50-100µl) resulting in too few functional copies of TALEN protein. Another explanation might be that the tested pair (only one pair was tested *in vivo*) has a very bad efficiency in order of target binding and another pair is more effective. Or thirdly that the gene itself is hard to target and mutations occur only in very low numbers and more injections and screenings would be necessary to find a positive hit.

Earlier injections, however, were carried out with higher concentrations (300-400ng/µl), but were still unsuccessful. For those an older generation of TALENs were used which are less efficient *in vitro* than the newer generation. Results so far indicate that the newer TALEN architecture is also more effective *in vivo*, as no mutations were found using the older one. One plausible explanation might be that the new TALENs have a shorter backbone, which most likely improves translational efficiency. In medaka it has been shown that shorter constructs are more effective than longer ones, but still both architectures work [57]. In

vertebrates translation of long mRNA may not be as problematic as in invertebrates. Since *Platynereis* is an invertebrate it might not be able to successfully translate long TALEN molecules and this giving an explanation why the older design is not working in this organism [Bannister, S. et al. script in preparation].

4.3. *PDU R-OPSIN* EXPRESSING CELLS LIKELY PLAY A ROLE IN ENTRAINING THE ANIMAL'S CIRCADIAN CLOCK

As mentioned earlier r-opsin expressing cells are located at various cephalic and non-cephalic regions in *Platynereis dumerilii* [12]. The r-opsin-GFP worm line in our culture also expresses *nitroreductase* (NTR) in rhabdomeric PRCs and by treatment with Metronidazole this cell type gets completely ablated (protocol established by Vinoth Babu Veedin-Rajan, a member of Tessmar-Raible group). The advantage of this method over laser ablation is that all r-opsin expressing cell-types are killed and therefore backup of other r-opsin expressing cell clusters is not possible (i.e.: loss of cerebral eyes can be compensated by e.g. the frontolateral eyelet). But still there is the ciliary PRC type which can sense the daily light cycle and compensate the loss of r-opsin. In *Drosophila melanogaster* are five photoreceptors and/or photopigments known which all contribute to the entrainment of the circadian rhythm. Mutants of one or more of these light receptive systems do lead to different changes in the circadian activity of flies. For instance the compound eyes are necessary for adjustment of the phase synchronisation, whereas cryptochrome plays a role in period lengthening under different day lengths [21]. In mice there is a similar story the retinal PRCs impart in entrainment and masking effects [45], but are not needed for phase adjustment [47] and period lengthening [46]. These latter features are thought to be covered by melanopsin, a photopigment which is located in the retinal ganglion cells [11]. Under constant light melanopsin knock-out mice show reduced period lengthening [48] and they have a weaker response to light pulses (phase shifts) [49]. That the different photoreceptive systems in *Platynereis dumerilii* also provide distinct features for clock entrainment in a similar fashion is thinkable. After drug treatment c-opsin expressing cells remain the only functional PRCs left in the organism, but since worms do not show a cyclic behaviour, ciliary type photoreceptors most likely do not play a role in circadian entrainment of *Platynereis*, or only contribute very little to it. Transplantation experiments showed the zeitgeber sensing organs are located in the prostomium, but cerebral eyes have been proven to be not necessary for entrainment to the lunar cycle by repeatedly destroying them with an electric epilation needle [55]. As mentioned above this method removes all r-opsin expressing cells in the organism, thus it is impossible to make a statement if and how the different r-opsin expressing compartments (cerebral eyes, frontolateral eyelet etc.) provide different features for entraining the circadian rhythm (like the compound eyes, ocelli and Hofer-Buchner-eyelet in *Drosophila*). Molecular

