



universität
wien

MASTERARBEIT/MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Developmental analysis and comparison of non-visual
opsins in medaka and zebrafish“

verfasst von / submitted by

Mariam Al-Rawi, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of
Master of Science (MSc)

Wien, 2020 / Vienna, 2020

Studienkennzahl lt. Studienblatt /
degree programme code as it appears on
the student record sheet:

UA 066 877

Studienrichtung lt. Studienblatt /
degree programme as it appears on
the student record sheet:

Masterstudium
Genetik und Entwicklungsbiologie

Betreut von / Supervisor:

Univ.- Professor Dr. Kristin Teßmar-Raible

Acknowledgments

I wish to express my sincere thanks to Kristin Tessmar-Raible for giving me the opportunity to work in her lab. She always had an open door and her support and encouragement were helpful and motivating. Seeing her deep knowledge and particularly her excitement regarding science was a big inspiration for me.

My biggest thanks go to Theresa, without whom I would not have come so far. Your patience, experience and humor made work a fun place to be and I will miss our weird jokes. My love goes to Aida and Dunja, who welcomed me immediately and were always there when I needed them. Thanks also to Pauline, my coffee partner in crime, Monika, Alex and the rest of the lab, who always had some interesting and weird conversation topics ready. Special thanks also to the CATastrophe group, who always had the best memes at hand.

To my family, I send all my love and hope they know how much I appreciate them all. Without my parent's support, I would not have made it so far. Karima, I thank for putting up with me for over 15 years and for enduring my illogical arguments. My love also to Neki, who is my favorite one-person bookclub and the hardest-working person I know. Lastly, special thanks go to Stefan and Yvonne, whose friendship I deeply value, and to Johanna and Isabella, who will take over the world one day.

Thanks to everyone who feels like I should have mentioned them but were not – I am probably sorry I forgot about you.

Abbreviations

BCIP	5-bromo-4-chloro-3'-indolyphosphate
CRISPR	Clustered regularly interspaced short palindromic repeats
DOL	Degree of labelling
dpf	Days post fertilization
Dre	<i>Danio rerio</i>
ERM	Embryo rearing medium
EtOH	Ethanol
FISH	Fluorescence <i>in situ</i> hybridization
hpf	Hours post fertilization
ISH	<i>in situ</i> hybridization
MeOH	Methanol
NBT	nitro-blue tetrazolium
Ola	<i>Oryzias latipes</i>
PAM	Protoadjacent motif
PFA	Paraformaldehyde
qPCR	Quantitative real-time PCR
RGC	Retinal ganglion cell
RT	Room temperature
SCN	Suprachiasmatic nucleus
sgRNA	single-guide RNA
smFISH	Single molecule FISH
SSC	Saline-sodium citrate
TMT	Teleost multiple tissue

Table of Contents

Acknowledgments	1
Abbreviations	2
Abstract	4
Abstrakt	5
Introduction	6
Opsins	6
Teleost as model organisms	10
Zebrafish	10
Medakafish	11
Comparing Zebrafish and Medaka	12
Aim of this project	14
Results	15
Developmental effects of the mutation of various TMT-Opsins in medaka	15
Regulation of gene expression by <i>tmt-opsin1b</i>	19
Troubleshooting <i>Tac1</i>	23
Visualizing transcripts with more sensitive methods	26
<i>Tmt-opsin1b</i> in zebrafish	29
Generating <i>tmt-opsin2</i> mutant with CRISPR/Cas9	31
Discussion	33
Developmental effects of the mutation of various TMT-Opsins in medaka	33
Regulation of gene expression by <i>tmt-opsin1b</i>	34
Visualizing transcripts with more sensitive methods	35
<i>Tmt-opsin1b</i> in zebrafish	35
Generating <i>tmt-opsin2</i> mutant with CRISPR/Cas9	37
Conclusion	37
Materials and Methods	38
Supplementary data	49
Developmental effects of the mutation of various TMT-Opsins in medaka	49
Visualizing transcripts with more sensitive methods	50
<i>Tmt-opsin1b</i> in zebrafish	51
Table of Figures	52
Table of Tables	53
References	54
Manuscript	58

Abstract

An important environmental cue for organisms is light, affecting molecular processes and their behavior. This information is processed by photoreceptors, which are encoded by opsins. In case of vertebrates, over a thousand different opsins have been found until today. Their types and functions are many, and alone in zebrafish over 40 different opsins have been found. These opsins are spread across the whole body, and many are expressed in more than one tissue. One group of these opsins are called TMT-Opsin, which are non-visual, ciliary type opsins. Research has already been done trying to characterize this group, with a special focus on *tmt-opsin1b* and *tmt-opsin2* in medakafish. This work focusses on these findings and tries to elucidate the workings of *tmt-opsin1b* and *tmt-opsin2* further. It could be shown that a knockout of these opsins does not lead to any developmental problems, ensuring that any observed results in regards to *tmt-opsins* stems from the knockout of the opsin itself, and not due to any issues in development. Furthermore, to support findings showing that *sst1b* is regulated indirectly by *tmt-opsin1b*, transcripts co-localizing with these genes were identified. In course of this, it could be shown that the influence of *tmt-opsin1b* on *sst1b* is very specific. For better visualization of transcripts in fish brains, efforts were undertaken to establish smFISH protocol in medakafish and first results were achieved. Additionally, knowing that *tmt-opsin1b* affects certain transcripts in medaka, a cross-comparison in zebrafish was started. There, it could be shown that the regulation of some transcripts by *tmt-opsin1b* is specific to medaka. To go further with this comparison, a *tmt-opsin2b* knockout-model was designed in zebrafish with CRISPR/Cas9.

Abstrakt

Für einen Organismus ist Licht ein sehr wichtiges Signal aus der Umwelt, das molekulare Prozesse und Verhalten beeinflusst. Diese Informationen werden von Photorezeptoren verarbeitet, welche von Opsinen kodiert werden. Im Falle von Vertebraten wurden schon mehr als 1000 verschiedene Opsine entdeckt. Sie werden in unterschiedliche Typen und Funktionen unterteilt, und allein in Zebrafischen hat man über 40 verschiedene Opsine gefunden. Diese sind über den ganzen Körper verteilt und unterschiedliche Opsine werden oft gleich in mehreren Geweben exprimiert. Einer dieser Gruppen ist als TMT-Opsin bekannt. Diese sind nicht visuelle Ziliar-Opsine. Es wurde bereits Forschungsarbeiten publiziert, die sich auf diese Gruppe konzentrieren, mit einem speziellen Fokus auf *tmt-opsin1b* und *tmt-opsin2* in Medakafischen. In dieser Arbeit hat man die Resultate dieser Forschung als Basis genommen und versucht, die Funktion von *tmt-opsin1b* und *tmt-opsin2* noch klarer zu machen. Es konnte gezeigt werden, dass ein Knockout von diesen Opsinen zu keinen Entwicklungsproblemen oder -verzögerungen führt. Dadurch kann davon ausgegangen werden, dass eventuelle Entdeckungen in Knockout-Modellen einen Einfluss von dem gefragten Opsin zeigen, anstatt Nachwirkungen von Abweichungen in der Entwicklung. Weiters wurde gezeigt, dass der zuvor entdeckte Einfluss von *tmt-opsin1b* auf *sst1b* spezifisch ist. Hierbei wurde die Expression von Genen, welche mit *sst1b* co-lokalisieren, in *tmt-opsin1b* Mutanten analysiert. Um die Expression von Genen in Fischen besser visualisieren zu können, wurde versucht smFISH in Medaka zu etablieren. Zusätzlich wurden erste Versuche unternommen, das bisher gewonnenen Wissen von *tmt-opsin1b* in Medaka mit Zebrafischen zu vergleichen. Hierfür wurden Transkripte, welche unterschiedliche Regulationen in *tmt-opsin1b* Medakamutanten zeigen, hergenommen und deren Orthologe in Zebrafisch *tmt-opsin1b* Mutanten verglichen. Hier konnte gezeigt werden, dass einige dieser Gene spezifisch bei Medaka von *tmt-opsin1b* reguliert werden. Schlussends wurden die ersten Schritte zur Generation eines *tmt-opsin2b* Mutanten in Zebrafischen mithilfe von CRISPR/Cas9 unternommen.

Introduction

The development of an organism is a complicated process that not only relies on the information stored within its cells, but is also affected by outside signals, i.e. environmental cues. They affect not only growing – developing – organisms, but also have an impact on the adult individual. Aside from biotic factors like food or predators, abiotic cues like photoperiod also play an important role (Gilbert, 2012). For the photoperiod to play a role, the organism has to be able to detect light, its intensity and also its absence.

When one thinks about light being processed by an organism, what comes to mind first is the visual perception of light. Photoreceptors located in the eye use the light input and are an integral part of processing and creating an image of the outside world (Davies, 2015). But apart from this visual aspect, light also affects movement, pigmentation and, less visibly, biological clocks. Of the various biological clocks, the circadian clock is the one that probably relies most on light. Core clock genes (like *period* and *cryptochrome*) show an oscillating pattern related to their exposure to light, meaning that this clock is set through photoentrainment (Tamai, 2007) (Moutsaki, 2003). In this case the detection of light and, thus photoentrainment, happens via non-visual photoreceptors. And though the visual systems in the animal kingdom can vary, these photoreceptors – be it visual or non-visual – are similar. The molecules involved in this shared photo-sensing machinery are opsins (Shichida, 2009).

Opsins

Opsins in general are G-protein coupled receptors (GPCR), with seven transmembrane helices. Chromophores can bind to a lysine, which is situated in the seventh helix, and change conformation. With this, the opsin gains photosensitivity (Porter, 2012)

So far, over a thousand opsins have been identified and can generally be grouped in seven subfamilies: Vertebrate visual and non-visual opsins, encephalopsin/tmt-opsins, G_q-coupled opsin/melanopsin, G_o-coupled opsins, neuropsins, peropsins and the retinal photoisomerase subfamily (Terakita, 2005).

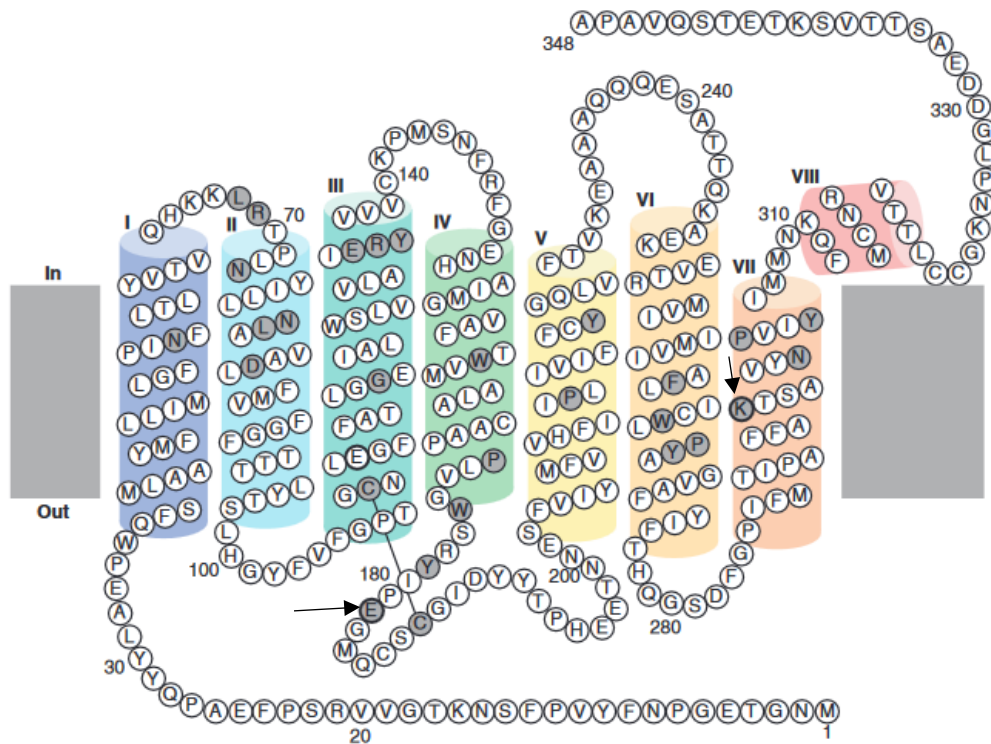


Figure 1 - Secondary structure of opsins. Opsins have seven transmembrane helices. Chromophores can bind to a lysine in the seventh helix, here the position (K296) is marked with a bold circle, as well as the counterion E113. Picture is a model of the bovine rhodopsin. (Terakita, 2005) Adapted to make the bolded aminoacids more visible.

In the case of non-visual opsins, it was thought for a long time that only one opsin was regulating the non-visual photoreception (Frøland Steindal, 2019). In this case it was melanopsin, which was first discovered in the late 90s in *Xenopus laevis*. A few years later an ortholog in zebrafish was isolated and further studied (Provencio, 1998), (Bellingham, 2002). In the past years, this has been investigated further, and Davies et. al. published a paper in 2015 where they looked extensively into the diversity of opsin-based photopigments as well as examined non-visual photoreception, using zebrafish (*Danio rerio*) as a model organism. By then, it could already be shown that zebrafish do not have one central clock system as mammals do, where the circadian pacemaker can be found in the suprachiasmatic nucleus (SCN). Instead, different cell and tissue cultures were shown to have a strong circadian oscillation of the *clock* gene (Whitmore, 2000). Being able to photo-entrain various tissues and organs leads to the assumption that all these cells must contain at least some sort of photoreceptor, reacting to light and starting downstream processes that include, apart from clock entrainment, DNA repair, stress responses and more (Davies, 2015). Analyzing the zebrafish genome showed a total of 42 different opsin genes – most of them

non-visual (Davies, 2015). Keeping in mind that tissue samples have already been shown to be light-sensitive, zebrafish organs and tissues were analyzed in regards of their opsin expression, which can be seen in Figure 2. A heatmap shows the varying expression patterns across all tissues. The eye, of course, expresses numerous different opsins – though perhaps surprising, this also includes non-visual opsins. A lot of different opsins are also expressed in the pineal complex, as well as in the brain. Other parts like skin, fin or even testis show opsin expression as well (Davies, 2015). Many opsins are expressed in more than one tissue, though their expression intensity might vary.

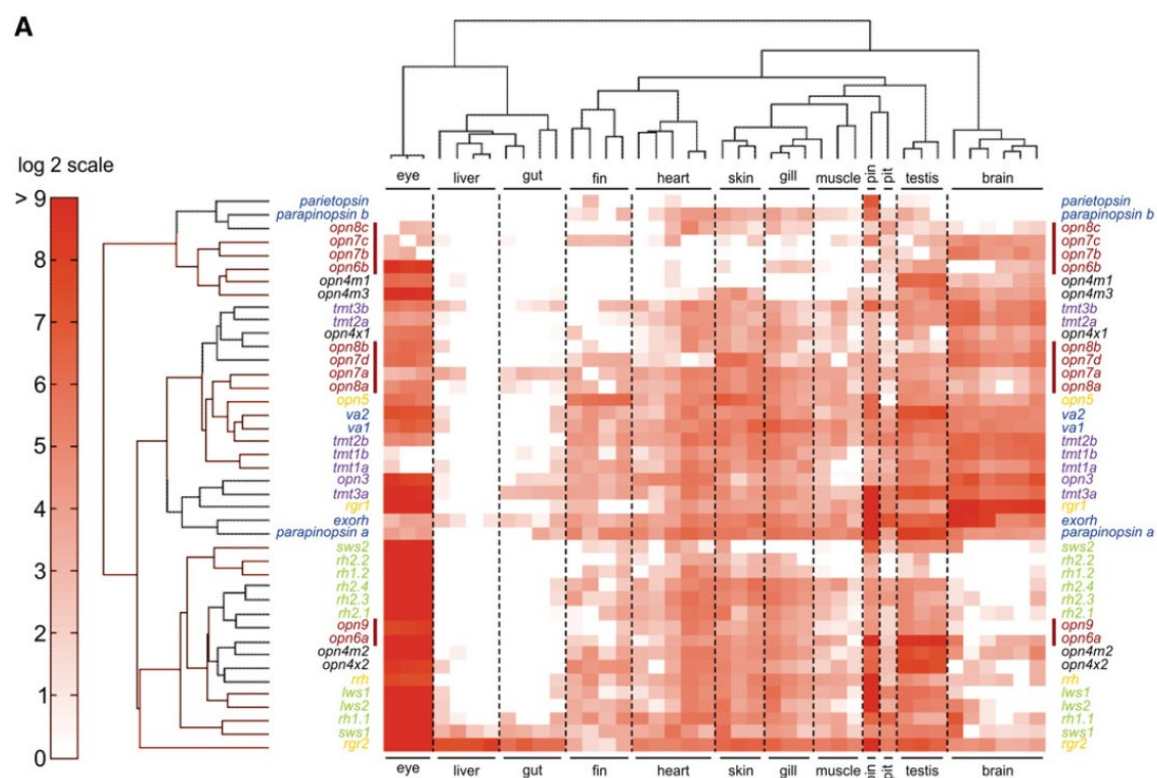


Figure 2 - Heatmap shows varying expression pattern of the various opsins expressed in Zebrafish tissue and organs (Davies, 2015)

An opsin first discovered in the mouse is Encephalopsin (Opn3), which could be first shown to be expressed in the preoptic area, the paraventricular nucleus of the hypothalamus and other areas of the brain, earning the group its name. (Blackshaw, 1999) Its homolog in zebrafish in turn could not only be found in neural tissue but in many more, and was thus named teleost multiple tissue (TMT) opsin (Moutsaki, 2003) (Sakai, 2015). An overview of the tissues expressing Opn3/TMT-opsins is shown in Figure 2. Phylogenetic analysis shows that there are four distinct groups within this Opn3/TMT-opsin group: Opn3, which is widely

distributed in vertebrates, including fish. The other three groups show different TMT-opsins clustering together, forming groups named TMT1, TMT2 and TMT3, as can be seen in Figure 3, (adapted from Sakai, 2015 and Fischer, 2013) with clearly separate clusters of the different TMT-groups.

Sequence analysis of the Encephalopsin and TMT groups showed that these are highly conserved across vertebrates, with Encephalopsins forming a close sister-group to the TMT-opsin groups (Fischer, 2013). A switch from the typical Schiff's base counterion from glutamate to tyrosine at position E113 is a feature that sets TMT-opsins apart from other photopigments – though this switch is something that can not only be found in TMT-opsins (Moutsaki, 2003). Encephalopsins, in turn, have not only a different counterion but also possess a different c-terminal domain, which is specifically conserved within this group. This points to the possibility that Opn3 might use different intracellular signaling pathways (Fischer, 2013).

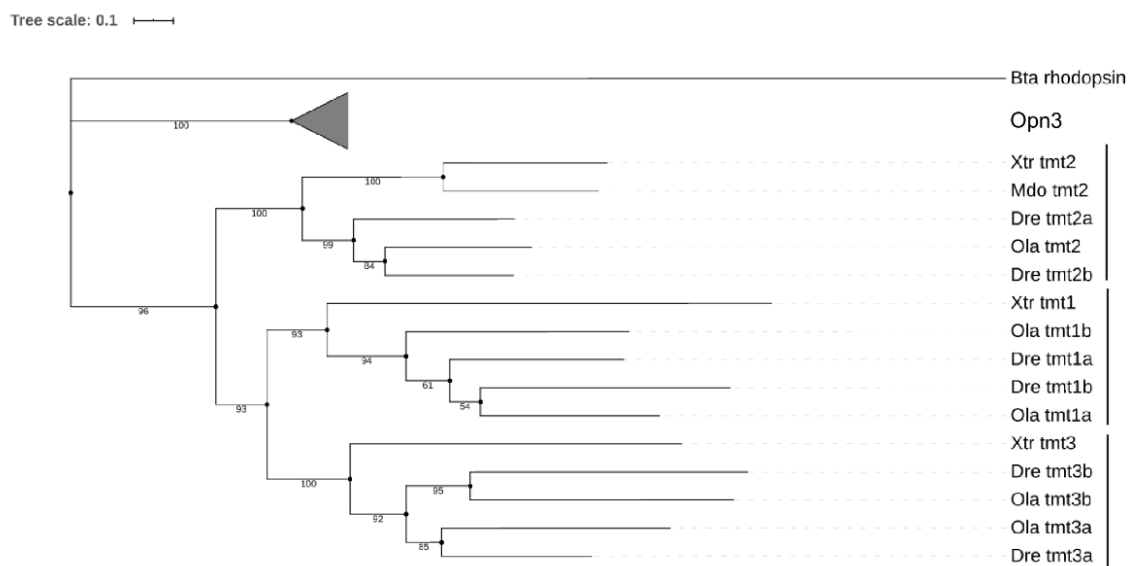


Figure 3 - Phylogenetic tree adapted from Sakai, 2015. Collapsed node shows OPN3 group. Maximum likelihood tree. Dre: Danio rerio, Ola: Oryzias latipes

The different TMT-opsins change to an active state with different characteristics. As an example, TMT1 opsin in medakafish (*Oryzias latipes*) absorbs visible light in its active state, while TMT2 opsin has a UV-light absorbing state with all-*trans*-retinal and in contrast to TMT1 can not photoconvert to another state or back to the original 11-*cis*-retinal state (Sakai, 2015). These characteristics are probably related to their different functions. More generally though, the TMT-opsins are

more sensitive to blue light than other opsins. They are expressed in the deep-brain and were shown to be co-expressed with motor – and interneurons. Fischer *et al.* were additionally able to show that in the medaka tectum, neurons that are co-localized with cells expressing *tmt-opsin1b* react directly to light input. This is supported by the mentioned sensitivity to blue light, meaning that neurons can sense a wider range on the light spectrum. These neurons can thus react fast to light-mediated changes in the environment in a cell-specific manner.

Various TMT-opsins are expressed in the same brain region in medaka as well as zebrafish (for example, *tmt-opsin1b* is expressed in the hindbrain and the posterior thalamic nucleus in both fish species) (Fischer, 2013). As mentioned below, a genome duplication in early teleost led to the emergence of thousands of different fish species, among them medaka and zebrafish, who are separated by thousands of years of evolution. Keeping this in mind this means that finding paralogs of TMT-opsins expressed in equal regions makes them highly conserved and draws the conclusion that they play a significant role (Fontinha, 2019).

Teleost as model organisms

Zebrafish

Danio rerio – also known as zebrafish – is a tropical fish native to South Asia. Since its introduction as a model organism in the 1980s, it quickly evolved to be one of the most used model organisms in science. In terms of popularity and use, statistics done by the Austrian Federal Ministry of Education, Science and Research shows it quite clearly: In 2019, zebrafish was the most used animal model organism right after the mouse (*mus musculus*), and almost twice as much used as the next in line – rats (*rattus norvegicus*) (BMBWF, 2020).

In the 1980s, genetic screens for *Drosophila melanogaster* have been published, successfully deciphering the early patterning process in the *Drosophila* (Nüsslein-Volhard, 1980) (Nüsslein-Volhard., 1984) (Wieschaus, 1984) (Jürgens, 1984). With these discoveries, the next step was to try and show the same thing in vertebrates, by screening for a genomic phenotype, nowadays known as the Tübingen/Boston screens, published in 1996 in *Development*, which dedicated an entire issue to zebrafish and these screens (*Development*, Volume 123). These genetic screens helped make zebrafish a popular organism to study vertebrate

development, genetics and more. They have a short generation time of about three months, and most importantly: the embryonal development can be observed early on, due to a development outside the mother, and the eggs and chorion being transparent. Within 72 hours, the embryonic development is concluded and the fish hatch. At around 10-12 weeks post-fertilization, they become sexually mature (Meyers, 2018). Furthermore, in 2001, the Wellcome Sanger Institute started the Zebrafish Genome project, and by now several genome assemblies of one zebrafish strain (Tübingen) exist and are updated regularly¹.

With the establishment of transgenic lines with transposons systems like *To/2*, *Sleeping Beauty* and the Cre-lox system (Kawakami, 2007) (Davidson, 2003), zebrafish became more interesting for a wider array of research. This broadened even more with gene-editing becoming more accessible and easier – first with transcription activator-like effector nucleases (TALENs), nowadays done via the CRISPR/Cas9 system (Meyers, 2018). Additionally, their ability to quickly repair cells and tissues including the heart, brain and fins make them popular in regenerative research.

Zebrafish is a valuable model organism also in the research of circadian rhythmicity and light sensitivity. As mentioned above, various zebrafish tissues were used to show that they all display steady oscillations of the *clock* gene, and thus refuting the idea of a centralized clock pacemaker (Whitmore, 2000). With this clock being set by light, studying the light-sensitivity of cells and tissues makes a promising field of research. Investigations showed that while evolving, zebrafish had a few gene duplication events, resulting in an increase of opsins, as mentioned above: a total number of 42 opsins could be verified in this organism (Davies, 2015).

Medakafish

A fish primarily found in Japan, medaka – *Oryzias latipes* – is a small fresh-water fish. It has been used as a model organism for over 100 years, and was actually the first vertebrate model, where the crossing over of X and Y chromosomes was shown in 1921 (Aida, 1921). Comparable to zebrafish, the development of medaka is easily observable due to transparent eggs, though it does take longer: While zebrafish larvae hatch within 72 hours, as previously mentioned, medaka only

¹ <https://www.sanger.ac.uk/data/zebrafish-genome-project/>

hatch at around 7-9 days post-fertilization (Iwamatsu, 2004). Similar to zebrafish, medaka saw a rise in use as a model organism with the establishment of transgenic lines (Ozato, 1986) and mutagenesis protocols (Shima, 1991).

In the 70s, a book named 'Medaka (Killifish)-Biology and Strains' by T. Yamamoto compiled most of the information known at that time about medakafish as a genetic model organism. The book included not only information about developmental stages, fertilization and more, but also mutant strains. There, the book summed up the discoveries of different spontaneous mutations and described mutants affecting body pigmentation and morphogenetic deformities (Shima, 2004) (Yamamoto, 1975). Nowadays, a lot of stable transgenic lines exist, including strains with mutations affecting pigmentation, and even a strain where the adult fish are still see-through (Wakamatsu, 2001).

Comparing Zebrafish and Medaka

When doing genetic analysis, one very important question is always whether the gene (function) is something that is specific to that organism or species, or is conserved over maybe thousands of years. With this in mind, looking into and comparing both zebrafish and medakafish is advantageous, considering their evolutionary distance. In the course of evolution, whole genome duplication events resulted in almost 25,000 different teleost species (Furutani-Seiki, 2004), meaning that around 110 million years lie evolutionary between zebra- and medakafish (Wittbrodt, 2002). Additionally, keeping both organisms in the lab is rather easy, since they require similar conditions regarding water and temperature. They develop in a rather similar fashion, and most established protocols are alike and might only need minor changes (Furutani-Seiki, 2004). In terms of studying Opsins, these fish offer themselves as advantageous models, considering the large number of Opsins they have, but also the fact that for instance medaka has less TMT-opsins than can be found in zebrafish (see Figure 3). It is then interesting to figure out what functions these Opsins actually hold. The *tmt-opsins* in medakafish could possibly have more responsibilities than their paralog in zebrafish. On the other hand, some of the additional *tmt-opsins* found in zebrafish could prove to have redundant functions. In any way, it will be fascinating to study their differences and even figure out why zebrafish for instance evolved to have more of these Opsins. In addition, facts like the seasonality of medaka and absence thereof in zebrafish makes for an interesting

comparison that could provide valuable insights to the function of certain photoreceptors. It has been shown that the reproduction of medaka can be influenced via the photoperiod – changing their light period from 16 hours light/8 hours dark to 8 hours light/16 hours dark leads to a steady decrease of reproduction, until it completely ceases (Koger, 1999). Furthermore, a number of genes and pathways have been implicated to be affected and/or activated in case of a change in photoperiod. Among them is *LDAIR*, which is one of the first genes activated if the photoperiod changes (e.g. moving from a short day to a long day), which has been shown to regulate *corticotropin releasing hormone receptor 2*, a gene known for its role in stress response (Nakayama, 2019). Incidentally, the corticotropin releasing factor has been shown to be regulated by light in zebrafish, having a role in camouflaging behavior of zebrafish larvae (Wagle, 2011). So, looking into how photoreceptors, and *tmt-opsin* especially regulates pathways and genes, and how seasonality plays into this all will certainly provide some interesting information.

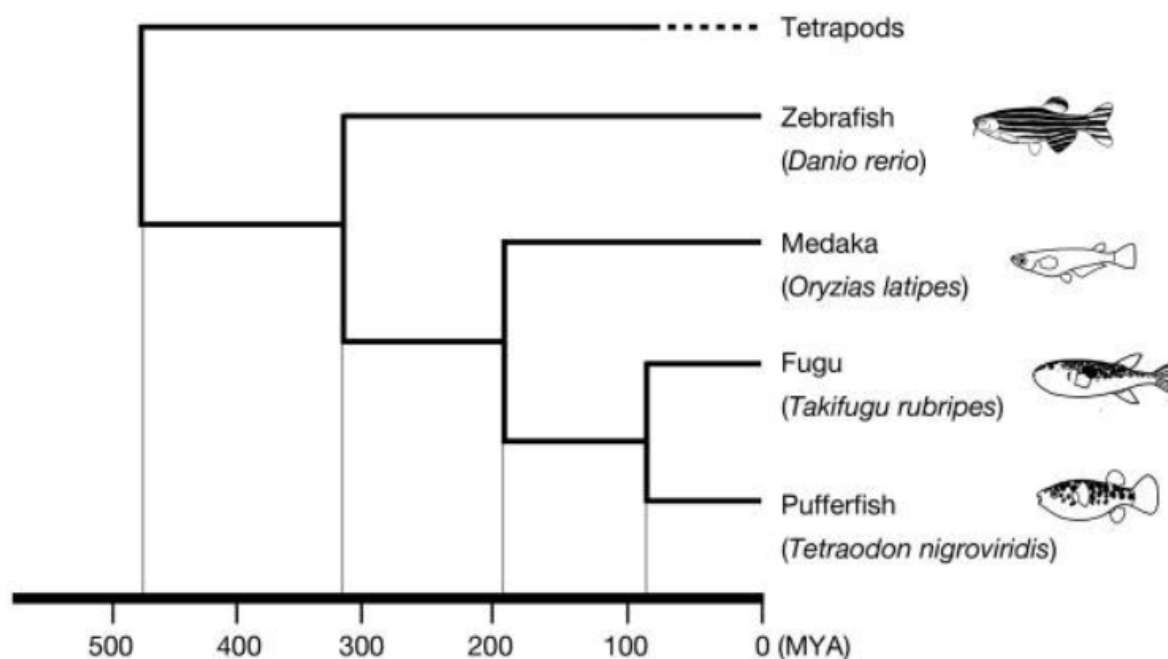


Figure 4 - Evolutionary distance of zebrafish and medakafish taken from Hashiguchi, 2006. X-axis: distance in Million Years. MYA: Million Years Ago

Aim of this project

Research shows that teleost contain a lot of different Opsin, all expressed in different tissues. Encephalopsins and TMT-Opsins form a group that has already been studied extensively in medaka and zebrafish, especially various *tmt-opsins*. However, the function and downstream effects of some *tmt-opsins* remain unclear. The goal of this project was to further investigate the functionality and downstream effect of the group of TMT-Opsin, as well as comparing these Opsins between zebrafish and medakafish.

This project focuses on *tmt-opsin1b* and *tmt-opsin2* specifically, and their function in medaka. Furthermore, first attempts are made to compare *tmt-opsin1b* in medaka and zebrafish. This comparison is done on molecular and developmental basis. To dive deeper into this comparison, *tmt-opsin2* mutants in zebrafish are created as well, using CRISPR/Cas9 mutagenesis.

Results

Developmental effects of the mutation of various TMT-Opsins in medaka

Studying the role of various Opsins in detail, their effect on light sensitivity, activity, molecular downstream effects and more can be researched. However, it is vital to first check whether the mutation has any effect on the development of the organism. This makes sure that any significant aberration observed in mutants later on (especially concerning larvae) does not have a developmental deviation as its cause.

Therefore, to ensure that the various mutations do not have any morphological effect, the development of medaka wildtype and homozygous mutant siblings (*tmt-opsin1b*, *tmt-opsin2*, *tmt-opsin1b/tmt-opsin2* double mutants) were observed until they hatched. The developmental stages, as well as morphological features, were identified and recorded over time using criteria first proposed by Iwamatsu T. in 2004.

Wildtype fish and mutants were collected during the same timepoint and first checked approximately 7-8 hours post-fertilization (hpf). From then on, embryos were controlled every 24 hours to make sure they all develop at the same rate.

