universität wien

MASTERARBEIT

Titel der Masterarbeit

„INVESTIGATION INTO THE ROLE OF MELANOPSIN IN THE ZEBRAFISH BRAIN“

verfasst von

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angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2014

Studienkennzahl lt. Studienblatt:	A 066 877
Studienrichtung lt. Studienblatt:	Masterstudium Genetik und Entwicklungsbiologie
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Introduction

How does vision work?

Vision provides animals with immediate and explicit information about their environment. The first eyes appeared in the early Cambrian, about 530 million years ago [1]. While plants and bacteria have a big repertoire of several light sensing molecules, animals possess very few such systems and only one of them enables visual perception [1]. The process of visual perception starts at the photosensitive cells in the retina. These cells express photoreceptors called opsins. Opsins are the first step in a pathway which converts light input into an electrical signal that is eventually transmitted by retinal ganglion cells to the optic tectum and results in the perception of an image. Based on their morphology the photoreceptors of animals can be divided into two classes, rhabdomeric and ciliary (Fig. 1). In rhabdomeric photoreceptors the apical cell surface is folded, providing an enlarged surface in which the opsins are located. In ciliary photoreceptors the ciliary membrane is folded for the same reason [2].

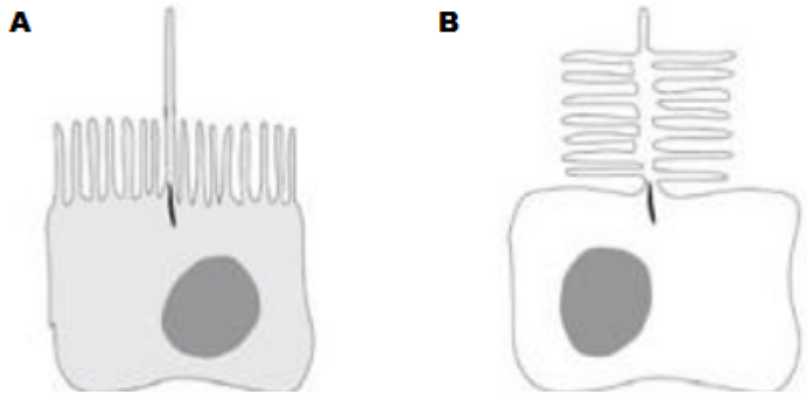

Figure 1: the two types of animal photoreceptors. A) rhabdomeric photoreceptor B) ciliary photoreceptor. (Fasolo, A., The Theory of Evolution and Its Impact. 2011: Springer).

The opsins found in rhabdomeric photoreceptors belong to the r-opsin gene family, those of the ciliary photoreceptors to the c-opsins. Rhabdomeric photoreceptors are mainly acting as visual photoreceptors in invertebrates, ciliary photoreceptors are the only photoreceptors used for image forming light detection in vertebrate eyes. [3].
Ciliary photoreceptors can be further subdivided into rods and cones. Vertebrates possess up to four different kinds of cone opsins (expressed in cone cells), which allow colour

vision and seeing under well lit conditions (photopic vision). These evolved even before the one rod opsin (expressed in rod cells), essential for vision under dim light (scotopic vision) [4]. In the evolutionary line of vertebrates both photoreceptor cell types, rhabdomeric and ciliary, were incorporated into the evolving retina. Thus, also rhabdomeric photoreceptors can be found in the vertebrate eye. While the ciliary photoreceptors became the main visual photoreceptor cells, the rhabdomeric photoreceptor cells transformed into retinal ganglion cells and are now involved in image processing and circadian entrainment [3] [5].

Photoreception

Opsins are seven transmembrane domain proteins which bind to the chromophore retinal, a vitamin A like protein retinaldehyde (Fig. 2). All opsins use the same chromophore as a ligand, thus the opsin determines at which wavelength the photoreceptor absorbs. The same chromophore, in combination with different opsins, can therefore be used to detect different wavelengths [4].

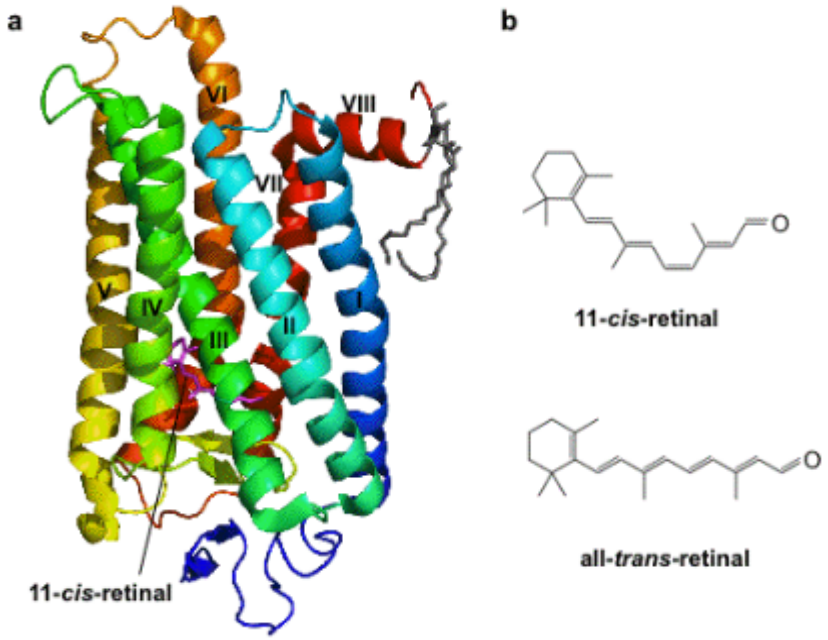

Figure 2: a) structure of bovine rhodopsin showing the seven alpha helical transmembrane domains and b) its chromophore retinal. (http://www.photobiology.info/Terakita.html, 29.09.2014).

Retinal is bound to the opsin in the *11-cis* form in darkness. Upon light absorption the chromophore photoisomerises by changing its conformation from *11-cis* to the *all-trans* form. This causes sterical changes in the opsin, resulting from the increased size of the *all-trans* form. The *all-trans* form interacts with transducin, a membrane bound G-protein.

The opsin activates the inactive heterotrimeric G-protein (consisting of an α, β and γ subunit) by converting GDP into GTP. Thereafter the α-subunit leaves the complex, bound to a GTP, and drives the chain of phototransduction. As one opsin can activate several hundreds of G-proteins the signal is amplified at this point [4] . After light absorption, the chromophore, in its *all-trans* form, has to be returned to its *11-cis* conformation. In vertebrate visual opsins it is separated from the opsin for that purpose and transported to the pigmentepithelium by transport proteins. There it is reisomerised and transported back to a photoreceptor cell, where it is build back into an opsin molecule [6].

Opsins have been shown to be expressed in tissues apart from the eye, even in animals like the sea urchin, which are eyeless, headless and supposedly brainless [7], indicating that opsins are also involved in non-visual processes. In addition to vision, non-visual light perception is vital for directly light regulated phenomena, like pupil dilatation or the entrainment of the circadian clock. Interestingly also a role for light in mechanotransduction was recently reported [8].

Non-visual light detection - Melanopsin

Melanopsin is a seven transmembrane domain G-protein coupled photoreceptor, which in mammals has been shown to be expressed in intrinsic photosensitive retinal ganglion cells (ipRGCs) [9]. The first indication that a non-visual photoreceptor that has a different function from the photoreceptors in rods and cones exists, came from mice [10].
Mice that have a genetic defect leading to no functional rods and a very small number of cones are virtually blind and don't show behavioural responses as a result of visual perception. Still their pupil reflex and photoentrainment does not show any differences when compared to wild type mice. To confirm these observations a knock out mouse line was created, which had absolutely no functional rods and cones (*rd/rd cl* mice). Interestingly, these mice could still adapt to the light cycle of their surroundings. That this new photoreceptor must be located in the eye was proven by showing that mice which had their eyes removed were unable to entrain their clock [11]. Finally melanopsin, expressed in a small subset of photosensitive retinal ganglion cells, was identified.
Melanopsin was first discovered in photosensitive dermal melanophores of *Xenopus laevis* where it is involved in the photic control of skin pigmentation [12]. It was subsequently shown to be expressed in a wide variety of species. While melanopsin knock out mice can still entrain their clocks, although strongly reduced, mice lacking rods, cones, and

melanopsin expressing pRGCs neither see nor show any circadian entrainment or other responses to light [11].

Melanopsins are rhabdomeric pigments. Rhabdomeric visual pigments are commonly found in invertebrates and melanopsins present in vertebrates show a strong similarity to non-vertebrate visual opsins [4]. Melanopsin expressing cells are thought to be derived of an ancestral rhabdomeric photoreceptor [4] [2]. Thus a separate class of opsins, belonging neither to the cones nor to the rods, plays a crucial role in non-visual responses to light and controls photoentrainment of the circadian clock and pupil constriction in mammals [13].

Non-visual light detection must be much older than vision, for vision requires a more complex system. But why did distinct photoreceptors for detecting irradiance and for image forming purposes evolve? Detecting light (intensity) and imaging the environment are quite different concepts. If an object is to be imaged, light detection has to be focused using a lens and restricted. In contrast, for irradiance detection it is necessary that light can be collected from all sides. While many organisms have extraocular photoreceptors, which makes light collection from all around relatively simple, mammals rely heavily on their eyes. To be able to fulfill both tasks, the mammalian eye has many cones and rods densely packaged. However, only few pRGC express melanopsin and they are distributed all across the retina [14]. The dendritic trees of these cells are quite large and widely spread, making them unpractical for relaying spatial information [15], but ideal for irradiance detection.

A gene duplication early in the lineage of vertebrates has led to the development of two melanopsin paralogs, *OPN4m* and *OPN4x* [16]. While mammals have only one melanopsin encoding gene (*opn4m*), non-mammailian vertebrates exhibit two classes of opsins, the group first discovered in *Xenopus laevis* (*opn4x*) and the mammalian variant. In zebrafish three melanopsin genes (*opn4.1*, *opn4a* and *opn4b*) are mammalian related, while *opn4xa* and *opn4xb* are *Xenopus* related [17]. All five of these melanopsins code for functional photopigments, having spectral peaks from 470 nm to 484 nm [18], which is similar to that of mammals around 480 nm [9]. Zebrafish embryos react to light before the retina has formed, indicating light sensing capacity in non-visual tissues [19] [20]. Unlike mammals, in zebrafish (and other non-mammalian vertebrates) melanopsin expression is not restricted to the retina. It is also found in other tissues, such as the brain [21], and *opn4.1* and *opn4xb* were found to be expressed in the neuromasts of the lateral line

system (Fig. 3).

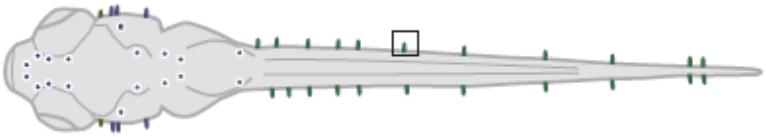

Figure 3: The lateral line in the zebrafish larvae. A dorsal view showing the distribution across the body (modified from Van Trump, W.J. and M.J. McHenry, 2008).

Fish rely on their lateral line to detect changes in water pressure. Behaviours like spawning and obstacle avoidance depend on this organ [22]. The sensory cells, the mechanosensory hair cells, are closely related to the auditory sensory organs.

Recently experiments in *Drosophila* have shown that rhodopsins have a function much different from their classical role in vision. A screen for mutants with defects in hearing revealed two rhodopsins playing a role in mechanosensation [8]. Melanopsins are the only true orthologues of invertebrate rhodopsins [16] and may therefore function in a very similar manner.

In *Drosophila* rhodopsin signals upon light activation as follows (Fig. 4): a G-protein gets activated, which in turn activates PLC (phospholipase C), which hydrolises the membrane bound protein PIP_2 (phosphatidylinositol 4,5-biphosphate). This generates IP_3 (inositol triphosphate), DAG (diacylglycerol) and a proton. About 1-2% of the lipids in the plasma membrane are PIP_2 molecules. DAG is a smaller molecule than PIP_2, this leads to membrane tension when PIP_2 is hydrolised and replaced by DAG [11]. This has been proposed to open TRP (transient receptor potential) channels through membrane tension [23]. The majority of these invertebrate opsins are reisomerised by absorption of a photon [2]. Evidence from studies on retinal explants and dissociated pRGCs, as well as cellular expression systems, indicates that the signal transduction in melanopsin expressing pRGCs also starts with the activation of a G-protein which is followed by activation of PLC and subsequent TRP channel opening, although the direct effect of PLC activation is not yet clear [7].

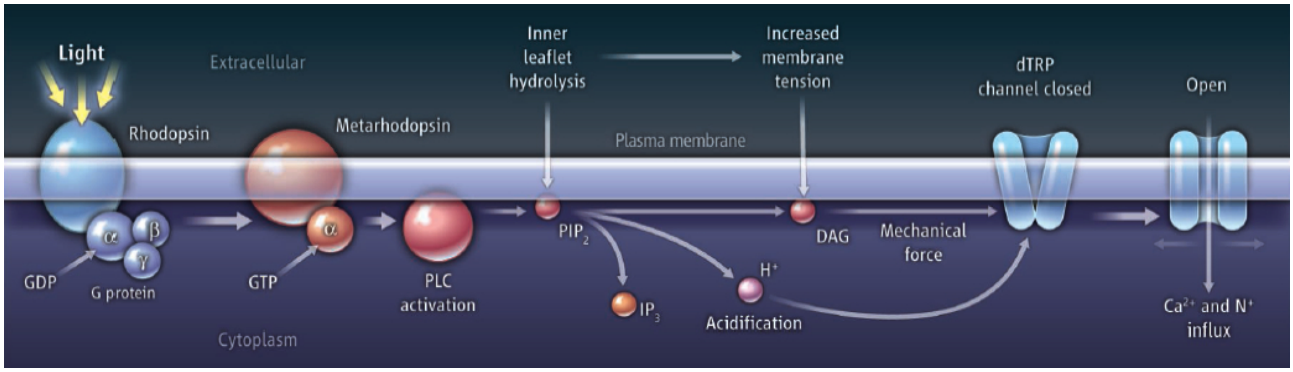

Figure 4: Light stimulation of rhodopsin in a fly photoreceptor cell. Hydrolysis of PIP_2 by phospholipase C (PLC), generates IP_3, DAG, and a proton. Conversion of PIP_2 to the slimmer molecule DAG on the inner leaflet of the membrane increases membrane tension, which opens dTRP channels. (Cell Signaling, Putting the Squeeze on Phototransduction, Liman, Science 12, October 2012).