data on how the clock genes are cycling after drug treatment, were still to be obtained, at the time I left the lab, but it is thinkable that they still oscillate as the organism itself (except *ntr*-expressing cells) should not be affected by Metronidazole. Although in fact the worms did not look very healthy shortly after treatment and as shown in fig.3.4, they did not behave "normally". This might not be caused directly by the drug, but more indirectly as Metronidazole is also a known antibiotic and may kill endosymbionts in *Platynereis'* gut and this is leading to disease. Since data of clock gene oscillation after ablation of r-opsin expressing cells is missing, to which the behavioural results could be compared, comparison of data obtained in DD seemed to be suitable. The reason for that is that in both cases the input signal is not available or recognizable, respectively, as the part of ciliary type PRCs seems to be negligible. Under DD conditions clock genes are oscillating free running for several days, furthermore also nocturnal behaviour and a period of approximately 24 hours can be observed. An explanation why the ablated worms are not behaving in a similar fashion as the ones under DD conditions might be that those results represent different time points. Under DD conditions feeding is not possible and the behaviour can be affected by hunger, if worms are not fed for more than five days. For this experiment worms are "incubated" 48 hours in complete darkness and then filmed for further 72 hours in darkness (behavioural data represents day 3-6 in DD). Combining the 48 hours in complete darkness during drug treatment and the 72 hours of recovery, behaviour of ablated worms was observed form day 6-9 without an input signal. At this point behavioural rhythmicity might have already dampened out and thus could not be observed. In conclusion, the role of r-opsin in entraining the circadian rhythm seems to be important and investigation of clock gene oscillations after loss of rhabdomeric PRCs would bring more insights.

4.4. LOCOMOTOR BEHAVIOUR IS ALTERED DURING FULL MOON

Various species synchronize their breeding behaviour with the help of the lunar cycle and this has been object of many studies [52]. Moon light was also identified as the major zeitgeber for spawning synchronisation of *Platynereis dumerilii* by mimicking lunar light in the culture environment and recording the spawning events [53, 54]. At full moon behavioural cycle of *Platynereis* is altered drastically regarding period (shortened) and amplitude (lowered). The fact that this result can be reproduced under free running conditions leads to the assumption that this is clock driven. The expression pattern of clock genes is also changed during full moon and free running full moon. The causation of the shortened period might be the up-regulation of the *Pdu-clk* gene [Zantke et al. unpublished]. It has been shown that artificial elevation of expression of the orthologous gene in Drosophila results in a shortened period as well [50]. The biological function of an increased activity during full moon stays unclear, especially because it is believed that the worms synchronize their spawning at

new moon to make it more difficult for predators to spot the swarm and feed on the worms before they can reproduce. If this hypothesis is correct, an increased activity, which includes leaving the safe tube and exposing oneself to a potent predator, would be futile. One possible explanation might be that those behavioural changes are only so harsh under culture conditions and less severe in natural habitat. The worms are exposed to constant illumination for one week, which is a much stronger input signal than the natural moon light, although it has been shown that light intensity does not play a role in entraining the circalunar rhythm [53]. However the moon does not shine the whole night, leading to a (shorter) period of darkness giving enough input to maintain phase and period adjustment. One interesting result of this study was that even though the behaviour was similarly altered under free running conditions as under constant light, the activity was still higher during the period of actual darkness than during light hours and not at the same level as it was under constant conditions. This hints a direct influence of the photic input on *Platynereis'* behaviour. Although the changes of behaviour might be amplified by cultural conditions, this method still gives good output data to investigate entrainment of the circalunar clock on another level (besides clock-gene expression and spawning patterns), especially if this method can be automated, because the approach in use now is relatively time-intensive.