Table 1 - Table shows the total amount of embryos analyzed. Developmental analysis between wildtype and mutant were done over two periods: (A) comparison between tmt-opsin1b wildtype and mutant, with an n = 20 for both groups. (B, C) Analysis between tmt-opsin2 wildtype and homozygous, as well as tmt-opsin1b/tmt-opsin2 homozygous fish. Tmt-opsin2 wildtype n= 27, tmt-opsin2 -/- n=26, tmt-opsin1b/tmt-opsin2 -/- n=19

DOB: 17.01.2020							
Date	Stage	tmt-opsin1b wildtype			tmt-opsin1b homozygous		
		n	died	hatched	n	died	hatched
17.01.2020	10 to 11	20	0	0	20	0	0
18.01.2020	19 to 20	20	0	0	20	0	0
19.01.2020	24 to 25	20	0	0	20	0	0
20.01.2020	30 to 31	20	0	0	20	0	0
21.01.2020	33 to 34	20	0	0	20	0	0
22.01.2020	35to 36	20	0	0	20	0	0
23.01.2020	39 to 40	14	6	2	16	4	0
24.01.2020	>40	14	0	12	16	0	16

DOB: 12.08.2020							
Date	Stage	tmt-opsin2 wildtype			tmt-opsin2 homozygous		
		n	died	hatched	n	died	hatched
12.08.2020	10 to 11	27	0	0	26	0	0
13.08.2020	19 to 20	20	7	0	25	1	0
14.08.2020	24 to 25	20	0	0	25	0	0
15.08.2020	30 to 31	20	0	0	25	0	0
16.08.2020	33 to 34	20	0	0	25	0	0
17.08.2020	35to 36	20	0	0	25	0	0
18.08.2020	39 to 40	20	0	0	25	0	0
19.08.2020	>40	20	0	20	25	0	25

DOB: 12.08.2020							
Date	Stage	tmt-opsin2 wildtype			tmt-opsin1b/tmt-opsin2 homozygous		
		n	died	hatched	n	died	hatched
12.08.2020	10 to 11	27	0	0	19	0	0
13.08.2020	19 to 20	20	7	0	13	6	0
14.08.2020	24 to 25	20	0	0	13	0	0
15.08.2020	30 to 31	20	0	0	13	0	0
16.08.2020	33 to 34	20	0	0	13	0	0
17.08.2020	35to 36	20	0	0	13	0	0
18.08.2020	39 to 40	20	0	0	13	0	0
19.08.2020	>40	20	0	20	13	0	13

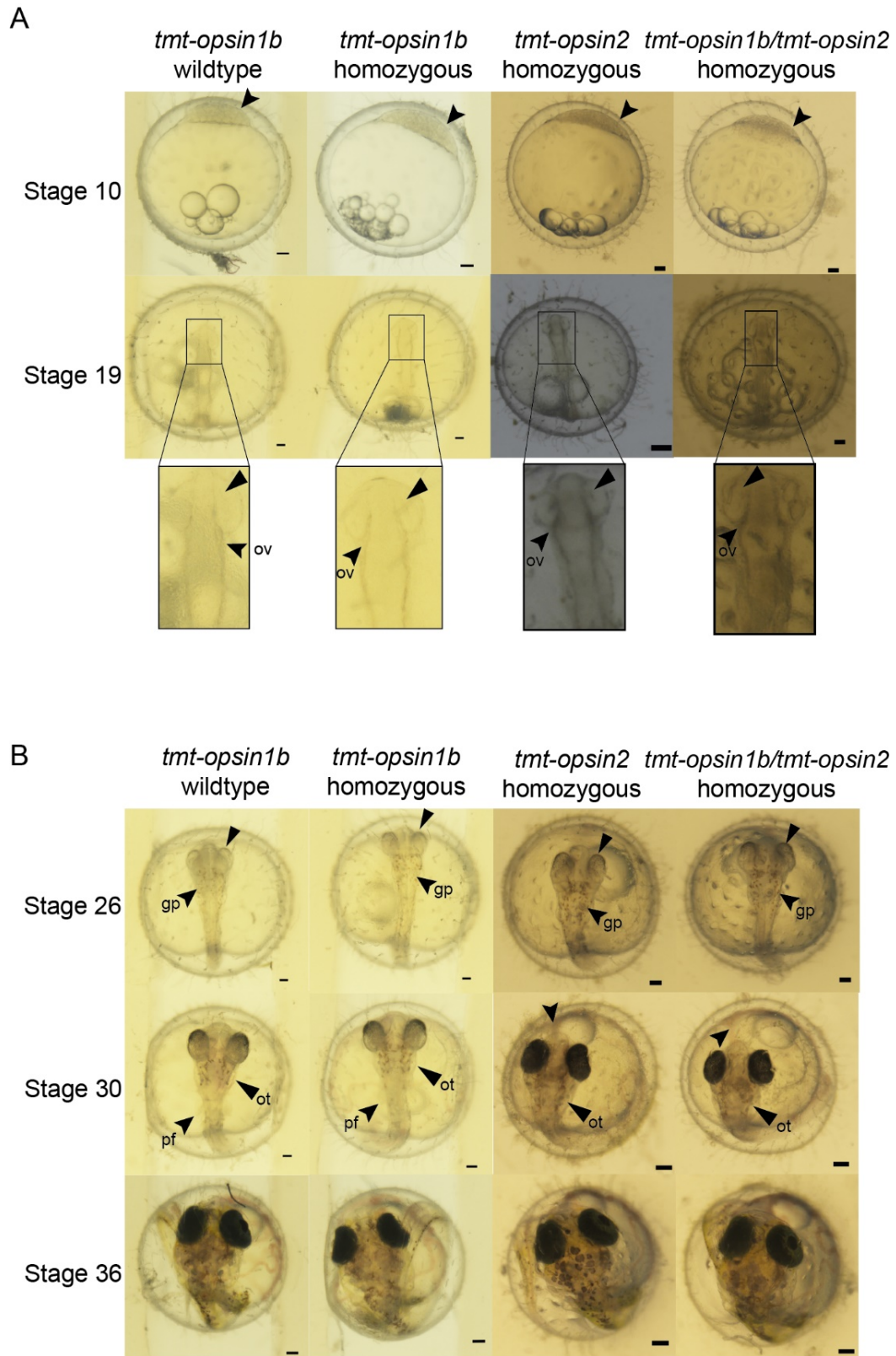


Figure 5- Embryonic development of medaka fish. Comparison between wildtype and *tmt-opsin1b*, *tmt-opsin2*, *tmt-opsin1b/tmt-opsin2* homozygous mutants. (A) Stage 10: Arrows indicate a thick blastoderm with smaller inner cells, typical for this stage. Stage 19 shows magnified embryo, to properly show the appearance of otic vesicles. A groove is forming in the optic lobe, indicated by arrow. (B) Stage 26: Choriodea are starting to cluster, as indicated, and guanophores are clearly visible. Stage 30 has apparent otholits, and for wildtype and *tmt-opsin1b* homozygous mutant the pectoral fin is apparent. Due to the angle the pectoral fin is not visible for the other two mutants, but here branches of arteries supplying the embryo are visible. Stage 36 shows a tail tip, that reaches past otic vesicle. At this stage, the guanophores are now distributed head to fin along the whole body. Scale bar: 500 μ m (ov: otic vesicle, gp: guanophores, pf: pectoral fin, ot: otholit)

The figure shows no morphological differences between the development of wildtype or the different *tmt-opsin* mutants. As indicated by arrows, morphological identifiers typical for a certain stage can be found in all mutations as well as in wildtype counterparts. Stage 10 shows as expected a thick blastoderm, with smaller inner cells. Interestingly, it could be observed that the homozygous fish (especially *tmt-opsin2* and *tmt-opsin1b/tmt-opsin2*) both seem to have small but numerous oil droplets, while the wildtype fish show the usual handful of bigger droplets (Not included in figure. See Supplementary Figure 17 for example). At stage 19 both wildtype and the different mutants show the appearance of otic vesicles, as indicated, as well as a groove in the optic lobes. With stage 26, the choriodea start to cluster and the guanophores are clearly visible. At stage 30, the pectoral fin can be nicely seen for the wildtype as well as the *tmt-opsin1b* homozygous mutant. Due to the angle the picture was taken at, the pectoral fin cannot be seen clearly for both *tmt-opsin2* and *tmt-opsin1b/tmt-opsin2*. However, for both, an artery branch supplying the larvae is clearly visible above the head. For all of them, the otholits are apparent as well. Furthermore, stage 30 *tmt-opsin2* and *tmt-opsin1b/tmt-opsin2* embryos seem to look slightly more developed than their compared *tmt-opsin1b* homozygous and depicted wildtype (e.g.: eyes have more pigmentation). Since these pictures were taken separately than the analysis for *tmt-opsin1b*, it is possible that there is a slight time difference when exactly those pictures were taken. While not shown in Figure 5, wildtype *tmt-opsin2* pictures were taken for comparison at the same time as well, and show the same degree of development as the *tmt-opsin2* homozygous fish show. It is therefore a matter of when the picture was taken, and not any developmental difference between these mutants. For stage 36, the tip of the tail reaches the otic vesicle and the guanophores are now distributed from head to tail.

Furthermore, a strain expressing GFP under the control of an *ath5* enhancer fragment was crossed with *tmt-opsin1b* homozygous mutant, and the resulting heterozygous, GFP positive, offspring were incrossed. *Ath5* promotes retinal ganglion cell (RGC) neurogenesis, which are the first cells generated in undifferentiated epithelium (Souren, 2009). Any deviation from the GFP expression would therefore point to any aberration in RGC development. Additionally, the axonal outgrowth and connection to the tectum would indicate the state of development for both RGC and tectum (Fontinha, 2019). By stage 36,

which corresponds to 6 days post fertilization (dpf), the tectum is covered by RGC axons (Yoda, 2004). Keeping that in mind, the larvae were tested for their GFP expression shortly before hatching, at stage 38 (8dpf). After hatching, they were genotyped. The overall numbers can be seen in Table 2.

Table 2 – Number of collected and analyzed *ath5::GFP* embryos

<i>tmt-opsin1b</i> <i>ath5::GFP</i>	n
Wildtype	19
Homozygous	18
Heterozygous	27

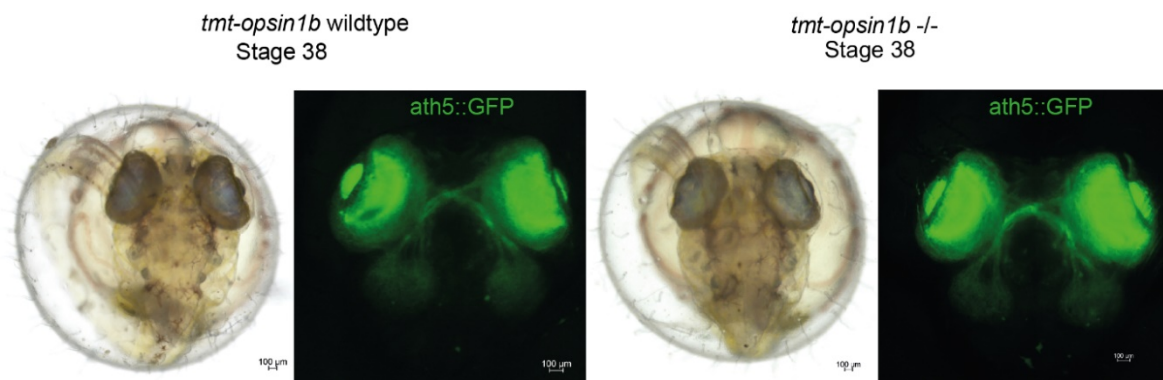


Figure 6 - Wildtype and *tmt-opsin1b* homozygous mutant shortly before hatching. GFP expression in the fish's retinal ganglion cells shows no noticeable difference in axonal projections. Scale bars: 100μm

As can be observed in Figure 6, the GFP expression between the wildtype and *tmt-opsin1b* homozygous mutants does not show any visible difference (Figure 6). This leads to the conclusion that the eye development as well as the development of the tectum is not affected by *tmt-opsin1b* mutation. Naturally, this has to be checked for the other mutants (*tmt-opsin2* and *tmt-opsin1b/tmt-opsin2* homozygous mutant) as well. The experiments for this are currently ongoing.

Regulation of gene expression by *tmt-opsin1b*

After making sure that mutations of these specific Opsins in medaka do not have a discernable effect on the development of the fish, the next step was to analyze any geno- or phenotypical changes possibly related to the knockout of the photoreceptors.

Quantitative RNA sequencing showed the differential regulation of pre-pro-hormone *sst1b* (also named *sst3* and *cort*) in the mid- and hindbrain of *tmt-*

opsin1b homozygous mutants (Fontinha, 2019). In addition, the expression of *sst1b* was shown to not overlap with *tmt-opsin1b*⁺ cells in the midbrain. Comparing the expression of *sst1b* in wildtype and *tmt-opsin1b* homozygous adult brains, a reduction of *sst1b* cells in the optic tectum could be observed, as well as an absence of *sst1b* around the hypothalamus. Furthermore, in the intermediate reticular formation of the hindbrain, a reduction of expression could be observed as well (Fontinha, 2019). This leads to the conclusion that *tmt-opsin1b* regulates *sst1b* – likely indirectly, since the *sst1b* positive cells seemed to be separate from the *tmt-opsin1b* expressing cells. With this knowledge, the next step was to see if the mutation actually reduces the cells expressing *sst1b*, or only reduces the amount of it. For this, the plan was to look at the regulation of transcripts co-expressed with *sst1b*. This kind of sequencing data for medaka was unfortunately not available, so data from the zebrafish larval brain was used to look for ortholog candidates (Raj, 2018). This data comes from droplet scRNA-seq done on one larval brain, to identify the different cell types and markers expressed. One cluster, shown to be located in the telencephalon, was of particular interest for this: the cluster included *sst3* (now called *cort* according to ENSEMBL and ZFIN, the ortholog to the medaka *sst1b*), as well as a few other genes. Of these, ultimately four were picked to look further into. The deciding factor was them having orthologs in medaka, a hopefully specific expression and, naturally, being co-expressed with *sst1b*. The genes chosen were: Glutamate Decarboxylase 2 (*gad2*), Purkinje Cell Protein 4 like 1 (*pcp4l1*), Neurogranin (*nrgna*) and Tachykinin Precursor (*tac1*).

RNA expression of *gad2*, *pcpl1* and *nrgna* was visualized using *in situ* hybridization on larvae at 10dpf. Staining could be shown for *gad2* and *pcp4l1* in the brain. *Nrgna*, in turn, did not show any specific staining, even with trying different staining conditions. For *tac1*, please refer to subpart 'Troubleshooting *tac1*'.



Figure 7 – Staining of *gad2*, *pcp4l1* and *nrgna* in medaka larvae. Staining for *nrgna* did not work and shows non-specific staining. *Gad2* and *pcp4l1* show specific staining in the brain, with *gad2* having a very broad staining. Scale: 500µm

To make sure of the gene expression of *gad2* and *pcp4l1* in the brain, *in situ* hybridization was performed on adult brain slices. Additionally, to make sure that the expression pattern of these genes was not affected by *tmt-opsin1b*, the *in situ* hybridization was done both in wildtype as well as *tmt-opsin1b* homozygous fish. The staining observed in the brain confirmed that both genes are, amongst other areas, expressed in the regions where *sst1b* was shown. Importantly, there is no visible difference in the expression when comparing wildtype and mutant fish. Unfortunately, it was not possible to obtain the exact same brain slices to compare the staining in wildtype and mutant, but a close approximation can be seen below.

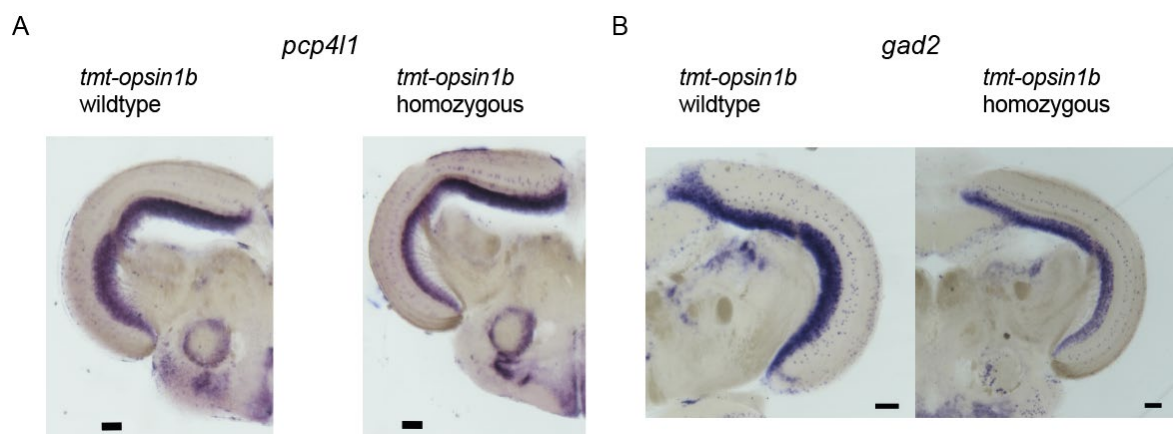


Figure 8 – Staining of *pcp4l1* and *gad2* in adult medaka brain slices of *tmt-opsin1b* wildtype and homozygous mutant. Staining in the optic tectum and the posterior hypothalamic region. Please note that the comparison of wildtype vs. mutants is not 1:1 since the slices are not 100% ident. Coronal sections, scale bar: 500µm.

To check whether these genes' expression co-localizes with *sst1b*, a fluorescence *in situ* hybridization was done where *gad2* was labelled fluorescently with Cy3, whilst staining for *sst1b* was done with an alkaline phosphatase.

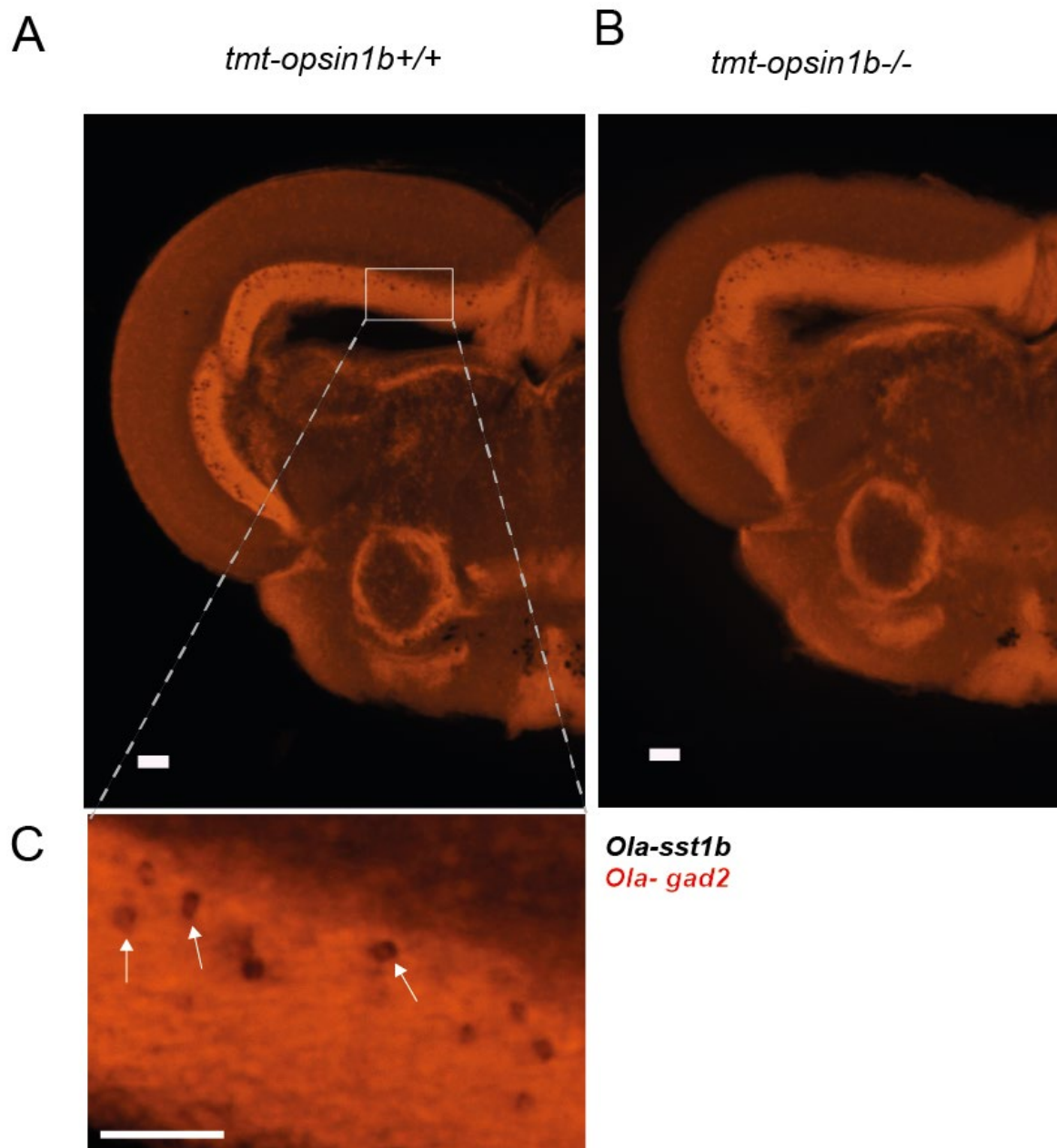


Figure 9 – Expression pattern of *sst1b* and *gad2* in coronal sections of adult medaka midbrain Comparison between (A) *tmt-opsin1b* wildtype and (B) *tmt-opsin1b* homozygous fish. (C) Co-localization of *sst1b* and *gad2* in tectal neurons, as indicated by arrows. Red staining: *gad2*. Scale bar: 200 μ m

As shown in Figure 9, co-expression of *gad2* and *sst1b* can be observed in the tectum (magnification in C, marked with arrows). Furthermore, comparing Figure 9A and B shows that *gad2* is not regulated differently in the mutant, while there is a change in the *sst1b* expression.

Troubleshooting *Tac1*

In turn, there were issues with *tac1* from the start. The staining for *tac1* in zebrafish has been shown to be rather specific (Ogawa, 2012), so particular focus was put on visualization of this gene. Using larval cDNA to amplify the gene was a decision made on the basis that most of these factors might be upregulated during development. Even with troubleshooting efforts, there was no conclusive amplification for *tac1* using this cDNA. Since *tac1* encodes for tachykinins, which are widely spread across the nervous system as neurotransmitters and -modulators (Ogawa, 2012), it was possible that they might mainly be expressed in differentiated tissue. Thus, the amplification of the gene was done again with adult whole brain samples as well as individual forebrain, midbrain and hindbrain samples. The whole brain sample did look more promising, but upon cloning and sequencing, the sequence did not align with the predicted *tac1*. So, two new primer pairs were designed and everything was repeated, with the same results. Again, troubleshooting, including different samples, nested PCR and more, was performed without any result.

The sequence alignments were re-done, and apart from finding a polymeric region located within the designed primers, sequences with other predicted ends were found. For those two sequences, two new primer pairs each were designed, cloned and sequenced, with bad sequencing results. An example for those sequencing results can be seen in Figure 10 below.

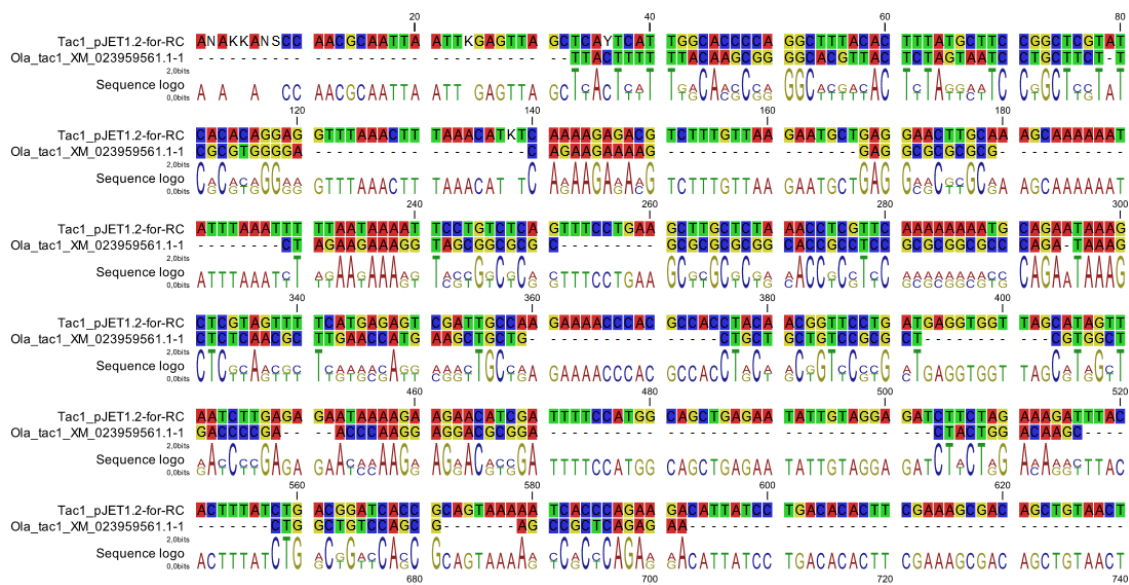


Figure 10 – Part of the alignment for *tac1*, aligning the sequencing result with the predicted sequence.

Finally, three different clones containing the medaka *tac1* were ordered². The expected length for this gene was around 400bp. Upon checking the length of the insert on an agarose gel, the insert placed at approximately 4.5kbp (Figure 11).

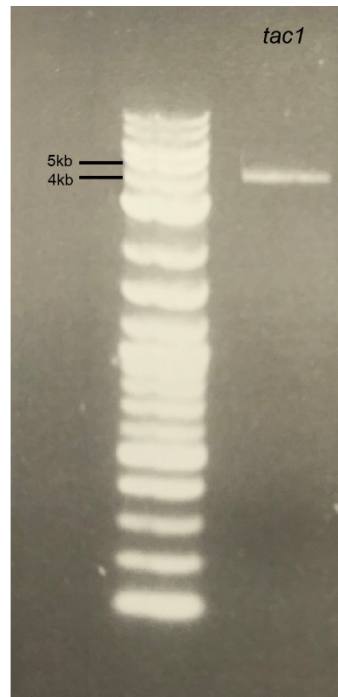


Figure 11 – Size of *tac1* insert at approximately 4.5kb

The end of the *tac1* sequence we got and aligned to our sequenced product always ended with a polynucleotide-stretch (in this case Guanin). It is likely that more stretches like this followed and the sequencing broke off in the middle of the sequence.

² Clones were ordered from NBRP Medaka (<https://shigen.nig.ac.jp/medaka/>)

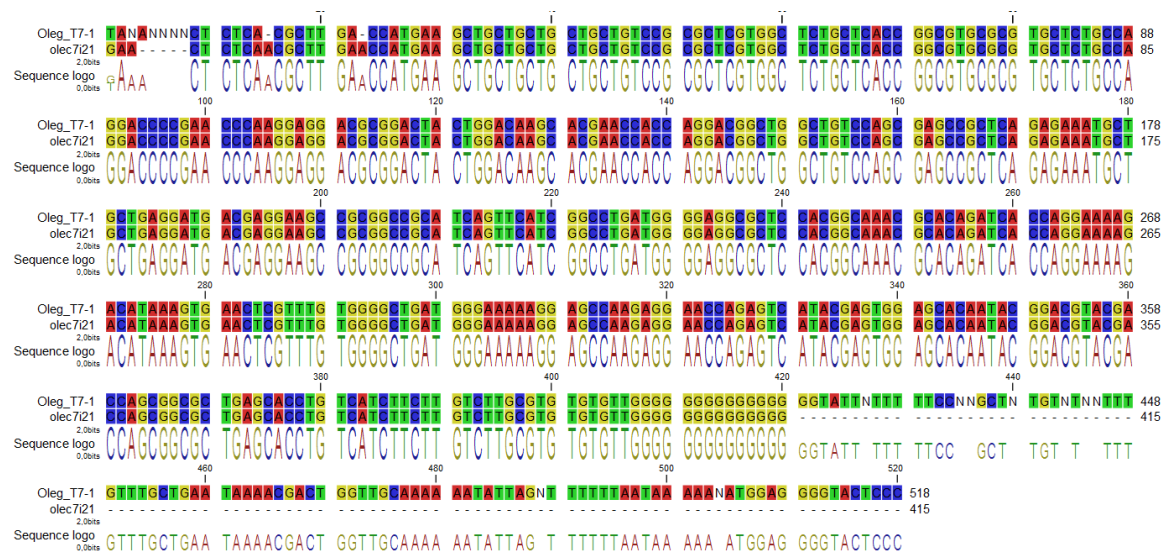


Figure 12 – Sequence alignment of *tac1* sequence with sequenced insert (Upper row). Sequence only goes a bit above 500bp, a lot shorter than the size shown through gel electrophoresis.

Trying to make dig- and fluo-labelled antisense probes from this was a challenge in itself and it was difficult to obtain working probes. Due to the low concentration of end-product, different approaches were tried to improve this. In the end, two different probes were tested: once without an RNA cleanup, and once with. The difference this clean-up makes can be seen below (Figure 13). Unfortunately, no specific staining could be observed and the *tac1* efforts were not continued due to time constraints.

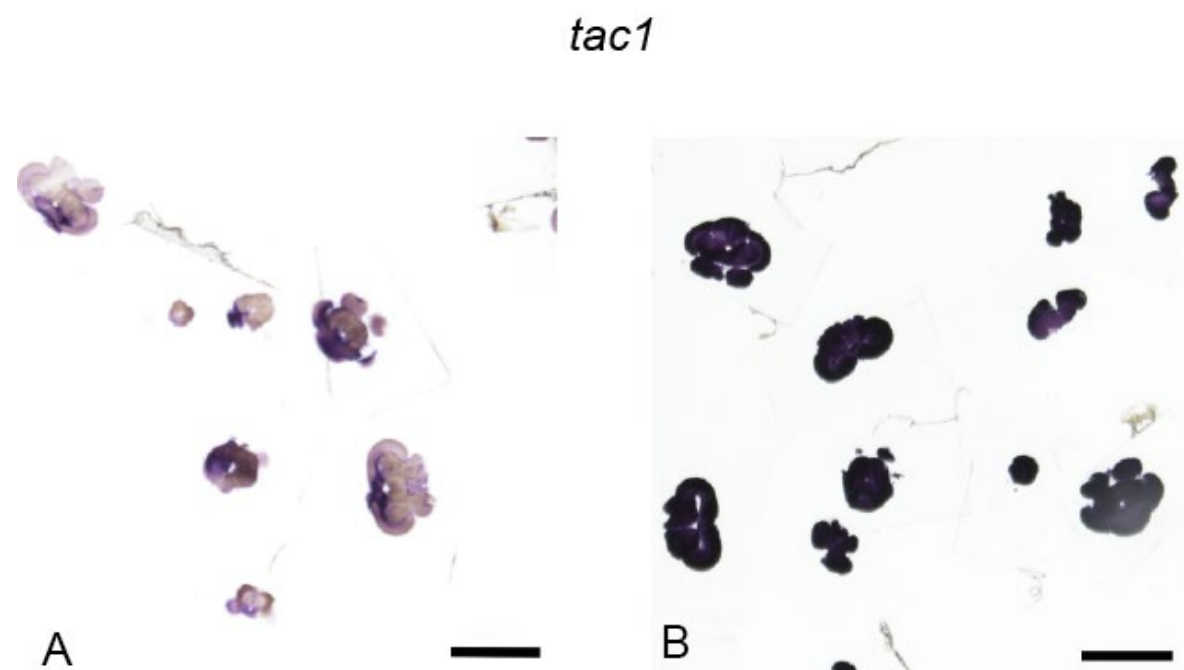


Figure 13 - Medaka adult brain stained for *tac1* with alkaline phosphatase. (A) unspecific staining of *tac1*. (B) unpurified *tac1* probe. Coronal sections. Scale: 5000µm

Visualizing transcripts with more sensitive methods

Trying to see the expression level of ubiquitously expressed genes like *gad2* is easily done with methods like *in situ* hybridization or immunohistochemistry. However, having a method at hand that is more sensitive and could potentially be used to quantify the expression of a certain gene is of great advantage. Furthermore, having a method where it is also possible to image more than 2 genes in the same sample could make for interesting results. So, the goal was to try and establish Single-molecule Fluorescence *in situ* hybridization (smFISH) in zebrafish and medakafish. With this method, it is possible to visualize single mRNA molecules. The challenge here was that the smFISH protocols available online have usually been established in other model organisms. One could be found that actually established the method in zebrafish embryo cryosections (Stapel, 2018) and formed the basis for this experiment.

The principle of smFISH is to design around 48 oligonucleotides with an approximate length of 20 bases. These stretches are then labelled individually with fluorophores. When these probes bind to their gene in question, this large number on one transcript produces a signal, which is easily distinguished from the background (Stapel, 2018) (Gaspar, 2017). For smFISH to work properly, thin tissue is necessary, which is a problem when it comes to fish. In case of the zebrafish embryo sections, Stapel et al. used 8-10µm thin sections.

The genes selected to create smFISH probes were: *sst1b*, *scn12aa* and *ChAT* for both zebrafish as well as medaka. The goal was to label them with different fluorophores and ultimately to be able to show the expression of all three genes at the same time in the fish brain.

There are three main steps to label these probes:

1. Synthesis of labeled ddUTPs
2. Labelling of oligonucleotides with created dye-ddUTPs
3. Purification

By measuring the absorbance of the labelled oligos, it is possible to calculate the concentration (c), the degree of labelling (DOL) (and thus the quality) as well as the recovery rate (R%).

$$c = \frac{OD_{260(labeled)} - CF_{260(dye)} * OD_{dye(labeled)}}{\epsilon_{(oligos)} + 9000}$$

$$DOL = \frac{OD_{dye(labeled)} / \epsilon_{(dye)}}{c}$$

$$R\% = \frac{c * V}{n_{initial}} * 100$$

Generally, probes with a DOL in the range of 0.9 and 1.0, are of good enough quality to be used for smFISH. In case the DOL is above or below these values, there are some troubleshooting steps that can be applied. To head off the most common error, which results in a DOL below 0.9, the ssDNA oligonucleotides are additionally split in two groups (even and odd numbered oligos). These two groups and a vial containing all oligos are labelled at the same time. The mixture with the better DOL is chosen. If the both the even and odd oligonucleotides have a better DOL than the combined tube, these labelled oligos can be put together and used for the procedure.

The probe generation was started with the oligonucleotides ordered against the medakafish genes. The fluorophores chosen were a red (Atto633), a green (Atto488), an orange (Atto550) and a yellow (Atto532) fluorescence emitting label.

Table 3 – Labelling of smFISH probes. Underlying colors indicate the fluorophores used: Red (sst1b): Atto633; Green (ChAT): Atto488, Orange (scn12aa): Atto550; Yellow (scn12aa): Atto532. Oligo column shows whether all oligos were labelled at the same time or even and odd numbered oligos were separately labelled. C: Concentration, DOL: Degree of labelling (ideally between 0.9 and 1), R%: Recovery rate (ideally 100%). Bold values have a good DOL and can be used.

Gene	Oligo	c	DOL	R%
sst1b	all	2,0118E-05	1,09	101
	even	2,21389E-05	0,93	111
	odd	2,56329E-05	0,90	128
ChAT	all	2,56306E-05	3,44	128
	even	1,94107E-05	3,36	97
	odd	1,56895E-05	3,45	78
scn12aa	all	1,07843E-05	2,04	54
	even	7,06937E-06	0,27	35
	odd	1,39731E-05	2,42	70
scn12aa	all	1,82471E-05	2,40	91
	even	3,3428E-06	2,42	17
	odd	1,45647E-05	2,43	73

The labelling was tested in different configurations. As can be seen in Table 3, labelling *sst1b* with Atto633 immediately showed good values, with the DOL being between 0.9 and 1, and the recovery rate being around 100%. Since these values were overall good, it was then decided to use the pooled even/odd labelled oligos as a probe for the smFISH procedure.

In contrast to this, the labelling with the other fluorophores did not work as well. An excerpt can be seen in Table 1, but there were multiple attempts to create probes for these genes. There were attempts to label *ChAT* and *scn12aa* with Atto488, Atto550 and Atto532, but the labelling was not yet successful (examples shown in Supplementary Data, Table 8). There are more troubleshooting steps left to figure this out, but at least for Atto488 it could already be observed that labelling with this dye usually results in a high free dye contamination (Gaspar, 2017). If the labelling with this will not work, it might be prudent to look for another labelling dye within a similar absorption spectrum.

