

SELECTIVE NEUROGENESIS IN THE ZEBRAFISH OLFACTORY EPITHELIUM

by

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B.S., Molecular Biology and Genetics, Boğaziçi University, 2013

Submitted to the Institute of Graduate Studies
in Science and Engineering in partial fulfillment
of the requirements for the degree
Master of Science

Graduate Program in Molecular Biology and Genetics

Boğaziçi University

2015

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Stefan Fuss, for his assistance and guidance over the course of my MSc life. He helped me along more than 2 years in every sense he can: He has taught me not only how to be a good scientist but also how to communicate with people, how to be a good friend, and even how to cook. His supervision and encouragement are beyond the price.

I would like to thank my teammates, Xalid Bayramlı, Burak Bali and Serdar Çapar from the so-called “team neurogenesis” in which we shared more than a project. The captain of the team Xalid Bayramlı has been like a big brother for me and saved my life many times. The other team members and my dearest friends, Burak and Serdar, were always there for me when I needed them. Each and every single of our adventures and memories together in different places like restaurants, pubs, beaches, seminars and hospitals are very precious to me.

I want to thank people from the fly lab; Çağrı Çevrim and Bahar Şahin for comforting and encouraging me at any time I needed. I also want to thank Ayşe Candayan for teaching me fly genetics – which I needed to learn for my internship – and for her friendly and warm conversations. I would like to thank my seniors, Kerem Uzel for teaching me the methods of handling the zebrafish embryos, Gizem Sancer for sharing the same concerns with me as a female scientist, helping me organizing each corner of the lab and fish room.

I also would like to thank my family for allowing me follow my dreams, supporting and believing in me all the time and loving me unrequitedly, and my roommate Esra Yıldız for taking me to peaceful places easing my mind. Last but not the least; I also want to thank my distant friends Şeydanur Tıkır and Nurşen Balekoğlu for always being with me and for their scientific talks in which we saved the world many times with our brainstorm. Finally, I wish to thank TUBITAK for supporting me. The work on this project was supported by TUBITAK project 113T038 to Dr. Stefan H. Fuss.

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ABSTRACT

SELECTIVE NEUROGENESIS IN THE ZEBRAFISH OLFACTORY EPITHELIUM

Neurogenesis in the adult nervous system is a limited process in higher vertebrates. The olfactory epithelium (OE) is an exception to this limitation and maintains the ability to regenerate lost neurons throughout the lifetime of the organism. Chemosensory neurons have a limited lifespan and need to be replaced continuously by new neurons. The zebrafish OE contains at least four morphologically distinct types of chemosensory cells that are molecularly characterized by their expression of different chemoreceptors. Even though a basic understanding of molecular events involved olfactory neurogenesis has emerged, little is known about the cell type specificity of this process. To address the question whether stem cell / progenitor activity in the OE is cell type-specific, an experimental paradigm to selectively ablate distinct subpopulations of chemosensory neurons *in vivo* was devised. To this end, transient transgenic zebrafish embryos expressing the bacterial nitroreductase (NTR) gene under the control of the cell type-specific OMP promoter were generated. NTR converts the prodrug metronidazole (MTZ) into a cytotoxic product that selectively kills the cells that express the NTR enzyme. The studies presented in this thesis aim to understand, if a selective loss of OMP expressing ciliated cells can be achieved and if this loss results in increased neurogenesis to selective replace the lost cell type. Results of MTZ treatment in early NTR-expressing embryos suggest that MTZ treatment efficiently and specifically kills transgenic neurons. However, the rate of ongoing neurogenesis during early embryonic development masks the effect of cell ablation, especially at later experimental time points. Transgenic constructs expressing the photo-convertible fluorescent protein dendra2 along with NTR should enable the distinction between previously existing cells and cells that were born after the onset of metronidazole treatment. Yet, the limited stability of converted dendra2 in sensory neurons limits this analysis to short experimental time windows.

ÖZET

ZEBRABALIĞI OLFAKTÖR DOKUSUNDA SEÇİCİ NÖROGENEZ

Yetişkin sinir sisteminde nörogenez sınırlı bir süreçtir. Olfaktör epiteli (OE) bu sınırlamada istisnadır ve organizmanın yaşamı boyunca kaybolan nöronları yenileme kapasitesini korur. Kimyasal algılayıcı nöronlar sınırlı bir ömre sahiptir ve sürekli yeni nöronlarla yer değiştirilmesi gerekmektedir. Zebrabalığı OE farklı kimyasal alıcı ifadelerine göre sınıflandırılabilen, morfolojik olarak birbirinden farklı en az dört farklı kimyasal algılayıcı hücrelere sahiptir. Olfaktör nörogenezinde temel moleküler mekanizmalar ortaya çıkarılmışsa da, bu olayın hücre tiplerine özel olup olmadığı hakkında henüz çok az şey biliniyor. Olfaktör dokudaki kök hücre aktivitesinin hücre tipine özel olup olmadığı sorusunu cevaplamak adına, canlı içerisinde belli hücre gruplarını seçici olarak öldürecek deneysel bir paradigma tasarlandı. Bu amaçla geçici olarak OMP promotorunun kontrolü altında bakteriyel NTR genini ifade eden transgenik zebrabalıkları oluşturuldu. NTR geni, metronidazol (MTZ) olarak bilinen ön ilacı hücre için toksin olan bir ürüne çevirir ve seçici olarak NTR genini ifade eden hücreleri öldürür. Bu tezde sunulan araştırmalar, OMP ifade eden silli hücreleri başarılı şekilde öldürülebileceğini ve bu kaybın seçici olarak öldürülen hücreleri tekrar yerine koymak için nörogenezde bir artışa sebep olacağını anlamayı amaçlar. NTR ifade eden embriyolar üzerindeki MTZ uygulama sonuçları, MTZ uygulamasının NTR ifade eden transgenik hücreleri özel ve verimli bir şekilde öldürebildiğini ileri sürüyor. Ancak embriyoların erken gelişim sürecindeki süregelen nörogenez oranı, özellikle deneyin ilerleyen zaman diliminde, hücre öldürülme etkisini maskeliyor. NTR ile birlikte ışıkla çevrilebilen florasan proteini, dendra2'yi ifade eden transgenik plasmid, önceden var olan hücreler ile MTZ uygulamasının başlangıcından sonra doğan hücreleri ayırmayı sağlaması bekleniyordu. Yine de, algı nöronlarındaki çevrilmiş dendra2 proteinin sınırlı kararlılığı, kısa deneysel zaman diliminde bu analizi sınırlandırıyor.

LIST OF ACRONYMS / ABBREVIATIONS

BAC	Bacterial Artificial Chromosome
bp	base pair
Cas3	Caspase 3
GBC	Globose Basal Cells
GFP	Green Fluorescent Protein
HBC	Horizontal Basal Cells
kb	kilo base pair
MTZ	Metronidazole
mut	mutant
NTR	Nitroreductase
OE	Olfactory Epithelium
OMP	Olfactory Marker Protein
OR	Olfactory Receptor
OSN	Olfactory Sensory Neuron
PCR	Polymerase Chain Reaction
SC	Sustentacular Cells
T2A	TaV 2A
TRPC2	Trans Receptor Potential Channel 2
UV	Ultra Violet
VNO	Vomer nasal Organ
wt	wild type

1. INTRODUCTION

1.1. Neurogenesis

Neurogenesis, the process describing the generation of mature and functional nerve cells from stem cells and undifferentiated progenitor cells, was initially considered to be a restricted phenomenon that only occurs during early development. However, in the early 1960s, it was discovered that neurogenesis continues in the adult mammalian brain, using [^3H]-thymidine labeling of newly synthesized DNA. Yet, in mammals adult neurogenesis is largely restricted to specific brain regions such as the hippocampus and the subventricular zone of the forebrain (Altman *et al.*, 1969). Neurogenesis a complex, multistep process in which the birth of new nerve cell is followed by differentiation, maturation, migration, and integration to the system (Fuentesalba *et al.*, 2012; Schmidt *et al.*, 2013). Although neurogenesis has been documented in several brain regions of rodents and non-human primates (Palmer *et al.*, 1995; Pencea *et al.*, 2001; Reynolds *et al.*, 1992), neurogenesis in the human brain is more restricted (Quinones-Hinojosa *et al.*, 2006; Boldrini *et al.*, 2009). Generally, the adult neurogenic potential appears to be a function of the evolutionary status of an organism, with lower organisms exhibiting a higher regenerative potential overall.

1.2. Neurogenesis in zebrafish

In contrast to mammals, lower vertebrates such as amphibians and teleost fish generally have a much higher capacity to regenerate both, axons (Meyer *et al.*, 1985; Stuermer *et al.*, 1992; Becker *et al.*, 1997; Doyle *et al.*, 2001; Matsukawa *et al.*, 2004) and neurons (Anderson *et al.*, 1983, 1987; Hitchcock *et al.*, 1992; Zupanc and Ott, 1999; Cameron, 2000). Zebrafish, a teleost, is also special in the sense that it can regenerate parts of the central nervous system, such as cerebellum, telencephalon, and olfactory bulb (Zupanc, 2006; Diotel *et al.*, 2013). Adult stem cells maintain their neurogenic activity in many proliferative areas within these brains areas. The production of new neurons is

supported by the persistence of radial glial cell in the adult zebrafish central nervous system (Pellegrini *et al.*, 2007; Onteniente *et al.*, 1983). A remarkable increase in cell proliferation can be observed and newly produced neurons repopulate the tissue when mechanical or chemical injuries damage neural tissue such as brain and spinal cord (Zupanc *et al.*, 2011, Kizil *et al.*, 2012). Considering the differences in the regeneration potential between mammals and fish, zebrafish constitutes an attractive model for regeneration studies that might lead to an understanding of how neurogenic activity can be regained or induced in mammals as well.

1.3. Anatomy of the olfactory tissue

The main olfactory system is one of the sensory systems that mediates the recognition of chemical substances from the environment by sending neuronal signals from the nose to the brain. The olfactory epithelium (OE) is a specialized neuronal tissue that consists of specialized populations of stem and precursor cells, immature and mature olfactory sensory neurons (OSNs), and a special type of glia, the sustentacular cells (Calof *et al.*, 2002). The first step of odor detection is initiated by the binding of a chemical ligand to chemosensory receptors in OSNs that is followed by subsequent signal transduction and action potential propagation to the brain (Figure 1.1; Axel, 1995). Interaction of odorants and OSNs occurs specialized ciliary protrusions that extend to the lumen of the nasal cavity and in which are rich in olfactory receptor protein.

One of the distinct characteristics of the vertebrate and some invertebrate olfactory systems is that each individual OSN expresses only single olfactory receptor (OR) gene from a large genomic repertoire of OR genes, a feature often referred to as the “one neuron-one receptor” rule in the olfactory system (Malnic *et al.*, 1999; Mombaerts, 2004). OSNs project a single, unbranched axon to the olfactory bulb and axons of the OSN that expresses the same OR converge onto the same target structures, called glomeruli (Ressler *et al.*, 1994; Vassar *et al.*, 1994, Wang *et al.*, 1996).

1.3.1. Neurogenesis in the olfactory epithelium

The OE is one of the most active neurogenic tissues and neurogenesis occurs throughout the lifetime of the organism. OSNs are directly exposed to the environment and are continuously prone to environmental insult. As a consequence of this vulnerability, OSNs have a limited life span and need to be renewed constantly to maintain the sense of smell. Life time estimates range from 30 to 90 days in rat and mouse (Graziadei and Graziadei, 1979; Mackay-Sim and Kittel, 1991; Tsai and Barnea, 2014).

The OE accommodates a community of proliferating cells; globose basal cells (GBC) which are thought to have stem cell potential (Calof *et al.*, 2002), a second responsive stem cell population, horizontal basal cells (HBC), which proliferate to injuries, and sustentacular cells (SC) which were implicated in signaling between stem cells and OE tissue (Leung *et al.*, 2007; Suzuki *et al.*, 2013).

GBCs are fast-cycling cells that are believed to be the direct precursors of immature OSNs and may contribute mainly to tissue maintenance as a consequence of the natural turnover of OSNs. They are residing in the basal layer in the rodent OE whereas immature and the mature OSNs are located more apically (Sato *et al.*, 2005). At least a subpopulation of GBCs has stem cell characteristics and contributes to ongoing neurogenesis by asymmetrical cell divisions. One daughter cell will differentiate into transit amplifying precursors, which migrate apically as they mature. The progenitor daughter then undergoes rounds of cell division producing immature OSNs (iOSN), which fully mature into OSNs (Calof *et al.*, 2002; Figure 1.1).

1.3.2. Neurogenesis in the zebrafish olfactory epithelium

Unlike the situation in the rodent epithelium, much less is known about neurogenesis in the zebrafish OE but the epithelial distribution of stem cells and precursors

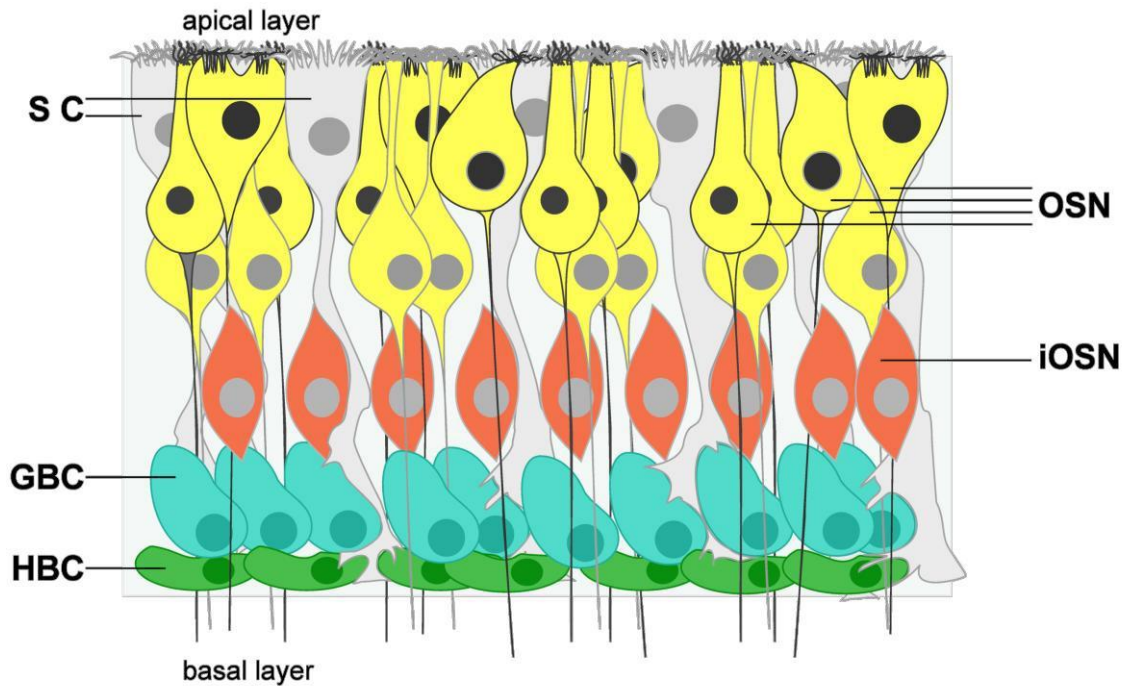


Figure 1.1. Cellular anatomy of the olfactory epithelium. Representation of the different cell types in the olfactory epithelium (adopted from Paschaki *et al.*, 2013): Globose basal cell (GBC) and horizontal basal cells (HBC), both considered as stem cells are located in the basal layer of the OE while mature immature (iOSN) and mature olfactory sensory neurons (OSN) adopt progressively apical positions.

appears to be organized differently. In the adult zebrafish OE, chemosensory neurons are restricted in the central region of the olfactory tissue and are surrounded nonsensory tissue. The sensory region stains positive for mature neuronal markers, such as olfactory marker protein (Celik *et al.*, 2002) or the pan-neuronal RNA binding protein HuC/D (Figure 1.2.a and b). The central sensory region is surrounded by peripheral non-sensory tissue, which is in part equivalent to the respiratory epithelium of the mammalian OE.

Cell proliferation assays in the zebrafish olfactory epithelium have shown that dividing cells reside in two specific regions, close to the peripheral sensory/non-sensory boundary and in the central curves between lamellae (Byrd and Brunjes, 2001, Iqbal and Byrd-Jacobs, 2010; Oehlmann *et al.*, 2004; Bayramli, unpublished). Cells generated in these proliferation zones then migrate towards the center of the sensory region while they

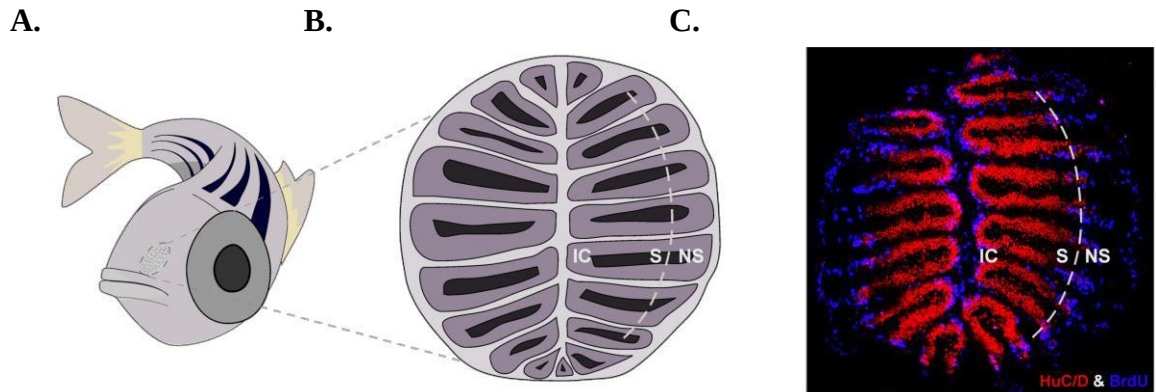


Figure 1.2. Depiction of adult zebrafish OE and spatial organization of the proliferating cells. (A) Illustration of the adult zebrafish (from left-anterior). (B) Closer view of the cross section of adult zebrafish OE. (C) Confocal stack of the OE demonstrating the spatial organization: HuC/D (in red) labels neurons, and BrdU positive cells (in blue) highlights cell proliferation (S/NS= sensory/non-sensory; IC= inner curve; Çapar, unpublished data).

mature (Bayramlı, unpublished). Thus, the baso-ventral migration of newly generated OSNs, which is typical for the rodent OE, is replaced by a radial migration in the zebrafish OE starting from two distinct proliferation zones, similar to the situation in the rodent vomeronasal organ (VNO; Calof *et al.*, 2002)

When the zebrafish OE is compared to that of higher vertebrates, it shares common features (Baier and Korsching, 1994; Weth *et al.*, 1996; Saraiva *et al.*, 2015) but also shows fish-specific peculiarities. In rodents, several anatomically distinct structures detect various chemical cues from the environment. One such chemosensory organ is the vomeronasal organ, which is specialized in the detection of pheromones and kairomones (Firestein *et al.*, 2001). In zebrafish a single chemosensory organ recognizes both, odorants and pheromones. Yet, comparative transcriptome analysis of zebrafish and mouse revealed that basic molecular and cellular signatures are conserved among both species (Saravia *et al.*, 2015).

In addition to the regionalized cell proliferation and maturation at each end of the sensory lamellae, induced neurogenesis appears to take place along the entire lamellae including the sensory region (Iqbal and Byrd-Jacobs, 2010; Çapar, unpublished).

Unpublished work from our laboratory shows that, when the tissue is damaged by irrigation with the toxic detergent Triton X-100, proliferation responses that are distinct from proliferation observed during maintenance of the tissue are induced. However, the nature of the cell populations that respond to acute injury are still not well defined and it awaits further investigation which type of precursors in the rodent OE they correspond to. It is likely but needs to be shown that different stem cell populations contribute to the production of the two different modes of neurogenesis.