Circadian Clocks

All organisms living on this earth are confronted with changes of their environment, be it differences in temperature, variation in the availability of food, the activity level of predators or others. However, one of the most dramatic and most reliably recurring changes is the alternating presence and absence of light caused by the earth's movement around the sun and its turning around its own axis. Circadian clocks have been shown to exist in a wide variety of species across kingdoms. They are endogenous and adapt to daylength.
The core clock is a self sustained mechanism that follows a roughly 24 hour cycle and is connected to an input pathway that sets and resets it, and an output pathway to elicit a rhythmic response [9]. The clock is (re)set by environmental conditions and able to keep up regular oscillations, even when the external cue is absent. The phase control is brought about by a synchroniser known as *zeitgeber*. The most common *zeitgeber* in nature is light [24]. Gehring and Rosbash [25] postulated that DNA damage caused by UV irradiation was the earliest *zeitgeber* and that it might not be a coincidence that especially blue light photoreceptors evolved to become involved in resetting the circadian clock. Organisms would have retreated deep into the sea during the day to avoid UV damage. Only blue light can penetrate that deeply, to indicate the time for returning to the surface. Many processes, for example gene expression, hormone secretion and activity levels, follow a regular, rhythmic pattern [15] as the circadian clock synchronises them with the changing environment.

Most core circadian clock genes which were initially discovered in mammals and *Drosophila* have been shown to be present and performing similar roles in zebrafish [26]. The circadian clock has been demonstrated to consist of transcriptional and translational feedback loops. BMAL and CLOCK are PAS-domain containing proteins, which form a heterodimer that acts as a transcriptional activator. This complex activates the transcription of several genes, among others *period* (*per*) and *cryptochrome* (*cry*). PER and CRY proteins form a dimer that moves to the nucleus and blocks the BMAL/CLOCK complex. PER and CRY proteins show peak expression during the day and get degraded at the end of the day. After PER and CRY levels drop, BMAL and CLOCK are released and bind to each other, thereby restarting the transcription of their target genes [24] (Fig. 5).

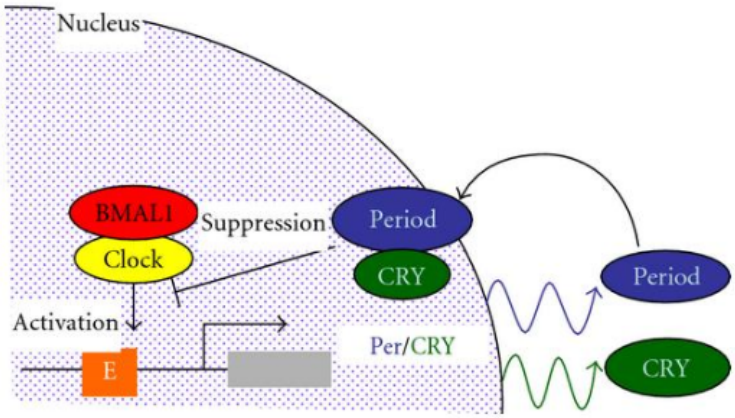

*Figure 5: negative feedback loop showing the interaction between CLOCK/BMAL and CRY/PER. (Ishida, N., PPAR Res, 2009. **2009**: p. 412949).*

Mammals have a master oscillator in the suprachiasmatic nucleus (SCN). It is composed of about 20,000 cells located in the anterior part of the hypothalamus and its function is to synchronise peripheral clocks in most organs and tissues [27]. Most zebrafish cells are directly light responsive [28]. This implies that the peripheral clocks are directly entrained by light and that the zebrafish circadian clock system is less (or not at all) centralised when compared to that of mammals studied so far [28].

Why we use zebrafish as a model organism

The zebrafish (*Danio rerio*) belongs to the family of the Cyprinidae and has its origins in the Indian subcontinent. There these freshwater fish inhabitate streams, but also stagnant waters, like for example rice fields [29]. Due to their preference for low water levels, zebrafish are exposed to light.

The zebrafish offers many advantages as a model organism in research, such as an extrauteral development, transparency of the embryo and the chorion and comparatively easy genetic manipulations (forward [30] and reverse [31] genetics). They have a relatively short generation time (three months) and under suitable conditions one female can produce several hundreds of eggs during one spawning event. The embryos develop rapidly, with all major organs being present, although not fully developed, at 36 hours post fertilisation [32]. The embryonic development can be easily observed, due to the embryos' transparency, under the microscope and follows a set pattern of cell divisions and defined stages. Embryos are light responsive already five hours post fertilisation and have been demonstrated to possess a functional circadian clock by the end of day one post fertilisation [19] [33]. A fully sequenced genome as well as numerous mutant strains [30] and transgenics (e.g. Gal4/UAS) are available for zebrafish.

Combining all these useful features with an easy maintenance and handling, zebrafish is a common and popular vertebrate model organism in various fields of research, such as genetics and developmental biology, and recently also photobiology.

Aim of this project

In this research project we aim to understand the role of zebrafish melanopsins in non-visual processes.
It is not clear yet if zebrafish melanopsin has a function in synchronising the circadian clock with its environment like in mammals. I will investigate its expression pattern in the brain to determine if it overlaps with the zebrafish core clock gene *per1b*. The expression pattern of *per1b* will also give information on how "centralised" the clock in the brain is.

Two melanopsin transcripts, *opn4.1* and *opn4xb*, were demonstrated to be present in the mechanosensory cells of the zebrafish lateral line by the Tessmar-Raible lab. Interestingly these two opsins belong to different groups (*opn4m* and *opn4x,* respectively). Furthermore photoreceptors in *Drosophila* have been shown to play a role in auditory processing [8]. These discoveries raise questions as to the exact function of melanopsins in the lateral line. Do they play a role as light receptors by, for example, modulating mechanosensory signals coming in from the sensory cells of the lateral line or do they have a function in mechanotransduction, which may be completely independent of light? Or are they involved in processing the sensory information in the brain?

I aim to confirm the expression of *opn4.1* and *opn4xb* in mechanosensory hair cells of the neuromasts by doing a fluorescent double *in situ* hybridisation.
In order to get insight into the functions and diversity of functions of zebrafish melanopsins I aim to analyse the expression of all five zebrafish melanopsins in larvae and adult brains. As melanopsin (*opn4a* and *opn4.1*) has been shown to be rhythmically expressed in the zebrafish retina [17], I will test *opn4.1* and *opn4xb* expression levels in the neuromasts of larvae taken at different times during the day.
Several behaviours could be modulated by melanopsins. Since we lack any understanding of the functions the *opn4.1* and *opn4xb* genes could play a role in, *opn4.1* and *opn4xb* mutants will be subjected to the following behavioural assays:

- dot assay (avoidance vs. approach)
- tapping assay (balance)

Another aim is to knock out another melanopsin gene (*opn4b*), by using the TALEN technology, in order to study its function.

Materials and Methods

Maintenance and breeding of zebrafish stocks

The zebrafish (*Danio rerio*) used were either Tübingen wild types (wt) or stocks with the respective mutation introduced into a Tübingen wild type background.
The fish are kept at a 16:8 LD cycle at a constant temperature of 28°C.

To obtain offspring a couple was set up in the evening in a mating box. Embryos were collected into a petridish the following morning and kept there in 1x E3 medium [34] until day 6, when they were connected to the system and started to be fed.

In situ hybridisations

Whole mount *in situ* hybridisation on six day old larvae

Larvae used for *in situ* hybridisations were PTU treated roughly 24 hours post fertilisation. PTU (1-phenyl 2-thiourea) inhibits melanogenesis by blocking tyrosinase and thereby preventing pigment formation [36].
Larvae were fixed at day six by putting them into 4% PFA (paraformaldehyde) in 1x PBS and keeping them overnight at 4°C.
Afterwards larvae were washed twice for 5 min in 1x PBST and subjected to methanol treatment (50%, 5 min, 100% 5 min) to permeabilise the larvae, and stored in 100% methanol at -20°C.

For *in situ* hybridisations the larvae were then rehydrated (75%, 50%, 25% methanol in PBS, 5 min each) and washed twice for 5 min in 1x PBST.
To further enable the probes to penetrate the sample larvae were treated with 10 mg/ml proteinase K for 16 min on a shaker and then rinsed twice for 2 min in 2 mg/ml glycine to inhibit proteinase K activity, thereby avoiding overdigestion of the sample. This was followed by a post fixation step in 4% PFA (30 min). Afterwards larvae were washed in 1x PBST three times 5 min.

The following steps were performed at precisely 65°C in a waterbath. This is the optimal annealing temperature of the probes, as empirically determined. Prehybridisation in Hyb-Buffer for two hours, followed by incubation with the probe overnight.

Probe synthesis

The probes were digoxigenin (DIG)-labeled antisense RNA probes and prepared as follows:

Plasmids (pJET or pGEMT) containing the DNA of interest were linearised, so that transcription could create antisense RNA. DNA was then transcribed into RNA using the promoter and corresponding RNA polymerase (SP6 or T7) which would give antisense RNA.

10.0 µl linearised DNA
2.0 µl DIG labeling mix
2.0 µl 10x RNA polymerase reaction buffer
1.0 µl SP6/T7 RNA polymerase
4.5 µl DTT (100 mM)
0.5 µl RiboLock (RNase inhibitor) incubation at 37°C for > 2 hours.

Addition of 1 µl DNAase I to remove plasmid DNA and incubation at 37°C for 15 min.

The reaction was purified using the Qiagen RNeasy Kit and eluted in RNase free H_2O. Quality of the RNA was checked on a 1.5% agarose TAE gel and quantity was measured using a nanodrop.

The remaining RNA was then mixed with 75 µl of Hyb-Buffer to create a stock solution which was further diluted (1:20) for hybridisations.

The final concentration of the probes ranged from ~2.5 to ~4.5 ng/µl.

Probes were removed after the overnight incubation and the following washing steps were performed:

2 x 30 min 50% Formamide/2x SSCT
3 x 10 min 25% Formamide/2x SSCT
1 x 5 min 2x SSCT
2 x 30 min 0.2x SSCT
after this the washes were performed at room temperature
1 x 5 min in 50% SSCT/50% MABT
1 x 5 min in MABT

Larvae were blocked in 2% Blocking Reagent (Roche) in MABT for several hours at room temperature or overnight at 4°C while gently shaking.

Incubation with the antibody (Anti-digoxigenin Fab fragments (150 U, Roche)) in 2% Blocking reagent (1:4000) was done overnight at 4°C while gently shaking.

The next day washes with MABT were performed, all on a shaker, starting with changing MABT every 15 min, to 30 min and up to one hour intervals at room temperature and finally overnight at 4°C.

For probe detection the larvae were transferred to six well plates and BM purple AP (alkaline phosphatase) substrate (Roche) was added.
The staining reaction took several hours in the dark, the exact time depending on the individual probes.
After colour development was optimal, staining was stopped by removing the staining solution and repeatedly washing the larvae in 1x PBST. Post fixation was done in 4% PFA overnight at 4°C. Larvae were then washed with 1x PBST several times and kept in 1x PBST in the dark at 4°C.

In situ hybridisation on adult brain sections

In situ hybridisations on adult brains were done as described above for whole mount larvae, with the following modifications:

Proteinase K digestion time varied, ranging from 25 to 14 minutes.

Brains were always blocked overnight and on the next day sliced into 100 µm thick sections using a Leica vibrating blade microtome (VT 1000S).
Herefore the brains were embedded in 3% agarose in PBS. After slicing the brains were again blocked for one hour before being subjected to the usual antibody treatment.

Fluorescent 2-colour _in situ_ hybridisation on six day old larvae

Larvae used for fluorescent double *in situ* hybridisations were fixed, stored and treated exactly as described above, up until probe hybridisation. The exceptions were that the larvae were screened for GFP expression at day 4, and only those positive for it were used for the experiment. An additional treatment of 5% H_2O_2 (15 min under light exposure) was

done to remove any remaining pigment (due to suboptimal PTU treatment).
Larvae were then incubated overnight in differently labeled probes:

- *opn4xb* probe + *gfp* probe
- *opn4.1* probe + *gfp* probe
- *gfp* probe (control)
- *opn4xb* probe only

Opn4 probes were labeled with digoxigenin, *gfp* probes were fluorescein labeled.
Gfp probes would target the *gfp* mRNA expressed in the neuromasts of the lateral line (among others) of these animals (see later).
Larvae were then washed in 50% Formamide/2x SSCT (2x 30 min), 2x SSCT (15 min), 0.2x SSCT (2x 30 min) at 65°C and later at room temperature in TNT (0.1M Tris pH 7.5, 0.15M NaCl, 0.1% Tween20) (3x 5 min).
Larvae were blocked in 2% Blocking Reagent (Roche) dissolved in TNT.