For the smFISH procedure itself, 26dpf wildtype medaka juveniles (Cab strain) were sacrificed and the cryosections were done with 16µm thickness. As mentioned above, the slices should be as thin as possible for the smFISH to work. However, there was the issue of getting these thin slices without them ripping. The smFISH protocol was done a few times, with varying thickness of 16µm to 20µm. Using 20µm definitely improved the transfer of the sections to the glass side and retaining a greater number of the slices while slicing and throughout the whole smFISH procedure. Regarding the staining, some positive *sst1b* cells could be shown in the midbrain (Figure 14). However, these positive cells were only seen during one run-through. This could not be replicated with 20µm thick slices, so the tissue might be too thick already. Additionally, a lot of slices were ripped and not recognizable anymore once it got to imaging, and some were simply lost (probably washed off) during the procedure. As mentioned above, the protocol by Staple et al., served as a foundation for this and had to be adapted, considering that they used zebrafish embryo, and not juveniles, as was chosen here. Moreover, the preparation of the fish samples (prior to slicing) had to be adapted and was based on a protocol for cryosections in *Platynereis dumerilii* (Veedin-Rajan, 2013). This still leaves a lot open for adaptation and changes within the sample preparation as well as the protocol itself. The first step towards a steadily

working protocol, however, is to make sure that the labelling of the probes works reliably.

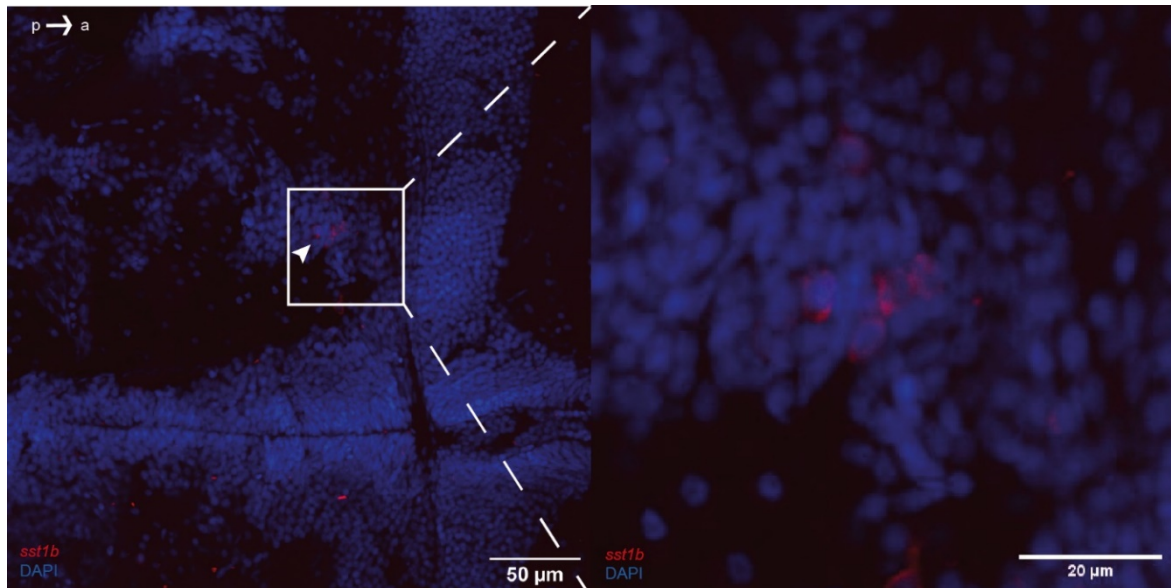


Figure 14 – *sst1b* positive cells in the midbrain of a medaka juvenile fish. *sst1b* staining in red.

***Tmt-opsin1b* in zebrafish**

Fontinha et al. showed that *tmt-opsin1b* affects the expression level of transcripts like *sst1b* and *apoEb* (*apoEb* data not published). A mutation of *tmt-opsin1b* was shown to affect *sst1b* expression especially in the mid- and hindbrain. When comparing the function of *tmt-opsin1b* paralog in zebrafish, an obvious start is to see if the same genes are affected by this Opsin, as they are in medakafish. Therefore, *sst1b* and *apoEb* transcription levels in the brain were analyzed with qPCR. Looking into the RNA sequencing data for medaka, other transcripts that are shown to be affected by *tmt-opsin1b* were chosen for zebrafish as well. RNA extracts of zebrafish *tmt-opsin1b* wildtype and homozygous adult fish (eyes, forebrain, midbrain, hindbrain) were used for the qPCR analysis. The chosen genes were *sst1b*, *apoEb*, *tph1a*, *grin1b* and *grin2aa*. In medaka, a downregulation of *sst1b* in the mid-/hindbrain of *tmt-opsin1b* mutants could be shown (Fontinha, 2019). Furthermore, *apoEb* was shown to be slightly upregulated in mid-/hindbrain in mutants, *tph1* being upregulated, and *grin2a* (paralog to *grin1b*) downregulated in the mutant forebrain. As can be seen in the Figure 15, a significant difference in the expression levels could not be shown for any of the genes in zebrafish. *Tph1a* shows a tendency towards downregulation in *tmt-opsin1b* homozygous fish in the midbrain, while both *grin1b* as well as *grin2aa*

seem to have a trend towards upregulation in the mutant hindbrain. (Expression levels in eye and forebrain can be found in the Supplementary data).

$+/+$ = *tmt-opsin1b* wildtype
 $-/-$ = *tmt-opsin1b* homozygous
 n = 6

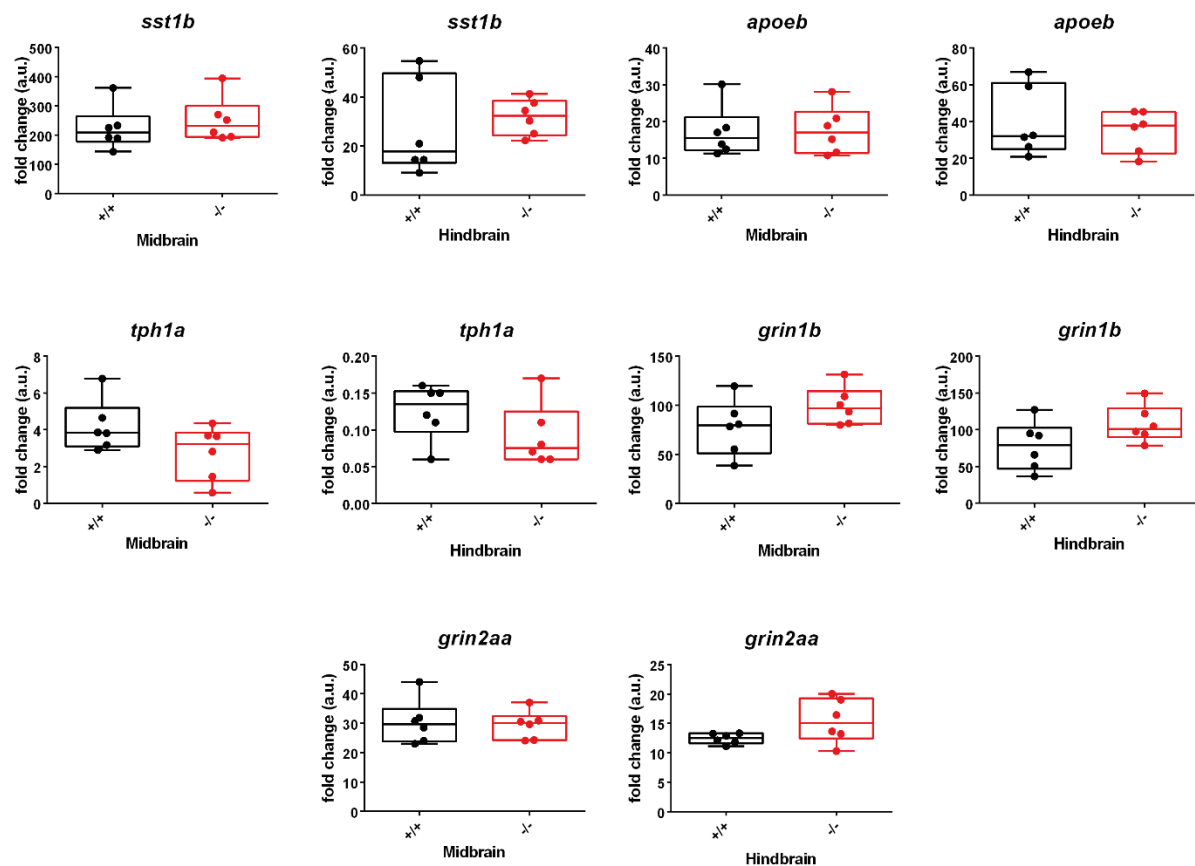


Figure 15 - Gene expression levels in adult zebrafish mid- and hindbrain

Table 4 - Analysis of gene expression levels in mid- and hindbrain. p-value calculated by: Unpaired t test with Welch's correction

	p-value	
	Midbrain	Hindbrain
<i>sst1b</i>	0.5351	0.5820
<i>apoEb</i>	0.9212	0.6055
<i>tph1a</i>	0.1114	0.1804
<i>grin1b</i>	0.1503	0.1112
<i>grin2aa</i>	0.8012	0.1058

Generating *tmt-opsin2* mutant with CRISPR/Cas9

As has already been mentioned, research has been done to look into the effect of *tmt-opsin1b* and *tmt-opsin2* in medaka. For example, it could be shown in medaka larvae that *tmt-opsin1b/tmt-opsin2* double homozygous mutants show phenotypes that are different to *tmt-opsin1b* mutants, having an additive effect to the phenotype (Fontinha, revised manuscript of Fontinha, 2019 attached).

Continuing to compare these findings with zebrafish, and to catalogue the functions of *tmt-opsins*, a knock-out model in zebrafish for *tmt-opsin2b* was created as well. This was done using CRISPR/Cas9. Two possible sequence-regions were chosen and single-guide (sg) RNAs created to get a functional knock-out model. It was predicted that it would affect the fourth transmembrane domain (of seven), and thus create a non-functional opsin.

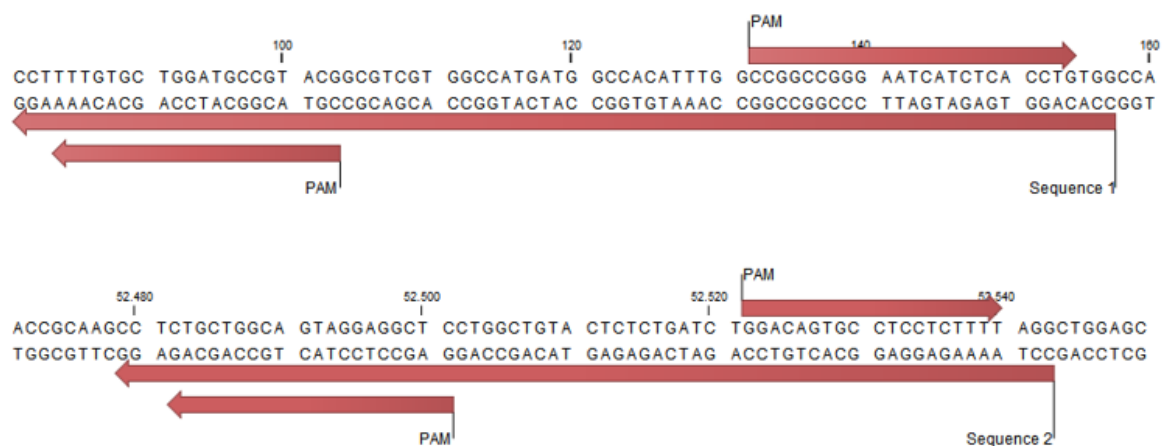


Figure 16 - Two regions were chosen to try and create a *tmt-opsin2* knock-out model in zebrafish. PAM regions to create the sgRNAs are indicated

With the help of a webservice³ possible target sites for the knockout were identified and two chosen. SgRNAs were created and the injection mix was prepared, with the sequence-region 1 being the first to be tried. Due to technical issues and time constraints, injecting embryos at the ideal one-cell stage was not possible. Most were already at 4-8 cell stage. For practice reasons, the injections still took place, likely creating a mosaic mutation pattern as a result. Over two days, around 30 eggs were injected, though most died within the first days. Wildtype embryos from the same clutch were kept as a control group and showed a better viability than the injected fish. A greater number of embryos has to be

³ <https://chopchop.cbu.uib.no/>, last accessed August 2020

injected (at the right age) to get enough fish with a working knockout of *tmt-opsin2b*. Both injection mixtures targeting different regions should be tried for this, to increase the probability to create mutants.

Discussion

Developmental effects of the mutation of various TMT-Opsins in medaka

To be able to properly study the function of TMT-Opsins, it first had to be ruled out that possible deviations observed in mutants come from developmental effects the mutation might have on the organism. For this, the development of *tmt-opsin1b*, *tmt-opsin2* and *tmt-opsin1b/tmt-opsin2* homozygous mutant embryos was observed until hatching and compared to the development of wildtype embryos, collected at the same time. Compared to the wildtype development and literature, it was possible to show that there seems to be no developmental effect on any of the observed *tmt-opsin* mutants. Less eggs were collected for *tmt-opsin1b/tmt-opsin2* mutants, and a few more died after the first day compared to the wildtype or other mutants, but the development still looks to be unaffected. It is possible that this is simply an issue regarding the parents. The mated pairs with the double mutation were a bit older than the other parents have been. Of course there is always the possibility that it has something to do with the mutation itself, but more fish have to be mated and analyzed to come to a proper conclusion regarding this. Apart from that, the only thing different that could be noted were the oil droplets. Generally, the oil droplets fuse while they move to the vegetal pole within the first hour of fertilization, and stay there as larger globules (as can be seen in Figure 5a, stage 10). According to Iwamatsu, 2004, the size '*usually varies according to differences in the temperature during and after oocyte maturation, in the time after ovulation and among the individual females.*' (Iwamatsu, 2004). The mating pairs were all held in same conditions, so a change in temperature is unlikely. This still leaves the option that the difference pertains to the different females. Additionally, it has to be stated that the importance of oil droplets is not yet clear. Experiments showed that embryos without oil droplets develop equally well into juveniles as their control group, though it has been suggested that the death rate of adult fish might be higher (Iwamatsu, 2008). Taking this into account with the fact that no developmental difference between the *tmt-opsin* mutants and wildtypes could be observed, rather points to the fact that this oil droplet state does not affect development and might also not be a sideeffect of the *tmt-opsin2* mutation.

But since this state of hundreds of small droplets could not be observed in wildtype at all, it would still be interesting to observe this further and also do a statistical

analysis concerning this. Continuing from there, fish expressing GFP were also analyzed. The GFP expression is under the control of an *ath5* enhancer, which promotes RGC neurogenesis. Making these neurons visible is a way to look for any deviation in neurogenesis and thus see an effect a *tmt-opsin* mutation would have on the development. The analysis was done on *tmt-opsin1b* mutants. It could be concluded that the development of the tectum and eye is not affected by this mutation. This still has to be checked for *tmt-opsin2* and *tmt-opsin1b/tmt-opsin2* mutants as well, and mutant strains expressing GFP under *ath5* control have to be created.

Regulation of gene expression by *tmt-opsin1b*

Analyzing RNA-sequencing of medakafish showed various transcripts, that look to be affected by *tmt-opsin1b*. Research already focused on *sst1b* and showed that in *tmt-opsin1b* homozygous mutants, the expression level of *sst1b* is reduced in the optic tectum, and are absent in the cells medial to the nucleus glomerulosus (Fontinha, 2019). To make sure that this is specific, other transcripts were identified that are supposed to be expressed in the same cells as *sst1b* in zebrafish. Orthologs were cloned, and two likely candidates chosen and co-stained with *sst1b*. Both *gad2* and *pcp4/1* showed staining in the regions where *sst1b* is also expressed. When comparing wildtype and *tmt-opsin1b* mutant, no difference in transcript levels could be observed. This means that this reduction of *sst1b* expression is likely a specific effect *tmt-opsin1b* has on this gene, and might even indirectly regulate it. Whilst *gad2* clearly co-localized with *sst1b* in the tectum, the staining itself was still very broad, as can be seen in the larval staining and coronal brain sections. A gene with a more specific expression pattern would have perhaps been able to show more clearly/easier a co-localization of *sst1b* and any possible effect a *tmt-opsin1b* mutation would have on *sst1b* expression levels. For this, *tac1* would have been the perfect candidate. Unfortunately, it was not possible to get a working *in situ* probe for this. Looking at the results the different troubleshooting options brought, it can be assumed that the problem of cloning and sequencing of this gene lies in part in the sequence itself. The sequence seems to have many poly-nucleotide stretches, which might trip the sequencing process, ending up with a sequence that might only be a third or half of the sequence length of the actual gene. It would still be interesting to not only figure out ways to obtain

the full sequence, but also to be able to generate probes and look at the expression pattern in the medaka brain.

Visualizing transcripts with more sensitive methods

Depending on the genes one is interested in, it pays to have different techniques for visualizing a expression patterns, depending on what should be seen. One of the newer methods is single-molecule FISH. Marking single RNA-molecules, it is more sensitive than some other methods, and has the added benefit of being able to stain for more genes than only two. Establishing a protocol in fish proved to be difficult. The thinner the tissue, the better, which is hard to achieve in medaka juveniles, for instance. 16µm is the lowest thickness that could be achieved, and there the cryosections were likely to rip or get lost throughout the protocol. Positively stained cells were able to be visualized in the midbrain, which means that the adaptations from the protocol taken from Staple et al. worked for juvenile fish. However, a lot of slices were still lost and thus the washing steps and mechanics may have to be changed a bit to account for that. Furthermore, labelling the probes still has to be adapted. Labelling with Atto633 created working probes, where the DOL and recovery were in a area appropriate for working probes. The other fluorophores proved to be more problematic. On the one hand, the labelling process itself can still be adjusted, and some troubleshooting steps suggested in protocols like Gáspár et al. were not tried in the course of this project. On the other hand, it was already noted by Gáspár et al. that fluorophores with 488nm seem to have a high free dye contamination, and are harder to label with. So looking for different flurophores is also an option. It can be concluded that establishing a working protocol in fish is definitely possible, as shown with positive staining results, even if the protocol still has to be altered further.

***Tmt-opsin1b* in zebrafish**

Research has deepened to try and elucdiate the function of *tmt-opsin1b* and *tmt-opsin2* in medaka. Taking these findings, a cross-comparison with zebrafish could be started. This is of particular interest with the fact that zebrafish and medaka are evolutionary distant and have differences like the seasonality of medaka. Comparing *tmt-opsins* could thus provide more information pertaining their function and evolution.

For this cross-comparison, some genes were chosen that showed a differential expression pattern in *tmt-opsin1b* mutants in medaka and their orthologs in zebrafish analyzed.

It could be shown that in medaka, *sst1b* expression is downregulated in mid- and hindbrain in *tmt-opsin1b* mutants (Fontinha, 2019). This could not be observed in zebrafish, which means that this regulation of *sst1b* by *tmt-opsin1b* is specific for medaka, and might even be linked to seasonality. This is corroborated by findings that when changing the photoperiod for medakafish, fish under a shorter light-regime show a significant reduction of *sst1b* expression levels (Fontinha, revised manuscript of Fontinha, 2019).

Regarding *apoEb*, unpublished results show an upregulation in *tmt-opsin1b* mutant midbrain. This could not be reproduced in zebrafish brains and is likely again specific to medaka. *ApoEb* is a marker for microglia cells. Murine microglia gene expression has been shown to be affected by blue light (Cheng, 2016), and seem to have a role in postnatal neuronal development, where cone photoreceptor loss was observed when blocking a pathway microglia use for neuron interaction (Jobling, 2018). So there seems to be a connection between microglia cells and light/photoreceptors, and it would be interesting to see in what way *tmt-opsin1b* is regulating or affecting this transcript, and why it differs to zebrafish.

The other genes (*tph1a*, *grin1b*, *grin2aa*) did not show any significant up- or downregulation either in zebrafish. However, the results do show a trend. The analysis was only done with only an n=6. It might be possible that adding more biological replicates are necessary to determine a significant regulation difference. If they do prove to be regulated differently, it would certainly make for interesting results: In medaka, *tph1* was shown to be upregulated in the *tmt-opsin1b* mutant forebrain, while the data for zebrafish shows a tendency to a downregulation in the midbrain of *tmt-opsin1b* mutants. *Grin1b* and *grin2aa* showed a tendency towards upregulation in the hindbrain of *tmt-opsin1b* mutants, which differs to what could be seen in medaka, where *grin2a* is downregulated in forebrain of *tmt-opsin1b* mutants. Before drawing any conclusions it is vital to make sure that the results are actually significant. If they are, they difference in regulation is certainly interesting. Should this not be the case, these genes being regulated by *tmt-opsin1b* are again specific to medaka.

Generating *tmt-opsin2* mutant with CRISPR/Cas9

To be able to further compare published and unpublished results obtained from *tmt-opsin2* medaka findings and observations with zebrafish, the corresponding mutation, which would be *tmt-opsin2b*, has to be generated as well. This would be of great interest, so that findings and results obtained from this can be brought into the bigger picture regarding species specific or photoreceptor-specific function.

Conclusion

This work contributes towards a better understanding of *tmt-opsins1b* and *tmt-opsin2* in the teleosts zebrafish and medaka. The work deepened on what has already been discovered by Fischer et al. and Fontinha et al., and managed to shed more light on the function and regulating effects of *tmt-opsin1b* in medakafish, as well as making sure that no developmental effects are caused by mutations of both *tmt-opsins*. Furthermore, an effort for cross-comparing the results with zebrafish was started, where various transcripts affected by *tmt-opsin1b* in medaka were looked at in zebrafish mutants and analyzed. To take this comparison further, a *tmt-opsin2b* knockout model for zebrafish was designed.

This project, with its foundations in the aforementioned discoveries by Fischer et al. and Fontinha et al., dug deeper into TMT-Opsins, with a special focus on *tmt-opsin1b*. While a few findings could be made, it just mainly showed how little is still known about the functions and responsibilities of TMT-Opsins. The results of these experiments certainly provided interesting results that can be looked into with more detail to create a clearer picture of *tmt-opsin1b* in medakafish and zebrafish.

Materials and Methods

Fish husbandry

Work with fish was done according to Austrian and European guidelines for animal research. Zebrafish (*Danio rerio*) strains AB and Tübingen and medakafish (*Oryzias latipes*) strains Cab were kept in a constant recirculating system at 28°C, with a photoperiod of 16h light/8h dark. Collected embryos were kept at 28°C until hatched. For medakafish, they were kept in Embryo Rearing Medium (ERM; 0.1% NaCl, 0.003% KCl, 0.004% CaCl₂, 0.016 % MgCl₂, 0.017 mM HEPES and 0.0001% methylene blue). Zebrafish embryos were kept in E3 Medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl, 20.33 mM MgSO₄, 10-5% Methylene Blue)

Fish were fed with brine shrimp (*Artemia*) and flake food. Fish larvae are fed with dust food and Rotifers.

Embryo Staging

For staging, the eggs were collected approx. 2 hours after lights on, and only taken directly from the female to ensure the correct age. At around 8hpf (hours post fertilization) eggs were controlled for unfertilized/dead eggs, and moved into molded tracks of 3% agarose/PBS with ERM medium, for easy analysis. Starting at ~8hpf, eggs were observed every 24 hours until hatching. Eggs were kept in the molded tracks with ERM medium at 28°C with usual 16h light/8h dark cycle.

Note: Embryo collection for staging done with help of Theresa Zekoll, MSc. Collection and analysis of ath5::GFP fish done with help of Dr. Kristin Tessmar-Raible.

Cloning of medaka genes

A BLAST search using the zebrafish *gad2*, *pcp4l1*, *gad2* and *nrgna* identified predicted homologous sequences, homology was confirmed by reciprocal back-blast. cDNAs were cloned following standard protocols, using Phusion Polymerase (NEB) and cloned in pJET1.2 (Thermo Fisher). Inserts were verified by sequencing.

Table 5 - Primer design for cloning of medaka genes

Gene	Primer (5'-3')	Sequence	Accession number
<i>pcp4l1</i>	FW1	AGC TGG CAT CCA CAA ACT CCG ACA AC	MT239386
<i>pcp4l1</i>	REV1	CGC CTC TAA CCA AGG GGG GTA GCT G	MT239387
<i>pcp4l1</i>	FW2	CAG CCA GAT GAC TGC AGC TGA TAA C	MT239388
<i>pcp4l1</i>	REV2	TGC CAG TTT CCA TGA CCA CCG TCT CC	MT239389
<i>nrgna</i>	FW1	ATG TCA GCG CAT TTC TTC ACC GCT C	
<i>nrgna</i>	REV1	TTG CTG GTT TGC TCA TAT TCC AGT GG	
<i>nrgna</i>	FW2	CCT GCG GGT CCC TCG GGG GCT CGG	
<i>nrgna</i>	REV2	ATT TAA ATC AGT CAT TTC ATC CTG GG	
<i>gad2</i>	FW1	ATG GCA TCT CAC GGG TTC TGG TCT C	MT267535

<i>gad2</i>	REV1	TTA TAG ATC ATG GCC GAG GCG TTC A	MT267536
<i>gad2</i>	FW2	AGA TAA TGC GGG GGC CAA CAC CGG G	MT267537
<i>gad2</i>	REV2	GTT GGT AGC TGA CCA TAG TTG TGC C	MT267538
<i>tac1</i>	FW1	CCC CTT TTC CTT CCA GTT CAA AGC TC	
<i>tac1</i>	REV1	TGT GGG GTG GTG CAA GTG TGC ACT CCC	
<i>tac1</i>	FW2	ATG AAG CTG CTG CTG CTG CTG TCC GC	
<i>tac1</i>	REV2	CTG TCA TGC TTA GAA GGT AGT TGT GTC	
<i>tac1</i>	FW3	GAA CCT GAA GTT ACT TTT TTA CCA C	
<i>tac1</i>	REV3	TAC AGG TAG AGT CAT GAC CAG AAC A	
<i>tac1</i>	FW4	ACC ACA GGG GCA CGT CAC TCT AG	
<i>tac1</i>	REV4	ATT CTG CTG GTA CAA ACC AGA CGG G	
<i>tac1</i>	XM1_FW1	CGC GGC GCC CAG ATA AAG TGT GTC	XM_024294204.1
<i>tac1</i>	xm1_REV1	TGA AGT TTG TTG ATA GAG TCC TTT AAT	XM_024294204.2
<i>tac1</i>	xm1_FW2	CAG CGC GGA CAG CGC AGC TCT CAC	XM_024294204.3
<i>tac1</i>	xm1_REV2	GAA TGT TCA TGG TGG GGG GGT GAG G	XM_024294204.4
<i>tac1</i>	xm2_FW1	TTA CTT TTT TAC AAG CGG GGC AC	XM_023959561.1
<i>tac1</i>	xm2_REV1	TCA TCA CCA GAA CAT GAA AAC AG	XM_023959561.2
<i>tac1</i>	xm2_FW2	CAC GTT ACT CTA GTA ATC CTG CTT C	XM_023959561.3
<i>tac1</i>	xm2_REV2	CAG TCG TTT TAT TCA GCT AAC ATT T	XM_023959561.4

Adult Fish Brain Dissection

For dissections, adult fish were anesthetized with 0.2% tricaine and decapitated. Heads were immersed in ice-cold 1x PBS (Phosphate buffered saline) and brain was dissected and immediately transferred to ice-cold 4% PFA/PTW (Paraformaldehyde/1x PBS with 0,1% Tween-20). Brains were kept shaking gently over night at 4°C. The next day they were washed 5x5' in PTW, rinsed in 100% MeOH, transferred into fresh 100% MeOH and put on -20°C for at least a week before use.

Probe generation

Probes were generated using previously cloned plasmids as template. Genes of interest were amplified with PCR using OneTaq Quick-Load 2X Master Mix with Standard Buffer (NEB). To generate sense or antisense probes, the appropriate sp6-adding primer was used with a primer for pJET1.2. PCR product was purified following the protocol for "QIAquick PCR Purification Kit" (Qiagen) and checked on a 2% agarose gel.

In Vitro Transcription (IVT) at 37°C for 4 hours:

- 10µl of template
- 2µl NTP-DIG labelling mix/NTP-Fluorescein labelling mix (Roche)
- 2µl 10x RNA polymerase reaction buffer
- 1µl sp6 RNA polymerase
- 4.5µl DTT (100mM)
- 0.5µl RNase inhibitor (Ribostock)

Following this, 1µl DNase was added and incubation was done for 10' at 37°C. Probes were then purified according to the "RNeasy Kit" (QIAGEN) and eluted in 50µl RNase-free water. After concentration was measured and probes were analyzed on agarose gel, hybridization mixture (Hyb+, see ISH protocol below) was added to the probes and were stored at -20°C.

***In situ* hybridization (ISH) on adult brain sections**

Rehydration and washes for 5' each in 75% MeOH/PTW, 50%MeOH/PTW, 25%Me/OH/PTW, 2x 5' 1X PTW. Then, brains were digested with fresh 10 µg/ml Proteinase K (Merck, USA) for 35 minutes, washed with 2mg/ml Glycine and fixed in 4% PFA/PTW, again washed 5x5' 1x PTW, then followed by 4 hours prehybridization at 65°C (waterbath) with Hyb+ solution (50% Formamide, 5x SSC (pH=6.0), 0.1% Tween-20, 0.5 mg/ml torula yeast RNA, 50 µg/ml Heparin). Incubation with DIG-RNA probe/Fluo-RNA probe in 200µl Hyb+ overnight at 65°C. Washing at 65°C with Formamide/SSCT mixtures (Saline sodium citrate with 0.1% Tween-20). Brain was sliced for coronal slices with 100 µm. Slices were made using a vibratome (Leica VT1000S, Leica Biosystems, Germany). Brainslices were then blocked for 1 hour min. in 10% sheep serum/1x PTW, followed by incubation with 1:2000 dilution of anti-DIG-alkaline phosphatase-coupled antibody (Roche, Switzerland) in 10% sheep serum overnight. Detection of DIG-probes was made in staining buffer (in 10% polyvinyl alcohol) supplemented with nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolylphosphate (BCIP) (Roche).

Gad2 and *pcp4/l1* were co-detected fluorescently with Akoya Biosciences Tyramide Signal Amplification Plus Cyanine 3:

After the alkaline phosphatase staining, samples were washed for 1-2 hours in PTW, before blocking in 10% sheep serum/PTW, followed by incubation with 1:50 dilution of anti-Fluorescein-horseradish peroxidase (Roche, Switzerland) in 10% sheep serum overnight. Brains were then washed for 2 hours in TNT Wash Buffer (0.1 M TRIS-HCl (pH=7.5), 0.15 M NaCl, 0.05% Tween®20). Detection of brains was done with 1:50 dilution of TSA Plus Stock Solution Cyanine 3 in 1x Amplification Diluent for 1.5 hours.

RNA Extraction

For RNA extraction, dissected brains or brainparts were snap-frozen, and a metal bead (PeqLab Biotechnologie) and 700 µl TRI Reagent® (Sigma-Aldrich, St. Louis, USA) were added. This was homogenized for 2 minutes at 30 Hz using a tissue lyser (Qiagen). RNA extraction was done using Direct-Zol RNA MiniPrep (Zymo Research, USA), according to protocol. CDNA was synthesized with the Qiagen QuantiTect Reverse Transcription Kit, and eluted in 30µl nuclease-free water.

Quantitative PCR (qPCR)

Primers (Zebrafish):

Gene	Primer Forward (5'-3')	Primer Reverse (5'-3')	Ensembl Gene ID
<i>grin2aa</i>	CAT CTC TGG AGC TTT GTG GAA	TGG AAA ACC CCA TAG TGC TC	ENSDARG00000034493
<i>grin1b</i>	GAC GTA GGT CGT CCA AAG ACA	TTC TCT CTT CAC TTT CAC ACC ACT	ENSDARG00000025728
<i>tph1a</i>	ATT CCA TGA ACC TCG GAC TG	CTT CGT GAA AGC AGA TTT GTT C	ENSDARG00000029432
<i>apoEb</i>	AAG AAA CGC CTG AAC AAG GA	TCC TTC ACT GCA TCA GCA TT	ENSDARG00000040295
<i>cort</i>	CAG CAG CTG TGC TTC CAC T	GAG CTC CTT ACG CTC TTT ACT CA	ENSDARG00000031649

QPCRs were done using Applied Biosystems™ QuantStudio™ 3 Real-Time PCR System.

qPCR protocol

- 10µl Luna® Universal qPCR Master Mix (NEB)
- 1µl Primer FW (10µM)
- 1µl Primer REV (10µM)
- 2µl cDNA
- 6µl H₂O

Two technical replicates per sample were analyzed, and the expression of each gene was normalized to *beta actin*. Transcript levels and fold changes were calculated using the Δ -Ct method and the fold change calculated (fold change = $2^{-\Delta CT}$)

CRISPR/Cas9

guideRNA:

sgRNA were designed using Chopchop (<https://chopchop.cbu.uib.no/>; Labun, 2019)

Target gene: Zebrafish *tmt-opin2b*: ENSDARG00000027822

sgRNAs generated for knockout with CRISPR/Cas9 Nickase. Two sequences were chosen to try and generate a knockdown.