1.3.3. Selective neurogenesis in zebrafish olfactory epithelium

Different from the rodent OE, the single zebrafish epithelium harbors a variety of different chemosensory subsystems that are located in distinct anatomical structures in mouse and rat. Accordingly, the zebrafish OE is heterogeneous with respect to the cell types it harbors and to molecular marker expression that is characteristic for the different chemosensory subsystems. So far, a total of four distinct types of chemosensory neurons have been classified in the zebrafish OE (Sato *et al.*, 2005; Ahuja *et al.*, 2014; Hansen *et al.*, 2000). The ciliated and microvillus cells (Figure 1.3) are the two major types of OSNs and are very distinct from each other with respect to their morphology, signal transduction machinery, and axonal projection patterns. Cell bodies of ciliated OSNs are located closer to the basal lamina compared to the microvillus cells, which occupy apical sections of the OE. Ciliated OSNs express classical OR and TAAR chemoreceptors and olfactory marker protein (OMP), while the microvillus cells express VR-type receptors and transient receptor potential channel C2 (TRPC2; Sato *et al.*, 2005). Two other types of chemosensory neurons, crypt and kappe neurons were described in more functional and molecular detail more recently (Ahuja *et al.*, 2014; Parisi *et al.*, 2014; Catania *et al.*, 2003). Cell bodies of crypt neurons have a heart-like shape whereas the kappe neurons have a more hat-like appearance and show immunoreactivity to certain antibodies; crypt neurons can be recognized by their immunoreactivity to S100 (Parisi *et al.*, 2014) and TrkA

(Catania *et al.*, 2003) antibodies, while Go antibodies recognize kappe neurons (Ahuja *et al.*, 2014).

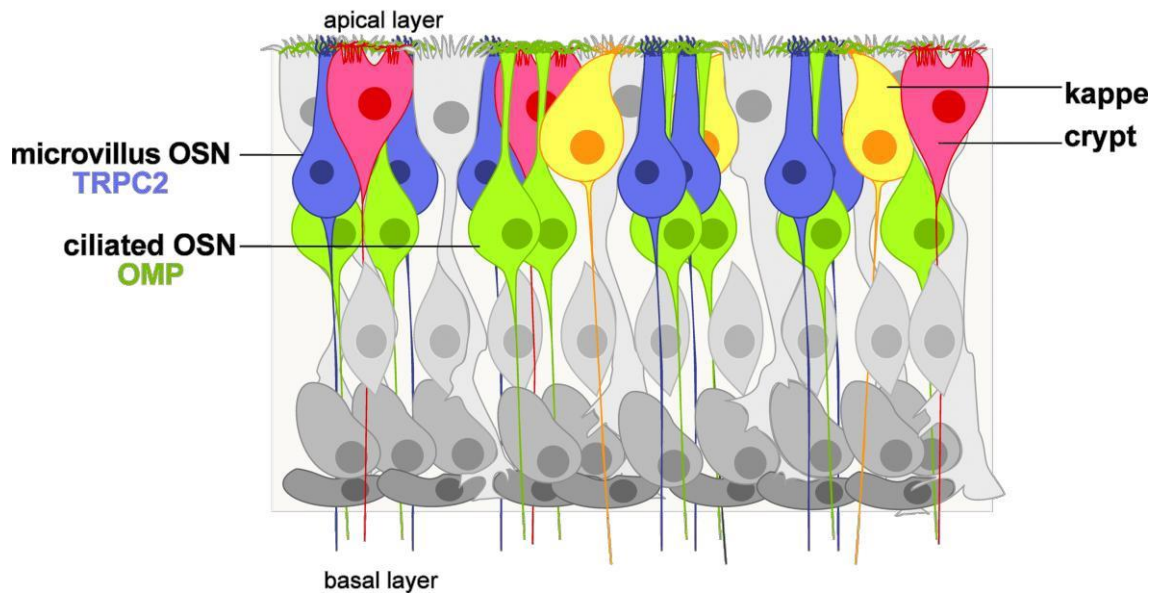


Figure 1.3. Neuronal subtypes in the zebrafish olfactory epithelium. There are four known-types of chemosensory neurons in the olfactory tissue of the zebrafish: Ciliated cells that express the marker OMP, microvillus cells that express the marker TRPC2, kappe neurons that show immunoreactivity against Go and crypt neurons that show immunoreactivity against S-100 or trkA antibody.

Despite their location in the same olfactory tissue, it is still unknown whether the morphologically and molecularly distinct cell types share common progenitors or stem cells or if they are generated by distinct cell lineages. A hint that, at least ciliated and microvillous cells may be generated by distinct lineages, comes from early developmental studies. Microvillous cells appear to arise from a population of Sox10-positive cells derived from the neural crest, whereas ciliated OSNs may be generated from the olfactory placodal field itself (Saxena *et al.*, 2013).

1.3.4. Induced cell ablation to study olfactory neurogenesis

In the adult zebrafish olfactory tissue, the dynamics of the cell proliferation was demonstrated using general ablation of all classes of mature OSNs via the irrigation of the detergent Triton X-100. De- and regeneration is fast, while the majority of mature OSNs disappeared within one day following chemical lesion, the tissue largely regenerates within 5 days after treatment (Iqbal and Byrd, 2010). The increase in cell proliferation occurs specifically in basal lamina of the epithelium (Iqbal and Byrd, 2010; Capar, 2015) similar to the rodent OE (Schwartz Levey *et al.*, 1991). The damage responsive cell population are believed to be predominantly HBCs (Leung *et al.*, 2007) or SC (Suzuki *et al.*, 2013).

1.4. Nitroreductase-mediated specific cell ablation method

Nitroreductase- mediated cell ablation is an efficient chemical-induced method for ablating a specific cell population (White *et al.*, 2013). The general strategy is to express the nitroreductase (NTR) enzyme in a specific cell population using cell type-specific promoters and to induce cell death through nitroreductase-mediated conversion of a prodrug into a cytotoxic product. The nitroreductase enzyme is encoded by the bacterial *nfsB* gene and it metabolizes the conversion of the nontoxic prodrug metronidazole (MTZ) into a cytotoxic compound in the presence of NADH or NADPH. The product of the conversion forms interstrand crosslinks in DNA and triggers apoptosis resulting in the death of the cell (Knocks *et al.*, 1988; Chen *et al.*, 2011).

Alternative methods, such as light-induced cell ablation rather than using a chemical have also been employed to achieve precise removal of specific cells. However, compared to light-induced cell ablation, chemical-mediated cell ablation is more advantageous because it can be applied to many cells or a group of experimental animals at the same time. In addition, the constant rate of ongoing cell ablation renders the method more similar to neurodegenerative diseases (White and Mumm *et al.*, 2013). The only restriction of the method is the “bystander effect” of the drug in which neighboring cells

can be influenced by nonspecific activity of the drug or by dying cells nearby. Thus, adjustment of drug concentration and application duration are essential for an effective cell ablation procedure before assessing the consequences of the ablation (Mathias *et al.*, 2014; White and Mumm *et al.*, 2013).

Another advantage is that the expression of NTR can be restricted to a specific cell population by using tissue-specific promoters and the cell ablation can be induced by application of the prodrug at a desired developmental time or for a defined interval. In addition, fusion or coexpression of NTR with a fluorescent reporter protein allows for simultaneous visualization of cells expressing the NTR, which is especially advantageous in organisms like zebrafish embryos, which are transparent. Zebrafish, especially embryos, are also an ideal model for chemical-induced cell ablation as the prodrug can be administered easily in the aqueous media. Because OSNs are in direct physical contact with the environment, the OE is one of the tissues that face the drug directly and should be well suited for chemical ablation (White and Mumm *et al.*, 2013).

Recently, a triple mutant version of NTR (ntr^{3mut} ; T41Q/N71S/F124T) was engineered to show improved catalytic activity with up to 50-fold increased efficiency over the wild-type (wt) form of NTR (Chen *et al.*, 2011). To better adopt the NTR system for use in zebrafish a codon-optimized version of the ntr^{3mut} was generated by changing the bacterial codons of the amino acids with those more frequent used in zebrafish in order to increase the rate of protein expression and enzymatic activity (Mathias *et al.*, 2014).

1.5. Cell tracing via the photo-convertible dendra2 protein

Cell labeling is crucial for bioimaging purposes and enables an understanding of a cell's location, motility, and fate over time. However, the fate of cells before and after a fixed time point, such as experimental intervention, cannot easily be distinguished with standard labeling techniques such as conventional fluorescent protein expression, bioluminescent probes, or radioactive isotopes. Therefore, a class of fluorescent proteins

that can change its emission spectrum irreversibly upon laser excitation was developed to overcome this shortcoming. The system is feasible for tracing cells over time and to understand whether a given cell existed before or persists experimental manipulation.

Several such photo-switchable proteins have been developed that can be utilized in cell tracking studies such as PA-GFP, which changes from invisible to green fluorescence (Patterson *et al.*, 2002), PS-CFP, which changes from cyan to green fluorescence (Chukadov *et al.*, 2004), and Kaede, which switches from green to red fluorescence (Hatta *et al.*, 2006). The most recent and most efficient protein appears to be dendra2 which is a green-to-red convertible fluorescent protein (Chudakov *et al.*, 2007).

A technical advantage of dendra2 is that it permits photoconversion by UV or short wavelength blue light, such as 488 nm, which is a common laser line, while most other photo-switchable proteins can be converted only by UV light (Chudakov *et al.*, 2007). For primary imaging of green fluorescent signals, 488nm excitation and 490-507nm detection is suggested. Converted dendra2 acquires excitation-emission maxima at 553- and 573nm. Therefore, the excitation of the red signal can be operated with 532-, 543-, 561-, or 568-nm laser lines and the detection is suggested at 550(570)–670 nm (Chukadov *et al.*, 2007).

Photo-switchable proteins made it possible to study the movement of cells – even proteins – by targeting one cell (or molecule), converting its emission spectrum and visualizing the converted cell over a defined time period after conversion. Moreover, the developmental dynamics of a tissue can also be assessed by switching the fluorescence of the entire tissue, and visualizing newborn cells, which express the unconverted isoform. This latter experimental set-up makes the photo-convertible proteins one of the most useful tools to understand cellular dynamics at the tissue level, especially in highly dynamic tissues, such as the olfactory tissue.

In this study, I intended to ablate one specific OSN cell population, the ciliated cells, by utilizing the NTR approach to study if neurogenesis in the zebrafish OE might be cell type-specific. Transgenic constructs containing either wild-type or triple mutant NTR were generated and expression in ciliated cells was driven by the well-characterized OMP promoter (Çelik *et al.*, 2002; Sato *et al.*, 2005). Different reporters were used to visualize NTR-expressing OSNs, GFP for visualization and dendra2 for cell tracking. Co-expression of the reporter protein and the enzyme was achieved by the use of a self cleavable peptide Tav-2A (T2A; Fu *et al.*, 2010), which was interspersed between NTR and the reporter. Finally, a Tol2 transposase vector was chosen as the backbone in which the core transgenic construct is flanked by transposable elements. The system allows for easy generation of transgenes through efficient integration into the genome by transposase-mediated recombination (Asakawa *et al.*, 2008).

In the experiments described in this thesis, I investigated the effect and consequences of different MTZ concentration and application regimes in olfactory tissue of zebrafish embryos. The results show that the olfactory tissue of a developing embryo is highly dynamic making it difficult to extract robust effect of the drug MTZ on OSN survival. Therefore, the photo-convertible feature of dendra2 protein was utilized during the treatment so that more comprehensive analysis of drug-induced cell death and addition of newborn neurons can be achieved.

2. PURPOSE

The olfactory epithelium (OE) is an impressive natural model of sustained life-long neurogenesis. Olfactory sensory neurons (OSNs) are in direct contact with the environment, prone to insult, and need to be replaced continuously to maintain a sense of smell. In addition to maintenance of the tissue, the OE is capable of mounting robust regeneration responses to acute injury. However, little is known about the molecular mechanisms that underlie this response and the specificity with which lost neurons are regenerated. The zebrafish OE harbors at least four morphologically different chemosensory subtypes but it is unknown whether neurogenesis would be cell type specific or if a common molecular pathway would give rise to all cell types.

The aim of the studies described in this thesis was to develop and to evaluate a tool to selectively ablate specific OSN subpopulations. The approach would allow studies targeting the question whether the zebrafish OE can selectively regenerate and compensate for the loss of cells from one specific cell type or whether degeneration of one cell type would induce a general proliferation response.

To this end, an expression system to specifically express the bacterial nitroreductase in ciliated OSNs under control of the well-characterized OMP promoter was developed and rigorously tested. Nitroreductase converts the prodrug metronidazole into a cytotoxic product that selectively kills the cells that express the transgene. As a first assessment of this tool, its ability to ablate cells, the time course of the effect, and critical technical parameters were tested.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Fish

Zebrafish, *Danio rerio*, of the AB or Tü genetic background obtained from the Zebrafish International Resource Center (ZIRC) were used in all experiments. The maintenance and the injection of transgenic constructs were carried out in the zebrafish facility at the Boğaziçi University Life science center.

3.1.2. Equipment and supplies

A detailed list of equipments and supplies including manufacturers' information can be found in Appendix A and Appendix B, respectively.

3.1.3. Buffers and solutions

All solutions, media and buffers used for zebrafish studies were prepared according to Westerfield (2007). Buffers and solutions for general molecular biology techniques were prepared according to Sambrook and Russell (2001) or were obtained from the manufacturer as part of a kit. A detailed list of buffers and reagent can be found in Appendix A.

3.2. Methods

3.2.1. Maintenance and breeding of fish

Zebrafish of the AB/AB and AB/Tü strains were kept at 28° C under a 14 hours light / 10 hours dark light regime. Housing densities were 5, 15 and 50 zebrafish in 1, 3, and 10 liters tanks, respectively. Adult fish used to obtain fertilized oocytes for microinjection of transgenic constructs were kept isolated as couples in 1 liter tanks. Artificial fresh water was prepared by dissolving 2 g sea salt (Instant Ocean), 7.5 g sodium bicarbonate, and 0.84 g calcium sulfate in 100 liters of reverse osmosis water. Tanks were kept in a professional housing systems (Stand Alone System, Aquatic Habitats) to provide appropriate aeration, five-stage filtration and UV sterilization of system water. Adult zebrafish were fed twice a day with either flake food (TetraMin, Sera Vipan) or live brine shrimp larvae (*Artemia sp.*) during morning and evening feedings.

3.2.2. Injection of the transgenic construct

For microinjection of transgenic constructs into one-cell stage zebrafish, specially designed mating tanks were utilized that are equipped with a separator wall to isolate male and female fish during overnight incubation in order to prevent premature mating. Subsequent to the removal of the separator wall, fish spawn and fertilized oocytes were collected from the bottom of double container tanks, which is protects the oocytes from predation by the parents. Fertilized eggs were aligned in an agarose injection mold and injections were performed using glass microcapillaries with the help of a micromanipulator and a Femto Jet Express pressure injector before completion of the first cleavage. DNA for microinjection was prepared as detailed in table 3.1 below.

Table 3. 1. Content of the injection solution

Content	Amount (ul)
Circular plasmid (final concentration 100 ng/ul)	7
0.5% phenol red	1
1M KCl	1
Transposase mRNA (250Ng/ul)	1
Total volume	10

3.2.3. Polymerase Chain Reaction (PCR)

In order to amplify desired DNA fragments for cloning purposes, polymerase chain reaction (PCR) was performed by using custom designed appropriate primers for the amplified DNA fragment. For most PCR reactions, the One Taq (NEB) PCR kit was used according to the manufacturers protocol. Colony PCR was used to check the success of DNA ligation and transformation on bacterial lysates directly. The composition of a typical PCR or colony PCR reaction is listed in table 3.2.

After assembly of the PCR reaction on ice, PCR conditions were set as follows: initial denaturation at 95 °C for 5 min, per cycle denaturation at 95 °C for 40 sec, annealing at appropriate temperature (melting temperature of the lowest melting oligonucleotide – 2 C) for 40 sec, elongation at 72 °C for 1 min/kb. Typically at least 28 cycles of amplification were performed, followed by a final elongation step at 72 °C for 10 min.

Table 3. 2. Contents of the PCR reactions

	Standard PCR	Colony PCR
Template (~100ng/ul)	1ul	Single colony picked with a pipette tip
Forward Primer (10 pM)	2.5ul	2.5ul
Reverse primer (10 pM)	2.5ul	2.5ul
dNTP (100 mM)	0.6ul	0.6ul
Taq buffer (5X)	10ul	3ul
Taq polymerase	0.5ul	1ul
MgCl ₂ (25mM)	-	2.5ul
Total volume	50ul	30ul

3.2.4. Digestion of the DNA with restriction endonucleases

For cloning of DNA constructs, plasmids and amplified DNA fragments were digested using restriction endonucleases from Promega, Invitrogen and New England Biolabs (NEB). Typically 5 units of enzyme were used for approximately 5ug DNA amount, and reactions were incubated at 37 °C for 1 hour for analytic digests or overnight for preparative digests.

3.2.5. Agarose gel electrophoresis

To visualize digested or amplified DNA fragments, gel electrophoresis on 1% agarose gels prepared in 1x TAE buffer was performed. Running conditions were set to 60-100 V for 1 to 2 h, or until DNA fragments could be distinguished clearly, using 1x TAE as running buffer. The 1kb DNA ladder (NEB, USA) was loaded to the same gel to estimate fragment sizes. For imaging purposes 0.5ug/ml Ethidium Bromide was added to the agarose gel directly, and the gels were visualized using a GelDoc XR imaging system

(Bio-rad, USA) under the UV light. Gel images were documented electronically as TIF files.

3.2.6. PCR and gel purification

In order to purify DNA fragments obtained by PCR reactions or recovery from agarose gels the High Pure PCR Purification kit (Roche, USA) was used according to the manufacturers' protocol.

3.2.7. Measurement of the DNA concentrations

The yield of DNA preparations was measured using a NanoDrop® Spectrophotometer in addition to direct visualization of the DNA on agarose gels.

3.2.8. Gibson assembly

Assembly of the two transgenic constructs (pOMP::*ntr*^{3mut}-T2A-GFP and pOMP::*ntr*^{3mut}-T2A-dendra2) was carried out using the Gibson Assembly kit (NEB, USA). In brief, the Gibson assembly reaction contains three enzymes; a 5'-exonuclease which removes nucleotides from the 5'-ends of DNA fragments, effectively creating 3' overhangs, a DNA polymerase which fills in the gaps of annealed DNA fragments, and a DNA ligase which seals the nicks between fragments. The system is efficient and allows the simultaneous cloning of up to five DNA fragments. DNA fragments to be ligated were prepared with at least 30bp overhangs by PCR amplification as described above (3.2.3.). Vector backbones were digested by restriction digest only. All fragments to be used in the subsequent assembly reaction were purified by High Pure PCR Purification kit (Roche, USA) prior to the assembly (3.2.6.). The suggested molarities of DNA fragments for optimal yield were between 0.02 - 0.1 pM for two fragments and between 0.2 - 1 pM for up to 5 fragments. Molarities were calculated using a dedicated online tool from the NEB

website (<http://nebiocalculator.neb.com/#!/dsdnaamt>) and DNA concentrations were measured using the NanoDrop® Spectrophotometer. The molarities were 0.01 pM for insert fragments and 0.03 pM for the vector backbone. The reaction mixture was prepared by combining 10x buffer, 2x Gibson enzyme mixture and the DNA fragments and the mixture was incubated at 50 °C for 1 hour. Subsequently 10 µl of the assembly reaction were transformed to chemically competent bacteria.

3.2.9. Ligation of DNA fragments

In the preparation of the one transgenic construct (pOMP::NTR-T2A-GFP), standard cloning techniques were used. Ligation of DNA fragments to endonuclease-digested vector backbones was performed by using 1 µl (20 units) T4 ligase (NEB) for an amount up to 100 ng of DNA in a total reaction volume of 10 µl. The molar ratios of insert to vector backbone were adjusted between 3:1 and 1:1, and the required amount of DNA was calculated in as described above. After the assembly of the reaction mixture, containing DNA fragments, ligase enzyme, and 10x buffer, the reaction mixture was incubated at 25 °C for 2 hours and transformed to chemically competent TOP10 bacteria.