5. APPENDIX

5.1. REFERENCES

1. Arendt, D., et al., *Ciliary photoreceptors with a vertebrate-type opsin in an invertebrate brain.* Science, 2004. **306**(5697): p. 869-71.
2. Raible, F., et al., *Vertebrate-type intron-rich genes in the marine annelid Platynereis dumerilii.* Science, 2005. **310**(5752): p. 1325-6.
3. Fischer, A. et al. The normal development of Platynereis dumerilii (Nereidae, Annelida) Front. Zool. 2010 Dec. 30; 7:31
4. Yau, K.-W. and Hardie, R.C. *Phototransduction Motifs and Variations.* Cell, 2009. **139**(2): p. 246-264.
5. Purschke, G. et al. Photoreceptor cells and eyes in Annelida. Arthropod Struct Dev, 2006. **35**(4): p. 211-30.
6. Purschke, G. and Bartolomaeus, T. *Morphology, Molecules, Evolution and Phylogeny in Polychaeta and Related Taxa.* Developments in Hydrobiology, 2005: p. 18.
7. Jekely, G., et al., *Mechanism of phototaxis in marine zooplankton.* Nature, 2008. **456**(7220): p. 395-9.
8. Arendt, D., et al., *Development of pigment-cup eyes in the polychaete Platynereis dumerilii and evolutionary conservation of larval eyes in Bilateria.* Development, 2002. **129**(5): p. 1143-54.
9. Arendt, D. and Wittbrodt, J. *Reconstructing the eyes of Urbilateria.* Philos Trans R Soc Lond B Biol Sci, 2001. **356**(1414): p. 1545-63.
10. Brökelmann, F. and Brökelmann, J. *Das Auge von Platynereis dumerilii (Polychaeta) Sein Feinbau im ontogenetischen und adaptiven Wandel.* Cell and Tissue Research, 1966. **71**(2): p. 217-244.
11. Hattar, S., et al., *Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity.* Science, 2002. **295**(5557): p. 1065-70.
12. Backfisch, B. et al. Stable transgenesis in the marine annelid Platynereis dumerlii sheds new light on photoreceptor evolution. PNAS 2013 Jan 110(1)
13. Kondo, T., et al., *Circadian Rhythms in Rapidly Dividing Cyanobacteria.* Science, 1997. **275**(5297): p. 224-227.
14. Branicky, R. and Hekimi, S. *What keeps C. elegans regular: the genetics of defecation.* Trends in Genetics, 2006. **22**(10): p. 571-579.
15. Mendoza, J., *Circadian Clocks: Setting Time By Food.* Journal of Neuroendocrinology, 2007. **19**(2): p. 127-137.
16. Eskin, A., *Identification and physiology of circadian pacemakers. Introduction.* Fed Proc, 1979. **38**(12): p. 2570-2.
17. Krispel, C.M., et al., *RGS expression rate-limits recovery of rod photoresponses.* Neuron, 2006. **51**(4): p. 409-16
18. Baylor, D.A., *Photoreceptor signals and vision. Proctor lecture.* Invest Ophthalmol Vis Sci, 1987. **28**(1): p. 34-49.
19. Berson, D.M., F.A. Dunn, and M. Takao, *Phototransduction by Retinal Ganglion Cells That Set the Circadian Clock.* Science, 2002. **295**(5557): p. 1070-1073.
20. Hannibal, J., *Neurotransmitters of the retino-hypothalamic tract.* Cell and Tissue Research, 2002. **309**(1): p. 73-88.

21. Rieger, D., Stanewsky, R., and Helfrich-Forster, C. *Cryptochrome, compound eyes, Hofbauer-Buchner eyelets, and ocelli play different roles in the entrainment and masking pathway of the locomotor activity rhythm in the fruit fly Drosophila melanogaster.* J Biol Rhythms, 2003. **18**(5): p. 377-91.

22. Hege, D.M., et al., *Rhythmic expression of a PER-reporter in the Malpighian tubules of decapitated Drosophila: evidence for a brain-independent circadian clock.* J Biol Rhythms, 1997. **12**(4): p. 300-8.

23. Helfrich-Forster, C., et al., *The circadian clock of fruit flies is blind after elimination of all known photoreceptors.* Neuron, 2001. **30**(1): p. 249-61.

24. Ouyang, Y., et al., *Resonating circadian clocks enhance fitness in cyanobacteria.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(15): p. 8660-8664.

25. Goodwin, B.C., *Oscillatory behavior in enzymatic control processes.* Adv Enzyme Regul, 1965. **3**: p. 425-38.

26. Novak, B. and Tyson, J.J. *Design principles of biochemical oscillators.* Nat Rev Mol Cell Biol, 2008. **9**(12): p. 981-91.

27. Bozek, K., et al., *Regulation of Clock-Controlled Genes in Mammals.* PLoS One, 2009. **4**(3): p. e4882.

28. Ueda, H.R., et al., *System-level identification of transcriptional circuits underlying mammalian circadian clocks.* Nat Genet, 2005. **37**(2): p. 187-92.