Sequence 1:

CCACAGGTGAGATGATTCCCGGCCGGCCAAATGTGGCCATCATGGCCACGACGCCGTA
CGGCATCCAGCACAAAAGG

sgRNA design with PAM sequence (Proto-adjacent motif) at 3' of target with 5'-NGG-3' (left and right side each have Top and Bottom Oligo):

left: TAGG CGTACGGCATCCAGCACAAA AAAC TTTGTGCTGGATGCCGTACG	right: TAGG CCGGCCGGGAATCATCTCACCTG AAAC CAGGTGAGATGATTCCCGGCCGG
---	--

Primers (for genotyping):

Primer FW: GTG TGT TAC CTT TTG TGC TGG A

Primer Rev: TGT GAT ATC CTG GAG GGA CTT T

Sequence 2:

CCTAAAAGAGGAGGCACTGTCCAGATCAGAGAGTACAGCCAGGAGCCTCCTACTGCCA
GCAGAGG

sgRNA design with PAM sequence at 3' of target with 5'-NGG-3' (left and right side each have Top and Bottom Oligo):

left: TAGGGAGCCTCCTACTGCCAGCAG AAACCTGCTGGCAGTAGGAGGCTC	right: TAGGGGACAGTGCCTCCTCTTTT AAACAAAAGAGGAGGCACTGTCC
---	--

Primers (for genotyping):

Primer FW: TTG ACT GTG TAC CAT AAA CGG G

Primer Rev: CTG GTA GTC CCA GGC AGA ATA C

Construction sgRNA expression vectors

Annealing of Top and Bottom Oligos each:

- 2µl Top oligo (5µM)
- 2µl Bottom oligo (5µM)
- 46µl Oligo Annealing Buffer (1x, Tris-HCl, NaCl, EDTA, H₂O)

Incubate at 95°C for 3 minutes. The temperature of the heatblock was re-set to 25°C and the sample was left in until it has cooled down to 25°C (approx. 1 hour)

Ligation into T7 sgRNA vector:

- 2µl annealed Oligo
- 1µl T7_sgRNA vector DNA (75ng/µl)
- 2µl 10x T4 DNA Ligase Buffer
- 2µl BSA (2mg/ml)
- 1µl BbsI Enzyme (NEB)
- 1µl T4 DNA Ligase (NEB)
- 11µl Nuclease-free H₂O

Incubation with PCR machine. Program:

- 3x 10' 37°C, 10' 16°C
- 10' 37°C
- 20' 65°C
- Hold 10°C

Transformation of 5µl of reaction into XL1-Blue competent *E.coli* (via heatshock), grow for 1 hour in 250µl SOC medium (Super Optimal broth with Catabolite repression). 100µl of transformed cells are plated on LB plates containing Ampicillin. Growth overnight at 37°C. 2 colonies are picked and miniprep broth is prepared (LB medium + Ampicillin), grown overnight at 37°C.

Plasmid purified with MiniPrep (QIAprep Spin Miniprep Kit, QIAGEN) and checked for positive ligation by digesting miniprep vs. original T7_sgRNA vector:

- 1µg plasmid DNA
- 1µl BbsI enzyme (NEB)
- 1µl 10x Buffer 2.1 (NEB)
- 7µl H₂O

Incubation at 37°C for 2 hours. Checked on 1.5% agarose gel vs. uncut controls. Ligated plasmid should not be cut with BbsI and should have the same size as uncut control.

sgRNA in vitro

Digest sgRNA with DraI:

- 5µg plasmid
- 1µl DraI enzyme (NEB)
- 2µl 10x Enzyme Buffer CutSmart (NEB)
- To 20µl H₂O

Incubation at 37°C for 2 hours. Load on 2% agarose gel to check, cut band with right size (~750bp) and do gel purification (QIAquick Gel Extraction Kit, Qiagen). Elute in Nuclease-free water.

In-Vitro Transcription (T7 Quick High Yield RNA Synthesis Kit):

- 18µl digested sgRNA vector DNA
- 10µl NTP Buffer Mix
- 2µl T7 RNA Polymerase Mix

Incubation at 37°C for 16 hours exactly. Add 30µl NF-Water, remove 2µl to check on gel. Add 2µl DnaseI, incubate for 15' at 37°C. Remove 2µl to check on gel. Transfer tubes to ice. Purification according to the "RNeasy Kit" (QIAGEN) and eluted in 30µl RNase-free water, moved column to new tube and eluted again in 30µl.

Check DnaseI – and Dnase+ samples as well as purified sample on gel. Check concentration via Nanodrop.

For injecting solution, sgRNA was diluted to a working concentration of 150ng/µl.

Injection solution:

- 2µl Cas9 (100ng/µl, CRISPR Cas9-D10A Nickase mRNA, Sigma-Aldrich)
- 1µl sgRNA left (150ng/µl)
- 1µl sgRNA right (150ng/µl)
- 1µl TRITC (Tetramethylrhodamine)
- 5µl H₂O

Injection:

Injections were done with a microinjection setup (microscope, microinjector,-manipulator) (<https://www.eppendorf.com/>).

Collected eggs from zebrafish AB wildtype strain. Eggs are lined up in tracks in a 2% agarose plate-mold for easier injection. Ideally, injection should happen at one cell stage. Fine-tipped needle is loaded with injection mix and should penetrate the yolk for ejection of injection mix.

smFISH (single molecule fluorescence *in situ* hybridization)

Probes:

Table 6 - Probes designed for smFISH. Ideally, 40-50 probes should be designed for a gene. However, zebrafish cort sequence is not long enough for this. Note: *sst1b* in medaka has undergone a few name-changes and is also known as *cort*

Gene medaka fish	DNA Sequence (5' -> 3')	Gene zebrafish	DNA Sequence (5' -> 3')
<i>scn12aa 1</i>	tgcagatgtggagctgaatc	<i>scn12aa 1</i>	tggcactgaagcggaagatg
<i>scn12aa 2</i>	cagatatttggtccagcttg	<i>scn12aa 2</i>	gatctgacatcgccatgaag
<i>scn12aa 3</i>	aatgcccgtgaaggtgtac	<i>scn12aa 3</i>	gccagtgaatgtatactcga
<i>scn12aa 4</i>	gacaataaagtcagccagt	<i>scn12aa 4</i>	cactcagtacaaaaccctct
<i>scn12aa 5</i>	aaattcagtgacgtaccca	<i>scn12aa 5</i>	atagccatgacgatgacac
<i>scn12aa 6</i>	tggttttcagacctggaatc	<i>scn12aa 6</i>	cgcagaactctgaacgttcg
<i>scn12aa 7</i>	gaagacggtcaggatcatca	<i>scn12aa 7</i>	gggatcaccgatattgtttt
<i>scn12aa 8</i>	atgaagagctgcagacctat	<i>scn12aa 8</i>	tgacagaaagccaggcttg
<i>scn12aa 9</i>	cgatccacatattaagcca	<i>scn12aa 9</i>	ccttgatcctcaattttg
<i>scn12aa 10</i>	agtcatagtttggattcctc	<i>scn12aa 10</i>	ctgctgtatccgcatattaa
<i>scn12aa 11</i>	aaccatgtaggttttccag	<i>scn12aa 11</i>	ctggttctgcattttatcga
<i>scn12aa 12</i>	atgaacaggtgttgagcac	<i>scn12aa 12</i>	gaagatgctcatttggttc
<i>scn12aa 13</i>	atgacgcccgtcaaatggtt	<i>scn12aa 13</i>	aacacctgagacgagtgact
<i>scn12aa 14</i>	ctcgcaacagtctgaaggat	<i>scn12aa 14</i>	cggttctgcccagagaaatg
<i>scn12aa 15</i>	tgccgatgatcttgatgaga	<i>scn12aa 15</i>	tggtcattctgacactctgg
<i>scn12aa 16</i>	aggagtggagaagtccttc	<i>scn12aa 16</i>	gcaggagaacacgtccaaat
<i>scn12aa 17</i>	gtaaattcctccattgcttt	<i>scn12aa 17</i>	ccgtgaagattccagtgaac
<i>scn12aa 18</i>	tcttcacgtctgatgactc	<i>scn12aa 18</i>	agcagtctgaacgagcgaag
<i>scn12aa 19</i>	atgtcaatgttcgacactg	<i>scn12aa 19</i>	gatgaagacgatgatggcca
<i>scn12aa 20</i>	caaagcaccactgctgagaa	<i>scn12aa 20</i>	ttttcccaaatagctgcat
<i>scn12aa 21</i>	actttgtcagcatattccag	<i>scn12aa 21</i>	acagggccagaaacagattc
<i>scn12aa 22</i>	cagtcacgatcagaaagt	<i>scn12aa 22</i>	cggcgctgatagattatcag
<i>scn12aa 23</i>	agaatacccagagagtttg	<i>scn12aa 23</i>	tggctatcgaaggttgctc
<i>scn12aa 24</i>	cgagcagcacattcatgatg	<i>scn12aa 24</i>	aaaccacagtggattcgagc
<i>scn12aa 25</i>	cttgccagcaaacaggttaa	<i>scn12aa 25</i>	gaaattccacgtctgactct
<i>scn12aa 26</i>	ttgttactatggtgatgct	<i>scn12aa 26</i>	tcagagaggctgatgacttc
<i>scn12aa 27</i>	tcattcatggctagacagtc	<i>scn12aa 27</i>	aacactctctggatccatg
<i>scn12aa 28</i>	aactgtggtccagtagaact	<i>scn12aa 28</i>	ccttgctgtgtgtttatatt
<i>scn12aa 29</i>	aaccctcttgaatcaacag	<i>scn12aa 29</i>	tgctccacaatttgatagca
<i>scn12aa 30</i>	tagagggttgatttctctgct	<i>scn12aa 30</i>	gtacgcaatccacttcagaa

<i>scn12aa</i> 31	agtatttcttctgttcttcc	<i>scn12aa</i> 31	cgatcagtgcatcactaca
<i>scn12aa</i> 32	catttgcttctgactgctcat	<i>scn12aa</i> 32	tgaatctgtcctattgaca
<i>scn12aa</i> 33	agacactcgggtggtgaagat	<i>scn12aa</i> 33	tcatgaatatgtcttgcca
<i>scn12aa</i> 34	gttccatgcaactgtgaaga	<i>scn12aa</i> 34	atccaagtttcttattgcg
<i>scn12aa</i> 35	caaggacaatccccacaata	<i>scn12aa</i> 35	tattgaggatggtctccatg
<i>scn12aa</i> 36	aatgttgacagtgctggca	<i>scn12aa</i> 36	tttcaattatccgccagg
<i>scn12aa</i> 37	cataggcaaagtgtgccatc	<i>scn12aa</i> 37	gtaaagtccttattccctta
<i>scn12aa</i> 38	taaattgtgtgacctcgaa	<i>scn12aa</i> 38	acagtaggagaccgatgtg
<i>scn12aa</i> 39	ttgggtttgcaatacgag	<i>scn12aa</i> 39	cgaagtttgccatgccaaaa
<i>scn12aa</i> 40	acaagatgtccagacagtgg	<i>scn12aa</i> 40	cgggttcgcaaaactggag
<i>scn12aa</i> 41	ttttggaagggttggtcatc	<i>scn12aa</i> 41	tggcgatgtacatgttgacg
<i>scn12aa</i> 42	gatgatgattgcagacacct	<i>scn12aa</i> 42	tcgaactctccaaaacttc
<i>scn12aa</i> 43	acaagatgtctctgtaacc	<i>scn12aa</i> 43	aatccttaggggttcggaaa
<i>scn12aa</i> 44	aagaagcttgcttcagttgc	<i>scn12aa</i> 44	gagttgatcttgttgggtt
<i>scn12aa</i> 45	atttccacattcactgtgg	<i>scn12aa</i> 45	gcgtttggtgaaagcgaaca
<i>scn12aa</i> 46	gctatcatagctgaggtg	<i>scn12aa</i> 46	ttgttgcttcaaacatcc
<i>scn12aa</i> 47	aacctctagatgttggtctg	<i>scn12aa</i> 47	ggccatagttctcttaatc
<i>scn12aa</i> 48	cctcgtagtttcaacggaa	<i>scn12aa</i> 48	cttcgatgtctctctgat
<i>chat</i> 1	gaagaccaataggcagggag	<i>chat</i> 1	ctttgattgttcccttttg
<i>chat</i> 2	agtgtgtcttccagatcagg	<i>chat</i> 2	tttgctcaaggggggaatg
<i>chat</i> 3	tcacacacctgaggtacatg	<i>chat</i> 3	atgcatgtcaggtacatgct
<i>chat</i> 4	ttgaattgctcttccgtgag	<i>chat</i> 4	ttggcttctgaactggtc
<i>chat</i> 5	ctgcttactatcttctgag	<i>chat</i> 5	gtgctccaaatttctctaca
<i>chat</i> 6	acgagtttgctctgcagaag	<i>chat</i> 6	acttttctgaaggggttcc
<i>chat</i> 7	catgtcattcagccaatagt	<i>chat</i> 7	aattggcctttgttcactt
<i>chat</i> 8	cagagcgactctgtgttta	<i>chat</i> 8	agtgtacactgtgtgtcaa
<i>chat</i> 9	atcacaggactggagttgac	<i>chat</i> 9	accataacaggactggagtt
<i>chat</i> 10	tgaagtctgtgtgggaag	<i>chat</i> 10	acatcactttgaccttgaa
<i>chat</i> 11	tttaaggagtcaatgggggc	<i>chat</i> 11	acgtccatcaattagagctt
<i>chat</i> 12	ctctgagattaagtgcaga	<i>chat</i> 12	cgtgaagacctgtgtact
<i>chat</i> 13	tccagaagagcttctgactc	<i>chat</i> 13	aagtgtctgttttctctct
<i>chat</i> 14	caatccacaggcaagtcacg	<i>chat</i> 14	ataactgtgtcttctgagc
<i>chat</i> 15	taggcgatgggatgtgaaca	<i>chat</i> 15	ctgattcttacctgccacta
<i>chat</i> 16	atcatgtgttcaggatctgg	<i>chat</i> 16	gcgaaggtttaccatcacgt
<i>chat</i> 17	gaactggttcttacaagcca	<i>chat</i> 17	cagctgagtgtaaaggctctt
<i>chat</i> 18	gttgatcaccacgttcagaa	<i>chat</i> 18	ttgatcagtagttgcgagc
<i>chat</i> 19	gtctcttctattgagtcgac	<i>chat</i> 19	ctcaatcatgtccagagagt
<i>chat</i> 20	cttgattttctccagctgaa	<i>chat</i> 20	tcatccaaacacaccagaca
<i>chat</i> 21	atgagaatgctgcgagcttc	<i>chat</i> 21	aaagctctgtgtgtcatt
<i>chat</i> 22	agtcctgttaattgattct	<i>chat</i> 22	caaactgcatgggtttgtcg
<i>chat</i> 23	aagacagaccagacacatgc	<i>chat</i> 23	gagagtgttcacagacgacg
<i>chat</i> 24	caaactgcatgggtttgtcg	<i>chat</i> 24	acaagcactattccctcaaa
<i>chat</i> 25	ccttcaaaaggcgagtgctc	<i>chat</i> 25	gtatctcagtagatactccg
<i>chat</i> 26	actcagtacactgcacaagg	<i>chat</i> 26	ctggaagttcactcatgtcg
<i>chat</i> 27	cccaatcatgtatttgagga	<i>chat</i> 27	aggaaagtttgatgtctgg
<i>chat</i> 28	gtttctggatctctggagaa	<i>chat</i> 28	ttctgtaatctgtctgtga
<i>chat</i> 29	gagttgtcagcagaagagc	<i>chat</i> 29	cattcatgtccagggttttt
<i>chat</i> 30	gacattcatgtccagattct	<i>chat</i> 30	tctttgccataaccagtga
<i>chat</i> 31	ctttccatagtcacagaat	<i>chat</i> 31	taagcatcagggtcatttt
<i>chat</i> 32	caaggcaacctggatgaagg	<i>chat</i> 32	aaactggagcgcaacttgga
<i>chat</i> 33	ccttctgaaaacggcgagta	<i>chat</i> 33	gacgtccatgacatctgtaa
<i>chat</i> 34	ctgatgaaagccaaagcctc	<i>chat</i> 34	tgaagcgccgattgatgc
<i>chat</i> 35	cttctttgagttgagttcc	<i>chat</i> 35	gaggagcgaatgtgtcgac
<i>chat</i> 36	tttgtctgtcagttatagc	<i>chat</i> 36	gccatagctttgacaaatgc
<i>chat</i> 37	gatgattgtctattgccatc	<i>chat</i> 37	ctcagcatcagtgattttgg
<i>chat</i> 38	gcaatctctcgaagacctag	<i>chat</i> 38	ttagttgggcttttatggc
<i>chat</i> 39	cttctccatcttcttctt	<i>chat</i> 39	gtgattgtctattgccattc
<i>chat</i> 40	tgatgaggtaagctgcatct	<i>chat</i> 40	tctgaaaacagctctggctt
<i>chat</i> 41	aaaacatctccacggtggtg	<i>chat</i> 41	agtgaagtggatgtgtgttg
<i>chat</i> 42	tccattttggacaacaggtc	<i>chat</i> 42	taggaacctggctgtagag
<i>chat</i> 43	agatgatgtggtctgactgg	<i>chat</i> 43	gaccatagcagcagaacatc
<i>chat</i> 44	tcatggaagctggacactga	<i>chat</i> 44	acacgcagaagaggatgtgg
<i>chat</i> 45	tgcagcaagtgtctcacaaa	<i>chat</i> 45	ccctcaagagtcttcacaaa

<i>chat</i> 46	cattcattgcacagatccat	<i>chat</i> 46	atttctgcacagatcttgc
<i>chat</i> 47	atttgatcattgtctgggc	<i>chat</i> 47	agctggtttaacttcagtg
<i>chat</i> 48	ctttaccttgcagactct	<i>chat</i> 48	tatgacttgctccattctt
<i>sst1b</i> 1	tcctacctcgagtttaaga	<i>cort</i> 1	gtgagtaagctgggtctt
<i>sst1b</i> 2	gggtgtgaaaacctcagctt	<i>cort</i> 2	gcaggaactttgaagtcctt
<i>sst1b</i> 3	gtcttctttagaagccatca	<i>cort</i> 3	aaggtcagtaagagtagtga
<i>sst1b</i> 4	gtaaccctcagtggaatcaa	<i>cort</i> 4	ggttgaagttaaagtgcggc
<i>sst1b</i> 5	actatgacgtctacacatct	<i>cort</i> 5	ggccaaaagctgcatactta
<i>sst1b</i> 6	gagcaggattcaggtctgac	<i>cort</i> 6	ccagcaagagagacactagg
<i>sst1b</i> 7	aacacacatctgctgaaggc	<i>cort</i> 7	gctgctctgacactatacag
<i>sst1b</i> 8	tttgtagtagcattgacca	<i>cort</i> 8	caactccctactttgtgctg
<i>sst1b</i> 9	gttaaccttatcgacacatt	<i>cort</i> 9	tgagctcctacgctcttta
<i>sst1b</i> 10	ggcagaccgtgaaatcattt	<i>cort</i> 10	agccagagatcagcttcaa
<i>sst1b</i> 11	cagaggaggggagatgaagg	<i>cort</i> 11	cagaacgctgtgtctactc
<i>sst1b</i> 12	tgtcattggagttgtagtg	<i>cort</i> 12	aaggggactggtgctatctc
<i>sst1b</i> 13	gggtgtatgacggtgtacaa	<i>cort</i> 13	ataatcgggtgtagacagcc
<i>sst1b</i> 14	ctctgctgttgacgttgagg	<i>cort</i> 14	gaagaagttttgcaggggg
<i>sst1b</i> 15	ccaactctttatactctc	<i>cort</i> 15	ccgtaacacgacgtgaagg
<i>sst1b</i> 16	cagatgtttctactgctcta	<i>cort</i> 16	gaggatcgttcaggacttg
<i>sst1b</i> 17	ttctcatcagttctggagac	<i>cort</i> 17	cagttcatgtcaaggtatca
<i>sst1b</i> 18	gaagaaagtcagtcagct	<i>cort</i> 18	attattacagtgagggtct
<i>sst1b</i> 19	actactcagagggtccagaag	<i>cort</i> 19	agaccttcttcagacactg
<i>sst1b</i> 20	tgagctggagtctgaacgac	<i>cort</i> 20	gactgtgtcagagtaagtc
<i>sst1b</i> 21	gttcacacaggaagggtgt	<i>cort</i> 21	gcaactatcttaacctgct
<i>sst1b</i> 22	gctacaggagcaagagaagg	<i>cort</i> 22	ccacagtaaatgagacatct
<i>sst1b</i> 23	tgcattgctgacaagagtg	<i>cort</i> 23	ttgaagctttcccaatgtt
<i>sst1b</i> 24	atgagagctgctaaccacac		
<i>sst1b</i> 25	ctgctctaacgctgaacaga		
<i>sst1b</i> 26	tggtcacatggatggagctt		
<i>sst1b</i> 27	tcagttccttacgctctttg		
<i>sst1b</i> 28	aagccagacaccagcttaag		
<i>sst1b</i> 29	catgttggtgtccagagatc		
<i>sst1b</i> 30	actctaattggctcctcgaag		
<i>sst1b</i> 31	gcagcgataacctgttatag		
<i>sst1b</i> 32	aagtttttgcaaggggcttt		
<i>sst1b</i> 33	ctgttagcacgaggtgaacg		
<i>sst1b</i> 34	aaggaggacaagatgtgggc		
<i>sst1b</i> 35	ctactacagttcatggggag		
<i>sst1b</i> 36	tgcacaggtggagatggttc		
<i>sst1b</i> 37	gtctcattaaagtccatgga		
<i>sst1b</i> 38	ccgaaagggaatggcactga		
<i>sst1b</i> 39	tcgatccatgctttattgt		
<i>sst1b</i> 40	agcaatcaactctgcttaca		
<i>sst1b</i> 41	gctgaacagttttctggac		
<i>sst1b</i> 42	gagtcattctaagttttgca		
<i>sst1b</i> 43	tttggtgcacagaaaaacccc		
<i>sst1b</i> 44	cactgttttttagggacaca		
<i>sst1b</i> 45	ttaggaacttctctccgtg		
<i>sst1b</i> 46	aatgcatttgactgagtgga		
<i>sst1b</i> 47	acgtttaggttttatgcagc		
<i>sst1b</i> 48	gctggaagaatgtttgtcgt		

Probe labelling (adapted from Gáspár, 2018)

Synthesis of labeled ddUTP:

- Dissolve 1 µmol Amino-11-ddUTPs in 50 µl ddH₂O (20 mM stock)
- Transfer Amino-11-ddUTPs into 1.5 ml tube
- Add 1 M NaHCO₃ pH 8.4 (final conc. 0.1 M in reaction mixture)
- Reconstitution of dye-NHS ester with anhydrous DMSO (final conc. 40 mM)

- Use reconstituted ester immediately for reaction with NH₂-ddUTP/NaHCO₃
- Incubate reaction mixture for 2-3 h at room temperature isolated from light
- Adjust mixture with nuclease-free ddH₂O to a final concentration of 5 mM

Labelled Oligonucleotides

- Prepare a 250 μ M mixture of all oligos targeting the same gene, as well as two mixtures dividing them into two mixtures with even and odd oligos

Oligo mix (250 μ M)	4.0 μ l
Dye-ddUTPs (5 mM)	1.0 μ l
5X TdT buffer	3.0 μ l
TdT enzyme (20 U/ μ l)	1.0 μ l
ddH ₂ O	6.0 μ l
total	15.0 μ l

Incubate at 37°C (lid of PCR machine at 37°C as well) for 16-18h (no more than that!)

Purification and analysis of labeled oligos

- Add 60 μ l 1 M NaAcetate (pH=5.5, 300 mM final concentration) and 125 μ l ddH₂O to probe
- transfer everything to 1.5 ml tube and add 800 μ l 100% EtOH (prechilled at -20°C)
- invert tube several times and incubate at -20°C for 15-20 minutes
- centrifuge for 20 minutes ($\sim$ 16,000g, 4°C)
- remove supernatant, wash with 1 ml of 80 % EtOH (prechilled at 4°C)
- vortex pellet until it dissociates from the bottom of the tube
- centrifuge for 5 minutes ($\sim$ 16,000g, 4°C)
- wash pellet (should dissociate from tubewall) with 1 ml 80 % EtOH
- transfer everything to new tube and repeat washing steps two more times
- remove as much EtOH as possible
- air-dry the pellet, resuspend in 50 μ l ddH₂O
- measure OD @ 260 nm and at the absorption maximum of the dye (e.g. at 633 nm for Atto633)
- calculate the average extinction coefficient (ϵ (oligos) in M⁻¹cm⁻¹) of the used oligos (can be found on the datasheet)
- the extinction coefficient of the dye (ϵ (dye)) and the relative absorbance at 260 nm of the dye (CF260) can be found on the data sheet of the dye
- calculate the concentration of the labeled oligos:

$$c = \frac{OD_{260(labeled)} - CF_{260(dye)} * OD_{dye(labeled)}}{\epsilon_{(oligos)} + 9000}$$

$$DOL = \frac{OD_{dye(labeled)} / \epsilon_{(dye)}}{c}$$

$$R\% = \frac{c * V}{n_{initial}} * 100$$

With V being the resuspension volume of the labeled oligos, $n_{initial}$ is the amount of oligos added to the labeling reaction (e.g. 1000 pmol). C = concentration, DOL = Degree of labelling, R% = rate of recovery. Ideally, the values should be $0.9 \leq DOL \leq 1.0$, with a R% near 100%.

Cryo sample preparation: (adapted from Stapel, 2018; Veedin-Rajan, 2013; Tomer R, 2010)

Fish used were medaka juveniles (Cab) at 26dpf

- Sacrifice fish
- Fix fish 4% PFA/PTW for approx. 4 hours on RT (room temperature)
- Put fish into 30% sucrose/PBS on 4°C, shaking
- Wait until they sink down (if they don't, incubation overnight should suffice)
- Take cryomold (Tissue-Tek), fill with O.C.T (Tissue-Tek), put fish in and orient them (can do as many as fit/want to slice at the same time)
- Put cryomold into isopentane standing in dry ice. Should freeze quickly. Molds can be stored at -80°C or continue on to slicing immediately
- Cryosection larvae at 16µm (or 20µm), transfer on to glass slide (Superfrost Microscope Slide, Thermo Scientific) and put the slices on -80°C overnight

Note for following protocol: make sure to not shake samples during washing steps and move everything carefully. Slices like to abandon their glass slides to vanish.

smFISH protocol adapted from Staple, 2018

Washes were done in a glass staining dish. Liquids were poured in and out carefully to not disturb slices.

Solutions:

Wash buffer: 10% Formamide, 2x SSC

Hybridization buffer: 10% dextran sulfate, 10 % Formamide, 1mg/ml torula yeast, 2x SSC, 0.02% BSA, 2mM Ribonucleoside Vanadyl Complex (NEB)

Hybridization buffer with probe: 100µl Hyb + 0.7µl 25µM probe

Procedure:

- Put slices immediately from -80°C into 4%PFA/PBS for 5' on RT
- Rinse 2x with PBS
- Rinse with icecold 70% EtOH
- Put into fresh 70% EtOH on 4°C for 4-8 hours

- Put into 2x SSC for 10'
- Exchange for 5µg/ml Proteinase K in 2x SSC for 10'
- Then 2x 5' 2xSSC
- Rinse with wash buffer
- Put into fresh wash buffer for 5' (for equilibration)
- Coat lid/box with parafilm, add ~1ml Hybridization buffer with probe per glass slide
- Put glass slide face down, 30°C without shaking, 14-16 hours
- Carefully lift slides off (pipette wash buffer to the corners of the slides so they get off more easily)
- Rinse with washbuffer
- Fresh washbuffer for 30' on 30°C
- Exchange for washbuffer with Hoechst for 30' on 30°C
- Put mounting medium (Vectashield) on slices, leave it for a bit and then cover with coverglass

Supplementary data

Developmental effects of the mutation of various TMT-Opsins in medaka

tmt-opsin2 homozygous
Stage 10

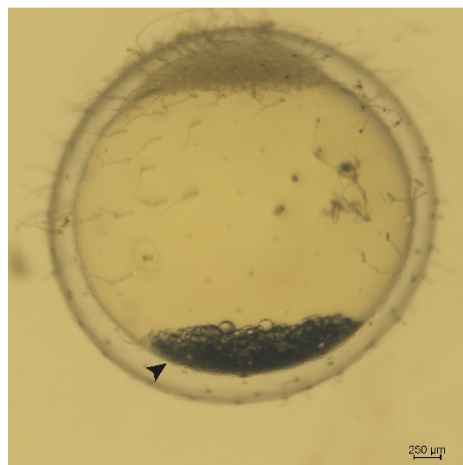


Figure 17 - *tmt-opsin2* homozygous mutant at approx. Stage 10. Developmentally, the egg is okay, showing a thick blastoderm with smaller inner cells. The droplets, however, are small and many, instead of the bigger few droplets that can usually be observed

Visualizing transcripts with more sensitive methods

Table 7 - Metrics used to calculate the efficiency of the labelled oligos to generate probes for smFISH

Atto633		
Cf260	0,04	
$\epsilon_{\max}$	130000	$M^{-1} cm^{-1}$
Atto550		
Cf260	0,23	
$\epsilon_{\max}$	120000	$M^{-1} cm^{-1}$
Atto532		
Cf260	0,2	
$\epsilon_{\max}$	115000	$M^{-1} cm^{-1}$
Atto488		
Cf260	0,22	
$\epsilon_{\max}$	90000	$M^{-1} cm^{-1}$

Table 8 - Example for labelling of oligos for scn12aa with different fluorophores

Gene	Oligo	c	DOL	R%
scn12aa 633nm	all	1,55E-05	0,88	77
	even	1,47E-05	0,89	74
	odd	9,81E-06	0,85	49
scn12aa 550nm	all	1,08E-05	2,04	54
	even	7,07E-06	0,27	35
	odd	1,40E-05	2,42	70
scn12aa 532nm	all	1,82E-05	2,4	91
	even	3,34E-06	2,42	17
	odd	1,46E-05	2,43	73
scn12aa 488nm	all	1,76E-05	3.61	88
	even	1,99E-05	2.71	100
	odd	2,08E-05	2.44	104

Tmt-opsin1b in zebrafish

+/+ = *tmt-opsin1b* wildtype

-/- = *tmt-opsin1b* homozygous

n = 6

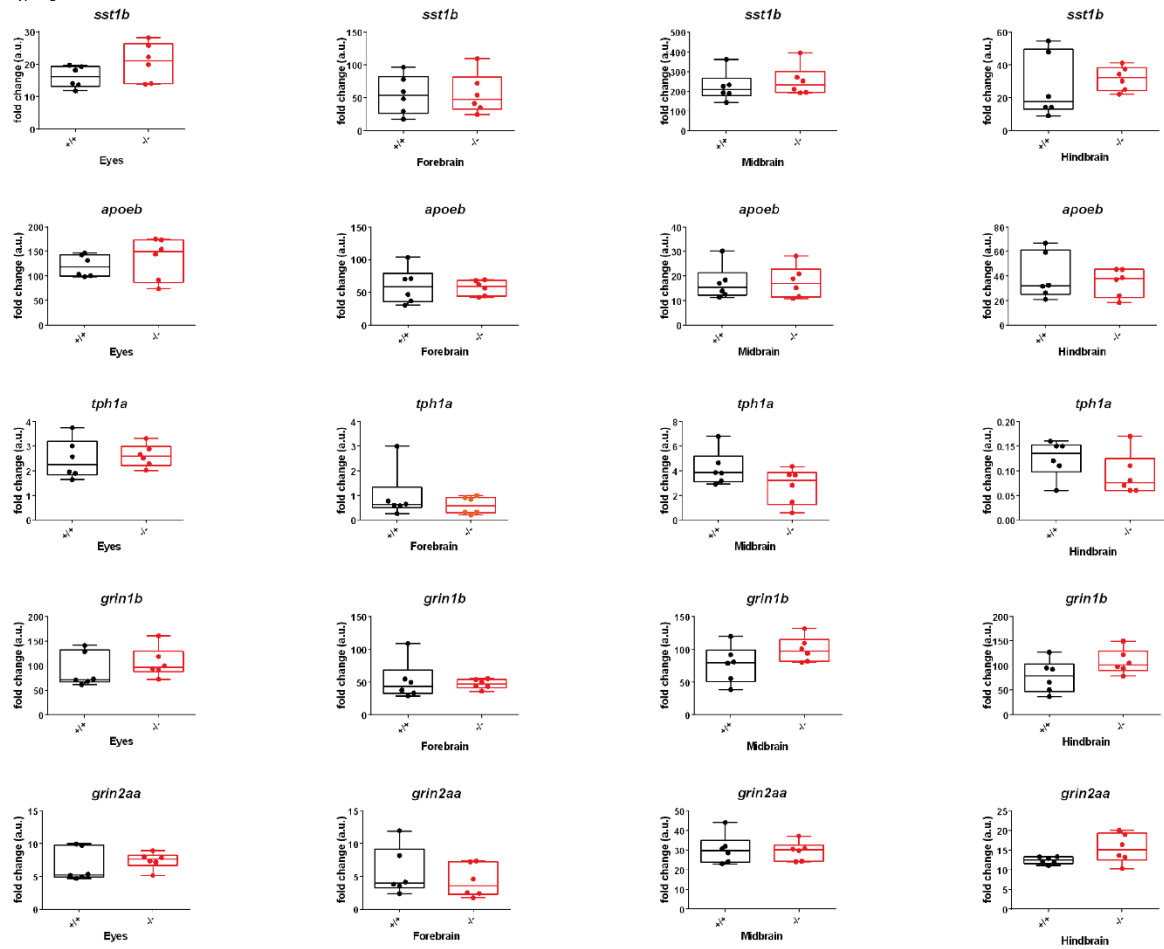


Figure 18 - Full figure showing the qPCR results of transcripts in Zebrafish wildtype and *tmt-opsin1b* homozygous mutants

Table 9 - p-values showing no significance in regulation of transcripts in *tmt-opsin1b* mutants

	p-value			
	Eye	Forebrain	Midbrain	Hindbrain
<i>sst1b</i>	0.1410	0.9453	0.5351	0.5820
<i>apoEb</i>	0.4651	0.8283	0.9212	0.6055
<i>tph1a</i>	0.7127	0.4106	0.1114	0.1804
<i>grin1b</i>	0.4194	0.6831	0.1503	0.1112
<i>grin2aa</i>	0.5211	0.4756	0.8012	0.1058

Table of Figures

<i>Figure 1</i>	7
<i>Figure 2</i>	8
<i>Figure 3</i>	9
<i>Figure 4</i>	13
<i>Figure 5</i>	17
<i>Figure 6</i>	19
<i>Figure 7</i>	21
<i>Figure 8</i>	21
<i>Figure 9</i>	22
<i>Figure 10</i>	23
<i>Figure 11</i>	24
<i>Figure 12</i>	25
<i>Figure 13</i>	25
<i>Figure 14</i>	29
<i>Figure 15</i>	30
<i>Figure 16</i>	31
<i>Figure 17</i>	49
<i>Figure 18</i>	51

Table of Tables

<i>Table 1</i>	16
<i>Table 2</i>	19
<i>Table 3</i>	27
<i>Table 4</i>	30
<i>Table 5</i>	38
<i>Table 6</i>	44
<i>Table 7</i>	50
<i>Table 8</i>	50
<i>Table 9</i>	51