3.2.10. Preparation of competent cells

Competent cells were prepared by the Rubidium Chloride method (obtained from mcmanus lab; <http://mcmanuslab.ucsf.edu/protocol/rubidium-chloride-competent-cell-protocol>). A single colony of TOP10 strain was picked from a freshly streaked bacterial plate and used to inoculate an overnight 5 ml starter culture. 1 µl of the starter culture were used to inoculate a 100ml culture and grown until an OD (0.6) was reached. The culture was chilled on ice for 15 min. and centrifuged for 10 min at 3 000 rpm at 4 °C. The pellet was resuspended gently by tapping in the remainder of the drained supernatant and mixed with 20 ml CT1 solution (30 mM potassium acetate, 10 mM CaCl₂, 50 mM MnCl₂, 100 mM RbCl, 15% glycerol, pH: 5.7) per 100 ml of initial culture. The mixture was incubated

on ice for 30 min and centrifuged at 3.000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 4 ml of CT2 solution (10 mM MOPS, 75 mM CaCl_2 , 10 mM RbCl, 15% glycerol, pH: 6.5) per 100ml of initial culture. The mixture was chilled on ice for 15 min, aliquoted and rapidly frozen in liquid nitrogen. Aliquots were stored at -80 °C until use. In order to determine the transformation efficiency the cells were transformed with up to 100ng μ g of DNA and the colony forming unit was calculated according to the formula:

3.2.11. Transformation of plasmids

For each transformation reaction, 50 μ l of competent cell were thawed first on ice, mixed with 10 μ l of ligation or Gibson assembly mixture (total DNA amount was up to 100 ng for each assembly) and incubated on ice for 30 min. The reaction was quickly heated to 42 °C for 90 sec, immediately chilled on ice for 2 min, resuspended in 500 μ l LB medium and incubated at 37 °C for one hour for recovery. The culture was spread on LB agar plates and incubated at 37 °C overnight.

3.2.12. Plasmid isolation

Plasmid DNA was isolated from bacteria using a commercial plasmid isolation kit (Thermo Scientific). To do so, 6 ml of overnight cultures were grown at 37 °C in LB medium containing appropriate antibiotics for selection.

3.2.13. Preparation of the transgenic constructs

3.2.13.1. Preparation of pOMP::NTR-T2A-GFP. The bacterial *nfsB* gene (653bp) was amplified by PCR from *E. coli* (TOP10 MRF⁺) genomic DNA using NTR_NcoI_F and NTR_XhoI_R oligonucleotides and ligated into the pGEM-T easy vector (Promega). The forward primer (NTR_NcoI_F) contained additional *NcoI* and the reverse primer (NTR_XhoI_R) *XhoI* restriction endonuclease recognition sites for cloning purposes. Then the NTR containing pGEM-T vector and a vector containing a sequence coding for T2A (57bp, vector p737, and dsRED reporter protein coding sequence) were digested with *NcoI* and *XhoI* restriction endonucleases and ligated together. The resulting plasmid was digested again with *NcoI* and inserted into the *NcoI*-digested Tol2 vector (pT2AL, Asakawa et al., 2008) containing pOMP::GFP (5731bp). The cloning strategy is depicted in Figure 3.1. Positive transformants were identified by colony PCR using NTR_NcoI_F (table 3.3) and GFP_R_5 primers. A single positive colony (colony 6, Figure 3.2) was confirmed by subsequent sequencing.

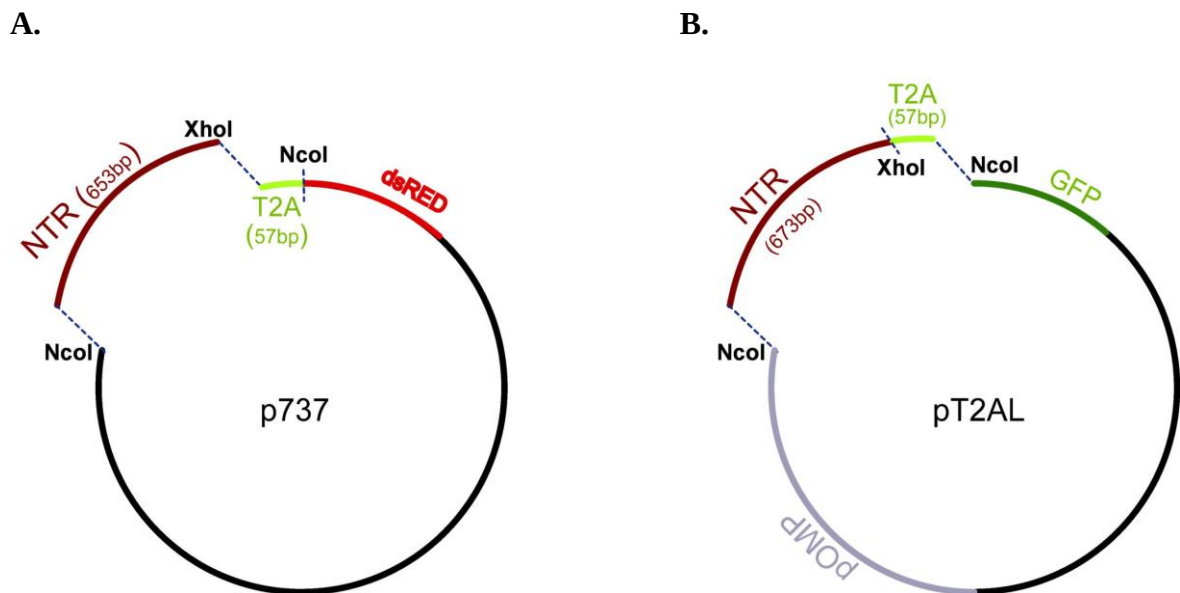


Figure 3.1. Cloning strategy of the transgenic construct pOMP::NTR-T2A-GFP. (A) A *NcoI* and *XhoI* double digested NTR fragment was inserted upstream of a sequence coding for T2A in the p737 vector. (B) In the following step, a *NcoI* digested NTR-T2A fragment was cloned between pOMP and GFP in Tol2 vector.

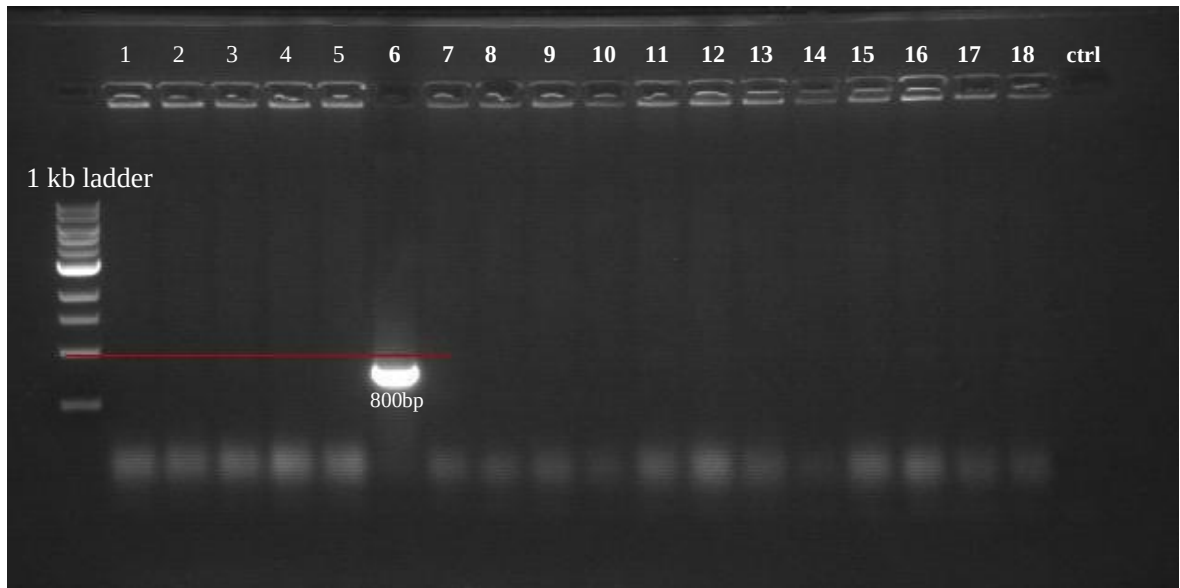


Figure 3.2. Colony PCR of pOMP::NTR-T2A-GFP performed with NTR and GFP_R_5. Expected band length is 800bp and last lane is the negative control.

3.2.13.2. Preparation of pOMP::ntr^{3mut}-T2A-GFP. The preparation of a pOMP::ntr^{3mut}-T2A-GFP construct was performed using Gibson assembly after the cloning of the triple mutant *ntr*^{3mut} (obtained from Jeff Mumm, John Hopkins Medicine) sequence into the T2A containing p737 vector in the same way as described for the construction of the wild type pOMP::NTR-T2A-GFP construct. Then the *ntr*^{3mut}-T2A fragment (760bp) was amplified by using *ntr*^{3mut}_5' pOMP overhang_F and T2A_5' GFP overhang_R primers designed to contain 30 bp of overlapping homology to the pOMP::GFP vector at each end (Table 3.3.). An *Nco*I-digested pOMP::GFP vector was used as the vector backbone. The assembly was carried out as described in section 3.2.8. The cloning principle of the construct is depicted in Figure 3.3. Colonies that tested positive by colony PCR using *ntr*^{3mut}_NcoI_F and GFP_R_5 primers were selected and verified by sequencing.

3.2.13.2. Preparation of pOMP::ntr^{3mut}-T2A-dendra2. The pOMP::ntr^{3mut}-T2A-dendra2 construct was also generated by Gibson assembly. A *ntr*^{3mut}-T2A (760bp) fragment was generated by PCR using NTR_F and T2A_R primers, which contain 30 bp of overlapping

homology from pOMP site and the homology from T2A site was provided by the dendra2_5' T2A overhang_F primer from dendra2 PCR product. The vector backbone was digested with *NcoI* and *ClaI* restriction endonucleases and was the same as for all constructs (pOMP::GFP). The detailed principle of the Gibson reaction is illustrated in Figure 3.4.

Table 3. 3. Primers used for generation of transgenic constructs.

Primer Name	#	Sequence
NTR_NcoI_F	315	5'-CCATGGATATCAT TTCTGTCGCCTTA-3'
NTR_XhoI_R	314	5'-CTCGAGCACTTCG GTTAAGGTGATGT-3'
<i>ntr</i> ^{3mut} _NcoI_F	431	5'- CCATGGACATCATCTCTGTGGCTCTG -3'
<i>ntr</i> ^{3mut} _R	430	5'- CTCGAGCACCTCTGTCAGGGTGAT -3'
<i>ntr</i> ^{3mut} _ 5' pOMP overhang_F	464	5'-CGGTAAGTTAAAAAACA ACAACCATGGACATC ATCTCTGTGGCTCTG-3'
T2A_5' GFP overhang_R	465	5'-CAGCTCCTCGCCCTTG CTCACCATGGTAGCA GGGCCGGGATTCTC -3'
T2A_R	471	5'-GGTAGCAGGGCCGGGATTCTC- 3'
dendra2_5' T2A overhang_F	472	5'-TGGAGGAGAATCCCG GCCCTGCTACCATGGAA CCCCGGAATTAACCT-3'
dendra2_5' tol2 overhang_R	473	5'-TATCTTATCATGTCTGG ATCATCATTTACTTG TACACGCCGCTGTCCG-3'
GFP_R_5	103	5' -AACTTGTGGCCGTTTACGTCGC -3'

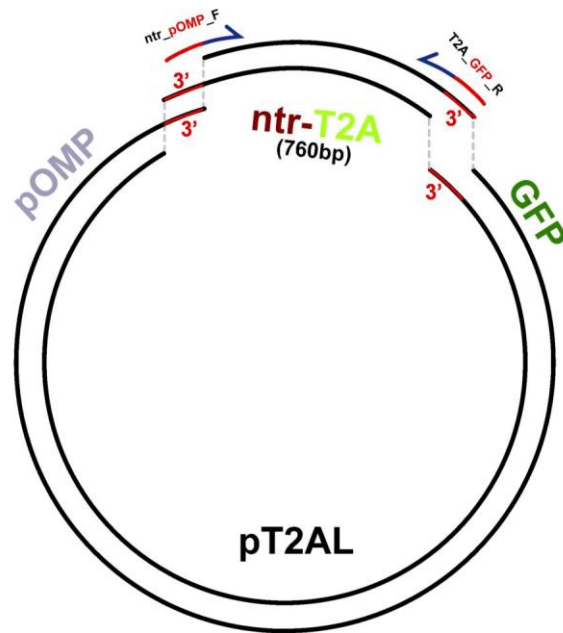


Figure 3.3. Cloning strategy of pOMP::*ntr*^{3mut}-T2A-GFP construct using the Gibson assembly method. Two fragments were assembled; *ntr*3mut-T2A (760bp) with pOMP and GFP overhang, pOMP::GFP and pT2AL vector backbone.

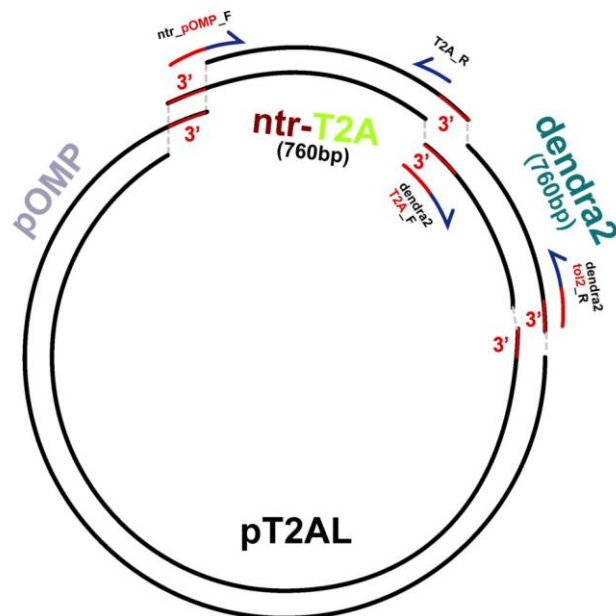


Figure 3.4. Cloning principle of pOMP::*ntr*^{3mut}-T2A-dendra2 construct with Gibson assembly. Three fragments were assembled; *ntr*3mut-T2A (760bp) with pOMP overhang, dendra2 (760bp) with T2A and Tol2 overhangs and pT2AL vector backbone.

3.2.14. Whole-mount antibody staining

Immunohistochemistry was performed against activated caspase 3 on whole-mount zebrafish embryos according to the protocol explained in Sorrels *et al.* (2013). Following to the fixation with 4% PFA, the embryos were dehydrated in 100% ice-cold Methanol and washed with PDT (0,1% tween-20, 0.3% triton X-100, 1% DMSO). Then 1:500 dilution of the rabbit α -Cas3 antibody (BD Biosciences, USA) used while the dilution of secondary antibody, Alexa Fluor® 633 (Goat anti-rabbit IgG conjugated to Alexa Fluor 633) was 1:800 in blocking solution (1% BSA, 10% heat-inactivated goat serum in PDT). Embryos were incubated in primary antibody overnight and prior to the secondary antibody incubation, PDT washing steps were repeated.

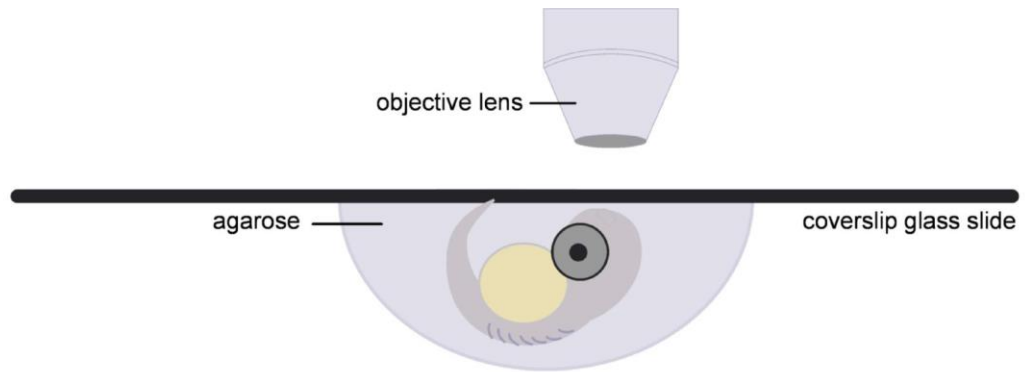
3.2.15. Imaging of the zebrafish embryos

Zebrafish whole-mount embryos were imaged at between 2 and 4 dpf for quantitative analysis of the numbers of transgene-expressing OSNs. Embryos were anesthetized in (75–125 mg/L) MS222 and mounted into a drop of low melting agarose onto a cover glass as depicted in Figure 3.5a. Mounted embryos were imaged with SP5-AOBS laser scanning confocal microscope with 20x or 40x objective lenses. (Leica, Germany). The illustration of the whole head and the documented confocal image format is also shown in Figure 3.5b.

3.2.16. Conversion of the dendra2 protein

Green fluorescent dendra2 protein in OSNs was converted to red fluorescence by applying UV fluorescent light for 30sec. Longer UV pulses showed phototoxic effect on the protein as reported earlier resulting in the loss of signal even the cells were expressing the dendra2 protein (Chudakov *et al.*, 2007; Figure 3.6).

A.



B.

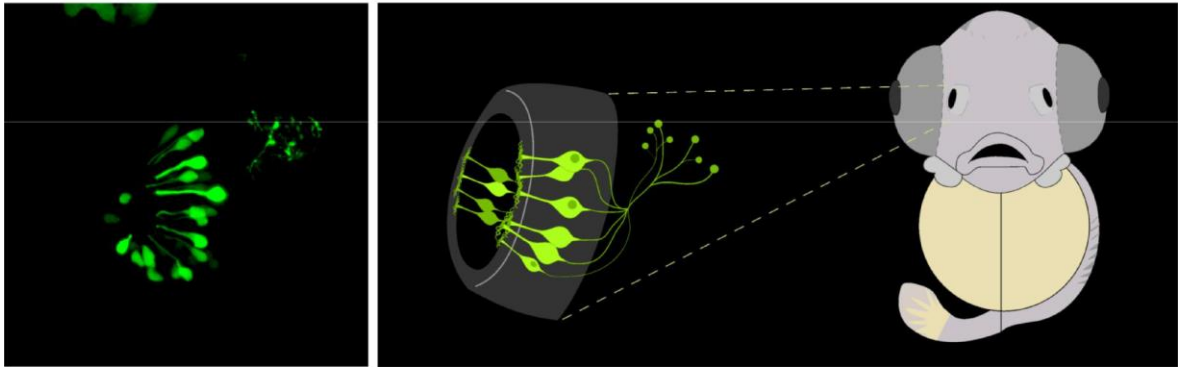


Figure 3.5. Visualization of the zebrafish embryos under the confocal laser microscopy. (A) Illustration of the mounting technique used for embryos in low-melt agarose, embryos were visualized from the anterior of the head. (B) Illustration of the zebrafish embryos seen from the objective of the microscopy (right), olfactory pit (middle), and olfactory pit confocal micrograph of the embryos expressing transgene GFP.