Probes for *opn4* and *gfp* were detected using the antibodies α-DIG-Fab-POD (150 U, Roche) and α-Fluo-Fab-POD (150 U, Roche) respectively.
The first antibody was added overnight (1:50 in blocking solution) at 4°C while gently shaking.
Detection of the antibodies was done using the TSA™ Plus Cyanine 3/Fluorescein System by PerkinElmer, Inc.
Unbound antibody was washed off by washing with TNT several times (10 min each). The samples were then rinsed in 100 µl TSA Amplification Diluent. Fluorescein Fluorophore Tyramide was diluted 1:50 in TSA Amplification Diluent and this staining solution was added. Larvae were stained for about one hour. After staining the larvae were washed several times in TNT, and in between subjected to an 1% H_2O_2/TNT treatment for 20 min to kill off the peroxidase.

Larvae were then blocked for one hour at room temperature in 1% Blocking Reagent (Roche) in TNT. Subsequently larvae were incubated in a 1:100 dilution of the second antibody overnight at 4°C.
Washes and staining were done as before, but with Cy3 Fluorophore Tyramide.
Additionally a Höchst staining of the nuclei was performed. Therefore a 1:10,000 dilution

(in 1x PBST) was applied for one minute and the samples then washed in PBST several times.

After the *in situ* hybridisation the larvae were put through a series of glycerol/PBS solutions (25%, 50%, 75% and 100% glycerol), mounted in ProLong® Gold antifade reagent (life technologies) and stored in the dark at 4°C.

Images were taken on a confocal microscope, which allowed looking at different and separate layers of the samples.

TALENs

TALENs (transcription-activator like endonucleases) are a useful tool to introduce targeted mutations into genomes. The transcription activator-like (TAL) effector from the plant pathogen *Xanthomonas spp*. is fused to the *Fok*I nuclease creating a construct able to recognise specific DNA sequences and introduce nicks (Fig. 6) [31]. In our lab we design our own TAL effectors, to target specific sequences.

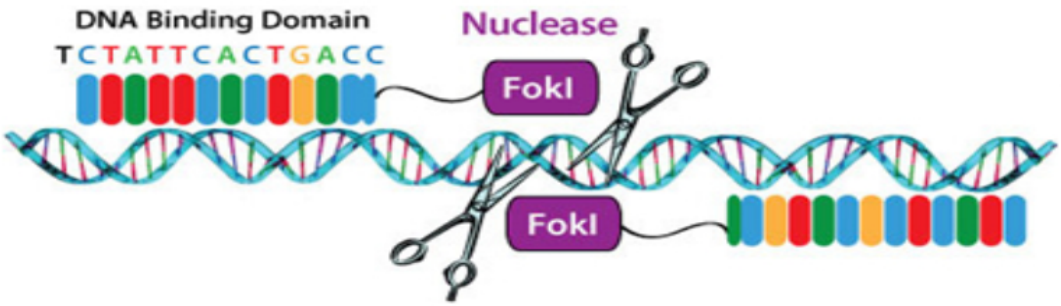

Figure 6: schematic representation of a TALEN pair. (Modified from http://www.creative-animodel.com/Products/Golden-Gate-TALEN-Assembly-Kit.html, 28.09.2014).

The DNA binding domain consists of up to 20 RVDs (repeat-variable di-residues). Each base is preferentially targeted by one special RVD (NI > A, NG > T, HD > C, NN > G). This construct is fused to the catalytic domain of the *Fok*I nuclease [31]. TALENs work in pairs, binding opposite DNA strands. This leads to the two nucleases creating a double strand break, which is less faithfully repaired than a single strand break, because the complimentary DNA strand cannot be used for repair and so the other chromosome has to be used. Therefore there is a good chance of the DSB leading to an insertion or a deletion after incorrect repair by homologous recombination. If these insertions or deletions are a number unequal to three they lead to a frame shift and will most likely disrupt proper gene function by, ideally, causing a premature stop codon.

Design and construction

TALENs were designed using the TALEN Targeter tool TAL Effector Nucleotide Targeter 2.0 by Cornell University (https://tale-nt.cac.cornell.edu/).

As a preset architecture Miller et al., 2011 was chosen (spacer 15-20, RVDs 15-20), NN as a G binding RVD. Off target sites were predicted by blasting the TALEN sequence against the *Danio rerio* genome. TALENs chosen had a minimal number of off target sites. A suitable, unique restriction enzyme recognition site within the spacer allows for subsequent screening. A successfully induced mutation would destroy the recognition site making it impossible for the enzyme to cut.

TALENs were created using an adapted version of the Golden Gate Protocol developed by Cermak et al. (N.A.R. V39 (12) e82, 2011). In this protocol up to ten RVDs of one TALEN are first ligated into a vector in one reaction, by subsequent cycles of restriction and ligation steps. In a subsequent step the RVD constructs are combined to create the full RVD sequence.

After construction the TALEN plasmids were linearised, so that only the coding strand was transcribed, and to avoid unspecific sequences. The reaction was purified using the Qiagen MinElute Reaction Cleanup Kit, DNA was then transcribed into capped mRNA using the mMESSAGE mMACHINE® kit by Ambion®.

Concentration was measured by nanodrop and quality of the RNA checked by running it on a 1.5% TAE gel. A single band indicates the size and shows that the RNA is not degraded.

RNA was diluted 80x for injections with RNAase free H_2O (0.6 µl per TALEN + 38.8 µl H_2O) and kept at -80°C.

Injections and genotyping

TALENs were injected at the one cell stage by delivering the TALEN mRNA (about 260 pg/µl) directly below the lifting cell.

1 µl of 3% TRITC-dextran (injection marker dye, SIGMA cat. No. D1819 in 0.2 M KCL) was added to the injection solution. 24 hours after injection the embryos were screened for fluorescence to confirm proper injection.

Primers were designed using the Primer3web website (http://primer3.ut.ee/) and positioned so that a PCR fragment with a unique restriction site, located where the TALENs will introduce a mutation, will be created. In the case of the wild type situation the

PCR fragment is cut in two equally long fragments, when a mutation destroys the restriction site the PCR fragment remains full length.

The first set of injected larvae were harvested at day three and tested for mutations, to determine the efficiency of the TALENs. When a TALEN pair was shown to efficiently introduce mutations, the injected animals were raised and genotyped as adults.
Fish were tested for mutations in the soma by fin-clipping (F1 generation) and for germline transmission by testing the offspring. In the latter case the potential carrier was mated to a wild type and the resulting larvae taken for testing at day three.
For fin-clipping the fish were anesthesised by putting them into a cup with water containing several ml of 0.4% Tricaine. After the fish were unable to move they were taken out of the water and a piece of the backfin was cut off with a scalpel.
The samples were digested in Embryo Lysis Buffer[1] containing proteinase K (20 mg/ml, 1:100).
The samples were incubated at 60°C overnight, proteinase K was then inactivated by heating the samples at 95°C for 10 min.
1:20 dilutions in ddH_2O were made and used as a template for the genotyping PCR reaction:

PCR-Mix/sample

2.5	µl Coral Load Buffer (10X)
5.0	µl Q-Solution (5x)
0.5	µl dNTPs (10 mM)
2.5	µl forward primer (5 µM)
2.5	µl reverse primer (5 µM)[2]
0.125	µl Hot Star Taq (5 U/µl)
1.5	µl DNA template
10.875	µl ddH_2O

1 Genetics, Genomics and Imaging in Medaka & Zebrafish; Singapore 2012, page 61
2 *opn4.1* FWD: 5' GGCCAAGCTTTAGAGTACTTACTGA 3', REV: 5' CTATGTATTCTGCCGGAGCC 3'
opn4xb FWD: 5' GCGTCAAAGCATGATTGTTC 3', REV: 5' ACGACGACAGGGTTTGAGTC 3'

PCR programme

98°C 5:00 min

98°C 20 sec

58°C 30 sec

72°C 1:30 min 41x

72°C 5:00 min

10°C infinite

Digestions to confirm presence or absence of the restriction site, and therefore respectively presence or absence of TALEN induced mutations, were set up as follows (examples given for *opn4xb* and *opn4.1*):

opn4xb	*opn4.1*
16.2 µl PCR product	12.6 µl PCR product
2.0 µl NEB CutSmart Buffer (10x)	2.0 µl NEB Buffer 4 (10x)
1.8 µl *Eco*NI (10,000 U/ml)	1.4 µl *Bsg*I (5,000 U/ml)
	4.0 µl H_2O

incubation at 37°C overnight.

The digested DNA was run on a 1.5% agarose *Sybr Safe* TAE gel.

The TALEN binding sites as well as the primers and the enzyme used for genotyping in the *opn4xb* and *opn4.1* genes are shown in Figure 7.

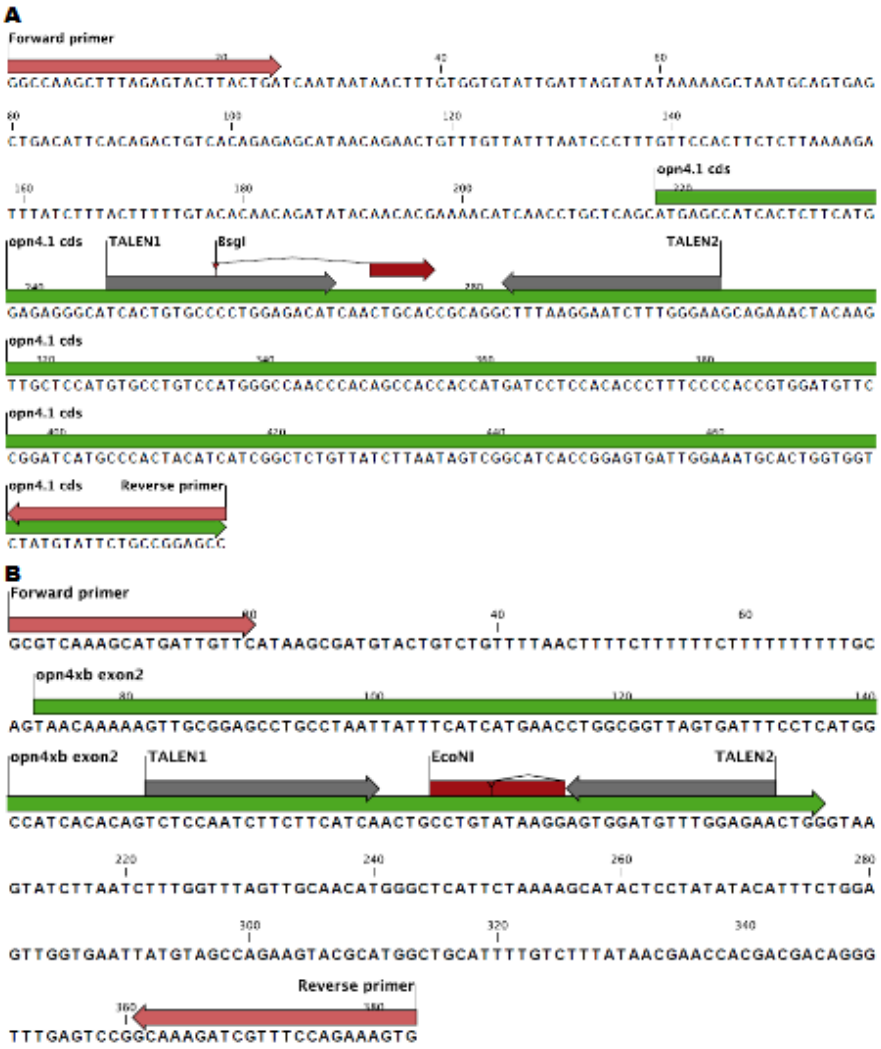

Figure 7: schematic representations of exons/coding sequences (green), primer binding sites (red), TALEN binding sites (grey) and respective enzymes. A) opn4.1 B) opn4xb.

The lesion induced in the *opn4xb* gene leads to an eight basepair deletion resulting in an early stop codon after the second (of seven) transmembrane domains, rendering the protein dysfunctional. In the case of *opn4.1* (which consists of a single coding sequence) a five base pair deletion leads to an early stop codon before the first transmembrane helix.

Inserting *KalTA4* into an iTAL backbone

TALENs are used to introduce targeted mutations into genomes. However, they also offer the opportunity for more advanced genome modifications. Reliably cutting TALENs can be used to insert a DNA cassette to knock in any gene of interest in front of a selected promoter. Using this technique one can label melanopsin expressing cells in zebrafish by inserting RFP (red fluorescent protein) in front of the melanopsin promoter.

An iTAL vector already available in the lab was used and its *EGFP-poly(A)* insert replaced by the *E2A-KalTA4* cassette from a vector received from the Del Bene lab [35].
KalTA4 is an improved version of the transcriptional transactivator *Gal4*. As in the classical construct *KalTA4* binds the *UAS* enhancer and drives the marker gene under its control. *E2A*, a peptide linker, ensures multicistronic expression. The ribosomal skipping sequence results in the protein (opn4m) being cut off during translation without the need for a stop codon.
This construct will be coinjected with TALENs into a transgenic line containing a *UAS* enhancer driving RFP expression (*Tg(UAS:RFP;cry1:eGFP)*). After the TALENs induce a double strand break, the construct should be inserted via ligation. If an in-frame insertion occurres, RFP will be expressed under the promoters of the respective melanopsin genes. Therefore the presence of RFP will provide information about the spacial expression of melanopsin.
This signal is expected to be much stronger than when only EGFP is inserted, as the signal will be amplified because *KalTA4* activates several *UAS* sites and can describe numerous times from each *UAS* site (Fig. 8). Melanopsin would be non-functional in these cells.