References

- Aida, T. (1921). On the Inheritance of Color in a Fresh-Water Fish, *APLOCHEILUS LATIPES* Temmick and Schlegel, with Special Reference to Sex-Linked Inheritance. *Genetics*, 6(6), 554-573.
- Bellingham, J., Whitmore D., Philp A.R., Wells D.J., Foster R.G. (2002). Zebrafish Melanopsin: Isolation, tissue localisation and phylogenetic position. *Molecular Brain Research*, 107(2), 128-136. doi:[https://doi.org/10.1016/S0169-328X\(02\)00454-0](https://doi.org/10.1016/S0169-328X(02)00454-0)
- Blackshaw, S, Snyder, SH. (1999). Encephalopsin: a novel mammalian extraretinal opsin discretely localized in the brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 19(10), 3681–3690. doi:[doi:10.1523/JNEUROSCI.19-10-03681.1999](https://doi.org/10.1523/JNEUROSCI.19-10-03681.1999)
- BMBWF. (2020). *Tierversuchsstistik 2019*. Vienna: Austrian Federal Ministry of Education, Science and Research. Retrieved July 27, 2020, from [https://www.bmbwf.gv.at/Themen/Forschung/Forschung-in-%C3%96sterreich/Services/Tierversuche/Tierversuchsstatik.html](https://www.bmbwf.gv.at/Themen/Forschung/Forschung-in-%C3%96sterreich/Services/Tierversuche/Tierversuchsstistik.html)
- Cheng, K. P., Kiernan, E. A., Eliceiri, K. W., Williams, J. C., & Watters, J. J. (2016). Blue Light Modulates Murine Microglial Gene Expression in the Absence of Optogenetic Protein Expression. *Scientific reports*, 6, 21172. doi:<https://doi.org/10.1038/srep211>
- Davidson, A. E., Balciunas, D., Mohn, D., Shaffer, J., Hermanson, S., Sivasubbu, S., Cliff, M. P., Hackett, P. B., & Ekker, S. C. (2003). Efficient gene delivery and gene expression in zebrafish using the Sleeping Beauty transposon. *Developmental biology*, 263(2), 191–202. doi:<https://doi.org/10.1016/j.ydbio.2003.07.013>
- Davies, W. I., Tamai, T. K., Zheng, L., Fu, J. K., Rihel, J., Foster, R. G., Whitmore, D., & Hankins, M. W. (2015). An extended family of novel vertebrate photopigments is widely expressed and displays a diversity of function. *Genome Research*, 25(11), 1666–1679. doi:[doi:10.1101/gr.189886.115](https://doi.org/10.1101/gr.189886.115)
- Fischer, R. M., Fontinha, B. M., Kirchmaier, S., Steger, J., Bloch, S., Inoue, D., Panda, S., Rumpel, S., & Tessmar-Raible, K. (2013). Co-Expression of VAL- and TMT-Opsins Uncovers Ancient Photosensory Interneurons and Motorneurons in the Vertebrate Brain. *PLoS biology*, 11(6). doi:[doi:10.1371/journal.pbio.1001585](https://doi.org/10.1371/journal.pbio.1001585)
- Fontinha, B.M., Zekoll, T., Gallach, M., Reithofer, F., Barker, A.J., Hofbauer, M., Fischer, R.M., von Haeseler, A., Baier, H. and Tessmar-Raible, K. (2019). TMT-Opsins differentially modulate medaka brain function in a. *bioRxiv*. doi:<https://doi.org/10.1101/698480>
- Fontinha, B.M., Zekoll, T., Al-Rawi, M., Gallach, M., Reithofer, F., Barker, A.J., Hofbauer, M., Fischer, R.M., von Haeseler, A., Baier, H., Tessmar-Raible, K. (2020). TMT-Opsins differentially modulate medaka brain function in a context-dependent manner. *revised version of bioarxiv publication*.
- Frøland Steindal, I. A., & Whitmore, D. (2019). Circadian Clocks in Fish—What Have We Learned so far? *Biology*, 8(1), 17. doi:<https://doi.org/10.3390/biology8010017>
- Furutani-Seiki, M., & Wittbrodt, J. (2004). Medaka and zebrafish, an evolutionary twin study. *Mechanisms of development*, 121(7-8), 629-637. doi:<https://doi.org/10.1016/j.mod.2004.05.010>

- Gaspar, I., Wippich, F., & Ephrussi, A. (2017). Enzymatic production of single-molecule FISH and RNA capture probes. *RNA (New York, N.Y.)*, 23(10), 1582–1591. doi:<https://doi.org/10.1261/rna.061184.117>
- Gáspár, I., Wippich, F. and Ephrussi, A. (2018). Terminal Deoxynucleotidyl Transferase Mediated Production of Labeled Probes for Single-molecule FISH or RNA Capture. *Bio-protocol*, 8(5), e2750. doi:DOI: 10.21769/BioProtoc.2750
- Gilbert, S. F. (2012). Ecological developmental biology: environmental signals for normal. *Evolution & Development*, 14(1), 20-28.
- Hashiguchi, Y., & Nishida, M. (2006). Evolution and origin of vomeronasal-type odorant receptor gene repertoire in fishes. *BMC evolutionary biology*, 6(76). doi:<https://doi.org/10.1186/1471-2148-6-76>
- Iwamatsu, T. (2004). Stages of normal development in the medaka *Oryzias latipes*. *Mechanisms of development*, 121(7-8), pp. 605-618. doi:<https://doi.org/10.1016/j.mod.2004.03.012>
- Iwamatsu, T., Muramatsu, T. & Kobayashi, H. (2008). Oil droplets and yolk spheres during development of Medaka embryos. *Ichthyological Research*, 55, 344-348. doi:<https://doi.org/10.1007/s10228-008-0048-z>
- Jobling, A. I., Waugh, M., Vessey, K. A., Phipps, J. A., Trogrlic, L., Greferath, U., Mills, S. A., Tan, Z. L., Ward, M. M., & Fletcher, E. L. (2018). The Role of the Microglial Cx3cr1 Pathway in the Postnatal Maturation of Retinal Photoreceptors. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 38(20), 4708–4723. doi:<https://doi.org/10.1523/JNEUROSCI.2368-17.2018>
- Jürgens, G., Wieschaus E., Nüsslein-Volhard C., Kluding H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. *Roux's Archives of Developmental Biology*, 193, 283–295. doi:<https://doi.org/10.1007/BF00848157>
- Kawakami, K. (2007). Tol2: a versatile gene transfer vector in vertebrates. *Genome Biology*, 8(S7). doi:<https://doi.org/10.1186/gb-2007-8-s1-s7>
- Kinoshita M, Murata K, Naruse K, Tanaka M. (2009). *Medaka: Biology, Management, and Experimental Protocols*. Wiley-Blackwell.
- Koger, C. S., Teh, S. J., & Hinton, D. E. (1999). Variations of light and temperature regimes and resulting effects on reproductive parameters in medaka (*Oryzias latipes*). *Biology of reproduction*, 61(5), 1287–1293. doi:<https://doi.org/10.1095/biolreprod61.5.1287>
- Labun, K., Montague, T. G., Krause, M., Torres Cleuren, Y. N., Tjeldnes, H., & Valen, E. (2019). CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Research*, 47(W1), W171–W174. doi:<https://doi.org/10.1093/nar/gkz365>
- Meyers, J. R. (2018). Zebrafish: Development of a vertebrate model. *Current Protocols Essential Laboratory Techniques*. doi:doi: 10.1002/cpet.19
- Moutsaki, P., Whitmore D., Bellingham J., Sakamoto K., David-Gray Z.K., Foster R.G. (2003). Teleost multiple tissue (tmt) opsin: a candidate photopigment regulating the peripheral clocks of zebrafish. *Molecular Brain Research*, 112(1-2), 135-145. doi:[https://doi.org/10.1016/S0169-328X\(03\)00059-7](https://doi.org/10.1016/S0169-328X(03)00059-7)

- Nakayama, T., Shimmura, T., Shinomiya, A., Okimura, K., Takehana, Y., Furukawa, Y., Shimo, T., Senga, T., Nakatsukasa, M., Nishimura, T., Tanaka, M., Okubo, K., Kamei, Y., Naruse, K., & Yoshimura, T.. (2019). Seasonal regulation of the lncRNA LDAIR modulates self-protective behaviours during the breeding season. *Nature ecology & evolution*, 3(5), 845–852. doi:https://doi.org/10.1038/s41559-019-0866-6
- Nüsslein-Volhard, C., Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature*, 287, 795-801. doi:https://doi.org/10.1038/287795a0
- Nüsslein-Volhard, C., Wieschaus, E., Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster* I: zygotic loci on the second chromosome. *Roux's Archives of Developmental Biology*, 193, 267-282. doi:https://doi.org/10.1007/BF00848156
- Ogawa, S., Ramadasan, P. N., Goschorska, M., Anantharajah, A., Ng, K. W., & Parhar, I. S. (2012). Cloning and expression of tachykinins and their association with kisspeptins in the brains of zebrafish. *The Journal of comparative neurology*, 520(13), 2991–3012. doi:https://doi.org/10.1002/cne.23103
- Ozato, K., Kondoh, H., Inohara, H., Iwamatsu, T., Wakamatsu, Y., & Okada, T. S. (1986). Production of transgenic fish: introduction and expression of chicken delta-crystallin gene in medaka embryos. *Cell differentiation*, 4, pp. 237-244. doi:https://doi.org/10.1016/0045-6039(86)90100-4
- Porter, M. L., Blasic, J. R., Bok, M. J., Cameron, E. G., Pringle, T., Cronin, T. W., & Robinson, P. R. (2012). Shedding new light on opsin evolution. *Proceedings. Biological sciences*, 279(1726), 3-14. doi:https://doi.org/10.1098/rspb.2011.1819
- Provencio, I, Jiang, G, De Grip WJ, Hayes WP, Rollag MD (1998). Melanopsin: An opsin in melanophores, brain, and eye. *Proceedings of the National Academy of Sciences of the United States of America*, 95(1), 340-345. doi:https://doi.org/10.1073/pnas.95.1.340
- Raj, B., Wagner, D. E., McKenna, A., Pandey, S., Klein, A. M., Shendure, J., Gagnon, J. A., & Schier, A. F. (2018). Simultaneous single-cell profiling of lineages and cell types in the vertebrate brain. *Nature biotechnology*, 36(5), 442–450. doi:https://doi.org/10.1038/nbt.4103
- Sakai, K., Yamashita, T., Imamoto, Y., & Shichida, Y. (2015). Diversity of Active States in TMT Opsins. *PloS one*, 10(10). doi:https://doi.org/10.1371/journal.pone.0141238
- Shichida Y, Matsuyama T. (2009). Evolution of opsins and phototransduction. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 364(1531), 2881–2895. doi:https://doi.org/10.1098/rstb.2009.0051
- Shima, A. & Mitani, H. (2004). Medaka as a research organism: past, present and future. *Mechanisms of development*, 121(7-8), 599–604. doi:https://doi.org/10.1016/j.mod.2004.03.011
- Shima, A. & Shimada, A. (1991). Development of a possible nonmammalian test system for radiation-induced germ-cell mutagenesis using a fish, the Japanese medaka (*Oryzias latipes*). 6, 2545–2549. doi:https://doi.org/10.1073/pnas.88.6.2545
- Souren, M., Martinez-Morales, J. R., Makri, P., Wittbrodt, B., & Wittbrodt, J. (2009). A global survey identifies novel upstream components of the *Ath5* neurogenic network. *Genome biology*, 10(9). doi:https://doi.org/10.1186/gb-2009-10-9-r92

- Stapel, L. C., Broaddus, C., & Vastenhouw, N. L. (2018). Detection and Automated Analysis of Single Transcripts at Subcellular Resolution in Zebrafish Embryos. *Methods in molecular biology (Clifton, N.J.)*, 1649, 143–162. doi:https://doi.org/10.1007/978-1-4939-7213-5_9
- Tamai, T. K., Young, L. C., & Whitmore, D. (2007). Light signaling to the zebrafish circadian clock by Cryptochrome 1a. *Proceedings of the National Academy of Sciences of the United States of America*, 104(37), 14712–14717. doi:<https://doi.org/10.1073/pnas.0704588104>
- Terakita, A. (2005). The opsins. *Genome Biol*, 6(213). doi:<https://doi.org/10.1186/gb-2005-6-3-213>
- Tomer R, Denes AS, Tessmar-Raible K, Arendt D. (2010). Profiling by image registration reveals common origin of annelid mushroom bodies and vertebrate pallium. *Cell*, 142(5), 800-809. doi:[doi:10.1016/j.cell.2010.07.043](https://doi.org/10.1016/j.cell.2010.07.043)
- Veedin-Rajan, V. B., Fischer, R. M., Raible, F., & Tessmar-Raible, K. (2013). Conditional and specific cell ablation in the marine annelid *Platynereis dumerilii*. *PloS one*, 8(9), e75811. doi:<https://doi.org/10.1371/journal.pone.0075811>
- Wagle, M., Mathur, P., & Guo, S. Corticotropin-releasing factor critical for zebrafish camouflage behavior is regulated by light and sensitive to ethanol. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 31(1), 214–224. doi:<https://doi.org/10.1523/JNEUROSCI.3339-10.2011>
- Wakamatsu, Y., Pristiyazhnyuk, S., Kinoshita, M., Tanaka, M., & Ozato, K. (2001). The see-through medaka: a fish model that is transparent throughout life. *Proceedings of the National Academy of Sciences of the United States of America*, 98(18), 10046–10050. doi:<https://doi.org/10.1073/pnas.181204298>
- Whitmore, D., Foulkes, N., Sassone-Corsi, P. (2000). Light acts directly on organs and cells in culture to set the vertebrate circadian clock. *Nature*, 404, 87-91. doi:<https://doi.org/10.1038/35003589>
- Wieschaus, E., Nüsslein-Volhard C., Jürgens G. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster* III: Zygotic loci on the X-chromosome and fourth chromosome. *Roux's Archives of Developmental Biology*, 193, 296-308. doi:<https://doi.org/10.1007/BF00848158>
- Wittbrodt, J., Shima, A., & Schartl, M. (2002). Medaka--a model organism from the far East. *Nature reviews. Genetics*, 3(1), 53-64. doi:<https://doi.org/10.1038/nrg704>
- Yamamoto, T. (1975). *Medaka (killifish): Biology and strains*. Tokyo: Yugaku-sha. .
- Yoda, H., Hirose, Y., Yasuoka, A., Sasado, T., Morinaga, C., Deguchi, T., Henrich, T., Iwanami, N., Watanabe, T., Osakada, M., Kunitatsu, S., Wittbrodt, J., Suwa, H., Niwa, K., Okamoto, Y., Yamanaka, T., Kondoh, H., & Furutani-Seiki, M. (2004). Mutations affecting retinotectal axonal pathfinding in Medaka, *Oryzias latipes*. *Mechanisms of development*, 121(7-8), 715-728. doi:<https://doi.org/10.1016/j.mod.2004.03.021>

Manuscript

Revised manuscript of Fontinha, 2019 (original version published 2019 on *bioRxiv*, doi: <https://doi.org/10.1101/698480>) submitted to *PLoS Biology*.

Date of the acknowledgement of receipt: 02.08.2020

Date of confirmation of being added as an author: 05.08.2020

TMT-Opsins differentially modulate medaka brain function in a context-dependent manner

Bruno M. Fontinha^{1,2#}, Theresa Zekoll^{1,2#}, Mariam Al-Rawi^{1,2}, Miguel Gallach³⁺, Florian Reithofer^{1,2^+}, Alison J. Barker⁵, Maximilian Hofbauer^{1,2,6}, Ruth M. Fischer^{1^^}, Arndt von Haeseler^{1,2,3,4}, Herwig Baier⁵ and Kristin Tessmar-Raible^{1,2,7*}

#,+ equal contribution

1 Max F. Perutz Laboratories, University of Vienna, Campus Vienna Biocenter, Dr. Bohr-Gasse 9/4, 1030 Vienna, Austria

2 Research Platform "Rhythms of Life," University of Vienna, 1030 Vienna, Austria

3 Center for Integrative Bioinformatics Vienna, Max F. Perutz Laboratories, University of Vienna and Medical University of Vienna, Dr. Bohr-Gasse 9, 1030 Vienna, Austria

4 Bioinformatics and Computational Biology, Faculty of Computer Science, University of Vienna, Vienna, Austria

5 Max Planck Institute of Neurobiology, Martinsried, Germany

6 loopbio GmbH, Wien, Austria

7 FENS-Kavli network of Excellence

present address:

^ IMC Fachhochschule Krems, University of Applied Sciences, Krems, Austria

^^ Boehringer Ingelheim RCV GmbH & Co KG, Vienna, Austria

Contributions to the manuscript of this author:

Cloning of *gad2* and *in situ* hybridization of *gad2* and *sst1b* were performed. Contributions to Supplementary Figure 2 and 7.

TMT-Opsins differentially modulate medaka brain function in a context-dependent manner

Bruno M. Fontinha^{1,2#}, Theresa Zekoll^{1,2#}, Mariam Al-Rawi^{1,2}, Miguel Gallach³⁺, Florian Reithofer^{1,2^+}, Alison J. Barker⁵, Maximilian Hofbauer^{1,2,6}, Ruth M. Fischer^{1^^}, Arndt von Haeseler^{1,2,3,4}, Herwig Baier⁵ and Kristin Tessmar-Raible^{1,2,7*}

^{#,+} equal contribution

¹ Max F. Perutz Laboratories, University of Vienna, Campus Vienna Biocenter, Dr. Bohr-Gasse 9/4, 1030 Vienna, Austria

² Research Platform “Rhythms of Life,” University of Vienna, 1030 Vienna, Austria

³ Center for Integrative Bioinformatics Vienna, Max F. Perutz Laboratories, University of Vienna and Medical University of Vienna, Dr. Bohr-Gasse 9, 1030 Vienna, Austria

⁴ Bioinformatics and Computational Biology, Faculty of Computer Science, University of Vienna, Vienna, Austria

⁵ Max Planck Institute of Neurobiology, Martinsried, Germany

⁶ loopbio GmbH, Wien, Austria

⁷ FENS-Kavli network of Excellence

present address:

[^] IMC Fachhochschule Krems, University of Applied Sciences, Krems, Austria

^{^^} Boehringer Ingelheim RCV GmbH & Co KG, Vienna, Austria

Contact Information

*kristin.tessmar@mfpl.ac.at

Keywords: non-visual opsins, behavior, modulation, transcriptome, teleosts

Abstract

Vertebrate behavior is strongly influenced by light. Light receptors, encoded by functional Opsin proteins, are present inside the vertebrate brain and peripheral tissues. This expression feature is present from fishes to human and appears to be particularly prominent in diurnal vertebrates. Despite their conserved widespread occurrence, the non-visual functions of Opsins are still largely enigmatic. This is even more apparent when considering the high number of Opsins. Teleosts possess around 40 Opsin genes, present from young developmental stages to adulthood. Many of these Opsins have been shown to function as light receptors. This raises the question, if this large number might mainly reflect functional redundancy or rather maximally enables teleosts to optimally use the complex light information present under water.

We focus on *tmt-opsin1b* and *tmt-opsin2*, c-Opsins with ancestral-type sequence features, conserved across several vertebrate phyla, expressed with partly similar expression in non-rod, non-cone, non-RGCs brain tissues and a similar spectral sensitivity.

The characterization of the single mutants revealed age- and light-dependent behavioral changes, as well as an impact on the levels of the preprohormone *sst1b* and the voltage-gated sodium channel subunit *scn12aa*. The amount of day-time rest is affected independently of eyes, pineal and the circadian clock in *tmt-opsin1b* mutants. We further focused on day-time behavior and the molecular changes in *tmt-opsin1b/2* double mutants, and revealed that – despite their similar expression and spectral features– these Opsins interact in part non-additively. Specifically, double mutants complement molecular and (age-dependently) behavioral phenotypes observed in single mutants.

Our work provides a starting point to disentangle the highly complex interactions of vertebrate non-visual Opsins, suggesting that *tmt-opsin*-expressing cells together with other visual and non-visual Opsins provide detailed light information to the organism for behavioral fine-tuning. This work also provides a stepping stone to unravel how vertebrate species with conserved Opsins, but in different ecological niches respond to similar light cues and how human generated artificial light might impact on behavioral processes in natural environments.

Introduction

Organisms are exposed to a large range of light intensities and spectral changes. While humans are well aware of the visual inputs from their environments, the range of intensity and spectral changes that animals, including humans, naturally undergo are less consciously experienced. Across the day, light intensity routinely differs by several orders of magnitude depending on the angle of the sun above the horizon and weather conditions (e.g. a 10^3 -fold difference occurs between a sunny day and the moments before a thunderstorm, which is a similar difference between a sunny day and the average office illumination)[1].

While vision evolved multiple mechanisms to compensate for these differences, allowing us to see almost equally well across about 10^6 orders of magnitude of light intensity [1], light differences have wider effects on physiology and behavior than solely impacting vision. This has been well documented for vertebrates, including mammals [2]. It prominently affects human (and other mammalian) physiology, mood and cognitive function via the entrainment of the circadian clock [3, 4]. In mouse it was shown that in addition to the circadian clock setting, light information conveyed via the perihabenular nucleus also directly affects brain regions that control mood [5], while moderate exposure to UVB light promotes the biosynthesis of glutamate and results in enhanced learning and memory [6]. Furthermore, photoperiodic effects on mammalian mood, cognition and fear behavior are well documented, which are for example connected with changes in transcript levels of *tyrosine hydroxylase* and *pre-prosomatostatin1* [7].

These far-reaching impacts of light on behavior and physiology are not just documented for mammals, but spanning across vertebrates. Seasonality, and in particular photoperiod, controls major hormonal changes connected to breeding, molt, and song production in birds (reviewed in [8]), while light modulates motor behavior independently of the eyes in frogs [9] and teleosts [10].

The past years revealed that Opsins, the proteins mediating light sensation in vertebrates, are present in various cell types inside and outside the vertebrate eyes, including specific brain neurons [11-14]. Zebrafish possess 42 *opsin* genes [15], and additional genomic and transcriptomic data suggest that *opsin* gene numbers are similarly high in other teleost species (<http://www.ensembl.org>).

Biochemical analyses, tissue culture assays and electrophysiological recordings on brain tissue suggest that most, if not all, of these Opsins can function as light receptors [14-18]. Light has been shown to reach deep brain regions of several mammals and birds [11, 19, 2]. Given the typically smaller sizes of fish brains, especially medaka and zebrafish, it is highly conceivable that light will reach cells inside the fish brain. Based on the expression of several Opsins in inter- and motoneurons in larval, as well as the adult zebra- and medaka fish brains we suggested that these Opsins could modulate information processing, depending on ambient light conditions [14].

Studies on young zebrafish larvae indeed implicate non-visual Opsins in specific light-dependent behaviors, such as the photomotor response present at 30 hours-post-fertilization (hpf) [20], for which however the exact identity of the Opsin(s) involved remained unclear. The suppression of spontaneous coiling behavior in larvae younger than 24 hpf is for its green light component mediated by VAL-opsinA [21], while melanopsins have indirectly been implicated to mediate part of the dark photokinesis response (in 5-7 days-post-fertilization (dpf) old larvae) [13]. The latter assay was further extended to investigate search pattern strategies of 6-7 dpf old larvae under extended periods of sudden darkness. In this assay Cas9/Crispr engineered *opn4a* mutants showed altered local search behavior [22]. Combinational analyses of enucleated wildtypes (wt) fish, *otpa* mutants and enucleated *otpa* mutants indicate a contribution of brain-expressed *opn4a* [22]. However, as there was no comparison between enucleated *opn4a* mutant and wt fish, the contribution of *opn4a* expressed in the eye versus the brain [23] remained unresolved.

While these studies have started to provide insights into the understanding of non-visual/deep brain Opsin functions, several important biological questions have remained entirely unaddressed.

First, non-visual Opsins are not just present during young larval stages, and their later functions remain unclear. How do the early functions compare to functions during later, juvenile stages, when the nervous system has further grown and differentiated?

Second, another line of evidence suggests that non-visual Opsins are involved in conveying light information for chronobiological functions, such as the circadian clocks autonomously present in different fish tissues [24, 25]. Outside the tropical/ sub-tropical zebrafish, non-visual Opsins have also been suggested to convey photoperiod information,

particularly to the reproductive system [26]. Is there evidence for such a function in a species for which seasonal cues are naturally relevant?

Third, one of the maybe most puzzling question concerns the number of Opsins in teleost fish. Why are there so many? Is it simply redundancy of the system or do these opsins control physiology and/or behavior in a more complex, possibly synergistic manner?

Here we focus on the functional characterization of two members of the Encephalopsin/TMT-Op sin (ETO) family, which is characterized by a particularly low rate of sequence changes over time and represents ancestral c- Opsins [14, 27]. One of its subfamilies (Encephalopsin) is conserved up to placental mammals [14, 28, 29]. We focused our analyses on medakafish, since these fish show a light-dependent seasonal breeding response as an adaptation to the photoperiod changes naturally occurring in their habitat [30, 31]. We decided to specifically focus on Tmt-opsin1b and Tmt-opsin2. Spectral analyses in cells and on purified proteins suggest that these Opsins have a similar wavelength preference, while also being expressed in close vicinity or even partly overlappingly ([14, 32] and this study), thus providing an interesting entry point for testing complementing vs. non-complementing functions. We generated medaka *tmt-opsin1b* and *tmt-opsin2* mutants, and analyzed mutant and sibling wildtype fish in multiple behavioral and molecular assays. Our functional work on even just two non-visual Opsins already indicates a complex, (at least in part) non-redundant light information processing system, which modulates a neurohormone and a voltage-gated sodium-channel subunit, as well as behavior. In addition, our work provides several examples how relatively small differences (in age and light intensity) manifests themselves in significant behavioral changes.

Results

***Ola-tmt-opsin1b* mutants exhibit light-dependent altered avoidance responses**

We started our investigation with medaka *tmt-opsin1b*, by generating several independent mutant alleles using TALEN technology (Fig. 1A). All three mutant alleles are predicted to result in an N-terminally truncated protein, prior to the first transmembrane domain (Supplementary Fig. 1A-D, G) and are thus likely functional null mutants. We confirmed the absence of functional protein with a previously generated antibody against *Ola-TMTopsin1b* [14] (Fig.1B). We next tested for noticeable developmental alterations by

detailed staging of the developing larvae according to morphological criteria [33]. We did not observe any difference between wt and homozygous mutant larvae (Supp. Fig 2A-C). We also crossed a strain expressing GFP under the control of an *ath5* enhancer fragment in the developing retinal ganglion cells [34, 35] with the *tmt-opsin1b*^{-/-} fish. The resulting heterozygous, GFP⁺ carriers were incrossed and their larval offspring analyzed for any deviations in the on-set of GFP expression (indicative of the timing of RGC differentiation), axonal outgrowth and connectivity to the tectum (indicative of RGC and tectal development). After hatching (7-9dpf) the fish were genotyped. As we again did not observe any differences between mutant and wildtype fish (n: wt=19 ; heterozygous mutant = 27, homozygous mutant = 18, Supp.Fig2D-G), we concluded that especially also eye and tectal development is unaffected by the *tmt-opsin1b* mutation.

Given that *Ola*-TMTopsin1b is expressed in the medaka optic tectum and reticular formation [14] (Fig. 1B, 4B,D), we started our functional analysis with an assay that requires these structures. It has been shown that the responses of frogs and fish to approaching stimuli of different sizes specifically require the tectum and reticular formation [36-40]. We adapted an assay in which the response of individual animals to displayed moving dots of different sizes (mimicking a range of potential prey and predator stimuli) is recorded and analyzed (here referred to as “avoidance assay”, Fig. 1C, Supplementary Videos 1 and 2). Such type of avoidance assays have been used for toads[36], larval *Xenopus*[37], mice[41], goldfish[42] and larval zebrafish[40]. Medaka larvae of different stages were collected in the morning (ZT2-5) and subjected to the avoidance assay. Newly hatched medaka larvae are immediately free-swimming and might best be compared to zebrafish larvae at about 7-8 dpf (constantly raised at 28°C), while later obvious developmental steps proceed similarly to zebrafish. Based on the responses of the larvae to the moving dots, we calculated an avoidance index (AI) for each dot-size: $AI = (\text{total number of avoidances}) - (\text{total number of approaches}) \text{ divided by } (\text{total number of dots presented})$. Each dot size was presented six times to each fish and care was taken to use different mutant alleles in the same trial (Supplementary Fig. 1H).

After blind response scoring and subsequent genotyping, we observed that *tmt-opsin1b* mutant fish exhibited a significantly elevated avoidance response compared to their wildtype siblings, particularly to dot sizes 20 degrees ($P = 0.0295$, Unpaired t test with

Welch's correction; $t = 2.3$; $df = 26.4$) and 30 degrees ($P = 0.0155$, Unpaired t-test; $t = 2.5$; $df = 40$), decreasing gradually towards bigger dots (Fig. 1D,E). Shuffling dot size order resulted in an overall shift of the response curve, presumably due to habituation of the fish to the larger dots that appeared earlier (compare Fig. 1D,F). Importantly, the elevated response of *tmt-opsin1b* mutants vs. wildtype siblings was maintained.

Given that different lab environments resulted in slightly different response curves of the mutant fish (Fig. 1D,E) and that TMT-Opsin1b should function as a light receptor in wildtype, we next tested if the observed elevated response in the avoidance assay would be altered by changes of light conditions. The difference of the avoidance responses present between wildtype and *tmt-opsin1b* mutants at 100% light intensity vanishes when the light intensity is reduced (Fig. 1G,H, $P = 0.0318$, Unpaired t-test; $t = 2.2$; $df = 40$, without changing the spectrum, Fig. 1I). Specifically, when we lowered the light intensity of the white background projected by the computer screen to 35% of the original light intensity, the mutant AI-curve and the wildtype AI-curve (compare Fig. 1E,G,H), became statistically indistinguishable ($P = 0.888$, Unpaired t-test; $t = 0.14$; $df = 22$). We also tested, if the observed changes in the behavioral responses observed between the different light intensity conditions might be due to contrast differences. For this, we analyzed the avoidance index of Cab wildtypes under different contrast conditions (Supplementary Fig. 1I) by testing the fish for their responses to dots of increasing luminance (keeping dot size constant at 20 degrees). 35% lower light levels at the computer screen correspond to a Michelson contrast of 0.93, a contrast that does not cause any observable behavioral differences compared to full light levels (Supplementary Fig. 1I), making a change of contrast an unlikely explanation for the observed drop in the avoidance index of *tmt-opsin1b* mutant fish at 35% ambient light intensity. These results suggest that *tmt-opsin1b* normally mediates constant responses, even under changing light conditions (for comparisons of the light spectra to natural light levels see Supplementary Figure 3).

***Ola-tmt-opsin1b* mutants exhibit strong age-dependent behavioral differences to wt in response to sudden light/dark changes**

The avoidance assay requires the tectum for proper responses, but it also requires normal functioning eyes. Like probably all Opsins, *tmt-opsin1b* is expressed in the eye, specifically in the amacrine layer [14]. We therefore next decided to use assays that do not necessarily

require eyes. Animals typically respond to changes in ambient illumination with rapid changes in movement[43]. Changes in motor behavior in response to light in blinded and pinealectomized minnows[10], eels [44] and lamprey tails [45] provided the first evidence for extraretinal, extrapineal “photomotor” behavior in vertebrates. Furthermore, in zebrafish larvae, short periods of sudden darkness result in an increased overall activity, which has been interpreted as ‘light-seeking behavior’ and termed ‘dark photokinesis’[13, 46]. Zebrafish larvae lacking the eyes and pineal organ still react to a sudden loss of illumination with an elevated locomotor activity and an undirected light-seeking behavior [13]. After a few minutes of continued darkness, zebrafish will subsequently decrease their amount of swimming, resulting in less distance moved during the remaining darkness time [13, 47].

We first evaluated the responses of free-swimming *tmt-opsin1b* mutant juveniles (20-22 dpf) to sudden light changes. Mutant vs. wildtype medaka were subjected to 30min each of white light vs. dark intervals (repeated three times, spectra: Supplementary Figure 3E, 4A), while the movement of the larvae was tracked automatically and evaluated using the Noldus EthoVision XT[®] software. Both *tmt-opsin1b* mutant and wildtype fish changed their swimming distance depending on the light / dark condition (Fig. 2A,B), and there was a significant difference between both during the dark phases (Fig. 2A,B). As there are more than 40 Opsins present in teleosts, we next wondered if this phenotype became stronger under light conditions that are more restricted to the maximal sensitivity of TMT-opsins. TMT-Opsins are functional photoreceptors with a maximal sensitivity in the blue light range (about 460nm [14, 32, 48, 49]). Thus, we next modified the assay by using monochromatic blue light (for spectra see Supplementary Fig. 3F, 4A). Under these light conditions *tmt-opsin1b* mutant fish exhibited a clear increase of activity during the light and dark phases of the assay compared to wt (Fig. 2C,D). Given this clear difference between white and blue light conditions, we next wondered if this response is specific to blue light. We assayed juvenile fish under lower intensity monochromatic blue, green and red light at as similar photon numbers as possible. Under these conditions we find that juvenile wt and *tmt-opsin1b*^{-/-} mutant fish assayed under blue light, but not under green or red light, exhibited statistically significant differences (Fig. 2E-G, H,J,L, for spectra see

Fig.2I,K,M, Supplementary Fig.3G-I). These results show that the response is specific and provides additional evidence for the blue light sensitivity of TMT-opsin1b.

Many behavioral and brain imaging experiments with genetically altered and/or transgenic zebrafish (as major functional vertebrate model system in neuroscience) are typically performed at early developmental stages when the fish have just started to swim freely (7dpf) or even earlier (e.g [50, 51]). As detailed in the introduction, also all analyses of non-visual photoreceptor responses in teleosts have so far only been performed at stages not older than 7dpf, and in most cases significantly younger [13, 20, 21] [22].

However, it is also obvious from multiple studies that significant improvements of sensory systems and behavior occur during subsequent development (e.g. [52, 53]).

We thus next tested how our results with juvenile fish compare to those at stages just post-hatching. The result was strikingly different. While there was also clearly a difference compared to wildtype, instead of swimming more, mutant young larvae (9-12 dpf) swam less than their wildtype siblings (Fig. 2N,O). This behavioral difference changed gradually during the days following post-hatching (Supplementary Fig. 4B-I), emphasizing that any molecular or cellular mechanism identified to control behavior has to be seen in connection with the developmental age of the tested organism.

Analyses of the “fast dark photokinesis” response rate (first two minutes after light cessation) revealed no difference in the amount of the response between *tmt-opsin1b* mutant and wildtype of either age, when the overall lowered or heightened mutant baseline level is accounted for (Supplementary Fig. 4J,K).