29. Bell-Pedersen, D., et al., *Circadian rhythms from multiple oscillators: lessons from diverse organisms.* Nat Rev Genet, 2005. **6**(7): p. 544-56.

30. Evans, S.M. Behavior in Polychaetes. The Quarterly Review of Biology, 1971. **46**: p. 379-405

31. Hauenschild, C. and Fischer, A. *Platynereis dumerilii. Mikroskopische Anatomie, Fortpflanzung, Entwicklung.* Großes Zoologisches Praktikum, 1969. **10**: p. 1-54.

32. Bogdanove, A.J. et al. *TAL effectors: finding plant genes for disease and defense.* Current Opinion in Plant Biology, 2010. **13:** p. 394-401

33. Boch, J. and Bonas, U. *Xanthomonas AvrBs3 family type III effectors: discovery and function.* Annual Review of Phytopathology, 2010. **48:** p.419-436

34. Boch, J. et al. *Breaking the code of DNA binding specifity of TAL-type III effectors.* Science, 2009. **326:** p.1509-1512

35. Moscou, M.J. and Bogdanove, A.J. *A simple cipher governs DNA recognition by TAL effectors.* Science, 2009. **326:** p. 1501

36. Christian, M. et al. *Targeting DNA double-strand breaks with TAL effector nucleases.* Genetics, 2010. **186**: 757–761

37. Li, T. et al. *TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain.* Nucleic Acids Res. published online, doi:10.1093/nar/ gkq704 (10 August 2010).

38. Engler, C. et al. *Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes*. PLoS One, 4, e5553

39. Cermak, T. et al. *Efficient design and assembly of TALEN and other TAL-effector based constructs for DNA-targeting.* Nucleic Acids Research, 2011. **39:** published online, e82 doi:10.1093/nar/gkr218

40. Zhang, X. and Bremer, H. *Control of the Escherichia coli rrnB P1 promoter strength by ppGpp.* The Journal of biological chemistry, 1995. **270**(19):11181-9.

41. Bridgewater, J.A. *The bystander effect of the nitroreductase/CB1954 enzyme/prodrug system is due to a cell-permeable metabolite.* Humane Gene Therapy, 1997. **8**(6):709-17.

42. Lee, G. et al. *Thymic Epithelial Requirement for γδ T Cell Development Revealed in the Cell Ablation Transgenic System with TSCOT Promoter.* Molecules and Cells, 2012. **34**(5):481-93.

43. Arendt, D. and J. Wittbrodt, *Reconstructing the eyes of Urbilateria.* Philos Trans R Soc Lond B Biol Sci, 2001. **356**(1414): p. 1545-63.

44. Helm, B. and Visser, M. *Heritable circadian period length in a wild bird population.* Proc. R. Soc. B, 2009. Published online doi: 10.1098/rspb.2010.0871

45. Mrosovksy, N. (a) *Contribution of classic photoreceptors to entrainment.* J Comp Physiol A, 2003. **189**:69-73.

46. Mrosovsky, N. (b) *Aschoff's rule in retinally degenerate mice.* J Comp Physiol A, 2003. **189**:75-78.

47. Freedman, M.S. et al. *Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors.* Science, 1999 **284**:502-504.

48. Ruby, N.F. et al. *Role of melanopsin in circadian responses to light.* Science, 2002. **298**:221-2213.

49. Panda, S. et al. *Melanopsin (Opn4) requirement for normal lightinduced circadian phase shifting.* Science, 2002. **298**:2213-2216.

50. Kadener, S. et al. *Circadian transcription contributes to core period determination in Drosophila.* PLoS Biol, 2008. **6**, e119.

51. Antoch, M.P. et al. *Functional identification of the mouse circadian Clock gene by transgenic BAC rescue.* Cell, 1997. **89**: 655-667.

52. Korringa, P. *Relations between the Moon and Periodicity in the Breeding of Marine Animals.* Ecological Monographs, 1947. **17**(3): 347-381.