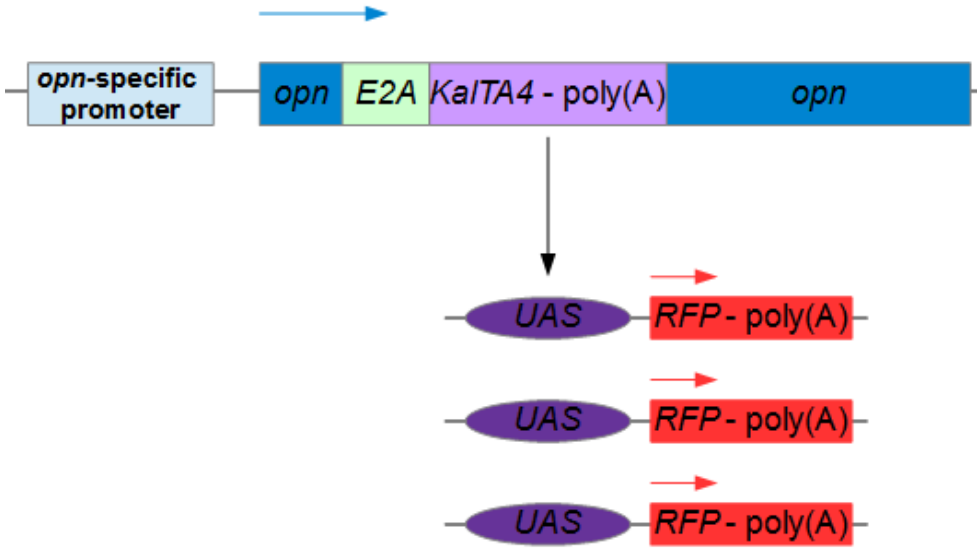

Figure 8: function of the KalTA4 cassette after successful in-frame knock in. Scheme modified from the classical Gal4-UAS system.

The *KalTA4* plasmid was used as a template for PCR. The primers were designed so that a *Bam*HI restriction site was added at the 5' end and a *Xho*I restriction site at the 3' end[3]. The PCR was set up as follows:

0.2	µl Phusion Polymerase
5.0	µl 5x Buffer HF
1.0	µl dNTPs (10 mM)
1.0	µl *KalTA4* fwd (5µM)
1.0	µl *KalTA4* rev (5µM)
1.0	µl *KalTA4* plasmid (1:1000)
15.8	µl ddH_2O

PCR programme

98°C	30 sec	
98°C	30 sec	
60°C	30 sec	
72°C	1:30 min	35x
72°C	1 min	

After the size of the PCR product had been confirmed by gel electrophoresis, the PCR was purified using the Qiagen MinElute Reaction Cleanup Kit.
The purified product was then digested with *Bam*HI and *Xho*I to generate sticky ends.

15.0	µl DNA	
1.0	µl *BamH*I (20,000 U/ml)	
1.0	µl *Xho*I (20,000 U/ml)	
3.0	µl CutSmart Buffer (10x)	
10.0	µl ddH_2O	incubation at 37°C, 2 hours

The reaction was purified using the Qiagen MinElute Reaction Cleanup Kit.
The original iTAL vector was digested with *Bam*HI and *Sal*I (*Xho*I and *Sal*I generate compatible ends).

3 *KalTA4* fwd: 5' TTATTGGATCCGGAGGAGGACAG 3'
KalTA4 rev: 5' TAAGACTCGAGTTAGGTCTCGAATTGCCCACTAGTTCTAGAGCGGC 3'

2.9	µg vector DNA	
1.0	µl *Bam*HI (20,000 U/ml)	
1.0	µl *Sal*I (20,000 U/ml)	
6.0	µl ddH_2O	incubation at 37°C, 2 hours

This led to the original *EGFP* being cut out of the vector. The reaction was run on a 1% TAE gel and the linearised vector was cut out and gel purified with the Qiagen QiaQuick Gel Extraction Kit.

The *KalTA4* cassette was then ligated into the vector:

1.0	µl linearised vector (36 ng/µl)	
4.0	µl *KalTA4* insert (42 ng/µl)	
1.0	µl T4 DNA ligase (400,000 U/ml)	
1.0	µl T4 DNA ligase Buffer (10x)	
3.0	µl ddH_2O	incubation at 4°C, overnight

The ligated product was transformed into *E. coli* XL-1 blue cells. 100 µl were plated on LB plates containing ampicillin (the vector having a resistance gene) and incubated overnight at 37°C.

Several colonies were used to inoculate LB medium (also containing ampicillin, 50 mg/ml, diluted 1:1000) and grown overnight (37°C, shaking).

The cultures were then minipreped and sent for sequencing to confirm correct insertion.

The final construct containing the *KalTA4* site contains two restriction enzyme recognition sites, for *Eco*RI and *Bam*HI, before the *E2A* sequence (Fig. 9).

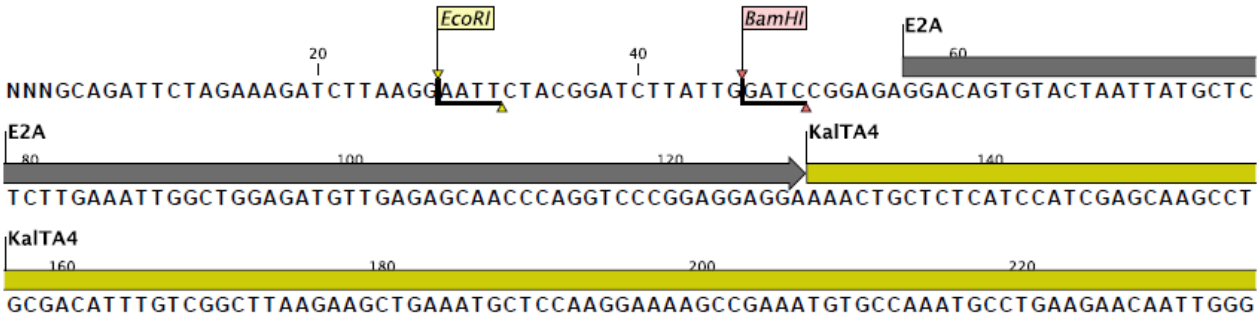

Figure 9: excerpt of the KalTA4 construct cloned into the iTAL vector.

For *opn4.1* and *opn4xb* oligonucleotides were designed which had TALEN1 (upper strand, 5' to 3') put on the lower strand (5' to 3'), making it our “left TALEN”. The same was done for TALEN2, making it our “right TALEN” (Fig. 10 for the oligonucleotides and compare to Fig. 7 for original TALEN pairs). Importantly, the spacer region in between the TALEN binding sites was not inverted. These oligonucleotides were used to create a TALEN binding site in the construct, so that it can be cut by TALENs and ligated into the *opn4* genome, that was cut with the same TALENs. When the TALEN sites are inverted, the sticky ends, after being cut, are still compatible with each other, thus the construct can be ligated into the genome. Thereafter the TALEN recognition sequence has been changed, due to the inversion of the TALEN binding sequences. Because of this the TALENs cannot cut and remove the construct after it has been inserted.

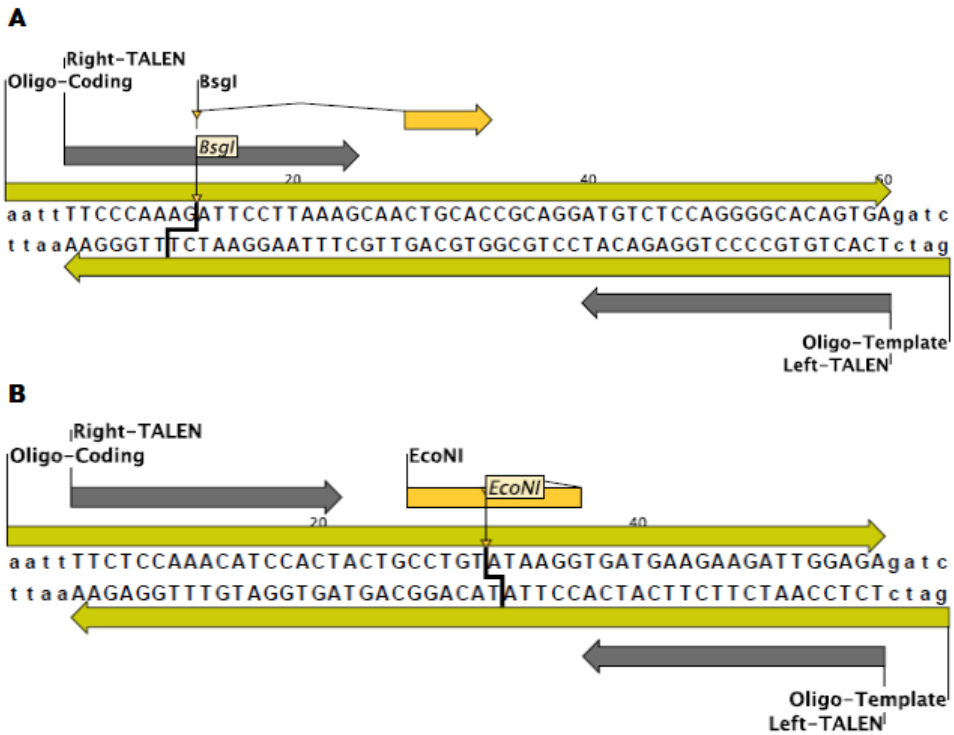

Figure 10: oligonucleotides designed to cause integration of the KalTA4 construct into the DSB created by TALEN activity. A) opn4.1 B) opn4xb.

The single stranded oligonucleotides were annealed to create double stranded molecules and ligated into the iTAL backbone containing the *KalTA4* cassette, using *Eco*RI and *Bam*HI overhangs designed onto the ends of the oligonucleotides.

The oligonucleotides were therefore diluted to a concentration of 5 µM each. 2 µl of each oligonucleotide were mixed with 46 µl of oligo-annealing buffer (10 mM Tris, 50 mM NaCl, 1 mM EDTA) and heated at 95°C for 3 min. The heatblock was then switched off and the

DNA left inside until it had reached room temperature. In order to clone the oligonucleotides into the *KalTA4* plasmid, the *KalTA4* plasmid was cut with *Eco*RI and *Bam*HI. The cut vector was gel purified and used to set up the ligation:

2	µl annealed oligonucleotides	
1	µl cut vector (74 ng/µl)	
1	µl T4 DNA ligase (400,000 U/ml)	
1	µl T4 ligase buffer (10x)	
5	µl ddH_2O	incubation at 4°C, overnight.

The final construct was then amplified using *E.coli* XL-1 blue cells as before and purified in an endotoxin-free maxiprep using the EndoFree Plasmid Kit from Qiagen. Sequencing confirmed the successful construction, the plasmids were diluted to a final concentration of 40 ng/µl to be coinjected with the respective TALEN pair.
Injected fish will show red fluorescence in those cells were the melanopsin gene is usually expressed. Obviously this only happens when an in-frame insertion occurrs. This can be easily screened for by looking at larvae at the age of five to six days post fertilisation under a fluorescence microscope. An out of frame mutation – even though not useful for visualising melanopsin expression – can still be screened for to determine if at least the integration event itself is taking place. This can be done by performing a PCR on the gene fragment. There are two possibilities: use a reverse primer binding in the *KalTA4* region to test for the presence of the construct or use the already well established genotyping primers. In the latter case the failure or success of integration can be determined by the size of the PCR product. This might not work, however, if more than one *KalTA4* construct had integrated for this would create a too large PCR fragment to be amplified.

Dot avoidance assay

Zebrafish is an animal model organism which can be used to study complex behaviour, and routine habits, such as feeding, resting and mating can be analysed. After the establishment of our mutant lines we moved on to study the phenotype by applying behavioural tests.
Our mutants (*opn4.1* and *opn4xb*) were subjected to a dot avoidance assay.
For this a heterozygous couple of was crossed and the progeny raised to day eight.
These larvae - siblings of yet unknown genotypes - were then put in “race tracks” (Fig. 11).

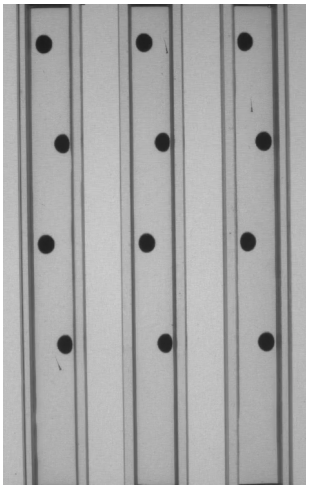

Figure 11: dot avoidance assay. 8 day old larvae were presented with dots of varying sizes, e.g. relative dot size 40.

These tracks were placed on a horizontal computer screen. The larvae were then presented with black moving dots of increasing size (relative sizes of 1, 5, 10, 20, 30, 40 and 50), each time six dots of the same size following each other. Before they were exposed to a series of a larger dot size, the animals were given a short resting period. During the assays the larvae were videorecorded. These videos were subsequently analysed to determine the larvaes' behaviour.

Two categories were scored: dot avoidance and dot approach. Every movement that was clearly not caused by the dot, such as ignoring of the dots as well as everything that could not be categorised beyond doubt was not taken into evaluation.

The major criteria for an approach or an avoidance were:

1. speed, the larvae must increase its swimming speed dramatically
2. direction, the larvae must clearly change the vector of its way to avoid or approach

only when both criteria were met the response was counted.

Genotyping was done only after scoring the videos to ensure complete objectivity (blind testing).