Finally, in order to test if the observed elevated locomotion levels are caused by generally altered stress or anxiety levels, we measured total cortisol levels.

We observed no difference between mutant and wildtype siblings at neither young larval nor juvenile stages (Fig.2P,Q), while our positive control, larvae exposed to mechanical disturbances (MD), showed a significant increase of cortisol levels (Fig.2P). This strongly suggests that the difference in locomotion apparent between mutants and wildtype is indeed mediated by the acute light changes involving *tmt-opsin1b*.

***Ola-tmt-opsin1b* mutants exhibit altered, partly eye-independent, daytime activity levels**

Given the relatively consistent changes over the entire period of the experiment, we next tested the free-swimming of *tmt-opsin1b* mutants across two consecutive days (blue light/dark, 16h:8h). *Tmt-opsin1b* homozygous mutant larvae and juvenile fish swam significantly more during the 1.5h period immediately after the lights went on (Fig. 3A-D). This difference was reduced or disappeared when the light conditions remained stable over the rest of the day (Fig. 3A,C).

Light is an important entrainment cue for the circadian oscillator. We thus next analyzed, if the mutation in *tmt-opsin1b* impacts on the phase or period length of the medaka circadian core clock. We selected two representative core circadian clock genes, *per1b* and *reverbB* [54], and tested their transcript oscillations in *tmt-opsin1b* mutant and wt larvae under light/dark (L/D) and constant dark (D/D) conditions. The timing of daily minima and maxima was indifferent between mutant and wt larvae, as was the overall cycling, clearly indicating that the *tmt-opsin1b* mutation has no impact on circadian phase or period length (Fig.3E,F).

Finally, we tested, if the increase in swimming upon sudden illumination would also occur in fish without eyes, by this testing for a possible functional contribution of *tmt-opsin1b* outside the eye (and pineal, as *tmt-opsin1b* is not expressed in the pineal [14]). Fish were enucleated and left to recover for 1 week before the trial onset. Importantly, the differences between *tmt-opsin1b* and their wildtype counterparts were still observable: while the trend was the same during both days, it reached statistical significance only during the second day (Fig. 3G,H). We thus conclude that *tmt-opsin1b*, at least in part, functions outside the eyes and modulates the responses of medaka behavior in response to different environmental light changes independently of the circadian clock.

***Ola-tmt-opsin1b* and *Ola-tmt-opsin2* mutants exhibit additive and non-additive responses to changes in environmental light**

Teleost, like zebrafish and medaka, possess more than 40 Opsins. Systematic analyses in zebrafish showed that most exhibit expression in the brain [15]. This raises the question if these opsins might just function redundantly or rather in a complex, non-redundant manner. We purposely started to approach this question by investigating an additional Opsin with similar characteristics as *Tmt-opsin1b*. Specifically, *Tmt-opsin2* is an evolutionarily conserved ETO relative of *Tmt-opsin1b* with highly similar spectral sensitivity and

absorbance characteristics [14, 32] and expression in adjacent, possibly partly overlapping, domains in mid- and hindbrain [14] (Fig. 4A-E).

We thus wondered, if mutating *tmt-opsin2* would reveal redundant or synergistic functions with *tmt-opsin1b*. Using TALE nucleases, we generated a large deletion in the *tmt-opsin2* gene (Fig. 4F), removing the first two transmembrane helices and resulting in a dysfunctional protein (Supplementary Fig. 1E-G). Larval and juvenile *tmt-opsin2* mutant and wildtype sibling fish were assessed for their responses during the light / dark phases across two days, as described above.

While under these conditions *tmt-opsin2*^{-/-} mutants do not display significant phenotypes, unexpectedly *tmt-opsin1b/tmt-opsin2* double homozygous mutants displayed phenotypes different from the *tmt-opsin1b*^{-/-} single mutants: while in larval stages adding the *tmt-opsin2* mutant to the *tmt-opsin1b* mutant resulted in a complementation of the *tmt-opsin1b* phenotype during the beginning of the light phases (Fig. 3A,B, Fig.4G,H, for individual, high temporal resolution graphs see Supplementary Figure 5A-J), the combination of these mutations lead to additive phenotypes in the same experimental test during juvenile stages (Fig. 3C,D, Fig. 4I,J, for individual, high temporal resolution graphs see Supplementary Figure 5A-J; compare position of purple bar graphs -also indicated by arrows- to the corresponding wt and single mutant graphs in Fig.4H and J).

These data suggests two main conclusions. First, the fact that the *tmt-opsin2* mutation can compensate for *tmt-opsin1b* loss shows that these photoreceptors do not function “just” redundantly. It suggests that light information received by the fish is fed into a complex light processing system and the output for behavior is not simply the sum of the input of all possible light receptors. Second, the functional interaction of the different opsin-based photoreceptors has a clear age-dependent component.

Mutations in *tmt-opsin1b* and *tmt-opsin2* induce transcriptional changes that impact on neuronal information transmission

We next wondered which molecular changes can be detected in the brain of the fish missing specific photoreceptors. It had previously been shown that differences in photoperiod cause changes on the transcript level in the rat brain, resulting in differences of neurotransmitter abundance [7]. We thus reasoned that quantitative RNA sequencing might be a possible

strategy to obtain an unbiased insight into the changes that occur due to lack of *tmt-opsin1b* or *tmt-opsin2* function.

We sampled three separate brain regions (eyes, forebrain and mid-/hindbrain, Fig. 5A) at ZT2-3 under a 16:8 hour white light/dark regime and sequenced stranded cDNA. It should be noted that for technical reasons part of the mid-/hindbrain sample includes a portion of the posterior hypothalamus, i.e. forebrain. The resulting sequences were mapped to the medaka genome (Ensembl version 96), reads mapping to annotated exons were quantified using edgeR (Bioconductor version 3.9) [55]. The result tables are deposited as Supp.Data 1,2. When comparing *tmt-opsin1b* and its wildtype siblings, one differentially regulated transcript caught our particular attention, the pre-pro-hormone *sst1b* (also named *sst3* or *cort*) in the mid-/hindbrain (Fig. 5B), a member of the somatostatin/corticotropin family. We next independently confirmed the quantitative RNAseq results using qPCR (Fig. 5C, black vs. red boxes for mid- and hindbrain). In this set of experiments, we separated the midbrain from the hindbrain tissue in order to obtain a more differentiated picture of the regulation. Given the differential effects of *tmt-opsin1b/tmt-opsin2* double mutants on behavior, we next wondered how the presence of both mutations would affect *sst1b* regulation. Adding the *tmt-opsin2* mutation compensated the downregulation of *sst1b* levels present in the *tmt-opsin1b* single mutant (Fig. 5C, purple boxes compared with others for mid- and hindbrain). In an analogous approach, we identified the voltage-gated sodium-channel subunit *scn12aa* as significantly regulated by the *tmt-opsin2* mutation (Fig. 5D). Again, while clearly visible in the single mutant (Fig. 5E, grey vs. blue boxes for mid- and hindbrain), the effect was compensated for in the *tmt-opsin1b/tmt-opsin2* double mutants (Fig. 5E, purple vs. blue boxes for mid- and hindbrain).

These molecular data further support the notion that the loss-of-function of non-visual Opsins does not necessarily lead to a simple summation effect originating from the single mutants. Again, this strongly corroborates the notion of non-redundant, complex light information processing, which can modulate the neuronal function on the level of neuropeptides (*tmt-opsin1b* regulating *sst1b*) and voltage-gated channels (*tmt-opsin2* regulating *scn12aa*).

The regulation of *sst1b* via *tmt-opsin1b* is non-cell autonomous

In order to gain deeper insight on the connection between *sst1b* expression changes in the mid-/hindbrain, *tmt-opsin1b* and responses to light, we next analyzed the expression of *sst1b*. In the midbrain, *sst1b* is expressed in several highly specific clusters of cells, none of which overlap with *tmt-opsin1b* (compare Figs. 6A and 4B, for detailed anatomical annotation of *sst1b* expression see Supplementary Figure 6, of *tmt-opsin1b* expression see [14]). This is particularly obvious for the tectum, in which *sst1b*⁺ cells are consistently located more dorsally within the *stratum periventriculare* (SPV) than *tmt-opsin1b*⁺ cells, and close to or possibly overlapping with *tmt-opsin2*⁺ cells (compare Fig.6A with Fig. 4B, anatomical annotation: [14], Supplementary Figure 6). Similar in the reticular formation of the hindbrain, likely all of the *sst1b*⁺ cells are separate from the *tmt-opsin1b*⁺ cells (Fig. 6B,4D, anatomical annotation: [14], Supplementary Figure 6). We thus conclude, that the changes of *sst1b* transcript levels are rather indirectly mediated by *tmt-opsin1b*⁺ cells upstream of *sst1b*-expressing cells.

We next analyzed where the regulation of *sst1b* transcripts occur. We performed *in situ* hybridizations on wildtype and *tmt-opsin1b* mutant adult fish brains for *sst1b* with specific attention to treating wildtype and mutant samples identically. We observed a specific reduction of *sst1b*⁺ transcript levels in the cells of the SPV layer in the optic tectum, the absence of *sst1b* staining in the dorsal periventricular hypothalamic zone surrounding the lateral recess and in cells medial to the nucleus glomerulosus in the medial preglomerular nucleus (arrows Fig. 6C). We also observed a reduction of *sst1b*⁺ cells in the intermediate reticular formation of the hindbrain (arrows Fig. 6D). In order to independently verify the specificity of the effects, we cloned *gad2*, whose ortholog is co-expressed with the zebrafish *sst1b* ortholog in neurons, based on scRNA analyses [56]. We confirmed co-expression of *sst1b* with *gad2* in medaka tectal neurons (Supplementary Figure 7), and also did not find any visible alteration in the expression of *gad2* in *tmt-opsin1b* mutants vs. wt brains (Supplementary Figure 7). Taken together, these *in situ* expression analyses confirm a likely specific effect of *tmt-opsin1b* on *sst1b* transcripts. They further suggest an indirect regulation of *sst1b* transcripts by *tmt-opsin1b*. We additionally identified that the tectal *sst1b*⁺ cells are GABA-ergic (by the expression of *gad2*), and hence likely convey inhibitory input to the tectal circuitry.

We likewise further analyzed *scn12aa* brain expression. *Medaka* *Scn12aa* is the common ortholog of the two separate amniote *Scn10a* and *Scn5a* groups (Supplementary Figure 8). Expression analyses of both mRNA (by *in situ* hybridization, Supplementary Figure 9A,B) and antibody staining (using a commercially available antibody with broad species reactivity generated against human *Scn5a*, Supplementary Figure 9C-H) suggest a broad, possibly ubiquitous brain expression. A specific set of neurons regulated by *tmt-opsin2* was not obviously detectable. It is hence plausible that the effect of *tmt-opsin2* is present in multiple neuronal populations and not restricted to few specific circuits.

Photoperiod regulates *sst1b* transcript levels via a *tmt-opsin1b* dependent mechanism

We finally aimed to further investigate the possible light dependency of the observed transcript regulation. Given that *sst1b* exhibits the more specific expression, we focused on this transcript. Specifically, we tested, if changes in the illumination regime impact on *sst1b* transcript levels in a TMT-Opn1b dependent manner. We chose a ‘photoperiod’- type light regime based on three reasons: First, any changes in transcription need sufficient time to occur. Second, we wanted to test a light regime that has obvious natural relevance to medaka [30], and third, *pre-pro-somatostatin* expression is regulated in response to exposure to short- and long-day photoperiods in adult rats [7].

We exposed wildtype fish to two different white light regimes (Fig. 7A,B). All fish were initially raised under a 16h (light):8h (dark) white light regime (Fig. 7B). One cohort remained exposed to this 16h:8h LD cycle (long day), while the other group was transferred to an 8h:16h LD cycle (short day). After one week eyes and brains were dissected at ZT 8 (long- day) and ZT4 (short-day) (blue arrowhead Fig. 7B) and analyzed by qPCR.

As *sst1b* is circadianly regulated with no phase difference between *tmt-opsin1b* mutants and wt fish (Fig.7C), we timed our brain part sampling to the middle of the respective light phases, an established sample timing strategy for photoperiod analyses in medakafish [57] (and see Methods for further reasoning).

A comparison of *sst1b* transcript levels at long vs. short day light regimes revealed significantly lower transcript level for the short day cohort, mimicking the *tmt-opsin1b* mutation (compare Figs. 7D vs. 7E, and red vs. black boxes Fig. 5B,C). Comparing the transcript levels of *sst1b* under long day vs. short day photoperiods in the *tmt-opsin1b* mutants revealed that this mutation leads to even stronger changes in *sst1b* transcript levels

(Fig. 7E). This is most apparent for the forebrain, where there are no changes between long- and short- day conditions in wildtype (Fig.7D), but a strong downregulation of *sst1b* under short-day in the mutant (Fig.7E). This shows that the regulation of *sst1b* by light depends on *tmt-opsin1b*, but – similar to the behavioral changes in the avoidance assay– suggests that *tmt-opsin1b* normally prevents specific neurophysiology changes to occur in response to environmental light changes, a “stabilizing function” that is perturbed in *tmt-opsin1b* mutants.

Discussion

The large number of *Opsin* genes uncovered over the past years in vertebrates, particularly in teleosts, is puzzling. Even more since many of these *Opsins* have been shown to encode functional light receptors and are expressed at various places beyond the rods and cones (e.g. [9-12, 14-17]). Here we have started to functionally tackle this diversity by investigating loss-of-function mutants of two non-visual Opsins of the Encephalopsin/TMT-Op sin family in medaka.

The results are multifaceted, complex and provide food for thought from multiple perspectives.

While first focusing on *tmt-opsin1b*, we show that this Op sin modulates fish swimming behavior in a light-dependent manner in several assays. Interestingly, in most cases do *tmt-opsin1b* mutant fish display enhanced behavioral responses. At present we can only speculate about the contribution of the different *tmt-opsin1b*⁺ neurons in the response circuitry. While the contribution of several *tmt-opsin1b*⁺ cells is unclear, it is highly likely that tectal cells are involved. This may suggest the following circuitry: *Tmt-opsin1b*⁺ tectal cells are positive for ChAT [14]. These cells connect –possibly monosynaptically (given the close vicinity and branching of ChAT tectal neurons- see [14])– to neurons marked by *sst1b/gad2*. We showed that *Tmt-opsin1b* affects the production of *sst1b*. A member of the somatostatin/corticotat in family in the mouse (SST, active form SST-14) has been shown to itself suppress excitatory inputs to downstream interneurons, resulting in enhanced visual gain and orientation selectivity [58]. It might hence be plausible to assume that tectal *sst1b* also exert suppressive effects. In *tmt-opsin1b* mutants *sst1b* is downregulated in tectal interneurons, which would reduce such a suppressive effect and result in enhanced behavioral responses. Alternatively, we showed that these *sst1b*⁺ neurons also express

gad2. It might thus also be imaginable that GABA-release could be modulated by *Tmt-opsin1* in these neurons. Thus, focusing on the tectal circuitry, there are at least two possibilities how loss of *tmt-opsin1b* results in increased activity. However, future work on specific neuronal circuits will be required to really understand the role of the different *tmt-opsin1b* expressing neurons.

We also showed that the response differences present in *tmt-opsin1b* versus wt fish, as well as the effects of the *tmt-opsin1b/tmt-opsin2* double mutants are strongly age-dependent. As the expression domains of *tmt-opsin1b* nor *tmt-opsin2* do not observably change between young larvae and adults [14], we think that the most plausible explanation for the age-dependency is the still on-going differentiation of the nervous system during young larval stages. Ontogenetic changes in behavior are very common and for instance well documented for the visual processing and social behavior in medaka fish [53, 59]. While the cellular and molecular causes still remain to be determined, it is clear that the phenomenon is common among vertebrates and even extends to humans across lifespan, where it has been shown to correlate with changes in glutamatergic and GABAergic systems [60, 61]. Especially the latter is interesting in the context of our analyses, as the *sst1b*⁺ neurons downstream of *tmt-opsin1b* function in the tectum are GABAergic and might hence be a possible subject to ontogenetic changes. Behavioral changes across life span are not just an important topic to better comprehend the extent of natural behavioral plasticity. It is also relevant to understand behavioral adaptations to natural environments, as ontogenetic changes are often intertwined with habitat shifts to balance the different risks and needs of individuals at different ages, often correlated with size differences [62, 63].

Another level of molecular modulation we uncover is the regulation of *sst1b* by photoperiod. Short-day photoperiods result in levels of *sst1b* in mid- and hindbrain that are equal to those of the *tmt-opsin1b* mutants. However, in the mutants these levels become reduced even further, and *sst1b* reductions also occurs strongly in the forebrain, where it is unaffected in wildtype under long- vs. short- day regimes. These data suggest that *tmt-opsin1b* normally prevents molecular alterations to happen when environmental conditions change.

Interestingly, different members of the *somatostatin/corticotropin* family are frequently connected to changes in light parameters. A change of the preprohormone transcript and peptide levels of Somatostatin1 has been reported for long vs. short photoperiods in adult rats [7], suggesting a possible evolutionary conservation of this regulatory relationship. Similar to the changes we observed for *sst1b* in medaka, SST1 amounts increase when the days are longer. In the case of the rat, this is correlated with a partial decrease of the enzyme *tyrosine hydroxylase* and the number of dopaminergic neurons, as well as an alteration in stress behavior[7]. However, in addition to the changes in the forebrain (posterior hypothalamus), as they were reported for rat, we find *sst1b* to be significantly regulated in mid- and hindbrain cells. It could be that this also extends to rat and certainly suggests, that brain regions outside the hypothalamus should be analyzed, in order to understand which effects photoperiod differences might exert on mammals. It will then be a question for the future to sort out, which molecular changes are related to which behavioral changes. Furthermore, *sst1.1* in zebrafish has been implicated in the regulation of behavioral search strategies upon sudden loss of illumination, a behavior that also depends on melanopsin-type light receptor *opn4a*. However, it remains to be clarified if *sst1.1* functions downstream or in parallel pathways to *opn4a*. It might be that different non-visual opsins relay different light information via different *somatostatin/corticotropin* members. Finally, one of our main aims was also to obtain first insight into the functional interaction of different photoreceptors in the organism. Unexpectedly for us, both on molecular and behavioral levels, the simultaneous loss of two *tmt-opsins* can compensate for the loss of each of them individually, suggesting that they act at least in part in opposite directions on the same downstream circuitry. In this respect the expression of *tmt-opsin1b*, *tmt-opsin2* and *sst1b* in the tectal region is again particularly interesting. *Tmt-opsin1b* and *tmt-opsin2* tectal expression is non-overlapping, both delimiting two distinct cellular populations, within two unique layers, while *sst1b* either overlaps or is directly adjacent to *tmt-opsin2* positive cells. This spatial expression raises the possibility that both *tmt-opsins* (and *sst1b*) might together contribute to the local neuronal circuit outlined above. Future work is needed to not only further delineate these interactions, but to also better understand their significant complexity in the context of natural light habitats and adaptations.

Online Methods

Medaka fish rearing and ethics statement

All animal research and husbandry was conducted according to Austrian and European guidelines for animal research (fish maintenance and care approved under: BMWFW-66.006/0012-WF/II/3b/2014, experiments approved under: BMWFW-66.006/0003-WF/V/3b/2016, which is cross-checked by: Geschäftsstelle der Kommission für Tierversuchsangelegenheiten gemäß § 36 TVG 2012 p. A. Veterinärmedizinische Universität Wien, A-1210 Wien, Veterinärplatz 1, Austria, before being issued by the BMWFW). Medaka fish (*Oryzias latipes*) strains were kept in a constant recirculating system at approximately 26-28°C in a 16h light / 8h dark cycle and bred using standard protocols [33]. Collected embryos were kept at 28°C until hatching in Embryo Rearing Medium (ERM; 0.1% (w/v) NaCl, 0.003% (w/v) KCl, 0.004% (w/v) CaCl₂ x 2 H₂O, 0.016 % (w/v) MgCl₂ x 6 H₂O, 0.017 mM HEPES and 0.0001% (w/v) methylene blue). Mutant lines were generated in and outcrossed at least four time to the Cab wildtype background. (*tmt-opsin1b* Δ+23 was outcrossed 13 times.) Where available, different mutant alleles were used interchangeably, thereby reducing the probability of effects induced by off-target mutations. Mutant versus sibling wildtype batches and batches from heterozygous crosses were position in random, alternating position relative to the respective light sources while grown prior to the assays. Parental fish grew in as similar fish room light conditions as possible.

Design and construction of *tmt*-TALENs

Transcription activator-like effector nucleases (TALENs) targeting the first exon of medaka TMT-opsin 1b gene (Ensembl gene ENSORLG00000013181) and TMT-opsin2 (Ensembl gene ENSORLG00000012534) were designed using the TALEN targeter prediction tool (<https://tale-nt.cac.cornell.edu/>; TAL Effector Nucleotide Targeter v2.0) according to the following parameters: equal left and right repeat variable diresidue (RVD) lengths, spacer length of 15–20 bp, NN for G recognition, only T in the upstream base and the presence of a unique restriction site in the spacer region. TAL effector modules were assembled and cloned into the array plasmids pFUS using the Golden Gate TALEN and TAL Effector kit (Addgene, Cambridge, USA) according to validated procedure (Cermak et al., 2011). The RVD sequence of the left *tmt-opsin1b*-TALEN was HD NN NG NG HD

HD HD NI NI HD NN HD NN NI NN HD HD NG binding to the genomic sequence 5'-CGTTCCCAACGCGAGCCT-3' and the RVD sequence of the right *tmt-opsin1b*-TALEN was NN HD NG HD HD NN HD NG NN HD NN NG HD HD HD HD NN NG binding to the genomic sequence 5'-GCTCCGCTGCGTCCCCGT-3' on the opposite strand featuring a unique BssHII restriction site in the 17 bp spacer region. The RVD sequence of the left *tmt-opsin2*-TALEN was NN NG NG NG NG HD HD NN NN NG HD NI NN NI HD binding to the genomic sequence 5'-GTTTTCCGGTCAGAC-3' and the RVD sequence of the right *tmt-opsin2*-TALEN was NI NG HD NN HD NG HD NG NN NN NG NG NN NI binding to the 5'-CATCGCTCTGGTTGA-3' genomic sequence. The final pCS2+ backbone vectors, containing the homodimeric FokI nuclease domains, were used as previously[64]. All final TALENs pCS2+ expression vectors were sequence-verified using 5'-TTGGCGTCGGCAAACAGTGG-3' forward and 5'-GGCGACGAGGTGGTCGTTGG-3' reverse primers.

Generation of capped TALEN mRNA and microinjection

Full-length TALEN plasmids were digested with KpnI, gel purified (Gel Extraction kit, Qiagen, Netherlands), and *in vitro* transcribed (Sp6 mMACHINE kit, Thermo Fisher Scientifics, USA), followed by a purification using the RNeasy Mini kit (Qiagen). The yield was estimated by NanoDrop (Thermo Fisher Scientific) and diluted to the final concentration for microinjection. Cab strain zygotes were microinjected with a mix containing 5 or 50 ng/μl of each transcribed TALEN mRNA and 0.6% tetramethylrhodamine (TRITC; Thermo Fisher Scientific) in nuclease-free water.

Genotyping of *tmt-opsin1b* and *tmt-opsin2*-TALEN mutated fish

Genomic DNA from hatched larvae or caudal fin biopsies were retrieved with lysis buffer (0.1% SDS; 100 mM Tris/HCl, pH 8.5; 5 mM EDTA; 200 mM NaCl; 0.01 mg/ml Proteinase K) at 60°C overnight and 1 μl of a 1:20 dilution of the lysate used in a PCR reaction (HotStarTaq, Qiagen). The sequence flanking the *tmt-opsin1b* TALEN binding sites was amplified using the forward 5'-GGGACTTTCTTTGCGCTTTA-3' and the reverse 5'-CAGGTCAGAGCGGATCTCAT-3' primers. 10 μl of the reaction was directly used for restriction digest using 4 units of BssHII enzyme (New England Biolabs, USA) in

a 20 µl reaction. Genotyping of the *tmt-opsin2* locus was made with the forward 5'-CGGTGAGCGATGTGACTG-3' and the reverse 5'-GGGAGATCTTTGTCCAGGTG-3' primers. Mutation caused by TALENs was assessed by analyzing the band sizes of the restriction digest product on a 2% agarose gel. Undigested bands were gel extracted and subcloned into pJet2-1 using the Clone JET PCR Cloning kit (Thermo Fisher Scientific) and sequence-verified.

Immunohistochemistry

Without clearing

Adult fish (> 2 months-old) were anesthetized in fish water containing 0.2% tricaine, decapitated, the brain dissected, fixed in ice-cold 4% PFA overnight and subsequently stored in 100% methanol for at least 1 week before use. After rehydration in successive steps of PBT (PBS with 1% Triton X-100), the dissected brains were fixed in 4% paraformaldehyde in PBT for 2 hours, embedded in 3% agarose and cut into slices of 100 µm thickness on a tissue vibratome, followed by 4 hours of blocking in 5% sheep serum at room temperature. Slices were then incubated for 3 days at 4°C with a polyclonal rabbit anti-Ola-TMT-1b antibody (1:250 in 1% sheep serum). After extensive PBT washes, sections were treated with the secondary antibody goat anti-rabbit Alexa Fluor® 488 (Thermo Fischer Scientific) in a 1:500 dilution in 1% sheep serum supplemented with 1:10000 of 4',6-diamidino-2-phenylindole (DAPI), during 2 days at 4°C. Following PBT washes, slices were mounted in proper mounting medium and pictures were taken on a Zeiss LSM700 confocal scanning microscope.

With clearing

The protocol was adapted from [65] with the following details: adult fish (> 2 months-old) were anesthetized in fish water containing 0.2% tricaine, decapitated, brain dissected, fixed in ice-cold 4% PFA overnight. After washes in 1x PBS at room temperature (RT), the brains were incubated in pre-chilled acetone O/N at -20°C, rehydrated in 1x PBS at room temperature and digested with 10ug/ml of Proteinase K (Merck #1245680100) for 10-12 min at RT, followed by 2x glycine washes (2mg/ml) (Roth #3908.3). Brains were washed in 1x PBS, immersed in Solution 1.1 (3 hrs, at 37°C) under gentle shaking, subsequently washed in 1x PBS O/N, embedded in 3% agarose and sliced (100 µm thickness) using a vibrating blade microtome (Leica VT1000S, Leica Biosystems, Germany). The slices were

then blocked for 1-2 hrs in 10% sheep serum / 1x PBS, followed by primary antibody incubation in 10% sheep serum at 4°C for 3 days (Anti-SCN5A antibody produced in rabbit (Sigma SAB2107930) 1:1000. After extensive 1x PBS washes, sections were incubated in a goat anti-rabbit Cy3 antibody (Thermo Fischer Scientific) (1:500 dilution) in 10% sheep serum, 1:10000 of DAPI were added, and incubated for 3 days at 4°C. Following PBS washes, slices were mounted in proper mounting medium and pictures were taken on a Zeiss LSM700 confocal scanning microscope.

Avoidance assay

The behavioral assay was essentially performed as described before [40]. A single medaka larva (9 - 12 dpf; unfed) or juvenile fish (20 - 22dpf; fed) was placed in a custom-built acrylic glass chamber (18 x 14 x 305 mm) filled with 1x ERM medium (1 cm high), having a transparent bottom and opaque upward walls. Five chambers lying parallel to each other were placed on a horizontal facing computer screen, allowing for the simultaneous recording of 5 free-swimming animals in the same behavioral trial. Stimuli consisted of moving black dots (except when testing for contrast, where different abstract grey values were used) on a white background travelling at a constant speed of 13.5 mm/sec in the same direction in every trial. Each behavior trial consisted of 7 blocks of 6 dots each, each block characterized by a unique dot size, presented in a size-ascending or in a pseudo-random order. Stimuli were generated on the computer screen using custom-written programs (Ubuntu). Two consecutive blocks were presented with a 19 sec interval, during which no stimulus was displayed. The size of the dots was characterized by its diameter (in pixels) and were calculated as degrees of the larva visual field, as described before[40]. A industrial camera (acA1300-30gm GigE; Basler AG, Germany), positioned 40 cm above the computer screen, allowed the visualization of the fish-dot interactions, recording videos at a frame rate of 33 frames per second for offline analysis. The fish were allowed to accommodate to the chamber for 5 minutes before the beginning of the trial, which is longer than in previous similar assays in zebrafish [40] and *Xenopus* [37], and by this likely ensuring a robust standardization across different experiments. The experiments were performed during the natural light part of the fish light/dark cycle, between ZT2 and ZT5 and a single animal was used only in one behavior trial to avoid possible habituation (e.g. [66] or learning. Each fish-dot interaction was scored either as an approach or an avoidance

when the fish swam toward or away from a moving dot, respectively [40]. A neutral interaction was scored when the dot entering the fish visual field cause no change in the initial swimming pattern of the animal or if the fish remained motionless. Approach behaviors consisted of swimming towards the dot, displaying a distinct attraction and predation behavior[66], whereas avoidance behaviors were faster than approaches, characterized by rapid swimming bouts away from the direction of the dot's movement. All video analysis were made prior to genotyping of the fish to shield the identity of the subjects from the observer. An avoidance index $[(A.I. = (\text{number avoidances} - \text{number approaches}) / (\text{number total of dots in a block}))]$ was used as a quantitative behavioral readout. All behavioral assays were made at a constant room temperature of 26°C, with the room lights switched off and the behavioral setup covered by a black light-impenetrable cover cloth, this way ensuring that the computer screen would be the only illumination source. The Michelson contrast [67] $((L_{\text{max}} - L_{\text{min}}) / (L_{\text{max}} + L_{\text{min}}))$ was used to quantify the relative difference in luminance between the moving dot and the computer screen background. L_{max} and L_{min} are luminance maximum and minimum, respectively. Medaka are known to have highly acute vision, as demonstrated by previous studies on the optomotor response [53, 68], showing excellent visual performance already from hatching [68].

Photokinesis assay

Individual larvae (9 to 12 dpf; unfed; kept at a 16h:8h light cycle at 28°C until hatching), or juvenile medaka fish (20 to 22 dpf; fed) were distributed randomly across a 6-well plate containing 10 mL of 1x ERM medium per well. A behavioral trial consisted of a behavioral paradigm that evaluates the animal swimming activity (i.e. distance moved) during alternating blocks of 30 minutes light and darkness (3 hours in total per trial)[69], at a constant temperature of 27°C. An initial acclimation phase of 5 min to darkness was used before the start of the trial. When assessing the swimming responses in a more naturalistic-like assay, a photoperiod of 16h light / 8h dark was used during 2 consecutive days, at a constant temperature of 27°C. Behavioral assessment was made between ZT3 and ZT12. DanioVision™ (Noldus, The Netherlands) hardware was used to track the distance moved by the animals in each trial. Larval motion was tracked at 60 frames/s over the trial. In an

alternate experiment, 1 min pulses of white light and darkness were presented to the larvae, during a total trial duration of 7 minutes (refers to Supplementary Fig. 2A). Video data was posteriorly analyzed offline by the tracking software EthoVision XT[®] (Noldus) to calculate the average distance moved by the animal every 10 sec of the trial. When comparing the dark photokinesis between *tmt-opsin1b* mutant and wildtype siblings (refers to Supplementary Fig. 2M,N), we normalized the different baseline levels by taking the average distance moved during the 5 minutes preceding darkness along the trial as a normalization factor. Each animal was used only once per trial, thus avoiding possible habituation biases. The ILT950 spectrometer (International Light Technologies Inc., USA) was used to measure the spectra and intensity of the different light sources. A light-impenetrable cloth was placed over the behavior setup, thus reassuring a lack of external light contamination.

Diel swimming test

Care was taken to start each trial at the precise light-to-dark transition, thus minimizing any deleterious effect that the shift to a new environment might impose on the natural behavior of the fish. Since scheduled food availability has an impact on the locomotor activity of fish [70], animals were kept unfed during the two consecutive days of the experiment, thus ensuring that the change in ambient light was the central feature impacting on swimming responses. The temperature was kept constant at 27°C. The setup, tracking and off-line analysis was made as previously described for the “photokinesis assays”, with the exception that the activity of the animals was averaged every 1 minute.

Enucleations

Enucleations were performed on 13 dpf wildtype and *tmt-opsin1b* mutant juvenile fish upon anaesthetization with 0.03% tricaine (MS-222) (Sigma, St. Louis, MO). After the surgery, fish were allowed to recover overnight in fresh rearing fish water at 26°C. After 1 week post-surgery, the fish were used for behavior testing. Handling control fish were anaesthetized for the same duration and placed afterwards in fresh fish water overnight.

RNA extraction and sequencing

For RNA-seq experimentation, the eyes, forebrain, midbrain and hindbrain (anatomical boundaries according to [71]) were dissected from age-matched wildtype and mutant *tmt-*

opsin1b and *tmt-opsin2* adult (>2 months old) medaka sibling fish. One metal bead (Peqlab Biotechnologie GmbH, Germany) and 350 µl RLT buffer (Qiagen) including 1% β-mercaptoethanol was added to the frozen tissue parts, followed by homogenization for 3 minutes at 30 Hz using the Qiagen tissue lyser. Total RNA was extracted using the RNeasy Mini kit (Qiagen) according to manufacturer's protocol. Three independent biological replicates, each made of 3 individual fish (total of 9 fish per genotype) were used. For *tmt-opsin1b*, 1 fish per independent mutation was used. The quality of total RNA was checked using the Agilent RNA 6000 Nano kit (Agilent, USA) and then enriched for poly(A)+ RNA using the Dynabeads® mRNA Purification Kit (Thermo Fisher Scientific). We used the SuperScript® VILO cDNA Synthesis Kit (Thermo Fisher Scientific) to generate strand-specific cDNA that was further sequenced on the Illumina HiSeq2500 platform by the VBCF NGS Unit (www.vbcf.ac.at) as 100 base single-end reads, resulting on a 15-61 million reads, on average, per biological replicate.

Differential gene expression

Sequences from each sample were mapped against the assembled chromosomes of the medaka genome (Ensembl version 96) using the read mapper NextGenMap [72]. After filtering for duplicates and low quality base, the stand-alone *featureCounts* tool [73] was used to count mapped reads per each transcript in each sample. The Bioconductor package edgeR (version 3.9) was used to analyze read count data and to identify differential expression levels (Benjamini–Hochberg method). We classified genes as being differentially expressed between genotypes when the differences in expression level between wildtype and homozygous mutant fish were significant at a false discovery rate (FDR) of 5%.