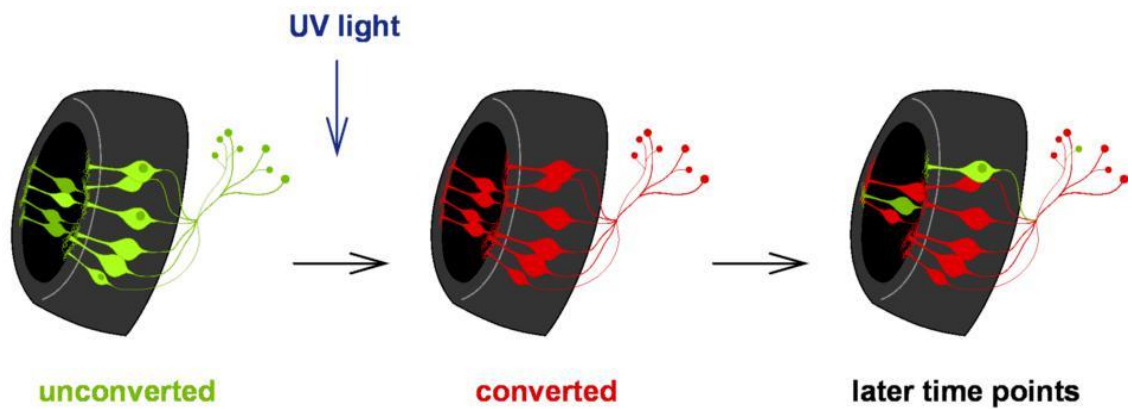


Figure 3.6. Principle of the dendra2 tracing experiments. Non-converted green cells were counted before conversion and the initiation of the MTZ treatment (left), then the cells were converted from green to red UV light pulse (middle) just before MTZ treatment and observed over the time course of the experiments. Green fluorescent cells after conversion indicates cells generated after conversion / treatment.

4. RESULTS

Major aim of the studies presented here was to establish a system that allows for the ablation of a specific neuronal subpopulation of sensory neurons in the zebrafish OE. To gain the required cell type specificity, transgenic constructs were driven by the promoter of the olfactory marker protein (OMP) gene, which is expressed exclusively in ciliated sensory neurons. To kill the cells, a sequence coding for the nitroreductase (*ntr*) gene was expressed in ciliated cells under OMP promoter control and a sequence coding for green fluorescent protein (GFP) was included in the constructs to visualize NTR-expressing OSNs. Two different isoforms, a bacterial-derived wild type, and a codon-optimized enhanced version of the NTR gene were used. NTR converts the prodrug metronidazole (MTZ) into a cytotoxic product that is supposed to result in cell death of NTR-positive OSNs. The various transgenic constructs were then injected into one-cell stage embryos and the number of the transgene-positive cells was analyzed over up to 48 h starting from two and three days post fertilization (dpf).

A range of MTZ concentrations pulse conditions were tested to develop an efficient protocol for ciliated OSN ablation in early embryos. The numbers of transgene-expressing cells were analyzed in two major ways. As a first approach, the dynamics of transgene-positive cells was observed simply by counting the number of transgene-positive cells in each individual OE and expressed as the average over all noses at different time points following the onset of drug exposure. As a second measure, differences in the number of transgene-positive cells were examined individually for each embryo and time point and results before and after MTZ treatment were compared as changes in the median of the distribution of differences in cell number. For instance, when the median of the difference in cell numbers in each nose was negative, it was considered as a general loss of cells and *vice versa*. Statistical tests were employed to gain confidence about changes in the number of transgene-positive cells upon MTZ-induced cell ablation: two sample, two-tailed Student's t-tests were applied for the average cell numbers, whereas a Kruskal-Wallis

rank-sum test was applied for the differences in cell numbers (for Kruskal Wallis test: $H(df) = h$ value, $p = p$ value; $df =$ degree of freedom).

4.1. Viability of MTZ-treated embryos

In order to assess whether MTZ treatment results in efficient ablation of NTR-expressing cells in the olfactory epithelium (OE), proper adjustments of the drug concentration and the duration of MTZ treatment are crucial. Although the optimal conditions may differ across tissue types, working conditions for cell ablation in zebrafish embryos have been reported with a concentration of 10mM MTZ and a pulse duration of 24 hours. Concentrations higher than 10 mM have been reported to be lethal (Curado *et al.* 2007). Thus, any specific effect of MTZ on individual NTR-expressing OSNs could be confounded by general toxicity of MTZ at high concentrations.

To establish ideal working conditions for the MTZ concentration and the duration of application empirically, batches of wild type embryos were continuously treated with 5, 10, 15 and 20 mM of MTZ starting from 3 dpf for a maximum duration of 1 week. During the experiment neither MTZ solutions nor the E3 medium were changed for experimental or control groups, respectively (Figure 4.1). Surprisingly, MTZ can be lethal even at the lowest concentration of 5mM after 4 days of continuous exposure. Increasing the MTZ concentration caused a further, concentration-dependent decrease in the viability of embryos. Under all conditions where MTZ concentrations higher than 5mM were used, embryos died within 4 days following the onset of treatment. At the highest concentration of 20mM 100% mortality was reached 3 days after first exposure to the drug. Thus, extended exposure to MTZ is toxic for zebrafish embryos regardless of the concentration within a range of 5 to 20 mM MTZ, with more lethal effects at higher MTZ concentrations. Nevertheless, under all conditions embryos survived the initial two days of exposure to MTZ, which may be a sufficient experimental time window for induced cell ablation experiments in the OE. A concentration of 10mM MTZ has been reported to be sufficient for effective ablation in other cells types of the zebrafish embryo, such as hepatocytes or

skin cells (Curado *et al.*, 2007; Chen *et al.*, 2011). Treatment of zebrafish embryos with 10 mM MTZ permits a longer experimental window of up to three days of continuous exposure to the drug, which might be sufficient to examine drug-induced specific cell loss in the zebrafish OE. Shorter incubation times, on the other hand, should reduce the overall toxic effect of MTZ on general viability.

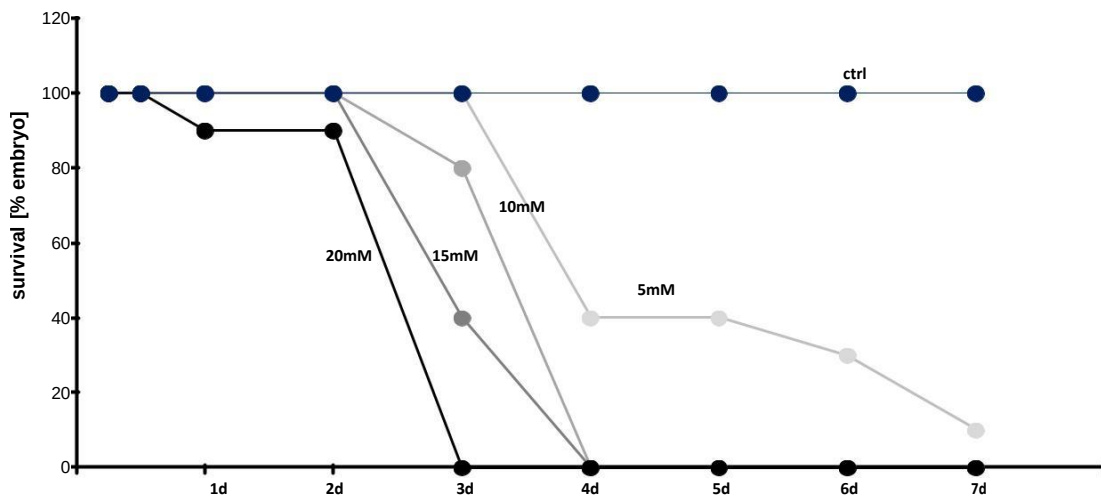


Figure 4.1. Effect of different metronidazole concentrations on embryo survival. Percentages of initial populations of embryos incubated in different concentration of MTZ (5, 10, 15 and 20mM) and E3 (ctrl) monitored over different time points.

4.2. Expression of NTR-containing transgenic constructs

For specific ablation of ciliated OSNs, which constitute a major subtype of chemosensory neurons in the zebrafish OE (Sato *et al.*, 2005), the well-characterized zebrafish OMP promoter (Çelik *et al.*, 2002; Sato *et al.*, 2005) was used to drive simultaneous expression of the nitroreductase enzyme (NTR) and the green fluorescent reporter protein (GFP). The wild type *nfsB* gene coding for NTR was directly cloned from *E.coli* (TOP10 MRF') genomic DNA and fused to a sequence coding for the green fluorescent protein (GFP) reporter through a sequence coding for the self cleavable peptide T2A (Fu *et al.*, 2010; Figure 4.2.a).

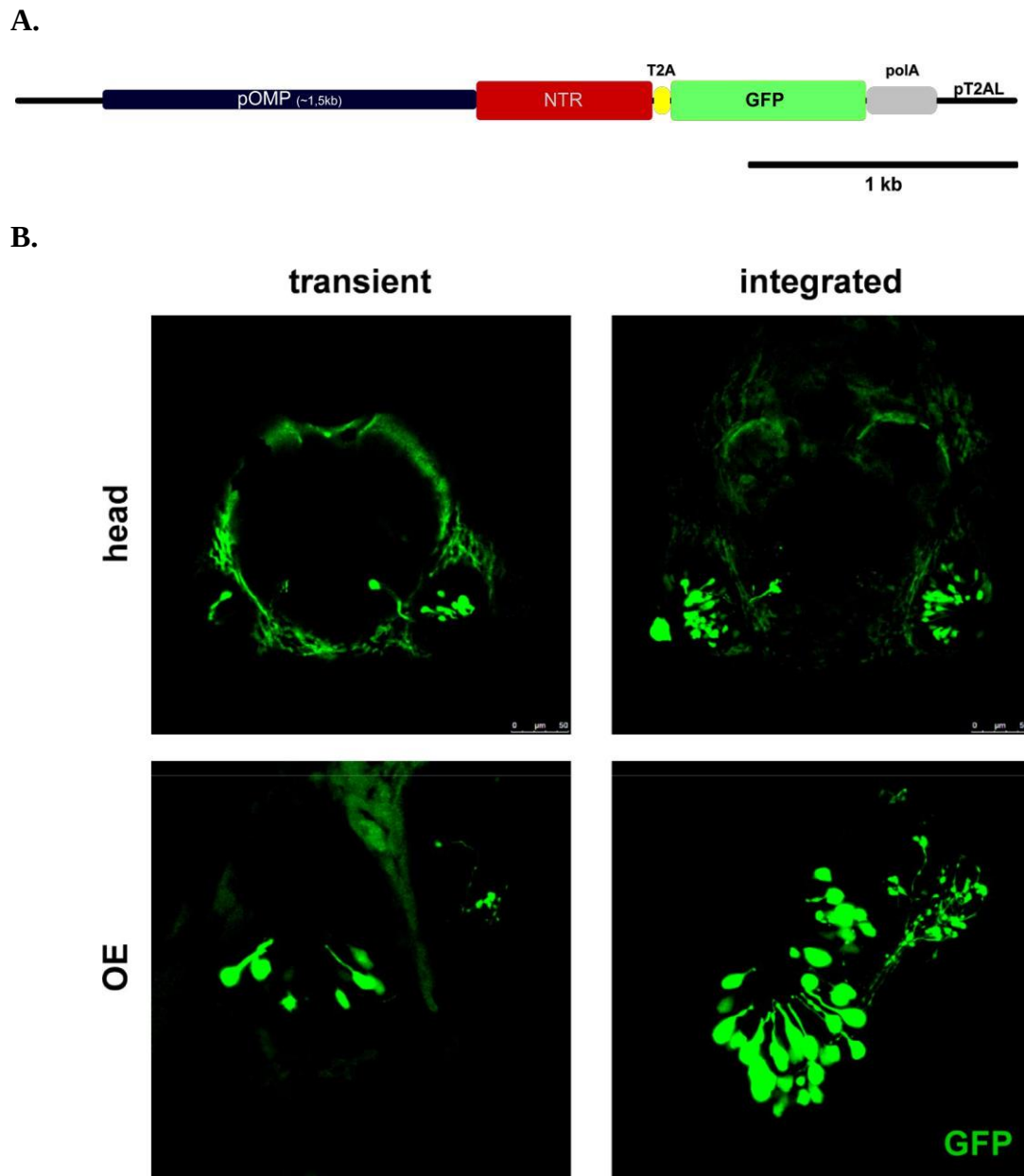


Figure 4.2. Phenotypes of pOMP::NTR-T2A-GFP-injected embryos at 3 dpf. (A) Overview of the pOMP::NTR-T2A-GFP transgenic construct. (B) Confocal stacks of pOMP::NTR-T2A-GFP expression of the entire zebrafish head (top row) and individual OEs (bottom row).

The resulting transgenic construct pOMP::NTR-T2A-GFP was injected into one-cell stage fertilized zebrafish oocytes, and transgene-expressing OSNs were analyzed at 3 dpf by fluorescent and confocal microscopy of whole-mount embryos. As expected, transgene-

expressing GFP-positive cells were predominantly observed in the OE but rarely in other tissues. In the OE, two major, quantitatively different phenotypes of transgene expression could be observed: embryos that expressed the transgene with strong intensity of the GFP reporter in few (1 to 20 OSNs) and embryos that expressed the transgene in many (> 20 OSNs, albeit with lower fluorescence intensity; Figure 4.2b). In the experiments reported here, the injected pOMP::NTR-T2A-GFP construct is flanked by Tol2 transposable elements and was co-injected with Tol2 transposase mRNA. The Tol2 systems allow for efficient integration into the zebrafish genome (Asakawa *et al.*, 2008), making it likely that the construct has been integrated into the genome by transposase-mediated recombination in some embryos but not in others. Thus, high expressors may represent embryos in which the transgene is integrated and stably expressed from the genome, whereas low expressors may represent embryos in which the transgene is expressed transiently from the injected plasmid DNA. This is further supported by the observation that in the phenotype classified as transient (less than 20 OSNs) the transgene-expressing cells showed stronger GFP fluorescence compared to cells observed in the phenotype classified as integrated (more than 20 OSNs) in which only moderate GFP fluorescence could be observed. (Figure 4.2b).

4.3. Effects of wild type NTR expression in OSNs

4.3.1. Effect of MTZ treatment on OSN morphology

Based on the results of the viability study (section 4.1) and conditions reported in the literature (Curado *et al.*, 2007), a first cell ablation experiment was conducted using the pOMP::NTR-T2A-GFP construct and continuous exposure to 10 mM MTZ for 24 hour. At this concentration and exposure duration 100% of wild type embryos (n = 10) survived the treatment.

When embryos were incubated in 10mM MTZ for 24 h, indications of treatment-induced cell death could be observed in transgene-expressing OSNs. As shown in the example in Figure 4.3, a loss of OSNs is apparent in the dorsal OE of MTZ-treated embryos but not in pOMP::NTR-T2A-GFP-injected untreated control animals. However,

the embryonic OE is highly dynamic and OSNs move positions across the OE during development. Because of this, and because transient expression may be lost over the time course of the experiment, the cause of OSN loss remains uncertain, unless individual cells are tracked over time. Another complication is that during early embryonic development new OSNs are continuously added to the OE, a circumstance that, at least numerically, compensates for lost neurons. Yet, a higher increase in the number of transgene-positive OSNs could be observed in control fish compared to MTZ-treated embryos (see section 4.3.2).

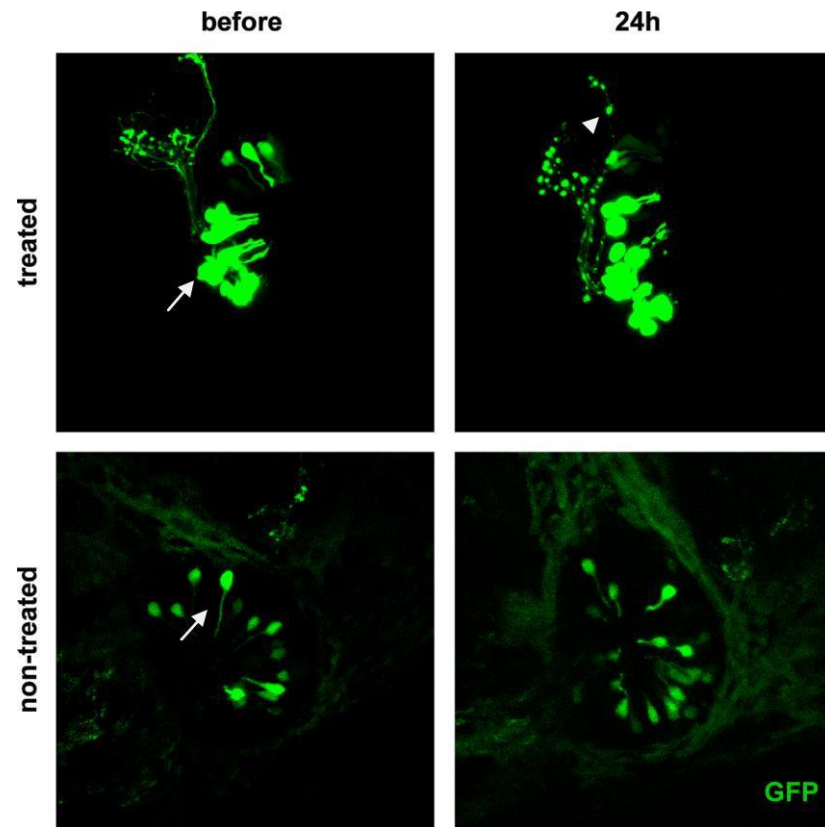


Figure 4.3. Phenotypic effects of continuous treatment with 10 mM metronidazole for 24 h. Images show confocal stacks through entire OEs visualized by pOMP::NTR-T2A-GFP expression (top row: MTZ-treated embryo; bottom row: untreated embryo). In each case, the same OE was visualized at 3 dpf the before and 24 h after treatment. The blebbing of axons in the treated OE is indicated with arrowheads top right.

A more direct indication of induced cell death is the morphological changes in transgenic OSNs that become visible upon MTZ treatment. Axons of transgene-expressing OSNs in MTZ-treated embryos showed distinctive blebbing along the length of the axon, which cannot be seen in untreated pOMP::NTR-T2A-GFP-injected control embryos. The axonal swellings most likely represent apoptotic events and the formation of apoptotic bodies along the length of the axon (Chen *et al.*, 2011).

4.3.2. Cell number analysis

To confirm the qualitative observations of the effect of MTZ treatment, 3 dpf pOMP::NTR-T2A-GFP-injected embryos were subjected to counts of transgene-positive cells before and after administration of 10 mM MTZ. Generally, stagnating or slightly increased cell numbers were observed in MTZ-treated animals as compared to untreated control animals, in which a general increase of transgene-positive cells could be detected.

As a first quantitative assessment, the number of transgene-positive OSNs was analyzed over 24 h in untreated control animals to establish the background rate of cell birth over the time course of the analysis (Figure 4.4). To do so, a total of 24 pOMP::NTR-T2A-GFP-injected embryos were individually followed starting from 3 dpf and analyzed at 6, 12, and 24 h intervals in the absence of MTZ. A marked increase in the number of GFP-positive OSNs was observed during the first 12 hours and the number of cells increased by 29.76% from 16.8 ± 10.6 cells (average $\pm$ SEM) at 3 dpf to 20.7 ± 9.6 cells at 6 h and 21.8 ± 13.17 cells at 12 h, respectively. When the change in cell number for all time points (6 h, 12 h, and 24 h) was analyzed as an alternative measure, the differences in transgene-expressing OSNs before and after treatment were mostly positive for each time point. The median of the differences was 4 for the 0 h / 6 h comparison, 3 and 4 for the 0 h / 12 h and 0 h / 24 h comparisons, respectively. However, the median of the change in cell number and the slopes between different time points for the average cell number per OE vary for different time points reflecting the dynamics of the expression system in early embryos

(Celik *et al.*, 2002). The rate of change was more pronounced during the early (0 h vs. 6 h) stages as compared to later developmental times.