The data scored was then numerised by calculating an avoidance index per dot size for each genotype, consisting of the averaged reactions to the dots.

$$\text{Avoidance index} = \frac{\text{number of avoidances} - \text{number of approaches}}{\text{number of dots}}$$

while this gives a value suited for comparison, it has one major weakness. A larvae that would e.g. avoid twice and approach twice is giving exactly the same result as a sibling being completely unresponsive. Therefore additionally a response index can be calculated, according to

$$\text{Response index} = \frac{\text{number of avoidances} + \text{number of approaches}}{\text{number of dots}}$$

For every sample point the standarddeviation was used to calculate the standard error of the mean (SEM), which was used to set error bars (+ and -). A T-test was later done to detect any statistical significance between two data points which were considered possible candidates due to their error bars.

Results & Discussion

In situ hybridisations on six day old larvae

In order to obtain insight into the identity of the tissues and organs the *opn4* genes are expressed in, *in situ* hybridisations were performed.

Staining in the larvae clearly shows the presence of *opn4xb* and *opn4.1* mRNA in the neuromasts of the lateral line (Fig. 12). Higher magnification images show the morphology of the typical hair cell structure, stereocilia protruding from the cell's apical surface, further confirming expression in mechanosensory cells.

It has been previously shown that melanopsin is expressed in cycles during the day in the retina, the temporal pattern depending on the length of the photoperiod. For *opn4.1* and *opn4a* rhythmic expression was shown [17]. To see if this is also the case for *opn4xb* expression in the neuromasts of the lateral line, I compared the expression levels, by *in situ* hybridisation, for *opn4xb* at four different times of the day. A difference between the four zeitgeber times tested (ZTs 3, 9, 15 and 21, 12:12 LD) was not detected. Thus *opn4xb* shows no rhythmic expression in the neuromasts and is therefore not under the control of the circadian clock at the transcript level.

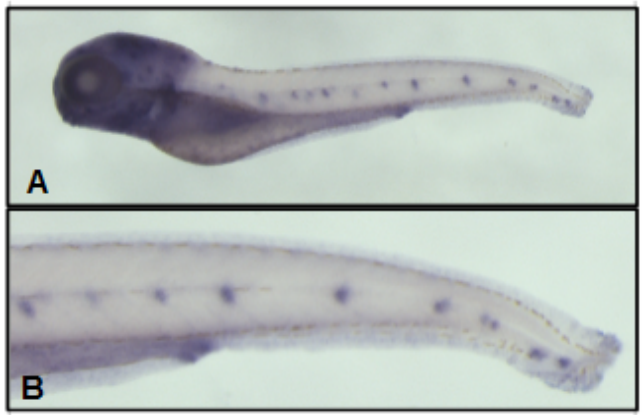

Figure 12: Alkaline phosphatase staining of opn4xb at ZT 9 in the whole larva (A) and the neuromasts of the lateral line (B).

The head shows strong staining, which may be background staining or brain specific. However, it proved quite difficult to reduce background staining in yolk and head by increasing blocking and washing times. The specific staining of the neuromasts by using *opn4xb* and *opn4.1* probes was clear, while the other three melanopsin genes are not expressed in the neuromasts of the larvae (nor anywhere else in the trunk). They were

found to be expressed in the retina (as previously reported [17] [18]) and also some staining in the head was detected, which might be located in the brain, however, the sensitivity of the *in situ* hybridisation is too low in this case to distinguish further details.

Expression of *opn4xb* and *opn4.1* in the neuromasts

To get further evidence that melanopsin is expressed in the neuromasts of the lateral line, a fluorescent double *in situ* hybridisation was done to show coexpression of *pou4f3* and *opn4* genes. *Pou4f3* is a marker for neuromast cells because it plays an important role in their differentiation.

A double *in situ* hybridisation on progeny from the *Tg(Brn3c:GAP43-GFP)* line [40] was done. This transgenic line carries the GFP (targeted to the membrane by GAP) under the *pou4f3* (*brn3c*) promoter/enhancer. GFP is expressed in transgenic embryos in the retinotectal projection, as well as in neuromasts in the inner ear and the lateral line.

We used confocal microscopy to determine whether the *gfp* mRNA and *opn4* mRNAs are coexpressed. The confocal microscope's scanning laser makes sections through the sample, thus this method is suitable for determining coexpression of markers in the same plane (in contrast to a normal fluorescence microscope, where only the whole sample can be imaged). The images clearly confirm overlaps of expression of *opn4xb* and *opn4.1* with *gfp* in the larvae's neuromasts.

Figure 13 clearly shows expression of *opn4xb* and *pou4f3* (shown by GFP), as well as their colocalisation. By merging the channels, yellow appears where red (*opn4*) and green (*gfp*) overlap. The same can be seen for *opn4.1.*

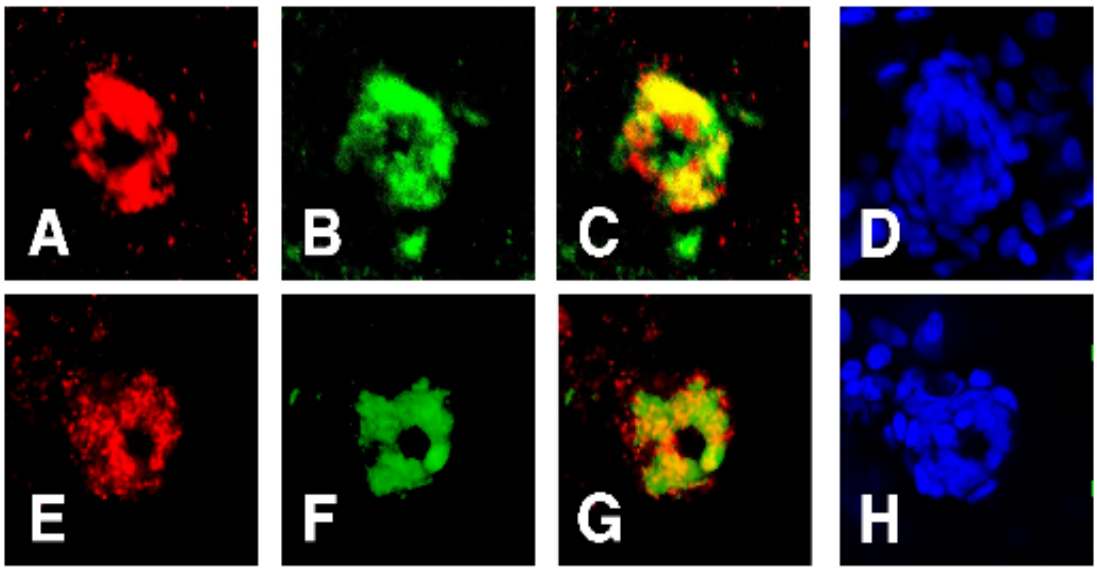

Figure 13: Lateral line neuromasts of 6 day old zebrafish larvae. A-D: opn4xb, E-H: opn4.1. Fluorescent staining against opn4xb (A) and opn4.1 (E), respectively, pou4f3 (B, F) in the same neuromasts, as well as the composite images (C, G). D, H: Höchst staining.

Figure 13 A shows the expression of *opn4xb,* E of *opn4.1*. Here, according to our observations in previous *in situ* hybridisations, *opn4.1* is again weaker expressed than *opn4xb*. In B and F *gfp*, expressed under the *pou4f3* promoter, known to be specifically expressed in the neuromasts, can be seen. Merging the two channels results in an overlap with yellow appearing where red and green are expressed in the same plane (Fig. 13 C,G). Höchst staining visualises the nuclei and shows the characteristic chimney structure of the neuromasts. The overlap between the melanopsins and the neuromast marker is not complete, but quite extended, this can be seen especially for *opn4xb*.

In situ hybridisation on adult brain sections

Melanopsins have been shown to be expressed in the brain [17]. Since larval brains seem to stain for *opn4xb* and *opn4.1*, we investigated if the five melanopsins are also expressed in the adult brain. As structures are easier recognisable due to the adult brain being fully developed their identification is facilitated.

All our melanopsin probes, for *opn4a*, *opn4b, opn4.1, opn4xa* and *opn4xb* show expression of these respective genes in the adult brain.

Figure 14 gives a selection of three brain slices for each melanopsin gene, to show their expression patterns.

The mRNA of *opn4xb* and *opn4.1* is clearly present in the periventricular gray zone of the optic tectum and along the caudal ventral zone of the periventricular hypothalamus and the posterior tuberal nucleus.
The staining doesn't appear to be random, clear patterns with sharp boundaries are visible. Both genes show a similar expression pattern, this may indicate that these genes are redundant, as also both are expressed in the neuromasts. In several independent experiments identical staining was obtained every time this *in situ* hybridisation was repeated, even with ever decreased proteinase K digestion times and ever increased washing and blocking steps, to minimise the chances of probe trapping or unspecific background staining.
Also hardly any background staining appeared in the *in situ* hybridisation with the probe against *opn4a*, which was done at the same time, indicating that the staining is unlikely to be unspecific. The expression of *opn4a* is more restricted and is located in the hypothalamus, labeling individual cells rather than whole areas. The medial expression is in the ventral zone of the periventricular hypothalamus. The small very lateral expession domain towards the base of the brain is in cells which have so far not been named. The other lateral, more dorsal and larger domain could be either the diffuse nucleus of the hypothalamus or in the preglomerular area (Fig. 14). To be certain one would need to apply a counterstain for cell body identification.

Opn4xa and *opn4b* were also demonstrated to be expressed in the brain. *Opn4b* (Fig. 14) may show labeling in the entopeduncular nucleus (ventral part). Also expression in the periventricular layer of the optic tectum can be seen, as well as the ventral zone of the periventricular hypothalamus.
We determined the identity of the areas in the brain where *opn4* genes are expressed using the Zebrafish Brain Atlas [41] and contacted the author to obtain his expert advice.

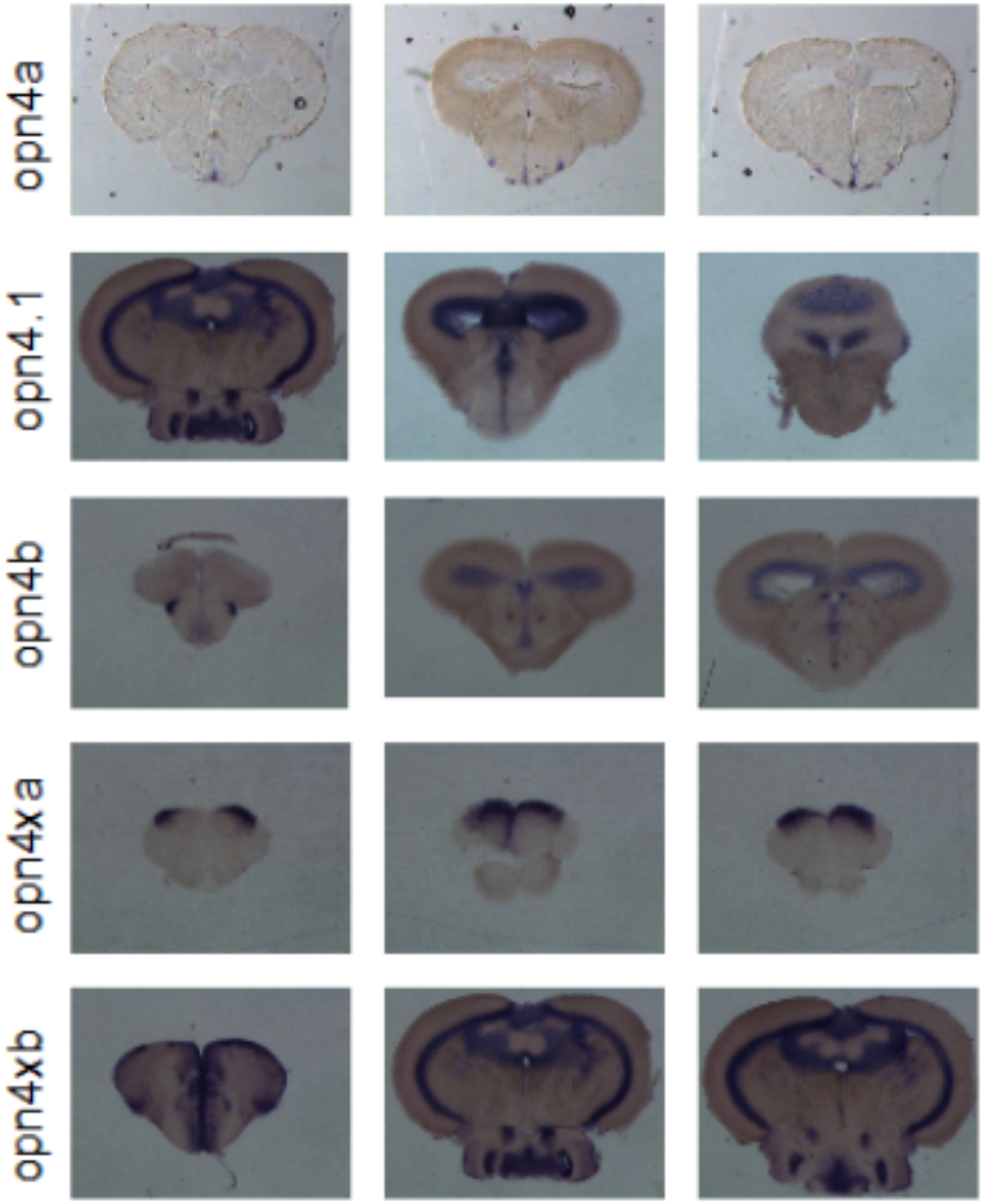

Figure 14: adult brain sections (100µm) for all five melanopsin genes after in situ hybridisation with antisense RNA probes and alkaline phosphatase staining. Magnifications: opn4a 5x, others 68x.

Table 1 gives an overview of expression patterns of all five melanopsins in the zebrafish central nervous system.