53. Hauenschild, C. *Photoperiodizitaet als Ursache des von der Mondphase abhaengigen Metamorphose-Rhythmus bei den Polychaeten Platynereis dumerilii.* Z Naturforsch, 1955. **10B**: 658-662

54. Hauenschild, C. *Lunar periodicity.* Cold Spring Harb Symp Quant Biol, 1960. **25:** 491-7.

55. Hauenschild, C. *Die Schwärmperiodizität von Platynereis dumerilii im DD/LD-Belichtungszyklus und nach Augenausschaltung.* Z Naturforsch, 1961. **16b**: 731 - 736.

56. Teßmar-Raible, K. and Arendt, D. *Emerging systems: between vertebrates and arthropods, the Lophotrochozoa.* Curr Opin Genet Dev, 2003. **13**(4):331-40.

5.2. ABSTRACT

Marine organisms that rely on external fertilisation need to synchronize their spawning to ensure successful mating. The organism *Platynereis dumerilii* exhibits such swarming events where numerous individuals mature and spawn at the same time. This is not only connected to daytime, but also to the lunar cycle, both resembling light signals. Swarming takes place nine to thirteen days after full moon, which could also be observed under culture conditions by mimicking the moon cycle artificially. Secondly this organism is only active during the night, meaning searching for food only occurs during darkness to avoid predators. To anticipate the correct time to spawn or feed and or the correlating geo-physical events respectively an internal timing mechanism called "clock" is evolutionary advantagous. A clock is entrained by environmental signals which provoke an oscillation of certain genes, which still continues even if the entrainment signal is not available for some time.

Platynereis possesses two photoreceptor systems based on opsins: the rhabdomeric type and the ciliary type, which could sense light as entrainment signal for the two different clocks. This study attempts to reveal if and how these two different systems contribute to entrainment of the worm's biological clocks. Therefore tools to visualize the photoreceptive cells and mutate the photoreceptor genes have been established. Labelling of ciliary photoreceptor cells with a GFP reporter construct was successful, although results were contradicting (and we were unsuccessful to develop an functional antibody) and no stable transgenic line was created. Transcription activator like effector nucleases (TALENs) as a tool to induce mutations were assembled with help of Golden Gate cloning in a fashion that they target ciliary opsin. Successful and specific cleavage of the target sequence could be demonstrated *in vitro*, but no mutations were observed *in vivo*. At last a method to observe and analyse the behaviour of *Platynereis dumerilii* was established to investigate the output stage of a biological rhythm. By using a camera system and defining and categorizing types of behaviour and scoring them, activity plots were created, thereby confirming previous studies about the nocturnal activity of *Platynereis*. After verifying the reliability of this approach behaviour was analysed under different light conditions and during different moon phases. Results of those experiments suggest that circadian behaviour of *Platynereis* is driven by the circadian clock, but the circalunar clock seems to have an impact on it, as during full moon the behaviour is severely altered. These alterations have also been observed without an external signal and hence are under clock control. Locomotor activity of worms that lost all their rhabdomeric photoreceptor cells because of chemical ablation has been analysed in a similar fashion. Three day after drug treatment the investigated animals showed no significant rhythmicity anymore, hinting an important role of this photoreceptor. In summary I had limited success in labelling ciliary photoreceptors and creating a method to

mutate c-opsin, but I was able to use a behavioural assay which revealed a possible role of r-opsin in maintaining circadian rhythmicity and the influence of the circalunar clock on it.