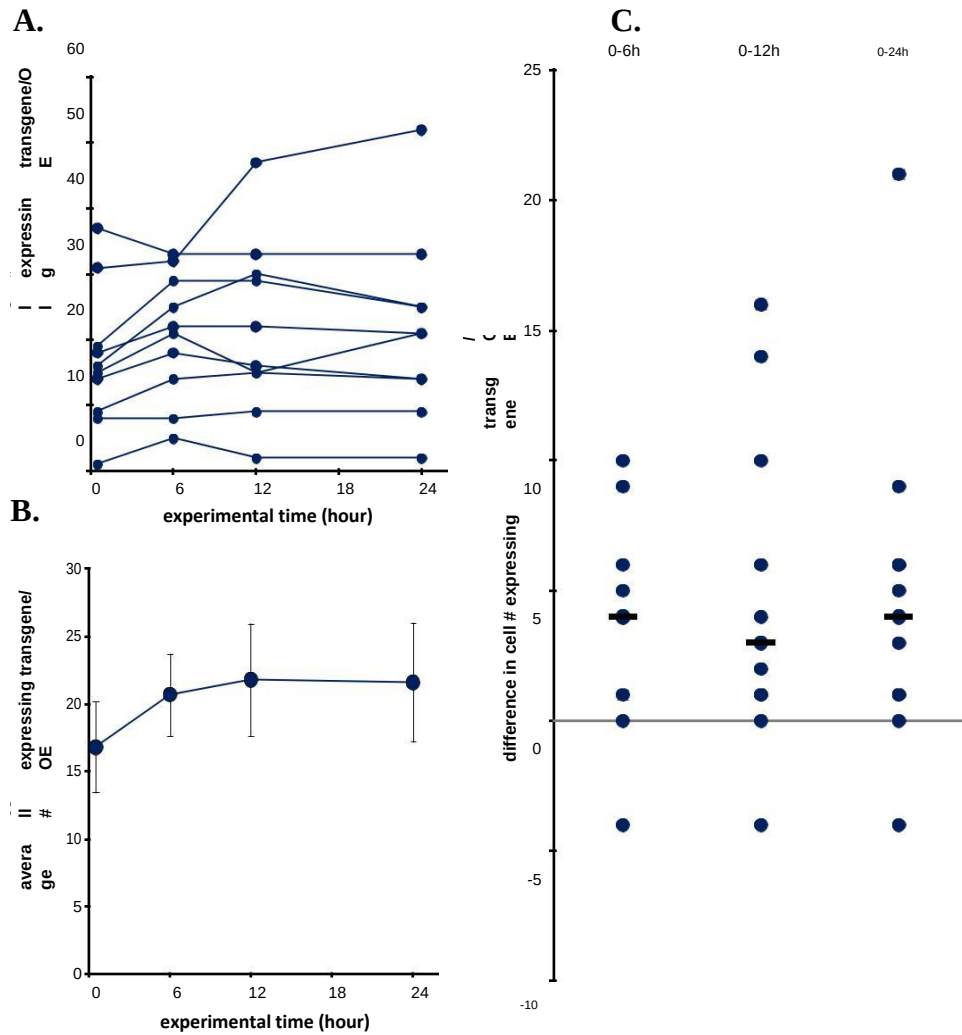


Figure 4.4. Developmental dynamics of pOMP::NTR-T2A-GFP expression between 3 and 4 dpf.

(A) Absolute cell numbers of transgene expressing OSNs/OE starting at 3 dpf. Embryos were grown in E3 medium for 24 h. (B) Average cell numbers of transgene expressing OSN/OE. (C) Difference plots showing the difference in the number of transgene expressing OSNs. The black bar represents median of each data set.

Next, we wanted to understand whether continuous exposure to 10 mM MTZ over the same time period leads to a difference in the dynamic of OSN numbers when compared

to the background rate of transgene expression over the same time period (Figure 4.5; n= 12 control embryos, 12 MTZ-treated embryos).

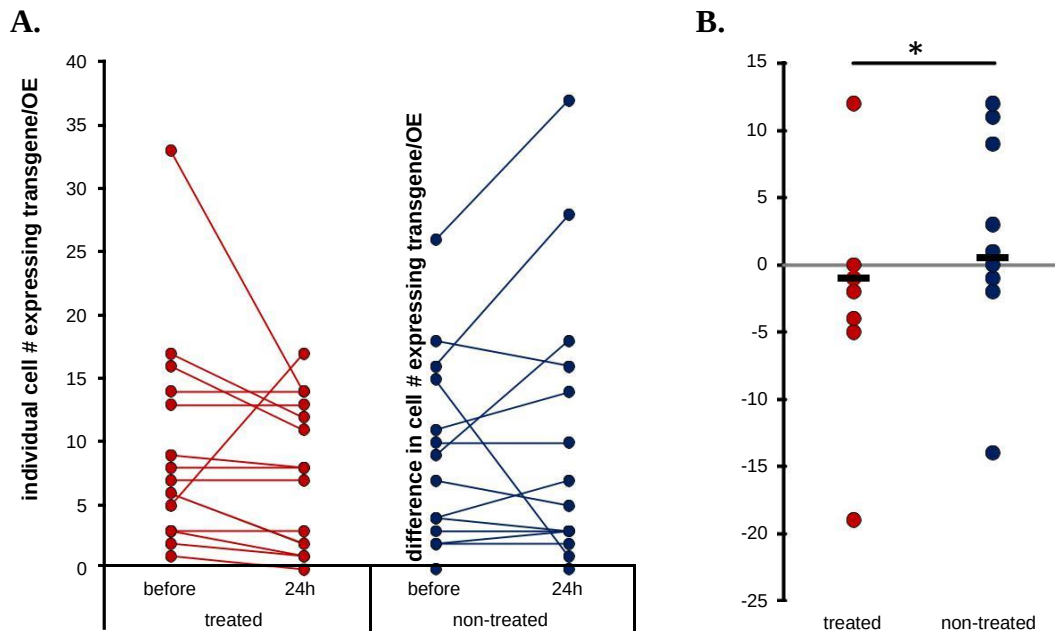


Figure 4. 5 Quantitative effects of treatment with 10 mM metronidazole for 24 h. (A) Absolute cell numbers of pOMP::NTR-T2A-GFP-expressing OSNs per OE / embryo before and after 24 h continuous treatment with 10mM MTZ (red: treated embryos; blue: untreated control embryos) (B) Plot of pOMP::NTR-T2A-GFP-expressing cell numbers before and after 24 h of 10 mM MTZ treatment (asterisk: Kruskal-Wallis test, p=0.03).

An overall decrease in cell numbers could be observed in most individuals of the experimental group while a more pronounced increase in cell number was evident in the untreated control group in which embryos were raised in standard E3 medium (Figure 4.5). However, exceptions to this behavior were also obvious in which a dramatic increase in cell number occurred in OE of the experimental group (from 5 to 17 OSNs) and a dramatic decrease occurred in the OE of the untreated control group (from 15 to 1 cells). When the change in cell number was plotted for experimental and control groups, a median of -1 was observed for the MTZ-treated group, indicating a general decrease in cell number, while a median of 0,5 was found for the control group, reflecting the addition of newly born transgene-expressing OSNs over the time course of the experiment (Figure

4.5b). The difference between the changes in cell number of experimental and control group was moderately significant (Kruskal-Wallis test, $H_{(1)} = 4.726$, $p = 0.03$).

Thus, treatment with 10 mM MTZ appears to alter the dynamic behavior of pOMP::NTR-T2A-GFP-expressing OSNs. However, the analysis of untreated control embryos revealed that during a 24 h interval between 3 and 4 dpf additional transgene-positive cells may occur. Thus, it may be that a loss of OSNs caused by MTZ treatment is compensated for by newborn OSNs during later time points of the experimental window. To gain better insight into this possibility, the change in the number of OSNs expressing the transgene was analyzed at different time points of the 24 h MTZ treatment period using the same 10 mM exposure conditions. A total of 18 transgene-expressing embryos were incubated in 10 mM MTZ starting from 3 dpf and the number of pOMP::NTR-T2A-GFP-expressing OSNs was quantified in individual embryos at 6, 12, and 24 h time points. For each time point different embryos ($n = 6$ embryos for each time point) were used because they were subsequently subjected to cell death analysis using immunohistochemistry against activated caspase 3. An untreated pOMP::NTR-T2A-GFP-injected control group ($n = 6$ embryos) was incubated in E3 medium and the same embryos were scored for the number of transgene-positive OSNs at 0, 6, 12 and 24 h time points before being processed by immunohistochemistry for activated caspase 3.

Generally, similar results were obtained as reported for the previous experiment (Figure 4.6a): after a 24 h incubation period, an overall decrease in cell number of 15.73% was observed for the experimental group, while a 28.57% increase was observed for the control group. Yet, when the change in cell number was analyzed for different time points, a stronger effect was observed during the initial 12 h period. When compared to the number of cells at the beginning of the experiment, an overall decrease of 5.13% could be observed after 6 hours, of 12.56% after 12 hours and of 15.73% after 24 hours. Thus, the rate of change was constant between the 6 h and 12 h time points with approximately 6% decrease per 6 h interval but declined thereafter and only a moderate decrease of an additional 3% was found over the remaining 12 h of treatment.

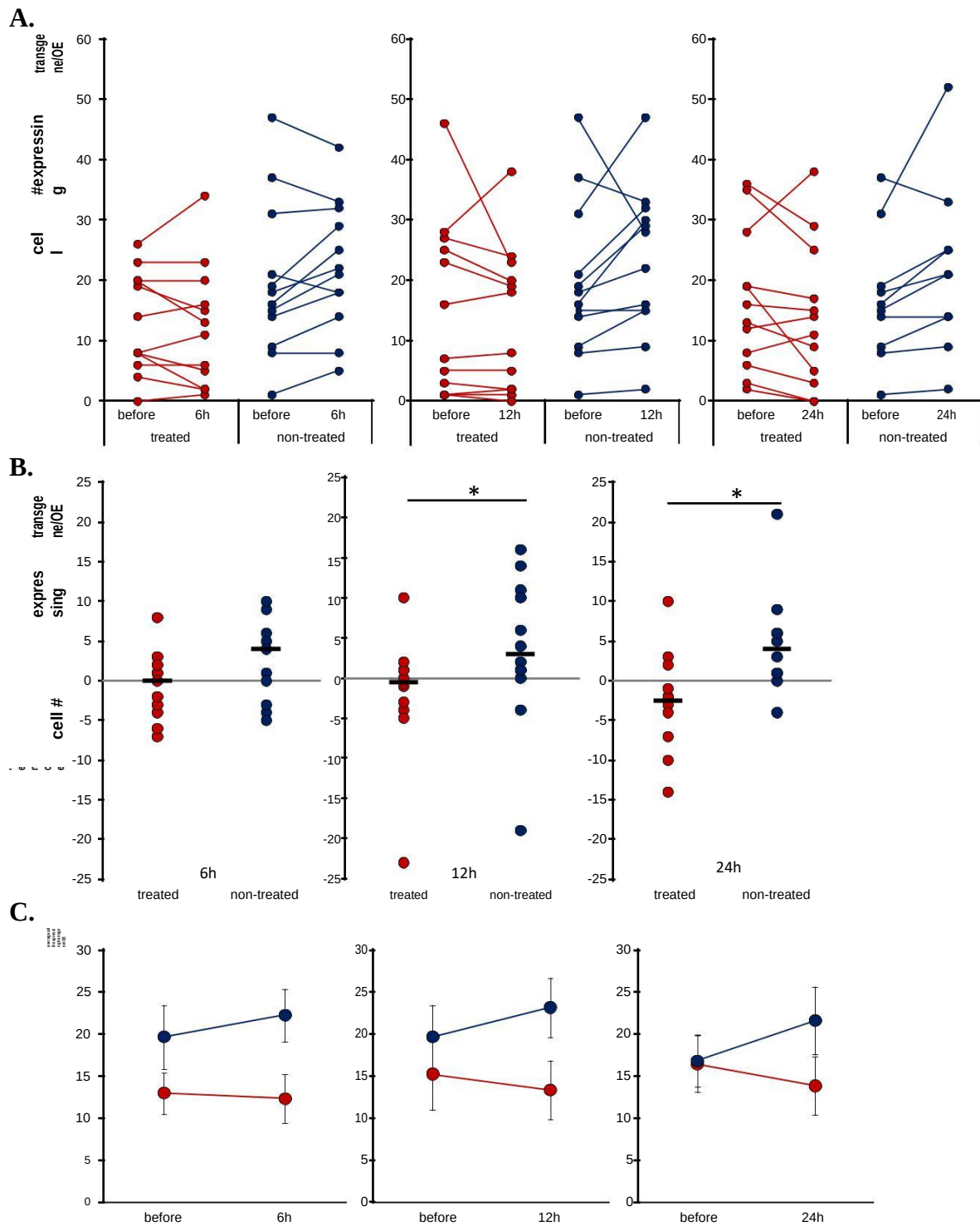


Figure 4.6. Time course of the effect of 10 mM metronidazole treatment over a 24 h time period. (A) Absolute cell numbers of transgene expressing OSNs per OE starting at 3 dpf. (B) Difference plots showing the difference in the number of transgene expressing OSNs. The horizontal bar represents median of each data set. (C) Average cell numbers of transgene expressing OSN/OE.

A similar trend but different temporal dynamics was observed when the difference in cell numbers was examined at different intervals following MTZ treatment. The medians of the difference in cell number decreased for each time point with 0, -0.5, and -2.5 for the 6, 12 and 24 h time points, respectively. However, a marked decline in cell number was first evident at 12 hours after the onset of MTZ treatment and becomes more pronounced at 24 h, while there was no significant difference between experimental and control groups at 6 h following MTZ exposure (Kruskal-Wallis rank-sum test, 0 h vs. 6 h: $H_{(1)} = 3.022$, $p = 0.082$; 0 h vs. 12 h: $H_{(1)} = 4.476$, $p = 0.034$; 0 h vs. 24 h: $H_{(1)} = 5.972$, $p = 0.015$; Figure 4.6b). Thus, cell death may occur late following the onset of MTZ treatment. However, a stronger dynamic of cell birth was observed during the first 12 h following the 3 dpf developmental time point at which the MTZ treatment was initiated. Thus, because of the combined effect of cell death and cell birth, MTZ-induced cell death needs to outweigh cell birth to become evident at the level of the entire OE, but might be more pronounced if individual cells were tracked over time.

4.3.3. Cell death analysis

So far MTZ-induced cell death was only inferred by the loss of pOMP::NTR-T2A-GFP-expressing OSNs over time. However, this method does not reveal the mechanism by which transgene-expressing cells are lost. A loss of cells could result from MTZ-induced cell death, but also from shut-down of transgene expression in individual OSNs. Thus, a more direct confirmation of cell death on a molecular level was needed. Activated caspase 3 is a common marker for programmed cell death (Reiner *et al.*, 1998). It is reasonable to assume that NTR-expressing OSNs undergo apoptosis upon MTZ treatment, similar to what was reported for skin cells (Chen *et al.*, 2011). Thus, MTZ-treated zebrafish embryos were examined using immunohistochemistry against activated caspase 3 and the number of cells expressing NTR and activated caspase 3 per OE was analyzed (Figure 4.7).

In Figure 4.7a activated caspase 3-positive and GFP-expressing cells of the 24 h treatment group are shown as a representative impression of the apoptotic effect of MTZ

treatment. Activated caspase 3 signals are localized to the nuclei of OSNs and most caspase 3-positive cells also express the transgene: 23% of the caspase 3-positive cells expressed pOMP::NTR-T2A-GFP, while 17% of pOMP::NTR-T2A-GFP -expressing cells stain positive for activated caspase 3.

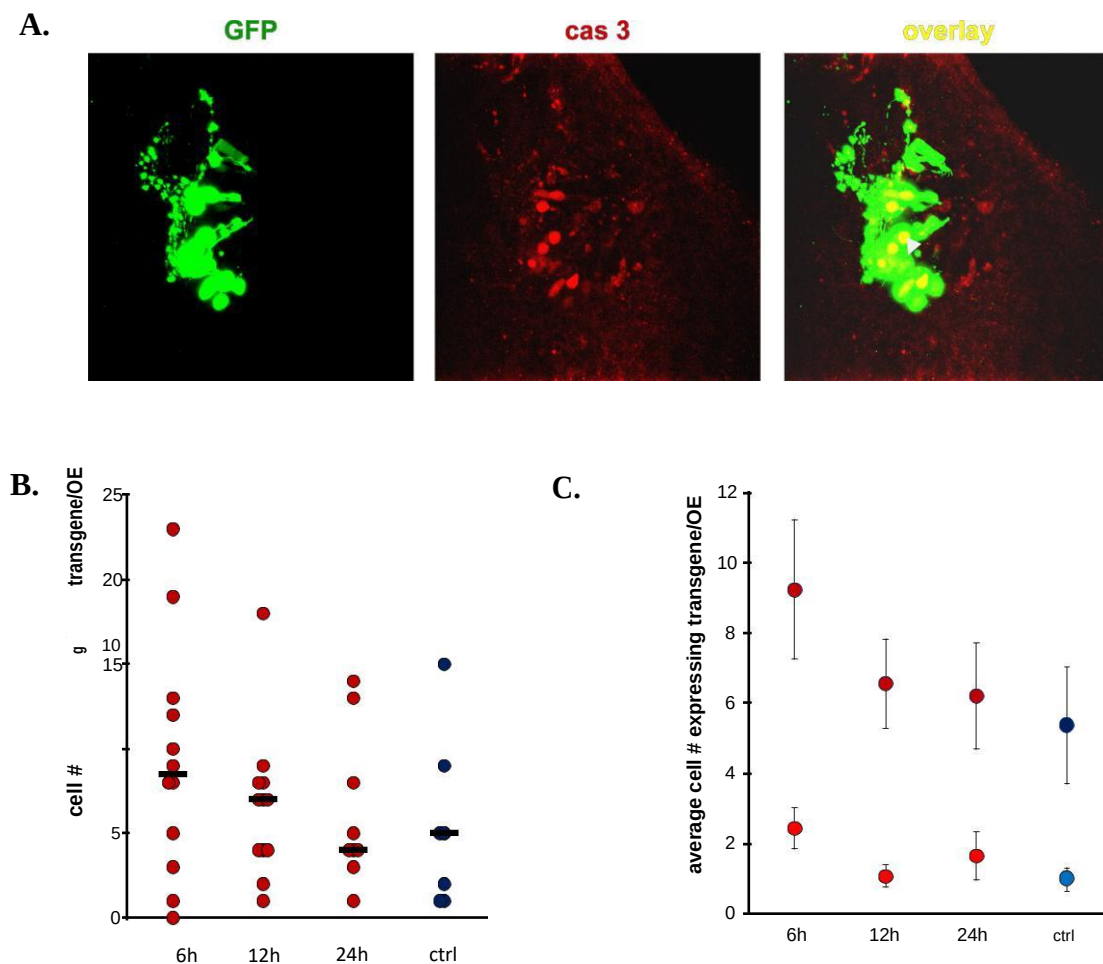


Figure 4.7. Metronidazole treatment stimulates apoptosis in transgene-expressing OSNs. (A)

Confocal micrographs of NTR-expressing OSNs (green), activated caspase 3 positive cells (red) and overlay of both channels (right). Arrowhead indicates the double positive cells. (B) Cell number of cas3-positive cells in each individual OE. (C) Average of cas3- (darker colors) and double-positive (lighter color) cell numbers at different time points.

When the number of caspase 3-positive cells in each OE was examined, an initial high number of caspase 3-positive cells can be found and the number of cells declines to control levels with the duration of the experiment. The highest number in the cells staining positive for either caspase 3 alone or caspase 3 and GFP were observed at 6 h after onset of MTZ treatment (9.25 ± 1.4) while the number of the caspase-positive cells decreased over time (6.58 ± 0.9 at 12 h, and 6.22 ± 1.51 at 24 h). Thus, the highest numbers of caspase 3-positive cells, as well as caspase 3-positive / transgene-expressing double-positive cells can be found early after the onset of MTZ treatment, similar to the rate change in the number of transgene-positive OSNs described above. It is surprising, however, that the fraction of double-positive cells is relatively small at every time point, including the initial 6 h period. It might be, however, that MTZ kills OSNs efficiently and cells stay double-positive only during a short interval. Therefore cells actively expressing the transgene may be young cells that only started expression of NTR shortly before analysis. Alternatively, cells may need a certain amount of time to accumulate sufficient amounts of NTR before becoming susceptible to MTZ and the number of apoptotic cells is a consequence of the number of cells that have reached the required levels of NTR.