Table 1: Areas of melanopsin expression in the zebrafish CNS

Gene Symbol:	Previous Gene Symbol(s):	Expression in retina [*]:	Expression in brain [†]:
opn4a	*opn4m1*	amacrine & bipolar cells	ventral zone of periventriclar hypothalamus & diffuse nucleus of hypothalamus (?).
opn4.1	*opn4c & opn4l & opn4m2*	horizontal cells & single cone photoreceptors	periventriclar layer of optic tectum, periventricular dorsal and caudal hypothalamus, corpus mamillare, torus longitudinalis, granule cells of valvula and corpus cerebelli, granule cells of lobus caudalis cerebelli, thalamus, posterior tuberculum, medial and posterior zone of dorsal telencephalon, & dorsal nucleus of ventral telencephalon.
opn4b	*opn4l2 & opn4m3*	amacrine, bipolar, horizontal & retinal ganglion cells	ventral part of the entopeduncular nucleus, lateral part of the ventral thalamus, periventricular ventral and dorsal hypothalamus, periventriclar layer of optic tectum, torus longitudinalis, periventricular pretectum, posterior tuberculum, dorsal tegmental nucleus (?), preglomerular nucleus, valvula cerebelli & corpus mammilare.
opn4xa	*opn4x-1*	amacrine, bipolar, horizontal & retinal ganglion cells	anterior pallial subdivision of the medial zone of the dorsal telencephalic area.
opn4xb	*opn4x-2 & opn4x1*	bipolar cells	periventriclar layer of optic tectum, periventricular dorsal and caudal hypothalamus, corpus mamillare, torus longitudinalis, granule cells of valvula and corpus cerebelli, granule cells of lobus caudalis cerebelli, thalamus, posterior tuberculum, medial and posterior zone of dorsal telencephalon, & dorsal nucleus of ventral telencephalon.

* Davies et al., Cell. Mol. Life Sci. (2011). † Wullimann, Rupp & Reichert, Neuroanatomy of the Zebrafish Brain, Birkhäuser (1996), and Wullimann personal communication.

Since melanopsins are known to be a crucial component of the input pathway into the circadian clock, we determined if the core circadian clock gene *per1b* is expressed in the same location of the brain. The results show that the clock gene *period* is rhythmically expressed in the zebrafish brain, the validity of these results was confirmed by other reports [42].

In zebrafish, *per1b* is expressed in a circadian manner, its expression peaking at ZT 21 (Fig. 15). Fish were raised in 12:12 LD conditions, the peak expression thus corresponds to the early morning. The brain sampled at ZT 3, where the second strongest expression was observed, was taken at noon, three hours after light onset.

The other zeitgeber times correspond to light conditions (ZT 9) and midnight, three hours after light off (ZT15). The results show that *per* expression largely overlaps with that of *opn4.1* and *opn4xb* and that *per* is expressed in a large part of the zebrafish brain. In contrast to mammals this may indicate a more decentralised clock system in the zebrafish. Although *in situ* hybridisations are not a quantitative method to determine gene expression levels, the oscillations of the mRNA show a high amplitude in the brains taken at the four zeitgeber times ZT 3, 9, 15 and 21. All stainings were stopped at the same time and no sample was allowed to stain longer than the others to avoid unrepresentative results.

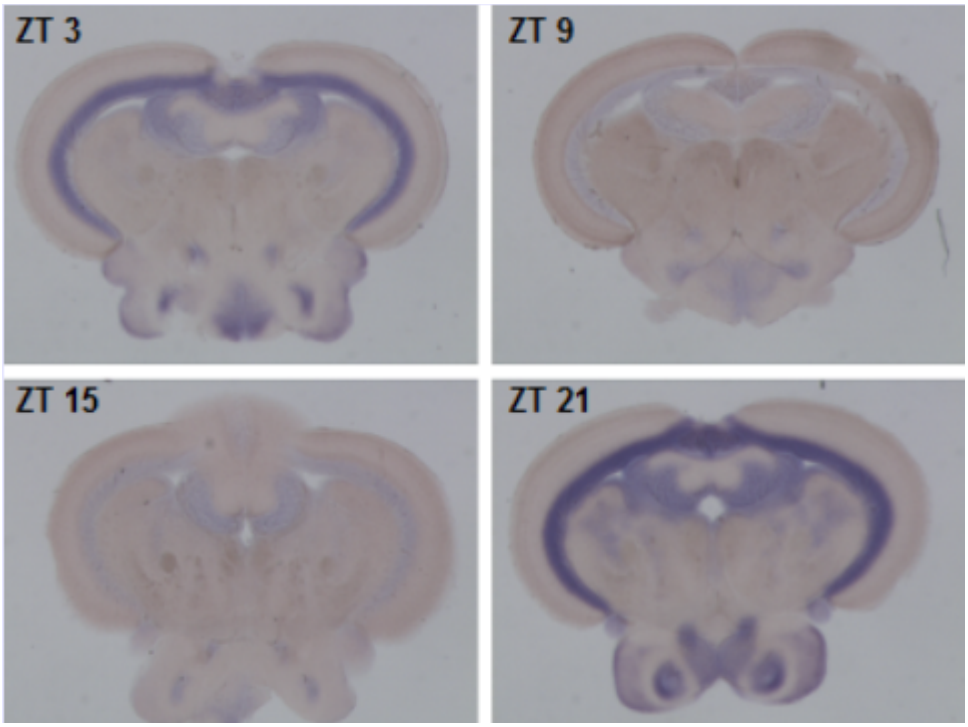

Figure 15: in situ hybridisation on adult brain slices (100µm). per1b shows oscillating expression levels.

That there is no central pacemaker in the zebrafish brain would not be surprising as most zebrafish cells can detect light directly. If every cell can reset its own clock, a master clock would be superfluous. The advantage of such a system is speed. If cells can react to light autonomously any response will be much faster than when a signal is send from a master clock to the periphery.
Most intriguing, the patterns seen for *opn4xb* and *opn4.1*, similar to each other, are also quite similar to the expression of *per1b* (Fig. 16).
This high degree of coexpression with a high amplitude core clock gene hints at a role for these two melanopsins in the input pathway of the circadian clock.

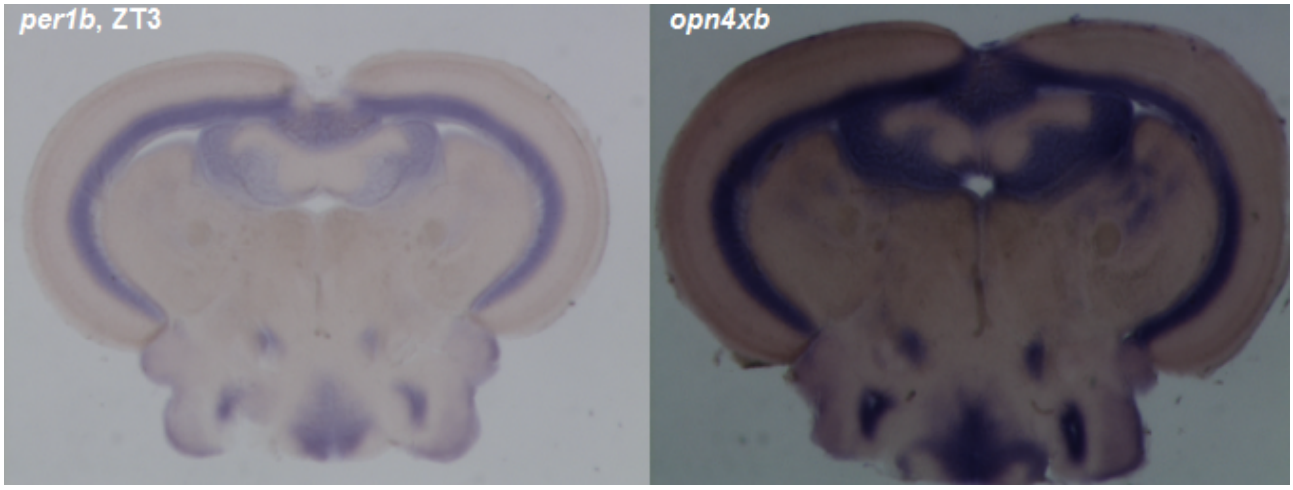

Figure 16: Comparing expression patterns in the adult brain. per1b and opn4xb show a very similar spacial expression. The same is true for opn4.1. Adult brain slices, 100µm.

It is an interesting fact that *opn4.1* and *opn4xb* – exactly and only those two out of five melanopsins being expressed in the zebrafish lateral line, show overlapping expression with a core clock component in terms of expression in the brain. If melanopsin's role in the neuromasts of the lateral line is a modulating one, like increasing or decreasing its sensitivity to water pressure changes, it would make sense to couple this to light receptors, for an increased sensitivity of the lateral line would be advantageous at a time where information input via the eye is strongly reduced.

Genotyping

In order to introduce mutations in the *opn4* genes, TALENs were generated and injected into zygotes. Subsequently these founder fish were screened when they had reached adulthood. Of the fish injected with TALENs targeting the *opn4xb* gene one mutant with germline transmission was recovered. The offspring was screened for the mutation via fin-

clipping, among 81 only one heterozygous male was found. This indicates that the TALENs only induced a mutation in a very small number of the founder male's germ line cells.

For *opn4.1*, 28 fish (offspring of the founder fish) were tested. In two F1 fish the mutations in the fins did not lead to a stop codon or a frameshift.

Three mutants having a stop codon were discovered in a subsequent round of screening (Fig. 17), one had an early stop codon. An early stop codon renders the *opn4.1* gene dysfunctional, because it will lead to a truncated protein omitting all its functional domains. To genotype a PCR product (494 bp) was designed that has the enzyme restriction site roughly in the middle, which would lead, when cut, to two similarly long bands (238 and 256 bp) appearing as one on the gel. Thus when a mutation is introduced the restriction site is changed giving a full length PCR fragment after digestion with the restriction enzyme. The strength of the uncut bands is weaker than the cut ones, as two larvae were pooled together and a PCR performed on them, leading to a mutant/wild type allele ratio of 1:3 if only one of them is mutant.

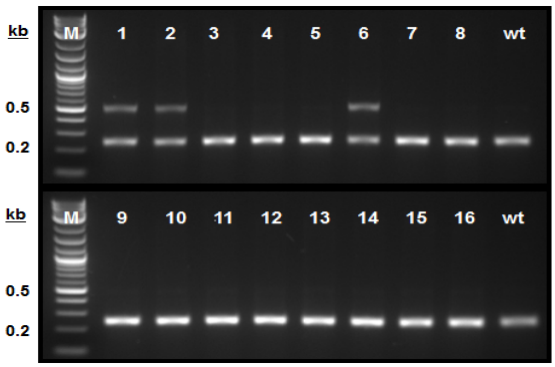

Figure 17: Male 1 offspring, opn4.1. 1.5% agarose. M_DNA-Marker, NEB 2-log ladder; lanes 1-16_samples 1-16, two larvae/sample;. wt_wt control. Samples 1, 2 and 6 show heterozygousity.

In the injected founder generation (P) some somatic mosaic mutants were found, but those would not pass the mutation on to their offspring. We also found injected fish that proved to be germline mutants, but had no mutation in their fins. In zebrafish the division between soma and germline is already present at an early stage [37]. Fin cells and germ cells have different precursors, therefore finding a mutant in the soma is not a guarantee that the mutation is also transmitted through the germline. The only way to test for germline transmission is to test the offspring of the founder fish, the F1 generation. Since it is very

unlikely that all cells of the germline are mutated, the founder fish is expected to yield a non-mendelian ratio of mutant offspring. When, however, one progeny of the founder fish is derived from a germ cell carrying the mutation, it will be heterozygous in all cells. Therefore, when this fish is crossed to a wild type, it should give 50% heterozygous offspring in the F2 generation.

For both genes *opn4xb* and *opn4.1*, the mutation frequency in the adult founder fish was lower than observed in the founders tested at day three post fertilisation. This could be due to lethality in the TALEN injected fish at a later stage. However, in both cases germline mutants were recovered. Founder fish were bred to wild type fish to recover heterozygotes. By incrossing the heterozygous fish homozygous mutants were obtained. To see if homozygosity for *opn4.1* or *opn4xb* leads to any dramatic effects, such as balance defects due to the genes' abolished function in the lateral line, offspring of heterozygous parents were subjected to a simple tapping assay in the first week post fertilisation. The dish with the larvae is tapped at the side and the behaviour of the animals is observed. The expectation was that homozygous mutants (roughly ¼ of all larvae) might show uncoordinated motility or circling behaviour due to defects in balance. These kinds of abnormal behaviours were reported for a zebrafish mutant line with defective hair cell function [38]. This assay failed to give any hints for balance phenotypes. There were no obvious differences between sibling larvae, neither for *opn4.1* nor for *opn4xb* mutants. It is still possible that the phenotype is weak and will become stronger with age, but is simply not detectable in three to six day old larvae, or that the *opn4.1* and *opn4xb* genes are redundant.
Thereafter the homozygotes and their siblings were subjected to more complex behavioural assays, such as the dot assay.

Design and construction of TALENs targeting the *opn4b* gene

TALENs targeting the *opn4b* gene were designed as described in *Materials and Methods* for four exons of the gene. TALENs targeting exon 3 and exon 5 were designed first, injected in the zygotes derived from a Tübingen wild type cross and the larvae screened at day three. 24 larvae survived and were screened after injections. Not a single mutation was introduced in either case. A second round of injections also failed to introduce mutations. Therefore I designed two new TALEN pairs against exons 7 and 8 (Fig. 18). Sequencing confirmed the correct order of the RVDs.