5.3. ZUSAMMENFASSUNG

Um die Wahrscheinlichkeit einer erfolgreichen Fortpflanzung zu maximieren müssen Meereslebewesen mit äußerer Befruchtung den Zeitpunkt der Paarung synchronisieren. Beim Borstenwurm *Platynereis dumerilii* kommt es zu einer Schwarmbildung, während der viele reife Würmer zugleich ihre Gameten abgeben. Dieses Schwärmen tritt immer nachts neun bis dreizehn Tage nach Vollmond auf. Dies kann auch im Labor mit einem künstlichen Mondzyklus beobachtet werden. Außerdem ist dieser Organismus nachtaktiv, um während der Futtersuche nicht so leicht von Fressfeinden entdeckt zu werden. Um den korrekten Zeitpunkt für die nächste Eiablage bzw. die korrelierenden geophysikalischen Ereignisse vorhersagen zu können und rechtzeitig mit der Metamorphose zum reifen Stadium zu beginnen brauchen Organismen innere Uhren. Die Tagesuhr besteht aus gewissen Genen die oszillieren und an die Lichtschwankungen des Tagesrhythmus (circadiane Uhr) oder des Mondzyklus (circalunare Uhr) gewöhnt werden, die Oszillation wird auch fortgeführt, wenn der äußere Einfluss nicht mehr da ist. Um die Lichtveränderungen zu erkennen besitzt *Platynereis* zwei Photorezeptorsysteme basierend auf Opsinen: den rhabdomerischen Lichtrezeptor und den ciliären Lichtrezeptor. Diese Arbeit versucht die Funktion dieser beiden Lichterkennungssysteme im Zusammenhang mit den beiden Uhren, die in Platynereis arbeiten, herauszufinden. Dabei wurde versucht ciliäre Photorezeptorzellen mit einem GFP-Reporterkonstrukt zu markieren, was auch gelang, jedoch konnten keine einheitlichen Ergebnisse erzielt werden. Die Generation von Antikörpern schlug fehl. Weiters konnte auch kein stabiler transgener Stamm etabliert werden. „Transcription activator like effector nucleases" oder kurz TALENs sind Konstrukte um gezielte Mutationen hervorzurufen. Solche mit c-Opsin als Ziel wurden mit Hilfe des Golden Gate Klonverfahrens erzeugt und *in vitro* auf Funktionalität und Spezifität getestet. Allerdings konnten *in vivo* keine Mutationen gefunden werden. Es wurde auch eine Methode entwickelt um das Verhalten von *Platynereis dumerilii* zu studieren. Dafür wurde mit Hilfe eines Kamerasystems die Würmer beobachtet und Arten von Verhalten definiert und kategorisiert und diese dann aufgezeichnet. Die entstandenen Graphen bestätigten vorherige Studien über das nachtaktive Verhalten des Organismus. Weitere Experimente wurden dann unter verschiedenen Lichtbedingungen und Mondphasen gemacht. Diese zeigten dass das circadiane Verhalten von *Platynereis* unter Kontrolle einer Uhr steht, aber auch von der circalunaren Uhr beeinflusst wird, da das Verhalten bei Vollmond stark verändert ist, auch wenn dieses Signal einmal nicht gegeben wird. In einem ähnlichen Experiment wurde untersucht wie sich der Verlust von rhabdomerischen Photorezeptorzellen, diese werden durch chemische Ablation abgetötet,

auf den Tagesrhythmus von *Platynereis* auswirkt. Drei Tage nach Behandlung mit der Chemikalie war kaum noch eine signifikante Rhythmik vorhanden, was auf eine Rolle dieses Photorezeptors hinweist. Zusammenfassend kann man sagen, dass ich eingeschränkten Erfolg hatte ciliäre Photorezeptorzellen zu markieren und c-Opsin zu mutieren, allerdings war es mir möglich eine Verhaltensanalyse zu etablieren und mit Hilfe dieser, eine mögliche Rolle von r-Opsin beim Einstellen der Tagesrhythmik gefunden habe.

5.4. ACKNOWLEDGEMENTS

5.5. Curriculum vitae

Heinrich Helmut Oberlerchner

Date of birth: 15.10.1986

Place of birth: Villach, Austria

<u>Education:</u>

2006- 2013   study of biology at Universität Wien with specialication in:

Mikrobiologie und Genetik

Cyto- und Entwicklungsgenetik

Diploma thesis at Department für Mikrobiologie, Immunologie und Genetik with the topic: *Tool development and behavioural analysis to investigate the influence of light on behaviour of Platynereis dumerilli*, Dr. Kristin Tessmar-Raible

1997-2005   BRG Spittal/Drau mit Matura (naturwissenschaftlicher Zweig)

2005-2006   Präsenzdienst