4.4. Effects of expression of an enhanced version of NTR in OSNs

The experiments described so far were carried out using the wild-type *nfsB* gene, which was directly cloned from the *E.coli* genome. During the time of the work reported here, an enhanced isoform of NTR that was engineered to contain three distinct mutations had been reported (Mathias *et al.*, 2014). In this version the sequence was changed to better reflect the codon usage in zebrafish (Mathias *et al.*, 2014). It was previously shown that a triple mutant form of the NTR improve the catalytic activity of the enzyme (Chen *et al.*, 2011), however, the latest version of NTR had even a higher efficiency (50-fold compared to wild type). Thus, in principle it should allow for shorter pulse of a 10 mM MTZ treatment with high efficiency and 4 h incubation in MTZ was shown to be sufficient for cell ablation (Mathias *et al.*, 2014).

To make use of the improved cell ablation system, the triple mutant NTR (ntr^{3mut}) was obtained from Jeff Mumm (Johns Hopkins Center of Medicine, Baltimore, USA) and cloned under control of the zebrafish OMP promoter and fused to a sequence coding for the reporter protein GFP through a sequence coding for the self-cleaving peptide T2A, similar to the strategy described above (Figure 4.8a). In comparison to the previous construct (Figure 4.2 a), the only difference is a replacement of the wild-type NTR with an adapted ntr^{3mut} version of NTR. Thus, the backbone of the vector, the promoter, and the reporter were identical.

4.4.1. Effect of 10 mM MTZ treatment for 4 h

Next, the enhanced NTR construct $pOMP::ntr^{3mut}$ -T2A-GFP was injected into 18 embryos and cells expressing the reporter gene were analyzed over a 48 h time interval starting at 3 dpf. In these experiments, 10 mM MTZ was administered during the initial 4 h and was replaced by standard E3 medium during the remaining time points of the experiment. As before, a decrease in cell number was observed for the MTZ-treated $pOMP::ntr^{3mut}$ -T2A-GFP-injected embryos during the first 4 h of treatment, while a slight increase in cell number was observed for the untreated but plasmid-injected control group. Over the initial 4 h of the experiment, the number of cells decreased by 8.2% from 14.6 ± 2.76 cells to 13.4 ± 2.88 cells for the experimental group but rose by 8.7% from 7.66 ± 2.36 cells to 8.33 ± 2.62 cells for the control group. (Figure 4.8b and d). However, this difference in cell numbers between experimental and control groups was not significant (Kruskal-Wallis test, $H_{0-4\text{ h}}(1)=1.513$, $p_{0-4\text{ h}}=0.219$; $H_{0-12\text{ h}}(1)=0.107$, $p_{0-12\text{ h}}=0.744$; $H_{0-24\text{ h}}(1)=0.766$, $p_{0-24\text{ h}}=0.381$; Figure 4.8c).

Interestingly, a slight increase in cell number from 13.5 ± 2.88 to 13.9 ± 3.26 cells was observed after 1 day for the MTZ-treated group, while the cell numbers of the control group slightly diminished from 8.33 ± 2.62 to 7.33 ± 2.33 cells. During later times of the experiment, the number of transgene-expressing cells further decreased in both groups to 9.2 ± 1.67 and 5.66 ± 1.94 cells for experimental and control groups, respectively.

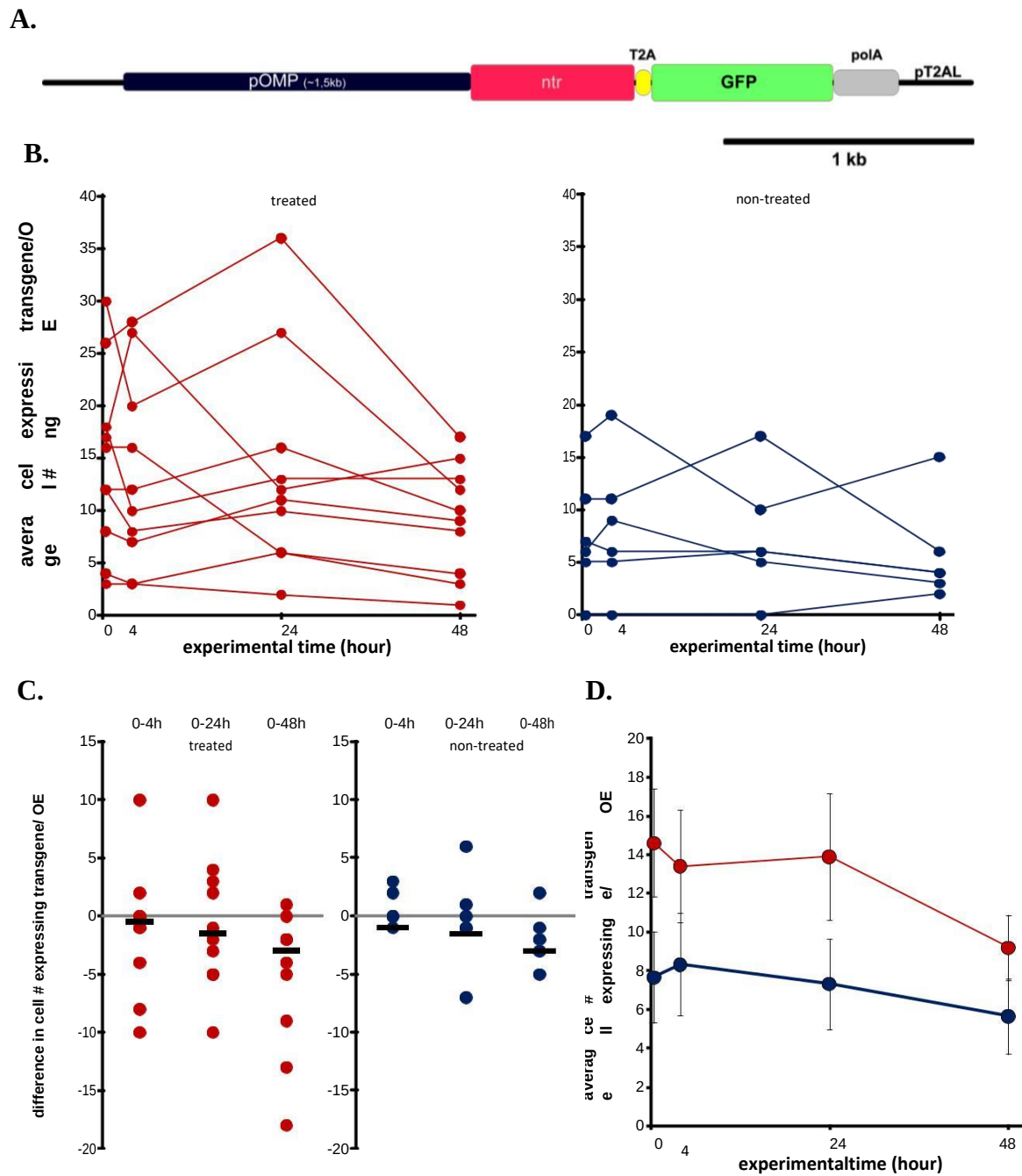


Figure 4.8. Effect of 10 mM metronidazole treatment for 4 h in OSNs expressing a mutant form of the nitroreductase gene. (A) Overview of the pOMP::*ntr*^{3mut}-T2A-GFP transgenic construct (B) Absolute cell numbers of *ntr*-expressing OSNs/OE starting at 3 dpf. (C) Plots showing the differences in the numbers of each OE. The black bars represent the medians. (D) Average cell numbers of *ntr*-expressing OSN/OE.

Thus, a short 4 h application of the drug may indeed kill transgene-expressing cells initially, but may not have a long-lasting effect and becomes overridden by the addition of new transgene-expressing OSNs during later time points. The late overall decrease seen after 48 h may not be the result of MTZ treatment because a similar decline was observed in non-treated control embryos and might be attributable to a decline of expression of the transgene between 3 and 4 dpf (Figure 4.8d).

4.4.2. Establishment of the background developmental dynamics

Because in previous experiments the number of cells expressing transgenic constructs most likely depended on a combined effect of MTZ-induced cell death and developmental addition of new OSNs, it was difficult to independently assess the effect of drug-induced cell ablation alone. In addition, the observed decrease in the number of cells expressing the transgene could also be the result of a shut-down of expression of the transgenic construct over the duration of the experiment, especially under conditions where the transgene is expressed only transiently from the injected plasmid DNA. In an attempt to overcome the latter problem, the experimental strategy was changed by incubating embryos starting from 2 dpf rather than 3 dpf because transgene expression is more stable during this interval (Çelik *et al.*, 2002). Similar to before, the number of cells expressing the transgenic construct in OSNs over the next 48 h (0 h, 4 h, 24 h and 48 h after treatment) was analyzed.

Similar to the analysis described for the wild type NTR construct the background rate of transgene expression over the experimental time window was established first. Therefore, 35 embryos were injected with the pOMP::*ntr*^{3mut}-T2A-GFP construct in 4 independent experiments and the number of transgene-positive OSNs were scored in individual embryos over time.

Generally, an increase in the number of transgene-positive cells was observed over the time course of the experiment (Figure 4.9a) and the total number of cells increased until the embryos reached the 4 dpf developmental stage (Figure 4.9b). The highest rate of change was observed for the initial 4 h of the experiment where the number of cells rose from an initial 6.22 ± 1.95 cells at 0 h to 7.77 ± 2.37 cells at 4 h, and numbers continued to rise to 10.45 ± 3.18 and 11.82 ± 3.87 cells at 24 h and 48 h, respectively. Thus, as before, a background addition of new transgene expressing OSNs takes place that complicates the analysis of induced-cell death following MTZ treatment, yet expression of the transgene may be more stable over the same interval (Figure 4.9c).

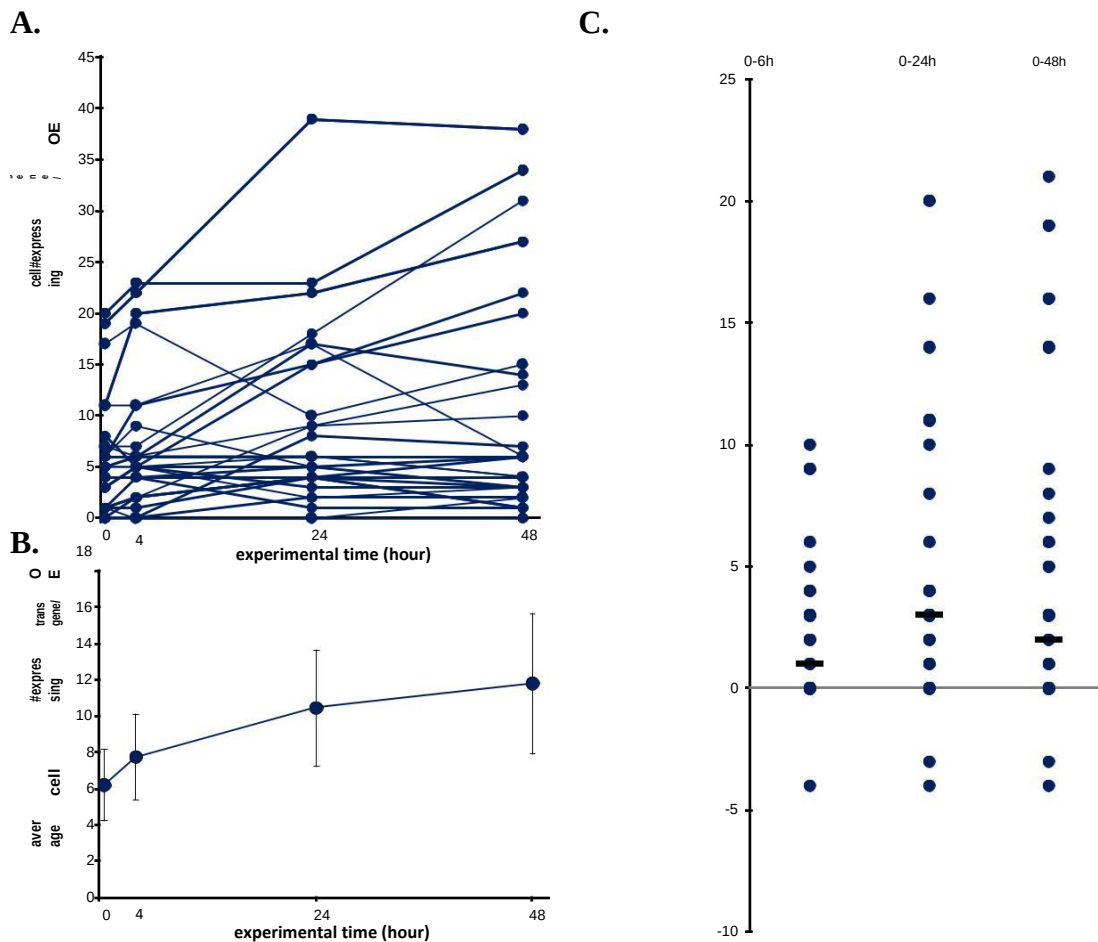


Figure 4.9. Developmental dynamics of pOMP::NTR-T2A-GFP expression between 3 and 4 dpf. (A) Absolute cell numbers of *ntr*-expressing OSNs/OE starting at 2 dpf. Embryos were grown in E3 medium for 48 h. (B) Average cell numbers of *ntr*-expressing OSNs/OE. (C) Plots showing the difference in the number of *ntr*-expressing OSNs. The horizontal bar represents median of each data set.

4.4.3. Effect of 20 mM MTZ treatment for 4 hours

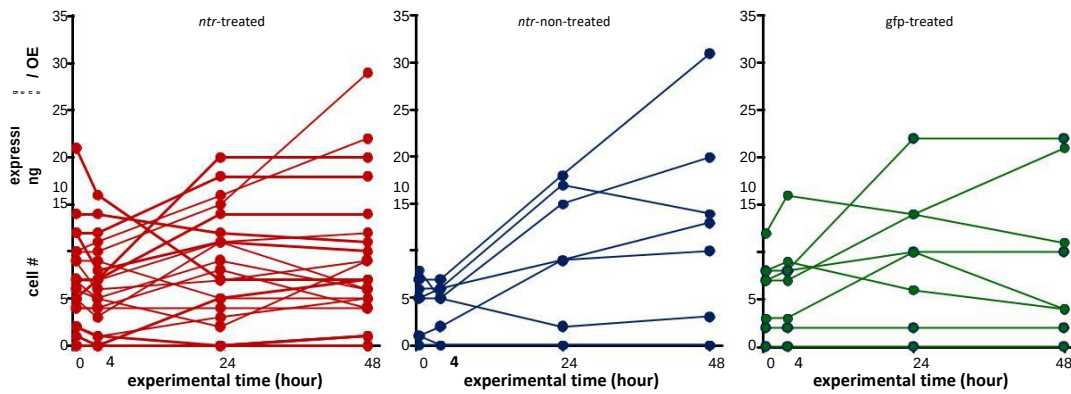
Treatment with 10 mM MTZ for 4 h was not sufficient to induce a prominent change in the number of NTR-expressing cells over a prolonged period of time (Figure 4.8). Thus, different concentrations of a short pulse of MTZ were tested to understand if more efficient ablation could be achieved. In order to investigate the toxicity and the bystander effect of the drug (White *et al.*, 2013; Mathias *et al.*, 2014), the reporter protein GFP alone under control of the OMP promoter (pOMP::GFP) was also injected and treated as a second control group.

When embryos were injected (pOMP::ntr^{3mut}-T2A-GFP experimental group: 12 embryos; pOMP::ntr^{3mut}-T2A-GFP control group: 4 embryos; pOMP::GFP: 4 embryos) and treated with 20 mM MTZ for 4 h, again an initial effect on the number of transgene expressing cells could be observed during the first 4 h of the experiment for the experimental group relative to the two control groups (Figure 4.10a, c). During the initial 4 h interval when the drug was present the cell numbers in the pOMP::ntr^{3mut}-T2A-GFP-injected 20 mM MTZ-treated group decreased from 6.12 ± 1.09 cells to 5.41 ± 0.98 cells (11% reduction), while the numbers changed from 4.12 ± 1.07 to 3.87 ± 0.9 and 5.87 ± 1.38 to 6.62 ± 1.77 in the untreated pOMP::ntr^{3mut}-T2A-GFP and treated pOMP::GFP control groups, respectively.

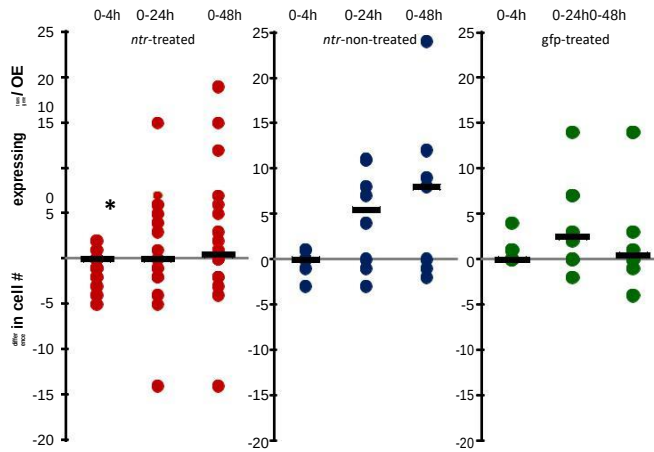
However, after the initial 4 h MTZ pulse, cell numbers started to increase for all groups, including the pOMP::ntr^{3mut}-T2A-GFP-injected MTZ-treated embryos (Figure 4.10c). The rates of change were similar for all groups over the 24 h and 48 h time intervals (the percent change rate were; pOMP::ntr^{3mut}-T2A-GFP / MTZ-treated: -11%, 36% and 12%, pOMP::ntr^{3mut}-T2A-GFP / untreated: 6%, 125% and 30%, pOMP::GFP / tretated: 12%, 47% and -5% in the time interval 0-4 h, 4 h-24 h, and 24 h-48 h respectively). These results show again, that a short 4 h pulse of MTZ may not have a long-lasting effect on ablating cells that reaches significantly beyond the duration of drug exposure. Instead, any initial drug-induced loss of OSNs may be outcompeted by the

generation of new cells at later time points of the experiment. Yet, it appears that exposure to 20 mM MTZ itself does not have any toxic effect on ciliated OSNs as revealed from embryos expressing the pOMP::GFP transgenic construct.

A.



B.



C.

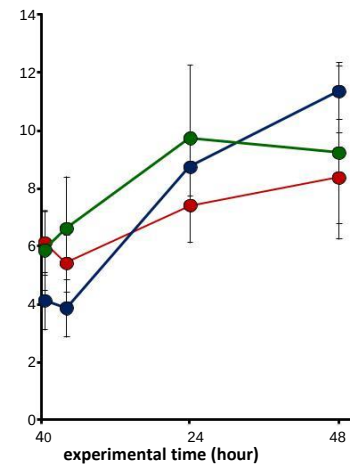


Figure 4.10. Effect of 20 mM MTZ treatment for 4 h in OSNs expressing ntr^{3mut} . (A) Absolute cell numbers of pOMP:: ntr^{3mut} -T2A-GFP-expressing (in red and blue) and pOMP::GFP-expressing (in green) OSNs/OE starting at 2 dpf. (B) Plots showing the difference in the number of OSNs expressing both constructs/OE. The black bars represent medians. (C) Average cell numbers of the cells.