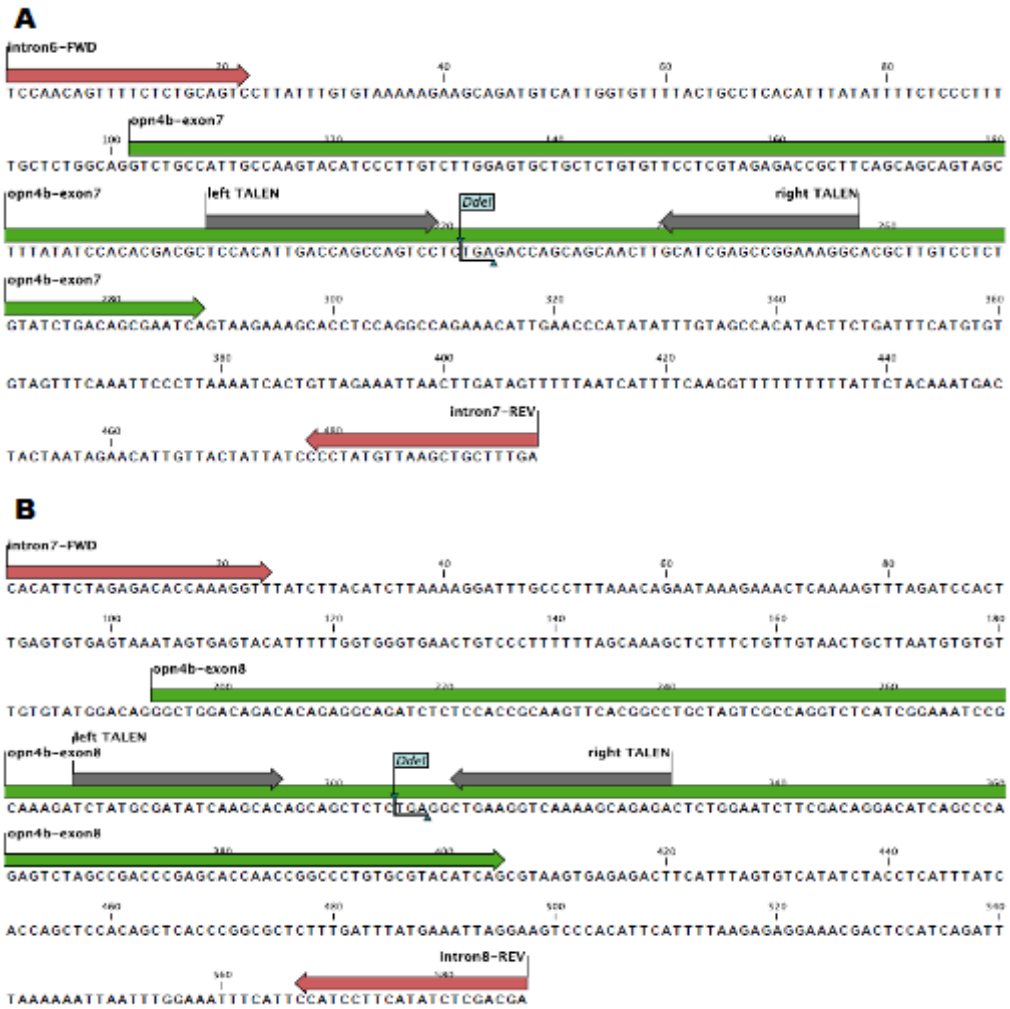

Figure 18:schematic representations of exons/coding sequences (green), primer binding sites (red), TALEN binding sites (grey) and respective enzymes for opn4b, A) exon 7 B) exon 8.

The quality of the RNA was tested via gel electorphoresis as can be seen in Figure 19. As the bands are quite strong compared to the smear (which is to be expected, since RNA gets degraded quickly when run on an agarose gel) the RNA was considered to be of high enough quality to be used for injections.

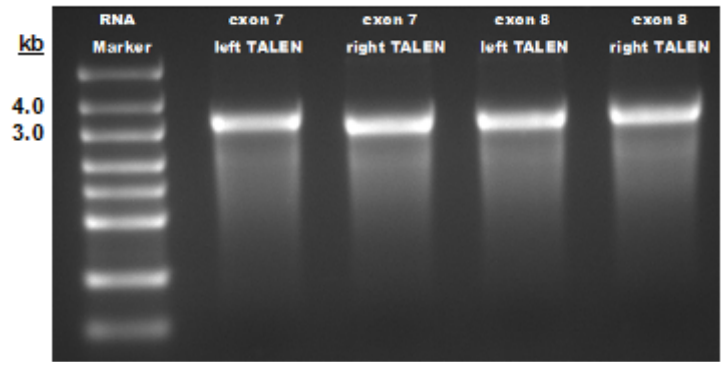

Figure 19: TALEN RNA, 1.5% agarose gel, RNA marker Thermo scientific RiboRuler HR. Expected fragment size ~ 3,200 bp for each TALEN part.

The new TALEN pairs were injected into offspring of Tübingen wild type crosses. At day three the larvae were taken for screening. Also in this case no mutations were introduced (Fig. 20).

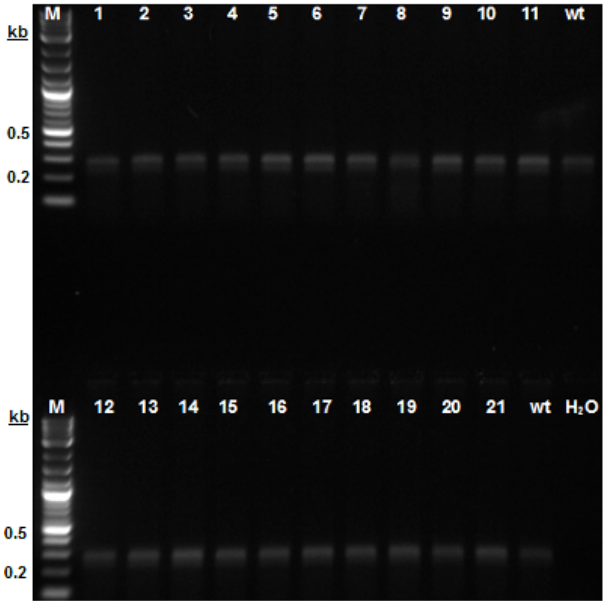

Figure 20: three day old larvae injected with TALENs against opn4b, exon7. 1.5% agarose, M_DNA-Marker, NEB 2-log ladder; lanes 1-21_samples 1-21, wt_wt control, H_2O_water control.

All four TALEN pairs designed to target the *opn4b* gene failed to introduce any mutations. The gene seems to be hard to target for the TALENs. In order to still introduce a mutation into this gene another genome modification technique will be used. The CRISPR/Cas9 system is a powerful tool to mutate the gene. As with TALENs this method relies on

introducing a double strand break. Here the Cas9 endonuclease can be guided to the target site by a short RNA molecule. This is in contrast to TALENs, which function through DNA-protein interactions [39]. Using the CRISPR/Cas9 system one could target several exons of the gene at once to determine the most efficient target site.

Labeling melanopsin expressing cells with a *KalTA4*-UAS system

By expressing RFP under the melanopsin promoter using a system that allows RFP to be integrated (in frame) in the TALEN target site, I aim to gain further information about melanopsin expression patterns. Using the *KalTA4*-UAS system, a modified variant of the GAL4-UAS system, one can obtain information on the protein expression level. For *opn4.1* and *opn4xb* a plasmid containing homologous DNA sequences of the TALEN target region, as well as the *KalTA4* cassette was created. The generation of the construct was successful as determined by sequencing (Fig. 21). This construct has all the necessary properties to be integrated at the site of a TALEN induced double strand break by the cell's DNA repair machinery.

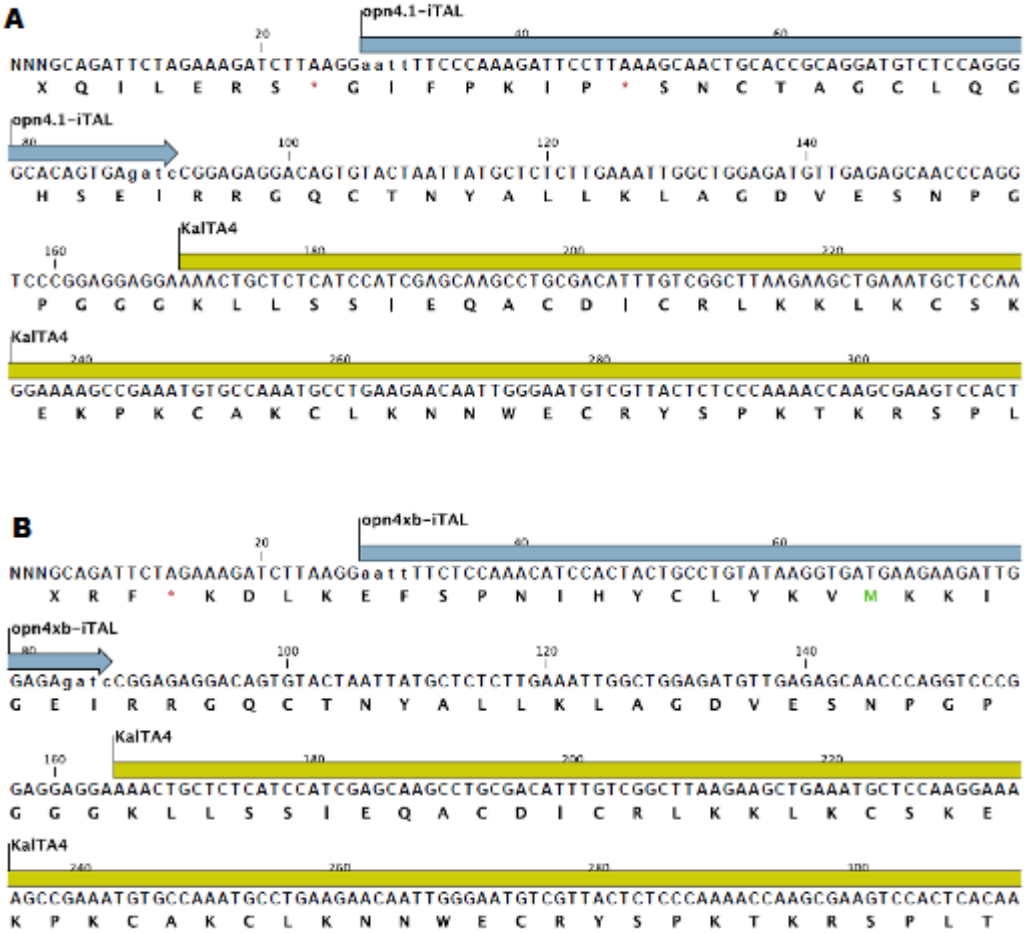

Figure 21: excerpt of the final constructs for insertion after TALEN induced DNA damage. A) opn4.1 B) opn4xb.

The construct was coinjected with the TALENs into the *UAS*-driver line and larvae inspected for RFP presence at day five. No fluorescence could be detected, neither in the case of the *opn4.1* nor the *opn4xb* specific construct. A PCR analysis using genotyping primers gave the expected band size for the wild type situation in all samples (Fig. 22). In case of a single integration event the size of the PCR product is increased by about 4.6 kb (construct + pJet vector). This fragment is still detectable via PCR, by using a long enough extension time. In order to introduce a smaller fragment into the genome, a second inverted TALEN site could be added at the 3' end of the *KalTA4* construct. Then only the intermediate part would be integrated, however the TALENs would have to cut the construct twice for this to work.

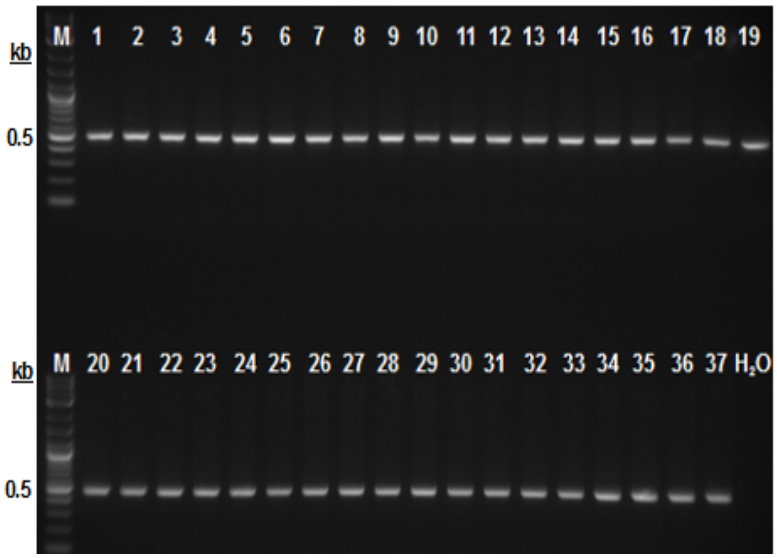

Figure 22: 4.1 KalTA4 injected larvae. M_DNA-Marker, NEB 2-log ladder; lanes 1-37_samples 1-37, H_2O_water control. No integration events have taken place.

The reason for the absence of integration in the first trial injections might be a very low efficiency. The chance that the TALENs induce a mutation and that the construct also gets integrated is expected to be lower than the chance of a TALEN pair inducing a mutation without integration.

Another possibility is that all components work fine, but that by chance no integration event happened. In this case injecting more animals would increase the chances of the desired insertion taking place. If a larvae with a successful integration could be recovered and raised to adulthood, this fish would then have to transmit the integration through its germline to establish a transgenic line. However, even expression for one single generation would be sufficient to get information about the location of *opn4* expression.