Quantitative PCRs

One metal bead (Peqlab Biotechnologie) and 700 µl TRI Reagent® (Sigma-Aldrich, St. Louis, USA) were added to the previously dissected and frozen eyes, forebrain, midbrain and hindbrain, followed by homogenization for 2 minutes at 30 Hz using the Qiagen tissue lyser. Total RNA was extracted from the different brain parts and eyes using the Direct-Zol™ RNA MiniPrep (Zymo Research, USA) according to the manufacturer's protocol. 80ng of total RNA was initially used to be transcribed into cDNA using the QuantiTect

Reverse Transcription kit (Qiagen) with random hexamer primers. Each cDNA was further analyzed in duplicate in a 25ul volume using a SYBR green-containing mastermix in the Applied Biosystems (Thermo Fisher Scientific) mastercycler. Intron spanning qPCR primers were designed with the universal probe library software from Roche. Expression of each gene was normalized to *beta-actin* transcript levels and fold changes were calculated.

Table 1: Forward and reverse primer sequences used for qPCR

Gene	Forward primer (5'-->3')	Reverse primer (5'-->3')	Ensembl Gene ID
<i>sst1b</i> (also named <i>sst3</i> or <i>cort</i>)	ggcttctgtggaggaca	cagacaccagcttaaggatca	ENSORLG0000002773 6
<i>beta-actin</i>	gtgctgtctttccctccatc	tagctgtctttctggcccat	ENSORLG0000001367 6
<i>scn12aa</i>	gcagatgacctgtcggaaact	ctgacagtgcctcagacagaa	ENSORLG0000001167 7
<i>perl1b</i>	ggggagagtcgtgtacgtgt	tgctgctgtagaaggtgctg	ENSORLG0000001937 0
<i>reverbB</i>	aggagcatccagcagaacat	ctggctgttttgctcatca	ENSORLG0000001643 1

Cloning of medaka *gad2*, *scn12aa*

A BLAST search using the Zebrafish *gad2* identified predicted homologous sequences, homology was confirmed by reciprocal back-blast (for *gad2*).

cDNAs were cloned following standard protocols, using Phusion Polymerase (NEB) and cloned in pJET1.2. Inserts were verified by sequencing.

Primers (5'-3'):

gad2

forward: atggcatctcacgggttctggtctc

reverse: ttatagatcatggccgagcggtca

scn12aa:

forward :cgccatgcagagagaagattag

reverse: gacactcgggtggaagattac

GenBank accession numbers: MT267535, MT239386, MT793601

***In situ* hybridization (ISH) on adult brain sections**

The generation of antisense digoxigenin (DIG)-labeled RNA probes and the ISH staining procedure on adult brain sections were performed as previously [14]. Briefly, adult fish (> 2 months-old) were anesthetized in fish water containing 0.2% tricaine, decapitated, and the brain dissected, fixed in ice-cold 4% PFA overnight, and subsequently stored in 100% methanol for at least 1 week prior to use. After rehydration in 1X PTW (PBS + 0.1% Tween-20), brains were digested with fresh 10 µg/ml Proteinase K (Merck, USA) for 35 minutes, followed by a 4 hours prehybridization step at 65°C with Hyb+ solution (50% Formamide, 5x SSC (pH=6.0), 0.1% Tween-20, 0.5 mg/ml torula (yeast) RNA, 50 µg/ml Heparin). Incubation with the specific DIG-RNA probe was made overnight at 65°C. Coronal whole-brain slices (100 µm thickness) were made using a vibrating blade microtome (Leica VT1000S, Leica Biosystems, Germany), blocked for > 1 hour in 10% sheep serum / 1x PTW, followed by incubation with 1:2000 dilution of anti-DIG-alkaline phosphatase-coupled antibody (Roche, Switzerland) in 10% sheep serum overnight. Detection of DIG-probes was made in staining buffer (in 10% polyvinyl alcohol) supplemented with nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolylphosphate (BCIP) (Roche). In addition, the *gad2* probe was co-detected fluorescently as described in [74].

Cortisol assay

Mechanical disturbance

Larvae were placed onto an orbital shaker (300rpm) for 5 minutes, subsequently sacrificed and snap-frozen in liquid nitrogen.

Cortisol extraction and measurement

The procedure for extracting cortisol from whole larvae was adapted from [75]. Briefly, the larvae were sacrificed and frozen in liquid nitrogen, and stored at -80°C until further processed. The samples were thawed on ice and 200ul sterile PBS was added. The samples were subsequently homogenized at RT for 1 minute at 30Hz (Tissue Lyser II, Qiagen). 20ul of each homogenate was removed for protein concentration measurements (Pierce™ BCA Protein Assay Kit, Thermo Scientific).

To the remaining 180ul, 1.4ml Ethyl acetate was added. The samples were subsequently centrifuged at 7000g for 15 minutes and the upper organic phase was collected within a glass vile. The samples were left to evaporate in a fume hood overnight. The dried samples were resuspended in 180ul ELISA Buffer (as provided by the Kit mentioned below).

The concentration of cortisol was measured using and enzyme-linked immunosorbent assay (ELISA) kit (500360, Cayman Chemical).

Phylogenetic Analysis

Sequences were aligned using the MUSCLE alignment algorithm (<https://www.ebi.ac.uk/Tools/msa/muscle/>). The resulting alignment was used to generate a maximum likelihood (ML) tree by the IQ-TREE web server [76], visualized using iTol (<https://itol.embl.de/>) and exported as .eps file. Coloration and text formatting was performed in Adobe IllustratorCC2017. The accession numbers can be found in the respective metadata table.

Statistics

The D'Agostino & Pearson normality test was used to asses data distribution. The F-test for comparison of variances for normally distributed data was used to assess the variance of the standard deviation. When comparing two normally distributed groups of data with the same standard deviation the unpaired t test was used. For comparing two groups of normally distributed data from with different standard deviations, the unpaired t test with Welch correction was used. The Mann-Whitney (M-W) test was used when comparing two non-normally distributed data sets. The software used was Prism version 8.

Data is presented as mean $\pm$ s.e.m. For all statistical tests used, a two-tailed *P* value was chosen: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$

Author contributions

B.M.F. and T.Z. performed the experiments and analyzed data, unless otherwise indicated. M.A-R. cloned *gad2* and performed the *in situ* hybridization experiments for *gad2* and *sst1b*. F.R. performed part of the qPCR experiments. M.G. and A.v.H mapped the transcriptome reads and performed the differential expression seq analyses. A.J.B. and H.B contributed equipment and analysis expertise for the avoidance assay. M.H. constructed the dot assay setup and contributed with software programming. R.M.F generated the *tmt-opsin1b* and *tmt-opsin2* mutants. B.M.F, T.Z. and K.T-R designed the study, interpreted the data and wrote the manuscript.

Acknowledgements

We thank Andrij Belokurov and Margaryta Borisova for fish care, Andrew Straw and the members of the Tessmar-Raible, Raible, von Haeseler and Baier labs for helpful discussions, Mario Wullimann for advice on medaka neuroanatomy, Mirta Resetar for preparation of fish brains and RNA for DEseq analyses and Sven Schenk for sharing the mRNA sequencing protocol. Marin Čagelj contributed with the illustrations on Figures 1C, 4A, 5A. This work was supported by the research platform “Rhythms of Life” of the University of Vienna and a FWF (<http://www.fwf.ac.at/>) SFB grant (#SFB F78) to K.T-R and A.v.H, a FWF (<http://www.fwf.ac.at/>) START award (#AY0041321), research project grant (#P28970), and funding from the European Research Council under the European Community's Seventh Framework Programme (FP7/2007-2013)/ERC Grant Agreement 337011 to K.T-R. A.J.B. and H.B. acknowledge funding by SFB 870 „Assembly and Function of Neural Circuits" and the Max Planck Society. R.M.F. was supported by a PhD fellowship of the Boehringer Ingelheim Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Financial Interests

Max Hofbauer is the CEO of loopbio GmbH, a company developing commercial animal behavioral tracking solutions. All other authors declare no competing financial interests.

References

1. Posch T, Freyhoff A, Uhlmann T. Das Ende der Nacht: Die globale Lichtverschmutzung und ihre Folgen. 1st edition ed. Weinheim: Wiley VCH; 2010. 154 p.
2. Hunt DM, Hankins MW, Collin SP, Marshall NJ. Evolution of visual and non-visual pigments. New York: Springer; 2014. 276 p.
3. Bedrosian TA, Nelson RJ. Timing of light exposure affects mood and brain circuits. *Transl Psychiatry*. 2017;7(1):e1017. Epub 2017/02/01. doi: 10.1038/tp.2016.262. PubMed PMID: 28140399; PubMed Central PMCID: PMC5299389.
4. Wahl S, Engelhardt M, Schaupp P, Lappe C, Ivanov IV. The inner clock-Blue light sets the human rhythm. *J Biophotonics*. 2019;12(12):e201900102. Epub 2019/08/23. doi: 10.1002/jbio.201900102. PubMed PMID: 31433569; PubMed Central PMCID: PMC67065627.
5. Fernandez DC, Fogerson PM, Lazzerini Ospri L, Thomsen MB, Layne RM, Severin D, et al. Light Affects Mood and Learning through Distinct Retina-Brain Pathways. *Cell*. 2018;175(1):71-84 e18. Epub 2018/09/04. doi: 10.1016/j.cell.2018.08.004. PubMed PMID: 30173913; PubMed Central PMCID: PMC6190605.
6. Zhu H, Wang N, Yao L, Chen Q, Zhang R, Qian J, et al. Moderate UV Exposure Enhances Learning and Memory by Promoting a Novel Glutamate Biosynthetic Pathway in the Brain. *Cell*. 2018;173(7):1716-27 e17. Epub 2018/05/22. doi: 10.1016/j.cell.2018.04.014. PubMed PMID: 29779945.
7. Dulcis D, Jamshidi P, Leutgeb S, Spitzer NC. Neurotransmitter switching in the adult brain regulates behavior. *Science*. 2013;340(6131):449-53. Epub 2013/04/27. doi: 10.1126/science.1234152. PubMed PMID: 23620046.
8. Dawson A, King VM, Bentley GE, Ball GF. Photoperiodic control of seasonality in birds. *J Biol Rhythms*. 2001;16(4):365-80. Epub 2001/08/17. doi: 10.1177/074873001129002079. PubMed PMID: 11506381.
9. Currie SP, Doherty GH, Sillar KT. Deep-brain photoreception links luminance detection to motor output in *Xenopus* frog tadpoles. *Proc Natl Acad Sci U S A*. 2016;113(21):6053-8. Epub 2016/05/12. doi: 10.1073/pnas.1515516113. PubMed PMID: 27166423; PubMed Central PMCID: PMC4889350.
10. von Frisch K. Beiträge zur Physiologie der Pigmentzellen in der Fischhaut. *Pflüg Arch ges Physiol*. 1911;138:319-87.
11. Foster RG, Follett BK, Lythgoe JN. Rhodopsin-like sensitivity of extra-retinal photoreceptors mediating the photoperiodic response in quail. *Nature*. 1985;313(5997):50-2. Epub 1985/01/03. PubMed PMID: 3965970.
12. Halford S, Pires SS, Turton M, Zheng L, Gonzalez-Menendez I, Davies WL, et al. VA opsin-based photoreceptors in the hypothalamus of birds. *Curr Biol*. 2009;19(16):1396-402. Epub 2009/08/12. doi: 10.1016/j.cub.2009.06.066. PubMed PMID: 19664923.
13. Fernandes AM, Fero K, Arrenberg AB, Bergeron SA, Driever W, Burgess HA. Deep brain photoreceptors control light-seeking behavior in zebrafish larvae. *Curr Biol*. 2012;22(21):2042-7. Epub 2012/09/25. doi: 10.1016/j.cub.2012.08.016. PubMed PMID: 23000151; PubMed Central PMCID: PMC3494761.
14. Fischer RM, Fontinha BM, Kirchmaier S, Steger J, Bloch S, Inoue D, et al. Co-expression of VAL- and TMT-opsins uncovers ancient photosensory interneurons and

- motoneurons in the vertebrate brain. *PLoS Biol.* 2013;11(6):e1001585. doi: 10.1371/journal.pbio.1001585.
15. Davies WIL, Tamai TK, Zhen L, Fu JK, Rihel J, Foster RG, et al. An extended family of novel vertebrate photopigments is widely expressed and displays a diversity of function. *Genome Res.* 2015;25(11):1666-79. doi: 10.1101/gr.189886.115. PubMed PMID: WOS:000364355600008.
 16. Jenkins A, Munoz M, Tardt EE, Bellingham J, Foster RG, Hankins MW. VA opsin, melanopsin, and an inherent light response within retinal interneurons. *Current Biology : CB.* 2003;13(15):1269-78. Epub 2003/08/09. PubMed PMID: 12906786.
 17. Davies WI, Zheng L, Hughes S, Tamai TK, Turton M, Halford S, et al. Functional diversity of melanopsins and their global expression in the teleost retina. *Cell Mol Life Sci.* 2011;68(24):4115-32. Epub 2011/08/13. doi: 10.1007/s00018-011-0785-4. PubMed PMID: 21833582.
 18. Nakane Y, Shimmura T, Abe H, Yoshimura T. Intrinsic photosensitivity of a deep brain photoreceptor. *Curr Biol.* 2014;24(13):R596-7. Epub 2014/07/09. doi: 10.1016/j.cub.2014.05.038. PubMed PMID: 25004360.
 19. Brunt EEV, Shepherd MD, Wall JR, Ganong WF, Clegg MT. Penetration of light into the brain of mammals. *Ann N Y Acad Sci.* 1964;117(1):217-24. doi: 10.1111/j.1749-6632.1964.tb48177.x.
 20. Kokel D, Dunn TW, Ahrens MB, Alshut R, Cheung CY, Saint-Amant L, et al. Identification of nonvisual photomotor response cells in the vertebrate hindbrain. *The Journal of Neuroscience.* 2013;33(9):3834-43. Epub 2013/03/01. doi: 10.1523/JNEUROSCI.3689-12.2013. PubMed PMID: 23447595; PubMed Central PMCID: PMC3600642.
 21. Friedmann D, Hoagland A, Berlin S, Isacoff EY. A spinal opsin controls early neural activity and drives a behavioral light response. *Curr Biol.* 2015;25(1):69-74. Epub 2014/12/09. doi: 10.1016/j.cub.2014.10.055. PubMed PMID: 25484291; PubMed Central PMCID: PMC4286461.
 22. Horstick EJ, Bayley Y, Sinclair JL, Burgess HA. Search strategy is regulated by somatostatin signaling and deep brain photoreceptors in zebrafish. *BMC Biol.* 2017;15. doi: ARTN 4
10.1186/s12915-016-0346-2. PubMed PMID: WOS:000392962100002.
 23. Matos-Cruz V, Blasic J, Nickle B, Robinson PR, Hattar S, Halpern ME. Unexpected diversity and photoperiod dependence of the zebrafish melanopsin system. *PLoS ONE.* 2011;6(9):e25111. Epub 2011/10/04. doi: 10.1371/journal.pone.0025111. PubMed PMID: 21966429; PubMed Central PMCID: PMCPMC3178608.
 24. Cavallari N, Frigato E, Vallone D, Frohlich N, Lopez-Olmeda JF, Foa A, et al. A blind circadian clock in cavefish reveals that opsins mediate peripheral clock photoreception. *PLoS Biol.* 2011;9(9):e1001142. Epub 2011/09/13. doi: 10.1371/journal.pbio.1001142. PubMed PMID: 21909239; PubMed Central PMCID: PMC3167789.
 25. Moore HA, Whitmore D. Circadian Rhythmicity and Light Sensitivity of the Zebrafish Brain. *PLoS ONE.* 2014;9(1). doi: ARTN e86176
10.1371/journal.pone.0086176. PubMed PMID: WOS:000330283100121.

26. Nakane Y, Ikegami K, Iigo M, Ono H, Takeda K, Takahashi D, et al. The saccus vasculosus of fish is a sensor of seasonal changes in day length. *Nat Commun.* 2013;4. doi: ARTN 2108
10.1038/ncomms3108. PubMed PMID: WOS:000323715700003.
27. Ramirez MD, Pairett AN, Pankey MS, Serb JM, Speiser DI, Swafford AJ, et al. The Last Common Ancestor of Most Bilaterian Animals Possessed at Least Nine Opsins. *Genome Biol Evol.* 2016;8(12):3640-52. doi: 10.1093/gbe/evw248. PubMed PMID: 28172965; PubMed Central PMCID: PMC45521729.
28. Blackshaw S, Snyder SH. Encephalopsin: a novel mammalian extraretinal opsin discretely localized in the brain. *J Neurosci.* 1999;19(10):3681-90.
29. Nissila J, Manttari S, Sarkioja T, Tuominen H, Takala T, Timonen M, et al. Encephalopsin (OPN3) protein abundance in the adult mouse brain. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol.* 2012;198(11):833-9. Epub 2012/09/20. doi: 10.1007/s00359-012-0754-x. PubMed PMID: 22991144; PubMed Central PMCID: PMC3478508.
30. Awaji M, Hanyu I. Seasonal Changes in Ovarian Response to Photoperiods in Orange-Red Type Medaka : Reproductive Biology. *Zoological Science.* 1989;6:943-50.
31. Koger CS, Teh SJ, Hinton DE. Variations of light and temperature regimes and resulting effects on reproductive parameters in medaka (*Oryzias latipes*). *Biol Reprod.* 1999;61(5):1287-93. Epub 1999/10/21. PubMed PMID: 10529276.
32. Sakai K, Yamashita T, Imamoto Y, Shichida Y. Diversity of Active States in TMT Opsins. *PLoS ONE.* 2015;10(10):e0141238. doi: 10.1371/journal.pone.0141238. PubMed PMID: 26491964; PubMed Central PMCID: PMC4619619.
33. Kinoshita M, Murata K, Naruse K, Tanaka M. *Medaka: Biology, Management, and Experimental Protocols*: Wiley-Blackwell; 2009.
34. Souren M, Martinez-Morales JR, Makri P, Wittbrodt B, Wittbrodt J. A global survey identifies novel upstream components of the *Ath5* neurogenic network. *Genome Biol.* 2009;10(9):R92. Epub 2009/09/09. doi: 10.1186/gb-2009-10-9-r92. PubMed PMID: 19735568; PubMed Central PMCID: PMC2768981.
35. Del Bene F, Ettwiller L, Skowronska-Krawczyk D, Baier H, Matter JM, Birney E, et al. In vivo validation of a computationally predicted conserved *Ath5* target gene set. *PLoS Genet.* 2007;3(9):1661-71. Epub 2007/09/26. doi: 10.1371/journal.pgen.0030159. PubMed PMID: 17892326; PubMed Central PMCID: PMC1988851.
36. Ewert JP. Neural Mechanisms of Prey-Catching and Avoidance Behavior in Toad (*Bufo-Bufo* L). *Brain Behavior and Evolution.* 1970;3(1-4):36-&. doi: Doi 10.1159/000125462. PubMed PMID: WOS:A1970I326400005.
37. Dong W, Lee RH, Xu H, Yang S, Pratt KG, Cao V, et al. Visual Avoidance in *Xenopus* Tadpoles Is Correlated With the Maturation of Visual Responses in the Optic Tectum. *J Neurophysiol.* 2009;101(2):803-15. doi: 10.1152/jn.90848.2008. PubMed PMID: WOS:000263120300027.
38. Khakhalin AS, Koren D, Gu J, Xu H, Aizenman CD. Excitation and inhibition in recurrent networks mediate collision avoidance in *Xenopus* tadpoles. *Eur J Neurosci.* 2014;40(6):2948-62. doi: 10.1111/ejn.12664. PubMed PMID: 24995793.
39. Semmelhack JL, Donovan JC, Thiele TR, Kuehn E, Laurell E, Baier H. A dedicated visual pathway for prey detection in larval zebrafish. *Elife.* 2014;3. doi: ARTN e04878
10.7554/eLife.04878. PubMed PMID: WOS:000346170300001.

40. Barker AJ, Baier H. Sensorimotor Decision Making in the Zebrafish Tectum. *Curr Biol.* 2015;25(21):2804-14. doi: 10.1016/j.cub.2015.09.055. PubMed PMID: WOS:000364262500022.
41. Yilmaz M, Meister M. Rapid Innate Defensive Responses of Mice to Looming Visual Stimuli. *Curr Biol.* 2013;23(20):2011-5. doi: 10.1016/j.cub.2013.08.015. PubMed PMID: WOS:000326317300022.
42. Preuss T, Osei-Bonsu PE, Weiss SA, Wang C, Faber DS. Neural representation of object approach in a decision-making motor circuit. *J Neurosci.* 2006;26(13):3454-64. doi: 10.1523/Jneurosci.5259-05.2006. PubMed PMID: WOS:000236363400010.
43. Fraenkel GS, Gunn DL. *The Orientation of Animals, Kineses, Taxes and Compass Reactions.* . New York: Dover Publications Inc.; 1961.
44. Van Veen T, Hartwig H, Müller K. Light-dependent motor activity and photonegative behavior in the eel (*Anguilla anguilla* L.). *Journal of comparative physiology.* 1976;111(2):209-19.
45. Young J. The photoreceptors of lampreys: I. Light-sensitive fibres in the lateral line nerves. *J Exp Biol.* 1935;12(3):229-38.
46. Burgess HA, Granato M. Modulation of locomotor activity in larval zebrafish during light adaptation. *J Exp Biol.* 2007;210(Pt 14):2526-39. doi: 10.1242/jeb.003939. PubMed PMID: 17601957.
47. Prober DA, Rihel J, Onah AA, Sung RJ, Schier AF. Hypocretin/orexin overexpression induces an insomnia-like phenotype in zebrafish. *J Neurosci.* 2006;26(51):13400-10. doi: 10.1523/JNEUROSCI.4332-06.2006. PubMed PMID: 17182791.
48. Sugihara T, Nagata T, Mason B, Koyanagi M, Terakita A. Absorption Characteristics of Vertebrate Non-Visual Opsin, *Opn3*. *PLoS ONE.* 2016;11(8):e0161215. doi: 10.1371/journal.pone.0161215. PubMed PMID: 27532629; PubMed Central PMCID: PMC4988782.
49. Kato M, Sugiyama T, Sakai K, Yamashita T, Fujita H, Sato K, et al. Two Opsin 3-Related Proteins in the Chicken Retina and Brain: A TMT-Type Opsin 3 Is a Blue-Light Sensor in Retinal Horizontal Cells, Hypothalamus, and Cerebellum. *PLoS ONE.* 2016;11(11):e0163925. doi: 10.1371/journal.pone.0163925. PubMed PMID: 27861495; PubMed Central PMCID: PMC4988782.
50. Naumann EA, Fitzgerald JE, Dunn TW, Rihel J, Sompolsky H, Engert F. From Whole-Brain Data to Functional Circuit Models: The Zebrafish Optomotor Response. *Cell.* 2016;167(4):947-60 e20. Epub 2016/11/05. doi: 10.1016/j.cell.2016.10.019. PubMed PMID: 27814522; PubMed Central PMCID: PMC4988782.
51. Kim DH, Kim J, Marques JC, Grama A, Hildebrand DGC, Gu W, et al. Pan-neuronal calcium imaging with cellular resolution in freely swimming zebrafish. *Nat Methods.* 2017;14(11):1107-14. Epub 2017/09/12. doi: 10.1038/nmeth.4429. PubMed PMID: 28892088.
52. Fero K, Yokogawa T, Burgess HA. The Behavioral Repertoire of Larval Zebrafish. In: Kalueff AV, Cachat JM, editors. *Zebrafish Models in Neurobehavioral Research.* Totowa, NJ: Humana Press; 2011. p. 249-91.
53. Carvalho PSM, Noltie DB, Tillitt DE. Ontogenetic improvement of visual function in the medaka *Oryzias latipes* based on an optomotor testing system for larval and adult

fish. Anim Behav. 2002;64:1-10. doi: 10.1006/anbe.2002.3028. PubMed PMID: WOS:000178657200001.

54. Cuesta IH, Lahiri K, Lopez-Olmeda JF, Loosli F, Foulkes NS, Vallone D. Differential maturation of rhythmic clock gene expression during early development in medaka (*Oryzias latipes*). Chronobiol Int. 2014;31(4):468-78. Epub 2014/01/25. doi: 10.3109/07420528.2013.856316. PubMed PMID: 24456338.

55. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26(1):139-40. Epub 2009/11/17. doi: 10.1093/bioinformatics/btp616. PubMed PMID: 19910308; PubMed Central PMCID: PMC2796818.

56. Raj B, Wagner DE, McKenna A, Pandey S, Klein AM, Shendure J, et al. Simultaneous single-cell profiling of lineages and cell types in the vertebrate brain. Nat Biotechnol. 2018;36(5):442-50. Epub 2018/04/03. doi: 10.1038/nbt.4103. PubMed PMID: 29608178; PubMed Central PMCID: PMC5938111.

57. Nakayama T, Shimmura T, Shinomiya A, Okimura K, Takehana Y, Furukawa Y, et al. Seasonal regulation of the lncRNA LDAIR modulates self-protective behaviours during the breeding season. Nat Ecol Evol. 2019;3(5):845-52. Epub 2019/04/10. doi: 10.1038/s41559-019-0866-6. PubMed PMID: 30962562.

58. Song Y-H, Hwang Y-S, Kim K, Lee H-R, Kim J-H, Maclachlan C, et al. Somatostatin enhances visual processing and perception by suppressing excitatory inputs to parvalbumin-positive interneurons in V1. Science advances. 2020;6(17):eaaz0517-eaaz. doi: 10.1126/sciadv.aaz0517. PubMed PMID: 32494634.

59. Isoe Y, Konagaya Y, Yokoi S, Kubo T, Takeuchi H. Ontogeny and Sexual Differences in Swimming Proximity to Conspecifics in Response to Visual Cues in Medaka Fish. Zoolog Sci. 2016;33(3):246-54. Epub 2016/06/09. doi: 10.2108/zs150213. PubMed PMID: 27268978.

60. Pinto JG, Hornby KR, Jones DG, Murphy KM. Developmental changes in GABAergic mechanisms in human visual cortex across the lifespan. Front Cell Neurosci. 2010;4:16. Epub 2010/07/02. doi: 10.3389/fncel.2010.00016. PubMed PMID: 20592950; PubMed Central PMCID: PMC2893712.

61. Siu CR, Beshara SP, Jones DG, Murphy KM. Development of Glutamatergic Proteins in Human Visual Cortex across the Lifespan. J Neurosci. 2017;37(25):6031-42. Epub 2017/05/31. doi: 10.1523/JNEUROSCI.2304-16.2017. PubMed PMID: 28554889; PubMed Central PMCID: PMC6596503.

62. Dahlgren CP, Eggleston DB. Ecological Processes Underlying Ontogenetic Habitat Shifts in a Coral Reef Fish. Ecology. 2000;81(8):2227-40. doi: 10.2307/177110.

63. Nunn AD, Tewson LH, Cowx IG. The foraging ecology of larval and juvenile fishes. Reviews in Fish Biology and Fisheries. 2012;22(2):377-408. doi: 10.1007/s11160-011-9240-8.

64. Bannister S, Antonova O, Polo A, Lohs C, Hallay N, Valinciute A, et al. TALE Nucleases mediate efficient, heritable genome modifications in the marine annelid *Platynereis dumerilii*. Genetics. 2014;197(1):19-31. doi: 10.1534/genetics.112.148254.

65. Pende M, Vadiwala K, Schmidbaur H, Stockinger AW, Murawala P, Saghafi S, et al. A versatile depigmentation, clearing, and labeling method for exploring nervous system diversity. Sci Adv. 2020;6(22):eaba0365. Epub 2020/06/12. doi: 10.1126/sciadv.aba0365. PubMed PMID: 32523996; PubMed Central PMCID: PMC7259959.

66. Matsunaga W, Watanabe E. Habituation of medaka (*Oryzias latipes*) demonstrated by open-field testing. *Behav Processes*. 2010;85(2):142-50. doi: 10.1016/j.beproc.2010.06.019. PubMed PMID: WOS:000282121400008.
67. Michelson AA. *Studies in optics*: Chicago, Ill. : The University of Chicago Press; 1927.
68. Beck JC, Gilland E, Tank DW, Baker R. Quantifying the ontogeny of optokinetic and vestibuloocular behaviors in zebrafish, medaka, and goldfish. *J Neurophysiol*. 2004;92(6):3546-61. doi: 10.1152/jn.00311.2004. PubMed PMID: WOS:000225164800038.
69. Kokel D, Bryan J, Laggner C, White R, Cheung CYJ, Mateus R, et al. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nat Chem Biol*. 2010;6(3):231-7. doi: 10.1038/Nchembio.307. PubMed PMID: WOS:000274565100016.
70. Lopez-Olmeda JF, Tartaglione EV, de la Iglesia HO, Sanchez-Vazquez FJ. Feeding entrainment of food-anticipatory activity and *per1* expression in the brain and liver of zebrafish under different lighting and feeding conditions. *Chronobiol Int*. 2010;27(7):1380-400. Epub 2010/08/28. doi: 10.3109/07420528.2010.501926. PubMed PMID: 20795882.
71. Anken R, Bourrat F. *Brain atlas of the Medakafish *Oryzias latipes**. Paris: Institut national de la recherche agronomique; 1998.
72. Sedlazeck FJ, Rescheneder P, von Haeseler A. NextGenMap: fast and accurate read mapping in highly polymorphic genomes. *Bioinformatics*. 2013;29(21):2790-1. doi: 10.1093/bioinformatics/btt468. PubMed PMID: 23975764.
73. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30(7):923-30. doi: 10.1093/bioinformatics/btt656. PubMed PMID: WOS:000334078300005.
74. Tessmar-Raible K, Steinmetz PR, Snyman H, Hassel M, Arendt D. Fluorescent two-color whole mount in situ hybridization in *Platynereis dumerilii* (Polychaeta, Annelida), an emerging marine molecular model for evolution and development. *Biotechniques*. 2005;39(4):460, 2, 4. Epub 2005/10/21. PubMed PMID: 16235555.
75. Bai Y, Liu H, Huang B, Wagle M, Guo S. Identification of environmental stressors and validation of light preference as a measure of anxiety in larval zebrafish. *BMC Neurosci*. 2016;17(1):63. doi: 10.1186/s12868-016-0298-z.
76. Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res*. 2016;44(W1):W232-5. Epub 2016/04/17. doi: 10.1093/nar/gkw256. PubMed PMID: 27084950; PubMed Central PMCID: PMC4987875.
77. Qayum HA, Klimley AP, Newton R, Richert JE. Broad-band versus narrow-band irradiance for estimating latitude by archival tags. *Marine Biology*. 2007;151(2):467-81. doi: 10.1007/s00227-006-0514-y.
78. Kiang NY, Siefert J, Govindjee, Blankenship RE. Spectral signatures of photosynthesis. I. Review of Earth organisms. *Astrobiology*. 2007;7(1):222-51. Epub 2007/04/05. doi: 10.1089/ast.2006.0105. PubMed PMID: 17407409.

Figure legends

Figure 1: Mutations in *Ola-tmt-opsin1b* cause light-dependent differences in an avoidance response test.

(A) Genomic locus of the *Ola-tmt-opsin1b* gene and corresponding mutant alterations. TALEN binding sites: black boxes; Exons: grey boxes; Inserted/substituted nucleotides: red; deleted nucleotides: “-”. (B) Confocal images of anti-Ola-TMT-1b (green) and nuclear DAPI (blue) staining in wildtype and *tmt-opsin1b* (D-2/D-2) mutant coronal tectal slices. SPV: stratum periventriculare. Scale bars: 20 μ m. (C) Schematic of the behavioral setup. (D) Size discrimination tuning curve of larvae reacting to moving dots presented in ascending size order. (E) Behavioral analysis as in (D), performed in a different laboratory with a different computer screen and surrounding (Light intensity = 2.2×10^{13} photons/m²/sec). (F) Avoidance responses to the dots displayed in a shuffled size manner. (G) Avoidance responses in the ascending dot size paradigm with background light reduced to 35% of its initial intensity (35% light intensity = 0.77×10^{13} photons/m²/sec). (H) Total avoidance (area under the curve) under different light intensities. (I) Spectra of the emitted computer screen light used for the 100% (black) and the 35% light intensity (orange). Data presentation: (D-G) mean ($\pm$ s.e.m.); (H) single bar: median, box: first and third quartile, whiskers: min and max; all assays done with 9-12 dpf larvae. a.u.: arbitrary units. For further details on mutants, responses to contrast changes, spectra and mutant vs. wildtype development see Supplementary Figures 1-3.

Figure 2: Mutations in *Ola-tmt-opsin1b* impact on activity levels upon light changes in a partly age-dependent manner

(A-G, H,J,L,N,O) Average distance moved and corresponding statistical analysis during alternating 30 minutes white light or monochromatic light/ dark intervals from juvenile fish (A-G,H,J,L) and larvae (O,P). Each data point (A,C,E-G,N) represents the mean ($\pm$ s.e.m.) distance moved for the preceding 10 seconds. Colored boxes on x-axis indicate light condition. (B,D,H,J,L,O) Locomotor activity measured as area under the curve: AUC during the entire light vs. dark intervals of the trial. **** $P \leq 0.0001$; ** $P \leq 0.01$. (I,K,M) Plotted spectra of the different light conditions used in E-G,H,J,L. The sum of photons is shown next to the respective spectrum. (P,Q) Cortisol levels measured as cortisol (ug/ml) over total protein levels (ug/ml) under baseline and mechanically disturbed (MD)

conditions from larvae (N) and juvenile fish (O). $*P \leq 0.05$. For all panels, (black) *tmt-opsin1b* and (red) *tmt-opsin1b* homozygous single mutant. Box plots: single bar: median, box: first and third quartile, whiskers: min and max; n.s.: no significance; a.u.: arbitrary units. For further details on spectra and age-dependent differences see Supplementary Figures 3, 4.

Figure 3: *Tmt-opsin1b* affects the amount of daytime rest, independently of eyes and the circadian core clock

(A,C,G) Average distance moved during 8 hours dark / 16 hours blue light periods for two consecutive days in larvae (A), juvenile fish (C) and enucleated juvenile fish (G). Each data point represents the mean ($\pm$ s.e.m.) distance moved for the preceding 1 minute. Colored boxes along the x-axis represent light condition. (B,D,H) Locomotor activity (measured as AUC) during the first 30min and 90min after light is turned ON during the 2 days trial. (B) $*P_{(8-9,5h)} = 0.0331$; $*P_{(32-32.5h)} = 0.0247$; $*P_{(32-33.5h)} = 0.0323$; (D) $*P_{(8-8.5h)} = 0.0330$; $*P_{(8-9,5h)} = 0.0299$; $***P_{(32-32.5h)} = 0.0002$; (F) $*P_{(32-32.5h)} = 0.0192$; $**P_{(32-33.5h)} = 0.0056$. (E,F) Relative mRNA levels of *per1b* (E) and *reverb* (F) measured from whole larvae entrained to 16:8 white light/dark cycles. Yellow and gray bars: light vs dark conditions during respective sampling. Gray arrows indicate the peaks and troughs of the curve. For all panels, (black) *tmt-opsin1b* and (red) *tmt-opsin1b* homozygous single mutant. Box plots: single bar: median, box: first and third quartile, whiskers: min and max; n.s.: no significance; a.u.: arbitrary units. For further details on spectra and individual, more time-zoomed movement plots see Supplementary Figures 3,5.