4.4.4. Effect of 20 mM MTZ treatment for 24 hours

Because a short pulse of MTZ treatment was not sufficient to show any long-lasting effect on ablating transgene-positive OSNs, pOMP::*ntr*^{3mut}-T2A-GFP-injected embryos (7 embryos) were incubated in 20mM of the drug for 24 hours continuously starting at 2 dpf and analyzed at different time points during the exposure (0 h, 4 h, 24 h, and 48 h). Similar to the experiments described above, GFP expressing ciliated cells, visualized by pOMP::GFP expression (n = 6 embryos analyzed), were included as toxicity control for the experiment. However, quite similar results to the 4 h pulse were observed. The number of cells decreased from an average of 3.36 ± 1.2 to 2.54 ± 0.88 during the early 4 h phase of the treatment in the experimental group but increased during later phases: 4.45 ± 1.13 after 24 hours and 5.36 ± 1.21 after 48 hours. However, the number of cells increased continuously for both non-treated pOMP::*ntr*^{3mut}-T2A-GFP-expressing and MTZ-treated GFP control groups (Figure 4.11 a, c).

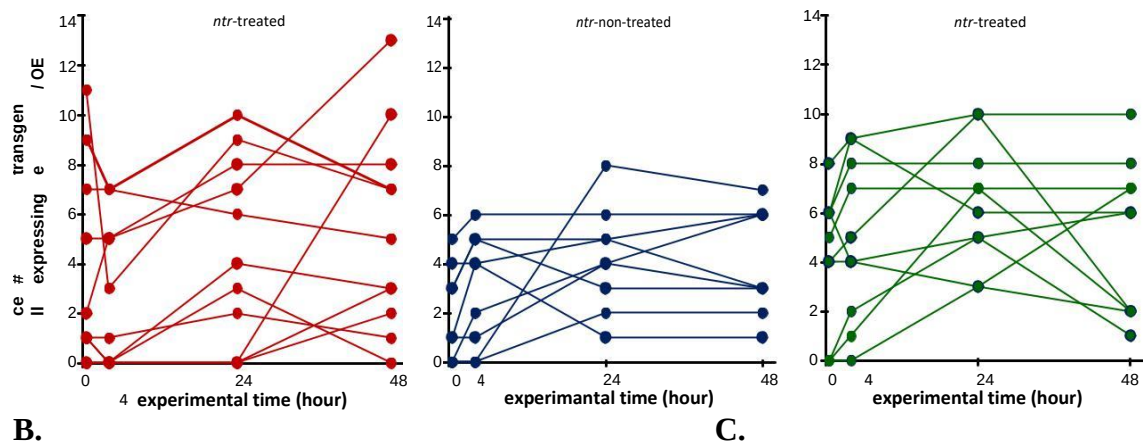
When the difference in cell number was analyzed, all differences were positive for the later time points (24 h and 48 h) for the treated experimental group and a significant difference between the treated and non-treated pOMP::*ntr*^{3mut}-T2A-GFP-injected embryos could only be found for the 4 h time point (Kruskal-Wallis test; $H_{0-4\text{ h}}(1)=5.57$, $p_{0-4\text{ h}}=0.018$, $H_{0-12\text{ h}}(1)=2.959$, $p_{0-12\text{ h}}=0.162$ and $H_{0-24\text{ h}}(1)=1.735$, $p_{0-24\text{ h}}=0.188$) (Figure 4.11c). The result is somewhat surprising as a longer lasting and more drastic effect was expected due to the continuous exposure to the drug. Yet, the stability of MTZ in solution is not known and it may be that the drug was only effective during the initial phase of the experiment.

4.4.5. Effect of continuous treatment with 50 mM MTZ

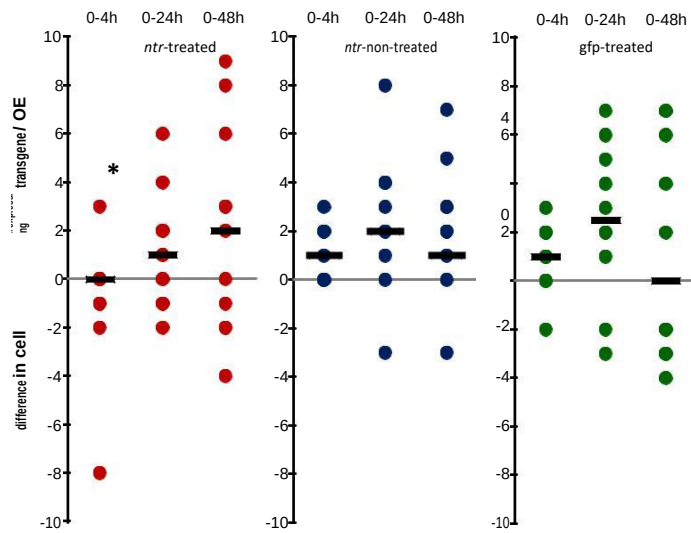
As a last condition, embryos were incubated with high, 50 mM concentration of MTZ continuously. However, in this experiment most of the fish died after one day of MTZ treatment as expected from initial mortality tests (Figure 4.1). A total of 20 embryos

were injected with pOMP::*ntr*^{3mut}-T2A-GFP and 7 embryos were injected with pOMP::*ntr*^{3mut}-T2A-GFP and 7 embryos were injected with pOMP::*ntr*^{3mut}-T2A-GFP. Of those 9 pOMP::*ntr*^{3mut}-T2A-GFP injected and 7 pOMP::*ntr*^{3mut}-T2A-GFP injected embryos were treated with the drug, while 11 embryos injected with pOMP::*ntr*-T2A-GFP construct were kept as control in E3 medium. All embryos survived the 4 h time point while only 3 MTZ-treated and 3 nontreated pOMP::*ntr*-T2A-GFP injected embryos survived longer.

A.



B.



C.

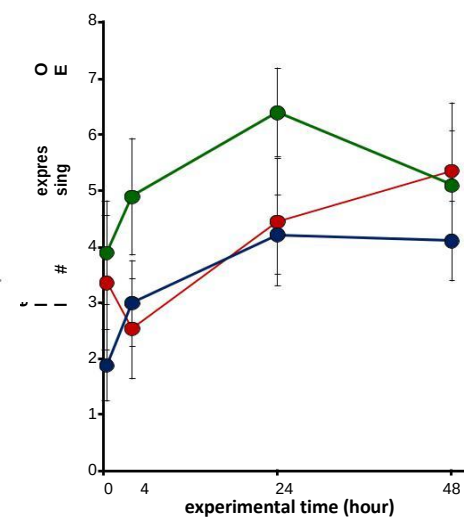


Figure 4.11. Effect of 20 mM MTZ treatment for 24 h in OSNs expressing *ntr*^{3mut}. (A) Absolute cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing (in red and blue) and pOMP::*ntr*^{3mut}-T2A-GFP-expressing (in green) OSNs/OE starting at 2 dpf. (B) Plots showing the difference in the numbers of OSNs expressing both constructs per OE. The black bars represent the medians. (C) Average number of OSNs.

When the number of cells of the surviving embryos was analyzed, a stagnation was observed for the pOMP::*ntr*^{3mut}-T2A-GFP-injected MTZ-treated group while an increase in cell numbers was obvious for pOMP::*ntr*^{3mut}-T2A-GFP untreated and treated and untreated pOMP::GFP control groups (Figure 4.12a, c). Interestingly, the rate of OSN cell birth is similar for all control groups (pOMP::*ntr*^{3mut}-T2A-GFP / untreated: 40%; pOMP::GFP / untreated: 18%; pOMP::GFP / treated: 28% after 4 hours, while the number of cells declined by 1.7% in pOMP::*ntr*^{3mut}-T2A-GFP / treated embryos (Figure 4.12b, c).

Thus, continuous exposure to high concentrations of MTZ may lead to effective OSN ablation. However, the conditions also lead to high mortality of embryos, which are not compatible with the experiment, as specific OSN cell death cannot be distinguished from general unspecific effects of the drug.

4.4.6. Effect of 50 mM MTZ treatment for 10 hours

A high 50 mM concentration of the MTZ appears to ablate cells effectively, albeit at high mortality rates. Thus, embryos were incubated in 50 mM MTZ for a shorter, 10 hour period and analyzed at 4 h, 24 h, and 48 h. Contrary to continuous exposure to 50 mM MTZ, survival rates were high (89%) for pOMP::*ntr*^{3mut}-T2A-GFP-injected / MTR-treated and untreated control groups. Surprisingly, however, all pOMP::GFP-injected embryos died after 4 h of MTZ treatment, most likely because of the quality of eggs, rather than experimental conditions.

When embryos were incubated at 10 mM MTZ for 10 h, an initial decline in the number of cells by 31% was observed for the experimental group at 4 h, while surprising increases in the number of cells by 34% at the 24 h and 123% at the 48 h time point were observed for the untreated control group. The slope of the change was different for MTZ-treated and untreated controls.

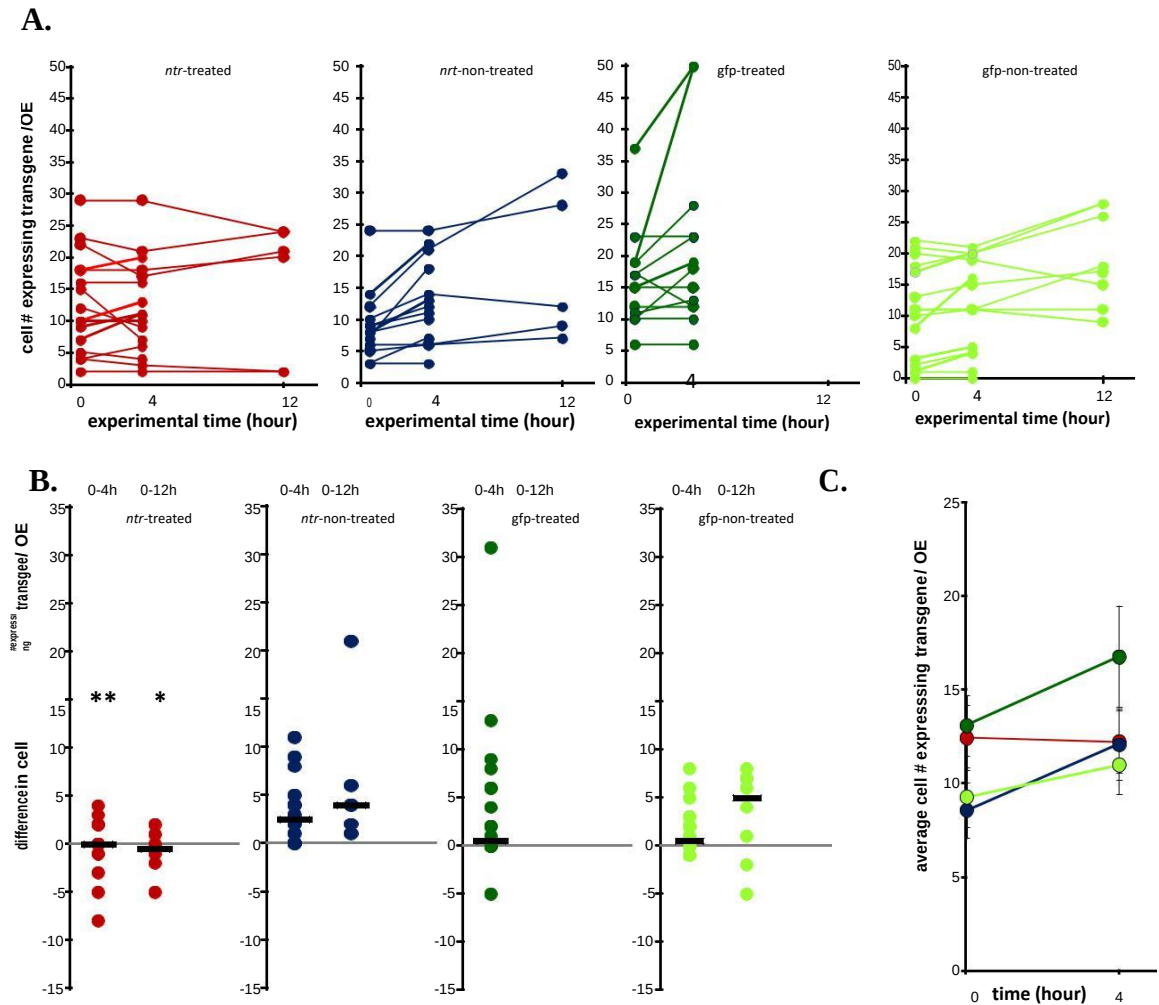


Figure 4.12. Effect of 50 mM MTZ continuous treatment in OSNs expressing *ntr*^{3mut} gene. (A) Absolute cell numbers of *pOMP::ntr*^{3mut}-T2A-GFP-expressing (in red and blue) and *pOMP::GFP*-expressing (in dark and light green) OSNs/OE starting at 2 dpf. (B) Plots showing the difference in the number of OSNs expressing constructs. Black bars represent the median. (C) Average cell numbers of OSNs expressing the constructs.

When the difference in cell number was analyzed, initially the median of the change in individual noses is negative, indicating a decrease in cell number (-4.5). Yet, it increases to 0 within 24 h after the onset of treatment and becomes positive with 4 cells by 48 h. Thus again, an initial decrease in the number of transgene-positive cells is followed by a later increase. Nevertheless the difference in cell number in the first 12 h of the treatment is significant (Kruskal-Wallis rank-sum test; $H_{(1)} = 7.707$, $p_{0-4\text{ h}} = 0.006$; $H_{(1)} = 7.232$, $p_{0-12\text{ h}}$

=0.007) while significance could not be reached for any of the later time point ($H_{(1)}=2.683$, $p_{0-24\text{ h}} = 0.101$; Figure 4.13b). Thus, 50 mM MTZ applied for a short 10 h period appears to have a significant effect on transgene-expressing OSNs, which induces only mild mortality, albeit only transitional.

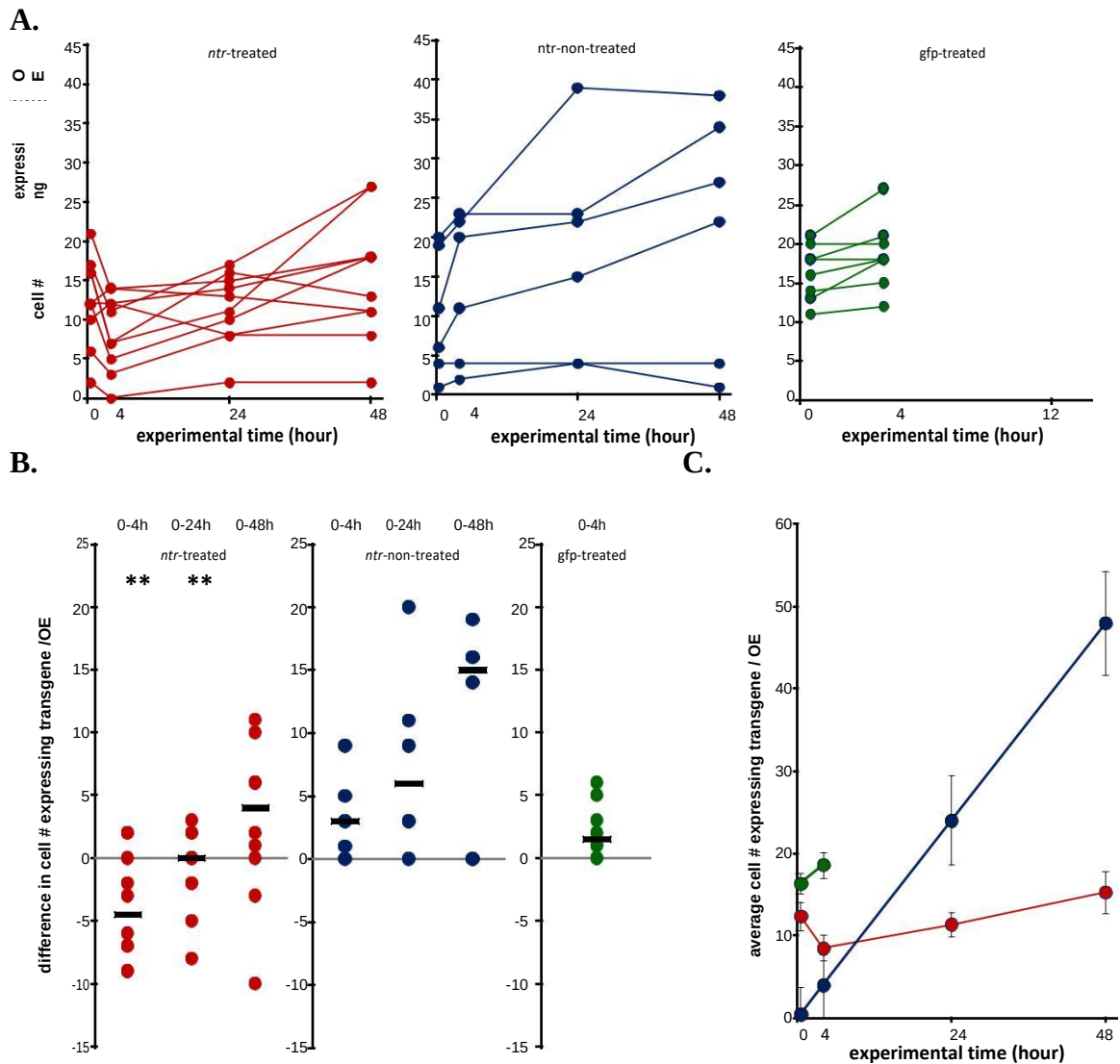


Figure 4.13. Effect of 50 mM MTZ treatment for 10 h in OSNs expressing a *ntr*^{3mut} gene. (A) Absolute cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing (in red and blue) and pOMP::GFP-expressing (in green) OSNs/OE starting at 2 dpf. (B) Plots showing the difference in the number of OSNs expressing both constructs/OE. The black bars represent the medians. (C) Average numbers of OSNs expressing constructs.

4.4.7. Effect of continuous treatment with 10 mM MTZ

Because high doses of MTZ had a lethal effect on the embryos and short pulses of MTZ treatment only showed transient effects, pOMP::*ntr*^{3mut}-T2A-GFP-injected embryos were treated continuously with 10 mM MTZ over a 48 h period. A total of 9 embryos were injected with pOMP::*ntr*^{3mut}-T2A-GFP (5 for experiment group and 4 for control group) and 4 embryos were injected with pOMP::GFP as second control.

The effect on the number of cells in this continuous treatment was somewhat similar to the 10 mM continuous treatment of embryos expressing the wild type pOMP::NTR-T2A-GFP (Figure 4.6). A slight increase in the average number of cells during the first 4 h of the treatment (30%), followed by a decline or stagnation thereafter (-16% after 24 hours and -10% after 48 hours compared to the 4 hour data point) (Figure 4.14a and c). Thus, under these conditions, continuous application of the drug had a longer lasting effect on the dynamics of pOMP::*ntr*^{3mut}-T2A-GFP-expressing OSNs. The flat profile of change in the average number of cells is in strong contrast to the continuous increase in the number of cells that is observed in both control groups, similar to previous experiments.

When the medians of the change in the cell number for each individual OE was analyzed, a near zero or slightly positive number is observed while increasing positive values can be found for untreated pOMP::*ntr*^{3mut}-T2A-GFP injected embryos (3, 6 and 15) and treated pOMP::GFP injected embryos (2, 9, and 15.5). Thus, continuous 10 mM exposure to MTZ appears to effectively slow down the developmental increase in transgene expressing OSNs, most likely because many transgene-positive cells die (Figure 4.14b). However, the difference in cell numbers between the experiment and control groups reached significance only for the 12 h time point (Kruskal-Wallis test, $H_{0-4\text{ h}}(1) = 0.765$, $p_{0-4\text{ h}} = 0.382$; $H_{0-12\text{ h}}(1) = 5.893$, $p_{0-12\text{ h}} = 0.015$; $H_{0-24\text{ h}}(1) = 2.671$, $p_{0-24\text{ h}} = 0.102$).