Behavioural assay – dot avoidance

The current theory states a separation between sensing and processing organs. The brain, traditionally thought of as a processing organ, may exhibit direct modulations of processes by light [43], indicating a sensing and processing brain. In order to determine whether the absence of one functional OPN4 protein leads to any detectable difference in behaviour, *opn4xb* and *opn4.1* mutants were subjected to a dot assay. In this assay their avoidances and approaches to different dot sizes, projected on a monitor placed under their translucent race track aquaria, were scored. Zebrafish larvae are known to show active avoidance behaviours already five days post fertilisation [32], this experiment was done at

day eight post fertilisation. The larvae, offspring of heterozygous parents, were genotyped only after analysis of the behavioural assay, ensuring a blind test. Statistically significant differences between mutant and wild type siblings in this assay were obtained with the *opn4.1* heterozygous incross offspring. For the *opn4xb* mutant it isn't possible to draw conclusions, for the number of homozygous mutants tested was simply too low.
Importantly fish recorded in the morning were far less responsive than fish recorded in the afternoon, showing the major impact of timing on behaviour. Progeny derived from *opn4.1* heterozygous parents (recorded during the afternoon) showed a significant difference, however only 30 fish could be recorded (Fig. 23).

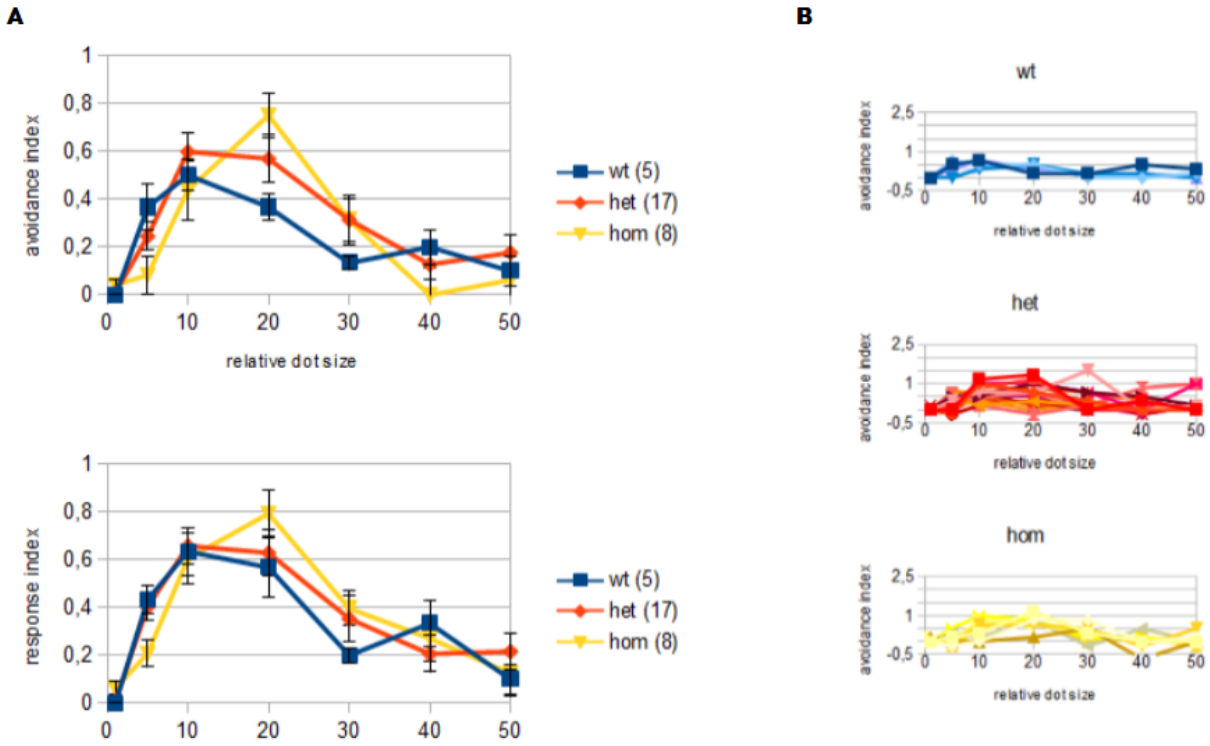

Figure 23: (A) avoidance and response index of opn4.1 heterozygous offspring. Error bars are the SEM of the SD. (B) All larvae are displayed in comparison to siblings of the same genotype.

The conceptual reasoning behind this experiment is that larvae would approach small dots expecting them to be food (larvae had to be fed in the days prior to the experiment) or simply out of curiousity. In contrast they would avoid the big dots, fearing them to be a predator or the shadow of a predator.
During the last dots the fish might well be already habituated to the stimulus, and at some stage they would realise that the dots are not actually harmful, which would reduce the avoiding stimulus. The avoidance index shows that the wild type fish seem to stop avoiding the dots quicker than the mutants. The statistical significance, based on the p-

value, is given for the difference between wild type and homozygous mutants at dot size 20 ($p = 0.0172$) of the avoidance index. Also the siblings of each genotype behave (except for some outliers) quite similar between each other (Fig. 23 B). This supports the validity of the averaged results. The overall responses are similar to each other, showing that the fish are equally reactive. The strongest response was by the homozygous mutants as they were presented with dot size 20, showing that they paid most attention to this dot size, but avoiding it rather than approaching.

Conclusion

The zebrafish model gives valuable insight into the expression of its melanopsin genes. Although little about melanopsin's exact function and possible involvement in the circadian system is known, a lot can be infered from data on mammals.

One of the most striking aspects is the common expression pattern of *opn4xb* and *opn4.1*. Not only are they the only melanopsins expressed in the neuromasts of the lateral line, these genes also show an expression in the adult brain which is very similar to each other, yet very different from the other three melanopsins. Since *opn4.1* and *opn4xb* are expressed in the optic tectum they might modulate processing there. Their overlapping expression with *per1b* points at a function in the input pathway of the circadian clock. *Opn4.1* and *opn4xb* are broadly expressed in the adult brain, which must be taken into consideration when interpreting data collected from the behavioural assays, for any effects might be due to melanopsin absence in the neuromasts, the retina, the brain or a combination thereof. Additionally, their similar expression pattern may point at a redundancy of their functions. As one gene might be able to substitute for the other when this is knocked out, double mutants could well show more severe phenotypes than the single mutants. Therefore we have crossed the *opn4.1* and *opn4xb* mutants to obtain the double mutant. In the dot assay, *opn4.1* mutants showed a different behaviour from their wild type siblings, this may suggest the gene to function in the modulation of behaviour. Several tests and assays are recommended to be done in the future. Since the *opn4b* gene could not be mutated using TALENs, the CRISPR system will be used as an alternative method to hopefully introduce a mutation. More fish will have to be tested with the dot assay to get more reliable results, to further confirm the data. The dot assay will also have to be repeated with an alternating dot size to see if the results can be confirmed. This will strengthen the conclusion that the fish indeed react differently to different dot sizes. As melanopsin could play a role in modulating behaviours the aim is to perform a range of behavioural tests. After this relatively simple assay the fish – single and double mutants – can hopefully soon be subjected to different behavioural tests to give insight into the phenotype and thus function of the two genes under study. One possibility would be to see how the fish are behaving in a virtual reality, where a broad range of stimuli can be presented to them. Another possibility is to test for differences in locomotor activity between mutants and wild type fish under different light conditions, as melanopsin may mediate the effect of light intensity on behaviour.

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Appendix

Summary

Melanopsin is a seven transmembrane domain G-protein coupled photoreceptor.
A gene duplication early in the lineage of vertebrates has led to the diversification into two melanopsin paralogs, *OPN4m* and *OPN4x*. While mammals have only one melanopsin encoding gene (*opn4m*), expressed in intrinsic photosensitive retinal ganglion cells, non-mammailian vertebrates exhibit two classes of opsins, the group first discovered in *Xenopus laevis* (*opn4x*) and the mammalian variant. In zebrafish (*Danio rerio*) three melanopsin genes (*opn4.1*, *opn4a* and *opn4b*) are mammalian related, while *opn4xa* and *opn4xb* are *Xenopus* related.
In this research project we aim to understand the role of zebrafish melanopsins in non-visual light dependent processes.
In order to obtain insight into the identity of the tissues and organs the *opn4* genes are expressed in, *in situ* hybridisations were performed.
Staining in the larvae clearly shows the presence of *opn4xb* and *opn4.1* mRNA in the neuromasts of the lateral line, further confirmed by fluorescent double *in situ* hybridisations using a neuromast marker. Interestingly, *opn4.1* belongs to the mammalian class of melanopsins, while *opn4xb* is *Xenopus* related. In addition we investigated if the melanopsins are expressed in the adult brain. As structures are easier recognisable due to the adult brain being fully developed, individual structures could be identified. All our melanopsin probes, for *opn4a*, *opn4b, opn4.1, opn4xa* and *opn4xb* show expression of the respective genes in the adult brain. Since melanopsins are known to be a crucial component of the input pathway into the circadian clock, we determined where the core circadian clock gene *per1b* is expressed in the brain. The results show that the clock gene *per1b* is rhythmically expressed in the zebrafish brain and that its expression pattern is very similar to that of *opn4.1* and *opn4xb*.
Furthermore, *opn4.1* and *opn4xb* mutants were subjected to behavioural assays, since several behaviours could be modulated by these melanopsins. In a dot assay larvae were presented with black moving dots of increasing size. Their behaviour, avoidance or approach, was scored. A statistically significant difference between mutant and wild type siblings in this assay was obtained with the *opn4.1* heterozygous incross offspring in a first run, but not for *opn4xb*. A tapping assay did not show any differences between mutant and wild type. This may be due to redundancy, as *opn4.1* and *opn4xb* are coexpressed.

Zusammenfassung

Melanopsin ist ein G-Protein gekoppeltes Transmembranprotein. Durch eine Genduplikation früh in der Evolution der Vertebraten entstanden zwei Melanopsin Paraloge, *OPN4m* und *OPN4x*. Während in Säugetieren nur ein für Melanopsin codierendes Gen (*opn4m*) erhalten geblieben ist, das in intrinsisch photosensitiven retinalen Ganglionzellen expremmiert wird, haben andere Vertebraten noch beide Varianten, die zuerst in *Xenopus laevis* entdeckte (*opn4x*) und die der Säuger. Im Zebrafisch (*Danio rerio*) gibt es drei Melanopsine die zur *opn4m* Gruppe gehören (*opn4.1*, *opn4a* und *opn4b*) und zwei, die enger mit den *opn4x* Genen verwandt sind (*opn4xa*, *opn4xb*).

Ziel dieses Projektes ist es, die Rolle von Melanopsin in nicht-bildgebenden, lichtabhängigen Prozessen zu verstehen.

Um einen Einblick in die Identität von Melanopsin expremmierenden Geweben und Organen zu bekommen, wurden *in situ* hybridisations durchgeführt. Die Präsenz von mRNA für *opn4xb* und *opn4.1* in den Neuromasten des Seitenlinienorgans bei Larven konnte gezeigt, und durch eine fluorescent double *in situ* hybridisation mit einem Neuromast-Marker bestätigt werden. *In situ* hybridisation an adulten Gehirnen zeigten die Präsenz von allen fünf Melanopsin Genen (*opn4a*, *opn4b, opn4.1, opn4xa* und *opn4xb*). Da das adulte Gehirn voll entwickelt ist, konnten individuelle Strukturen identifiziert werden. Da Melanopsin ein wichtiger Bestandteil des Input-Pathways der *Circadian Clock* ist, testeten wir wo das *Clock* Gen *per1b* im Gehirn expremmiert wird. Die Ergebnisse zeigen, dass *per1b* rhythmisch expremmiert wird und dass das Expressionsmuster denen von *opn4.1* und *opn4xb* sehr ähnlich ist.

Außerdem wurden *opn4.1* und *opn4xb* Mutanten Verhaltensanalysen unterzogen. Es ist gut möglich, dass das Verhalten der Fische von Melanopsinen moduliert wird, da bis jetzt noch nicht klar ist in welchen Funktionen *opn4.1* und *opn4xb* eine Rolle spielen. In einem *dot assay* wurden die Larven mit sich bewegenden, schwarzen Punkten zunehmender Größe konfrontiert und ihre Reaktion darauf, annähern oder vermeiden, gewertet. In einem ersten Durchlauf gab es statistisch signifikante Unterschiede zwischen *opn4.1* Mutanten und ihren wildtyp Geschwistern, allerdings nicht bei den *opn4xb* Mutanten. Ein *tapping assay* zeigte keine Unterschiede zwischen Mutanten und Wildtypen. Das könnte an einer möglichen Redundanz der *opn4.1* und *opn4xb* Gene liegen.

Acknowledgements

I want to thank Kristin Tessmar-Raible. By taking me into her lab as a master student she did not only provide me with the possibility to finish my studies, but also to learn a lot and to participate in first class research.

I am also immensely grateful to Marcus Dekens, for introducing me to the lab and the work, for supporting and helping me during my project and for giving me the opportunity to work independently. I profited a lot from his experience and guidance and am also grateful for the productive and at the same time very pleasant working environment he created.

A big thank you also to all the other members of the Tessmar-Raible labs, including Bruno Fontinha, for helping with the dot assay and other issues, Ruth Fischer, for advice on the *in situ* hybridisations, Stephanie Bannister, who designed the *KalTA4* project, Vinoth Babu Veedin Rajan, who helped a lot with microscopy and Agne Valinciute and Natalia Hallay, who helped with technical issues and more.

I consider the time spent in this lab as the one in which I gathered the most valuable experiences throughout my whole studies.

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Mai 2012	Josef Manner & Comp. AG, 1170 Vienna Microbial Laboratory/Quality control
June-August 2007	B&M Industrie & Gebäudereinigung, 1230 Vienna Office work & Administration

Work experience

April 2010 – December 2013	Licht für die Welt – Christoffel Entwicklungszusammenarbeit, 1120 Wien, freelancer