Figure 4: Individual and combined differential impact of *Ola-tmt-opsin1b* and *Ola-tmt-opsin2* on daytime rest levels

(A) Schematized lateral view of an adult medaka brain. Dashed lines: position of sections in B,C and D,E. NDIL: nucleus diffuses of lobus inferiosis (of hypothalamus). (B,C,D,E) *In situ* hybridization (ISH) of *tmt-opsin1b* (B,D) and *tmt-opsin2* (C,E) on coronal sections from Cab wildtype fish. SPV: *stratum periventriculare*. Scale bars: 100 μ m. For detailed anatomical annotations see [14] (F) Genomic locus of the *Olatmt-opsin2* gene and corresponding mutant. TALEN binding sites: black boxes; Exons: blue boxes; Inserted/substituted nucleotides: red; deleted nucleotides: “-”. (G,I) Average distance moved profile during a 2 consecutive days trial on a 16h:8h blue-light/dark photoperiod of (G) 10-13 dpf larvae and (I) 21-23 dpf juvenile fish. Each data point represent the mean ($\pm$ s.e.m.) distance moved for the preceding 1 minute. Colored boxes

along the x-axis represent light condition. **(H,J)** Locomotor activity (assessed by area under the curve) during the first 30 minutes or 90 minutes after light is turned ON along the 2 days trial of **(H)** larvae and **(J)** juvenile fish. Arrows highlight the data from the double-mutants to be compared to the respective single mutants and wt. For all panels: **** $P < 0.0001$. Box plots: single bar: median, box: first and third quartile, whiskers: min and max; n.s.: no significance; a.u.: arbitrary units. For further details on mutant, spectra and individual, more time-zoomed movement plots see Supplementary Figures 1,3,5.

Figure 5: *Ola-tmt-opsin1b,2* double mutants are compensated for differential transcript changes present in *Ola-tmt-opsin1b* and *Ola-tmt-opsin2* single mutants.

(A) Schematic drawing of the adult medaka brain in dorsal view. Dotted lines indicate the dissection boundaries (TE: telencephalon; P: pineal organ; OT: optic tectum; CC: corpus cerebelli). Note: part of the posterior hypothalamus is dissected with the midbrain, as it is attached to its ventral part and technically unfeasible to reliably separate. **(B)** Normalized transcript read counts (**** adjusted P-value < 0.001) for *sst1b*. **(C)** mRNA transcript levels for *sst1b* in brain areas and eyes. Decreased *sst1b*-levels are unique to *tmt-opsin1b*^{-/-} mutants and are compensated by the *tmt-opsin2*^{-/-} mutant. **(D)** Normalized transcript read counts (**** adjusted P-value < 0.001) for *scn12aa*. **(E)** mRNA transcript levels for *scn12aa* in brain areas and eyes. Increased levels of *scn12aa* are unique to *tmt-opsin2*^{-/-} mutants, and compensated for when both *tmt-opsin1b* and *tmt-opsin2* are jointly mutated. Box plots: single bar: median, box: first and third quartile, whiskers: min and max, each displayed data point corresponds to one individual biological replicate. a.u.: arbitrary units.

Figure 6: *sst1b* levels are reduced in specific cells in the tectum, posterior hypothalamus and hindbrain in *tmt-opsin1b*^{-/-} medaka brains.

In situ hybridization for *sst1b* performed on coronal sections of midbrain **(A)** and hindbrain **(B)** from Cab wildtype fish. Magnification of boxed areas of the optic tectum are displayed below. SPV: stratum periventriculare. Note that *sst1b* identify a distinct tectal population of dorsal cells and on the ventral area of the hindbrain. **(C,D)** Comparison of *sst1b* expression in wildtype vs. *tmt-opsin1b* mutant sibling coronal brain sections reveals specific reduction (denoted by arrows) in interneurons of the tectum and reticular formation, and in the posterior hypothalamus. Scale bars: 100um. For further details on anatomical annotations, co-localization of *sst1b* with *gad2*, as well as *Scn12aa* orthology relationships and localization see Supplementary Figures 6-9.

Figure 7: *tmt-opsin1b* sensitizes *sst1b* regulation to changes in photoperiod

(A) Schematic representation of the two different photoperiods assigned to different cohorts of *tmt-opsin1b* wildtype and *tmt-opsin1b* mutant adult fish. Black boxes: dark period; White boxes: white light period. Red arrowheads: feeding time; Blue arrowheads: time when fish were sacrificed and the brain dissections made. (B) Light spectrum of the light used in (A). (C) Relative mRNA expression of *sst1b* in larvae sampled during constant darkness after entraining with 16:8 hour white light/dark cycle at 27°C. (D,E) mRNA *sst1b* transcript expression levels in *tmt-opsin1b* wildtype (D), *tmt-opsin1b* mutant siblings (E). **** $P \leq 0.0001$, ** $P \leq 0.01$, * $P \leq 0.05$. Box plots: single bar: median, box: first and third quartile, whiskers: min and max. Each data point represents one biological replicate. For further details on spectra see Supplementary Figures 3.

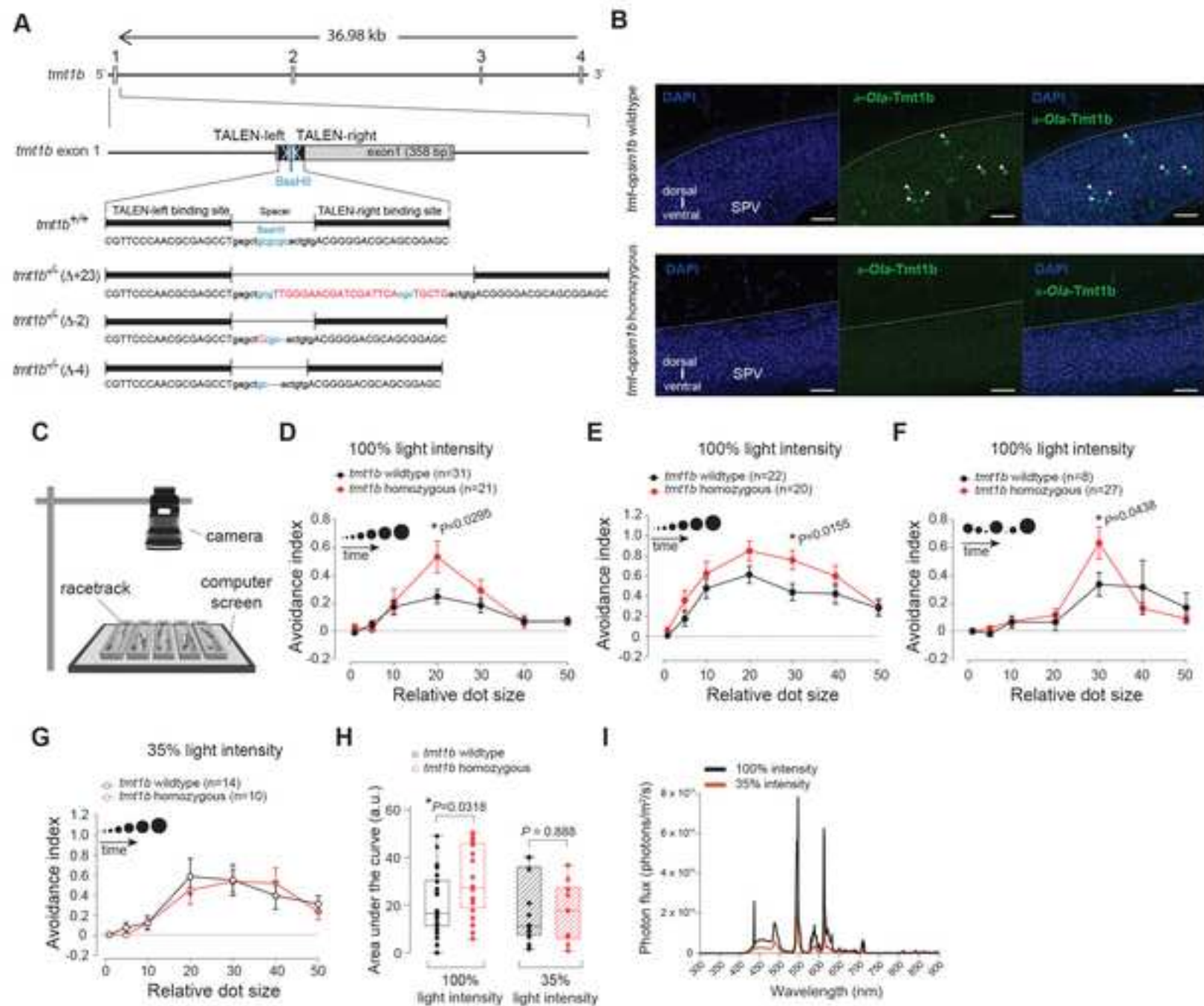


FIGURE 1

Figure 2

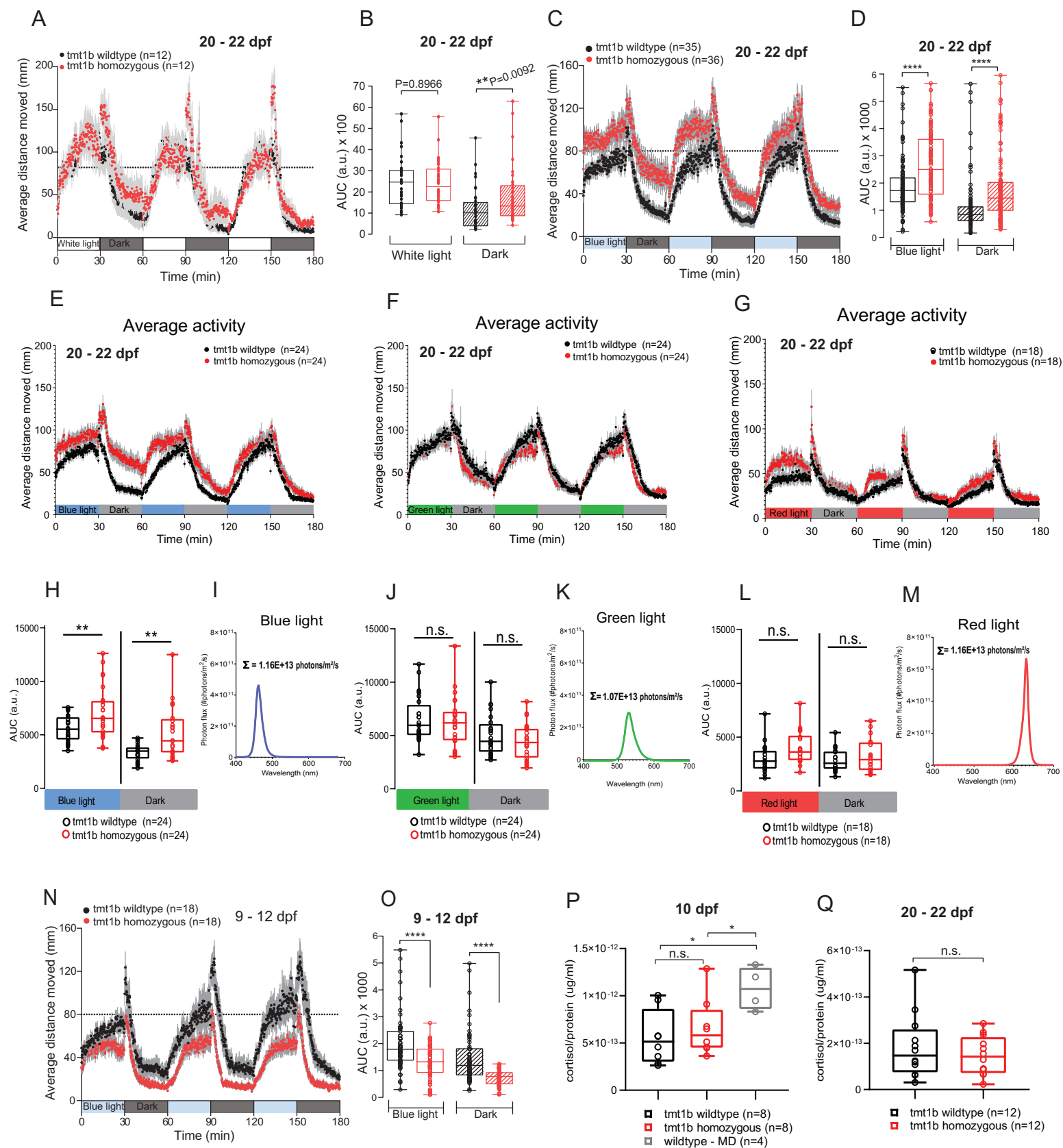


FIGURE 2

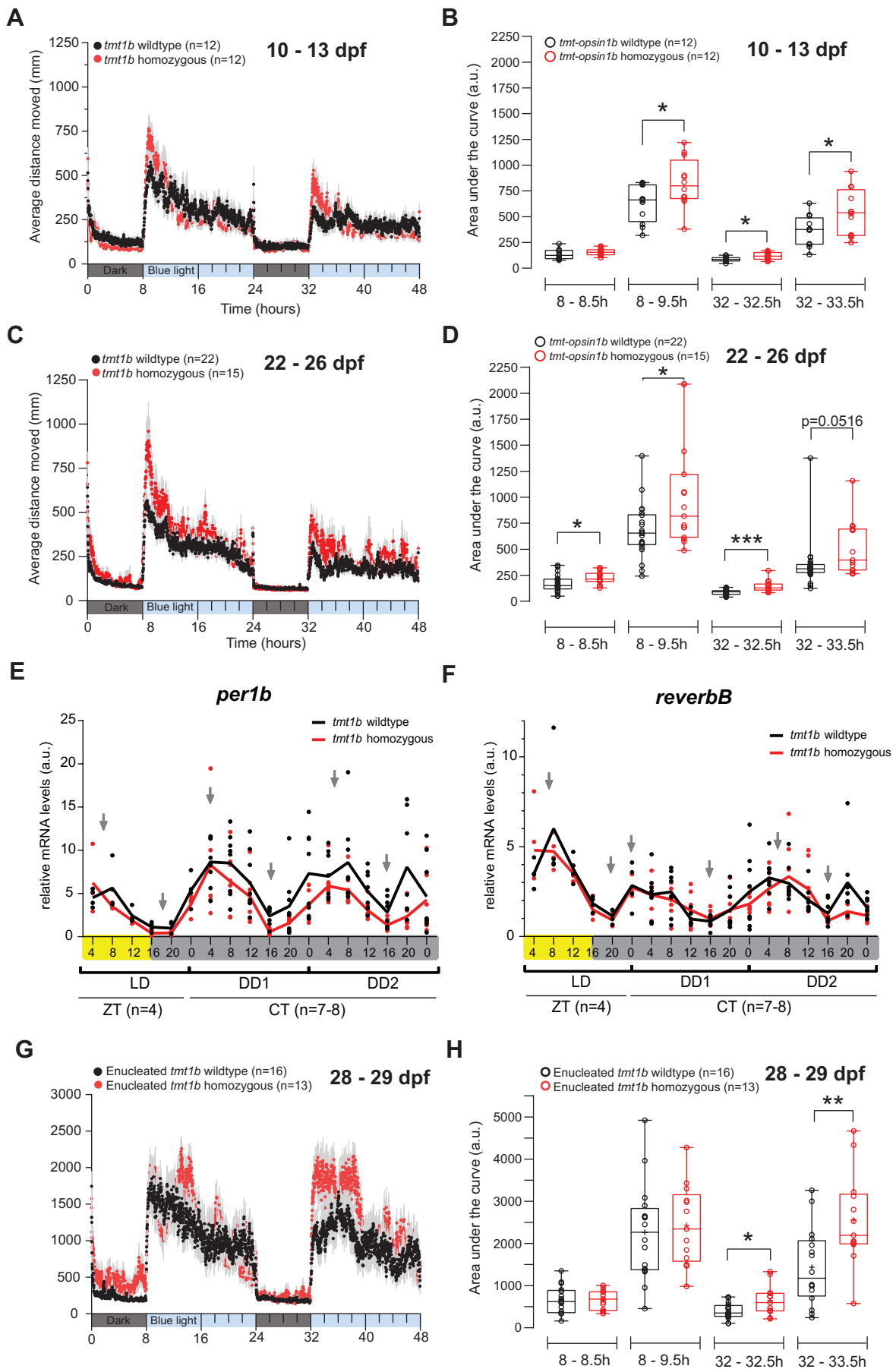


FIGURE 3

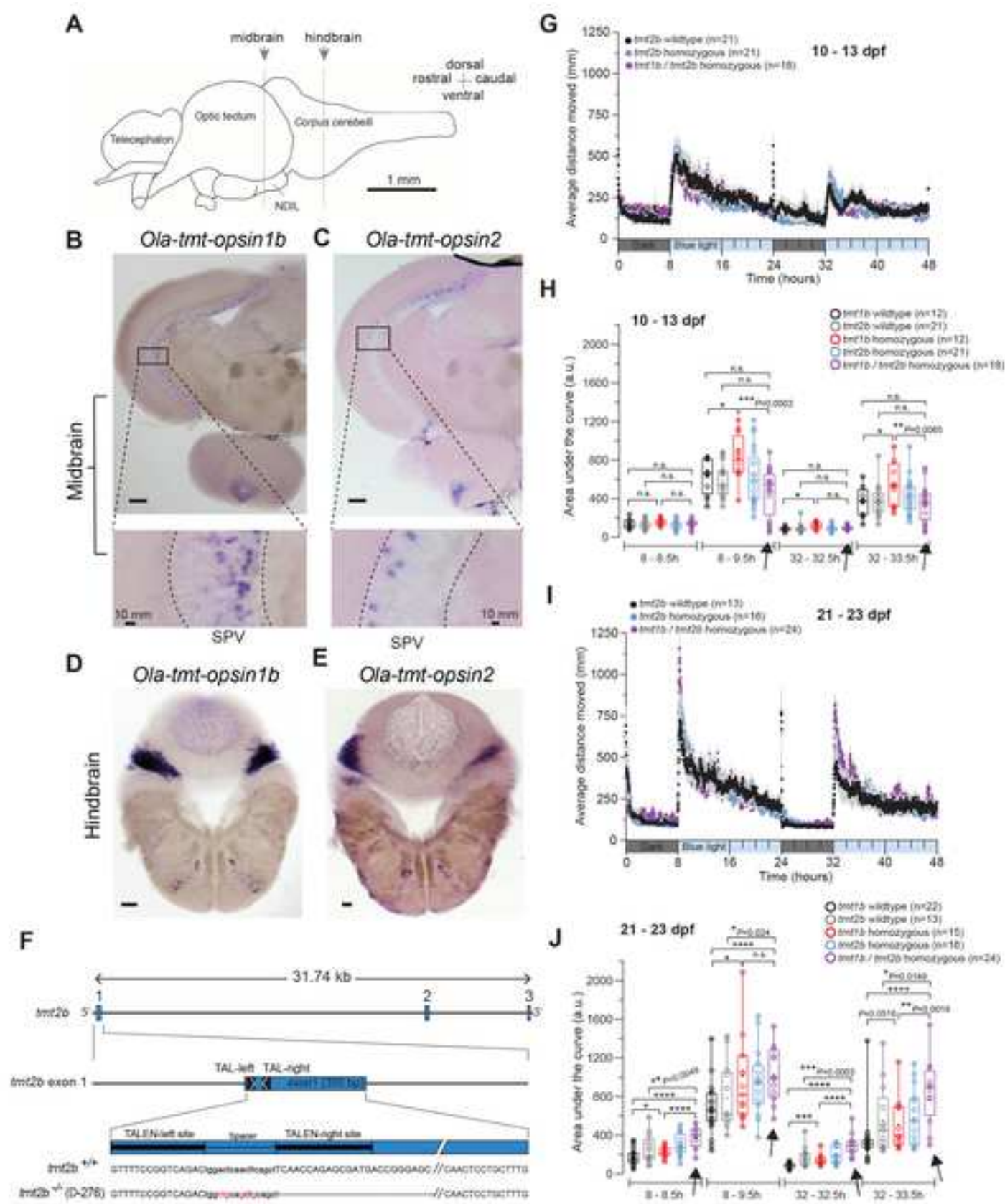


FIGURE 4

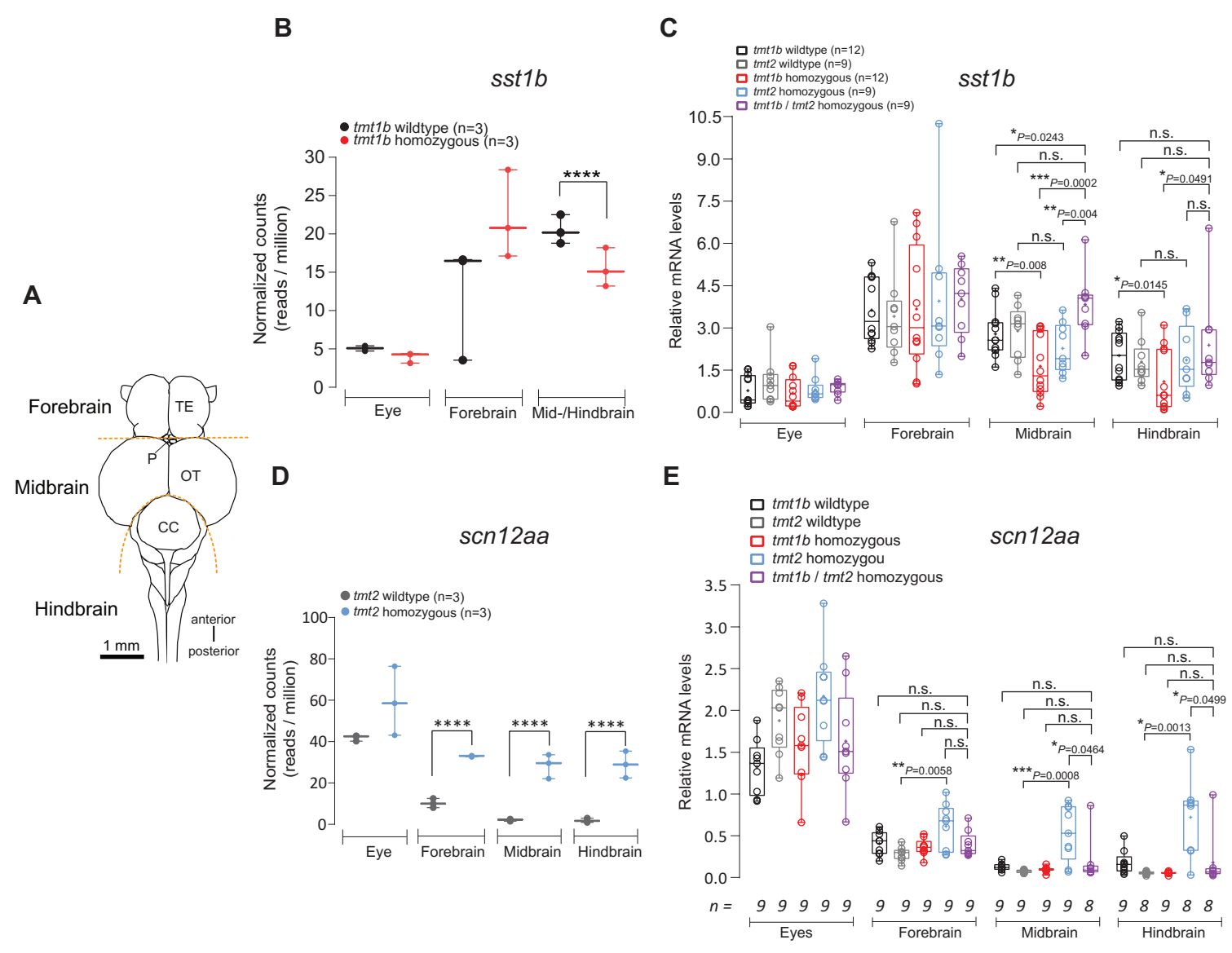


FIGURE 5

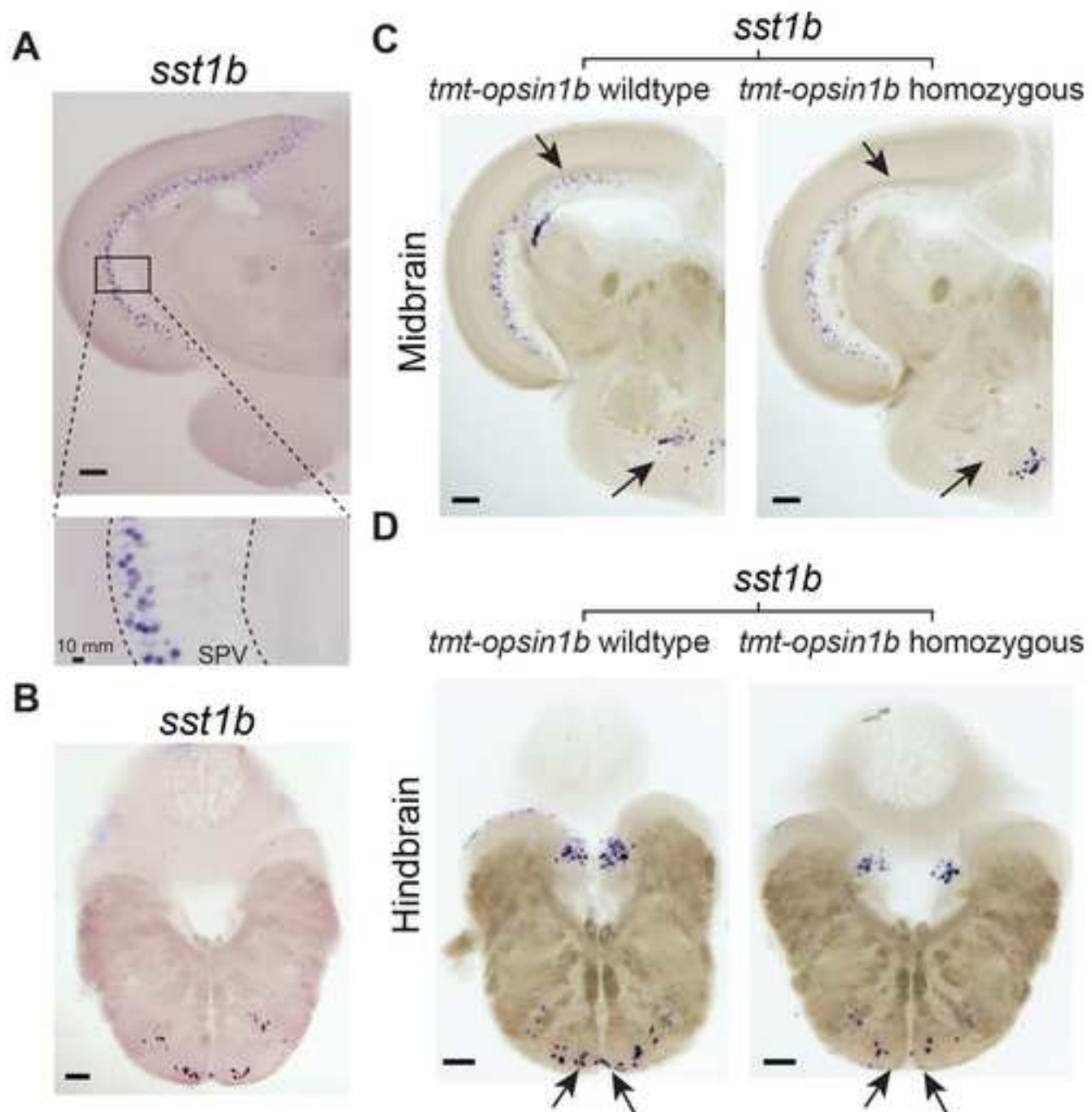


FIGURE 6

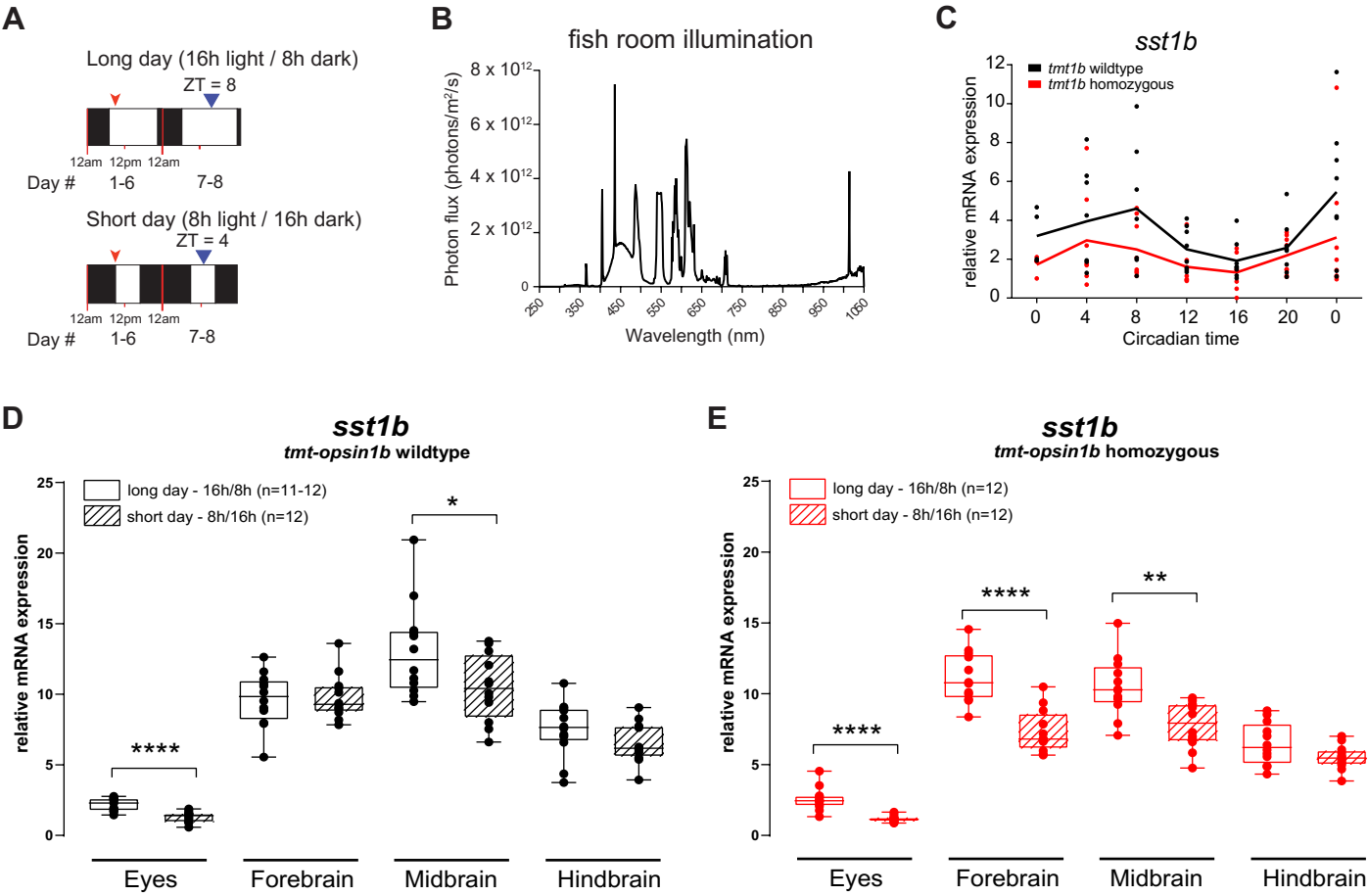
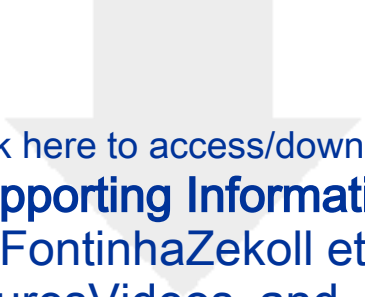


FIGURE 7

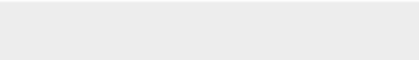
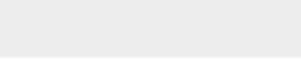


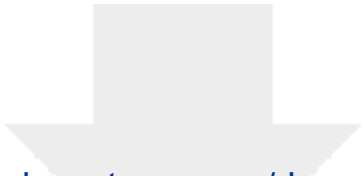
Click here to access/download

Supporting Information

FontinhaZekoll et

al_SuppFiguresVideos_and_legends.pdf

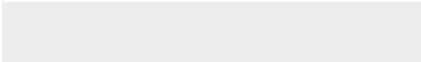


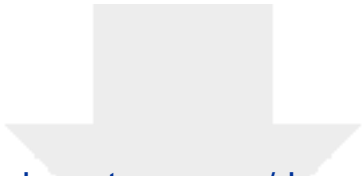


[Click here to access/download](#)

Supporting Information

tmt1b_Forebrain_DE_genes.xlsx

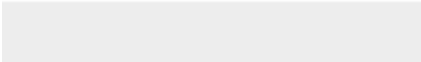




[Click here to access/download](#)

Supporting Information

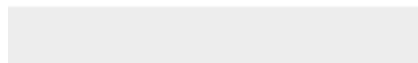
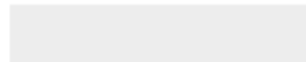
tmt1b_mid_hindbrain_DE_genes.xlsx





[Click here to access/download](#)

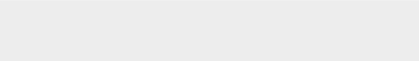
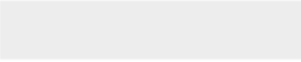
Supporting Information - Compressed/ZIP File Archive
supplemental videos_cropped.zip





[Click here to access/download](#)

Supporting Information - Compressed/ZIP File Archive
metadata_final.zip





[Click here to access/download](#)

Related Manuscript

FontinhaZekoll et al_ms_final2_main
fig_legends_trch.docx