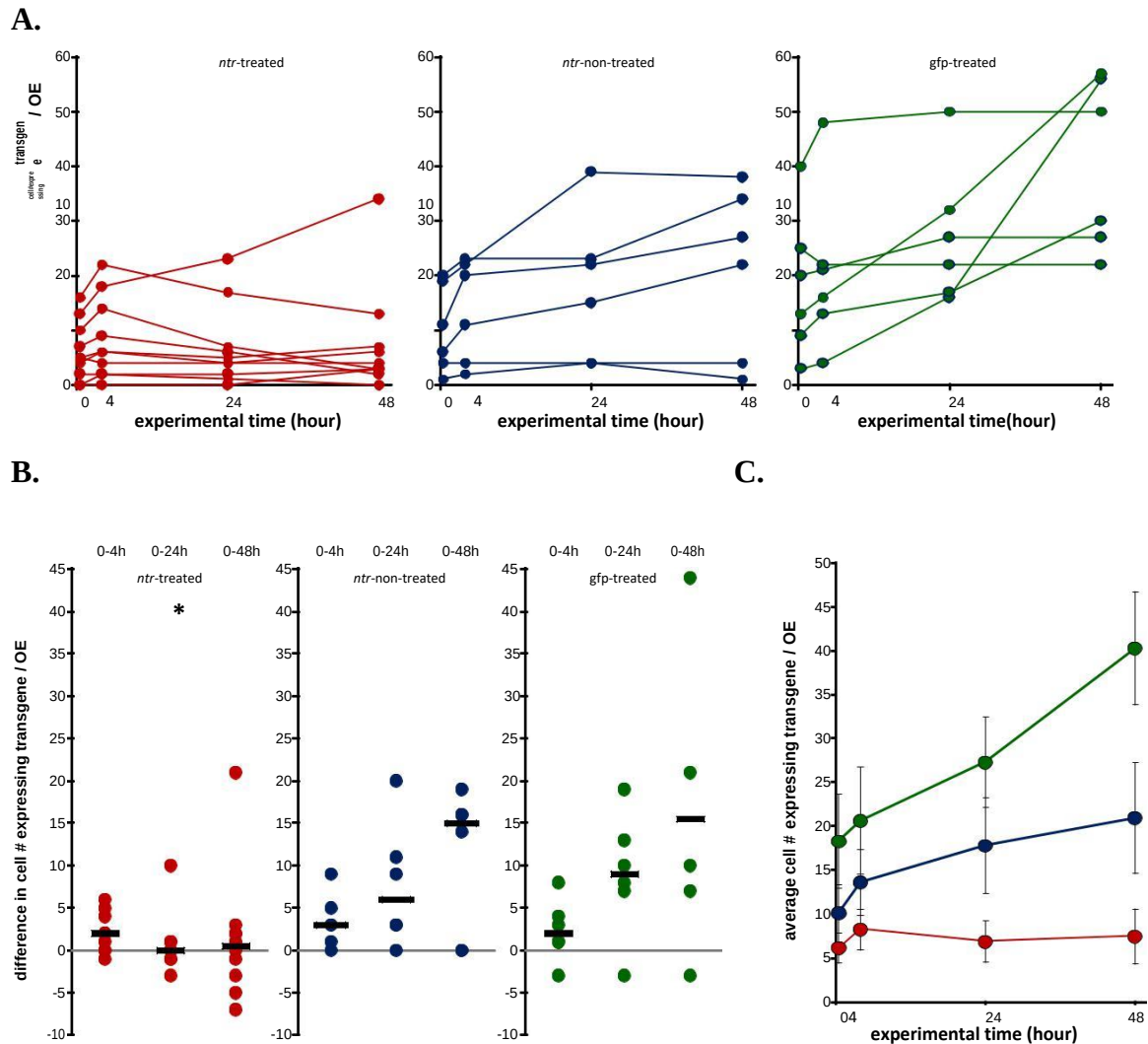


Figure 4.14. Effect of 10 mM continuous MTZ treatment in OSNs expressing *ntr* gene. (A) Absolute cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing (in red and blue) and pOMP::GFP-expressing (in green) OSNs/OE starting at 2 dpf. (B) Plots showing the difference in the number of OSNs expressing both constructs/OE. The black bars represent medians. (C) Average cell numbers of OSNs.

4.5. Tracking cells by photo conversion

Cell ablation experiments with different MTZ concentrations and duration of drug incubation showed that the olfactory system of the developing zebrafish embryo is

expectedly dynamic: new OSNs are continuously generated over the experimental period, any loss in OSNs through MTZ-induced cell ablation is offset by newly generated OSNs. For this reason MTZ-induced cell death cannot easily be detected by a decrease in cell number because dying neurons are continuously replaced by new cells after onset of the drug treatment. In order to better distinguish between cells that were present at the onset of drug treatment and cells that were generated after the drug was applied the photo-convertible fluorescent reporter dendra2 was utilized.

The emission spectrum of dendra2 can be converted from green to the red by a short pulse of UV or short-range blue light (Chudakov *et al.*, 2007). Thus, a new transgenic construct, pOMP::*ntr*^{3mut}-T2A-dendra2, containing the OMP promoter driving expression of the enhanced version of NTR and dendra2 was generated and injected into one-cell stage embryos (Figure 4.15a). Cells that already expressed the reporter gene at the beginning of the experiments, 2 dpf (time point 0 h) were converted from green to red fluorescence before embryos were treated with 10 mM MTZ. The numbers of converted and unconverted cells were subsequently counted at 4 h and 12 h time points.

Different from published reports, photo-conversion was not 100% and most green to red-converted cells retained green fluorescence. Moreover, because individual cells continue to express the transgenic construct and nascent unconverted dendra2 emits fluorescence in the green range, it is expected that even successfully converted cells regain green fluorescence over time (Figure 4.15b).

Because green to red-converted cells also show green fluorescence in addition to red fluorescence, green fluorescent cells represent both, cells that were present at the beginning of the experiment and newly produced cells, while red fluorescent cells represent cells that existed before drug application. Newborn cells are, thus, represented by the difference between the numbers for green and red fluorescent cells, although fading of the red-converted version of dendra2 cannot be ruled out.

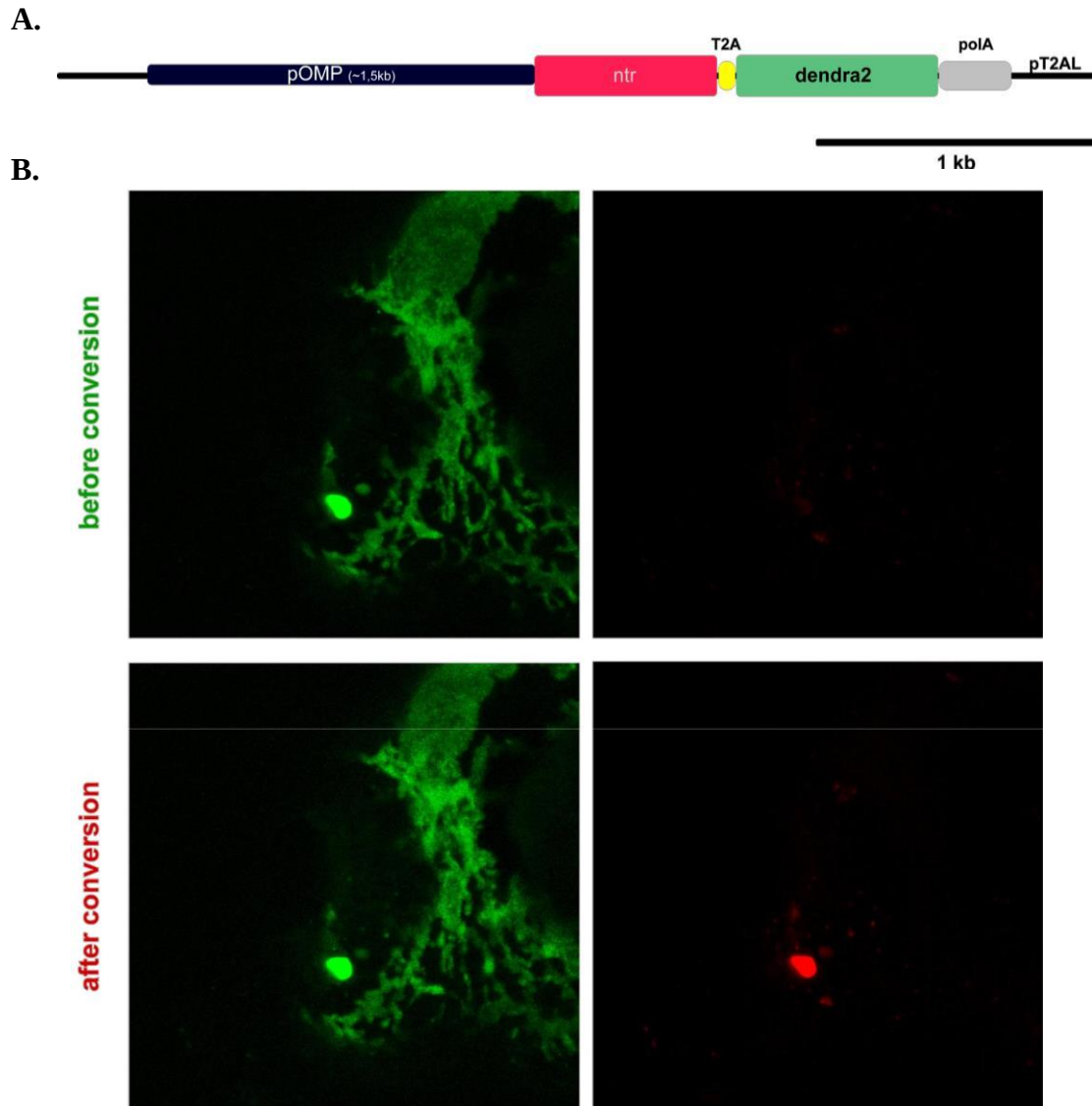


Figure 4.15. Dendra2 conversion in $pOMP::ntr^{3mut}$ -T2A-dendra2 expressing ciliated cells of 3 dpf embryo OE. (A) Overview of the $pOMP::ntr^{3mut}$ -T2A-dendra2 transgenic construct. (B) Confocal micrograph of dendra2-expressing OSNs red signal shows the activated dendra2, and the green signal (non-converted dendra2) does not abolish after conversion .

When the number of cells was analyzed for each individual nose, a dramatic decrease in number of cells emitting red fluorescence from 9.25 ± 1.56 to 3.37 ± 0.7 for experiment group and from 2.5 ± 0.28 to 1.5 ± 0.3 for the control group, was observed after 4 h of treatment, while no red fluorescent cells could be observed at 12 h for both experimental

groups. On the other hand, green fluorescent cells are either increasing or stagnating in the control group as expected (from 2.5 ± 0.2 to 2.75 ± 0.1 at 4 h and to 2.5 ± 0.3 after 12 h) or showed a decrease from 9.25 ± 1.56 to 7 ± 0.98 after 4 h and 6.875 ± 1.5 at the end of the experiment. (Figure 4.16a and c).

The overall number of transgene-expressing cells behaves similar (Figure 4.16 d). The medians for green fluorescent cells are negative for the experimental group, -1.25 for the 4 h time point and -2 for the 12 h time interval, while the medians of the control group were close to zero. However, since the number of the cells emitting red fluorescence decreased and eventually ceased entirely in an unexpected manner, the medians of the difference for each time point were negative: -6 and -8.5 for treated group and -0.95 and -2.97 for control group after 4 and 12 h respectively (4.16c). This result can be explained in two alternative ways; either some of the cells that were present at the onset of treatment were replaced by newly born cells, or the converted form of the dendra2 protein is not stable and decays rapidly over time.

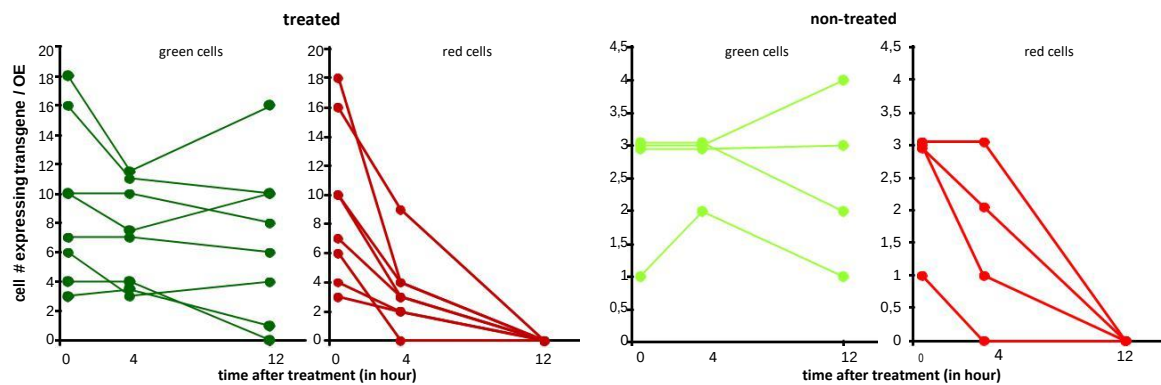
4.6. Stability of the photo-convertible reporter protein

Decay in the red fluorescent signal is more likely rather than death of all cells produced before the treatment, as the number of cells decreased significantly in the control group unexpectedly as well. To investigate this possibility, the stability of the converted dendra2 protein was examined. To do so, cells were converted from green to red fluorescence and the persistence of the red signal was monitored over time (Figure 4.17).

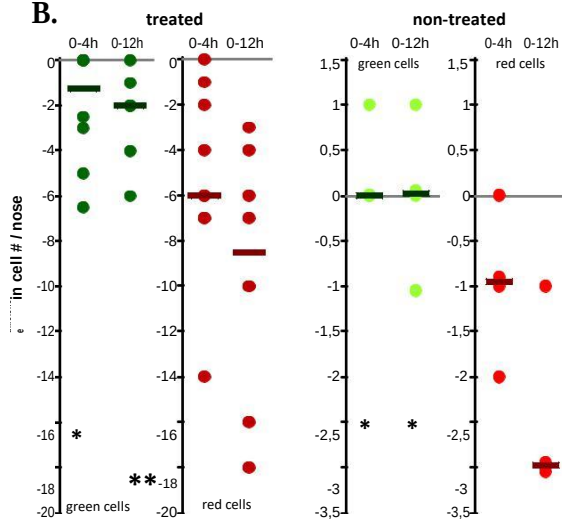
Since dendra2 is very sensitive to UV and the blue spectrum of the light, imaging the green signal was avoided 2 and 4 h after conversion. As suspected, faint signals emitted from smaller cells or newly produced cells decreased after 2 h and vanished completely after 4 h. In addition, even the signal of brightly fluorescent cells decays over time (Figure 4.17). Thus, the decrease in the number of cells observed in the previous MTZ experiment

may result from the low stability of the converted form of dendra2 but not only from MTZ-induced cell death. As conclusion, ambiguous stability of the activated dendra2 protein is not dependable to utilize in the tracing of the ciliated cell lineages, and the dramatic decrease in the cell number expressing activated dendra2 cannot be deduced as an effect of the MTZ as it might be combined consequence of the weak endurance of dendra2 and cell death.

A.



B.



C.

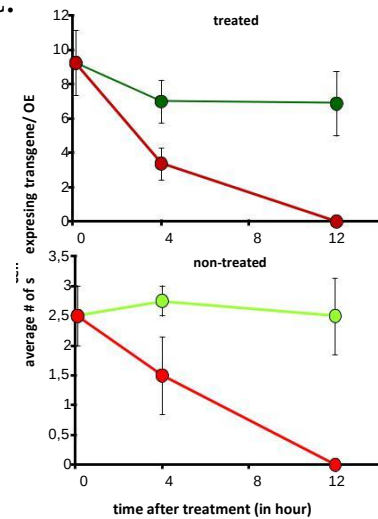


Figure 4.16. Effect of 10 mM continuous MTZ treatment in OSNs expressing dendra2 with *ntr*^{3mut}.

(A) Absolute cell numbers of pOMP::*ntr*^{3mut}-T2A-dendra2-expressing (non-converted cells in green, converted cells in red; conversion at 0 point of the experiment) OSNs/OE starting at 2 dpf. (B) Plots showing the difference in the cell number. The black bars represent medians. (C) Average numbers of OSNs.

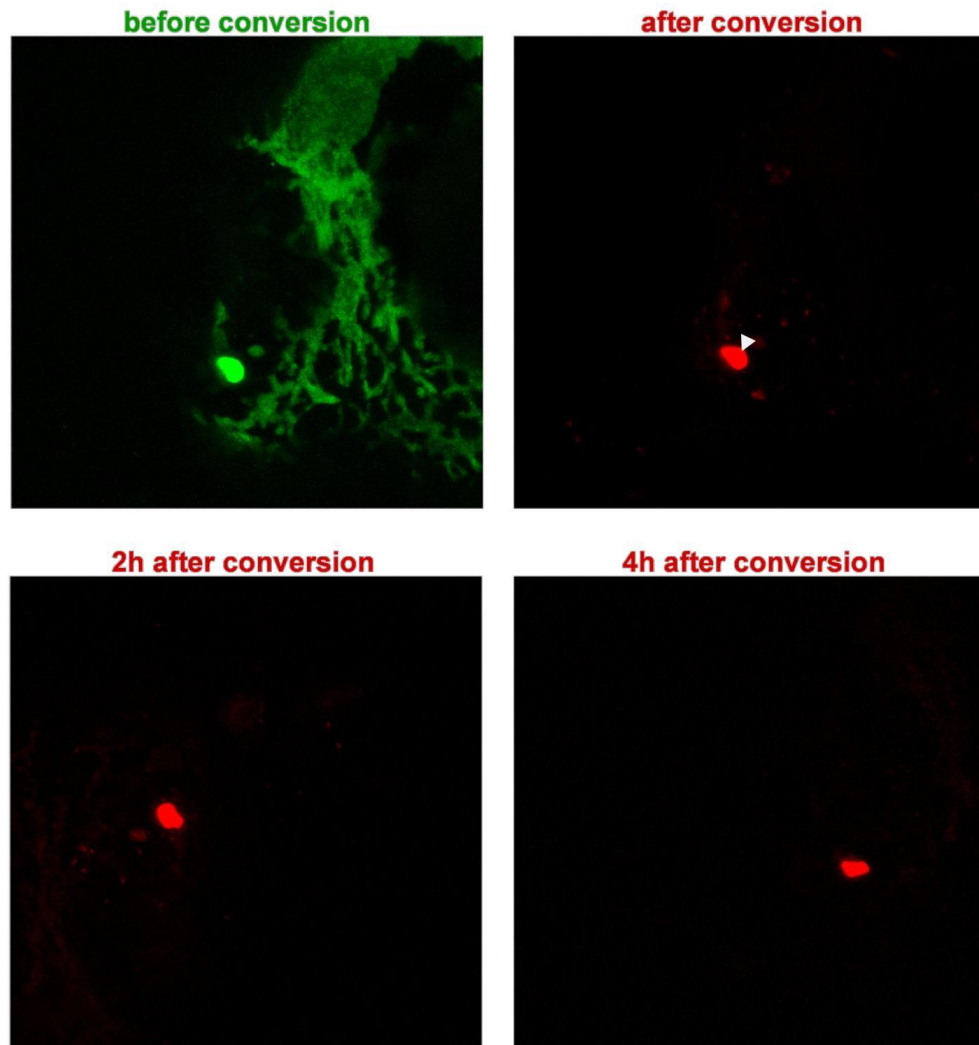


Figure 4.17. Dendra2 conversion stability in pOMP::*ntr*^{3mut}-T2A-dendra2 expressing ciliated cells of 3 dpf embryo nose at different time points. Confocal micrograph of dendra2-expressing OSNs (in green), red signal is showing the activated dendra2 and there are faint signals converted but lost during the time course of the experiment showing that the signal can be lost over time (indicated with arrow head).

In summary, in early zebrafish embryos the effect of the NTR-mediated cell ablation is short lived and appears at detectable levels only during the interval when the drug is present. The MTZ/NTR approach triggers cell death by apoptosis in OSNs but the dynamics of the early embryonic OE preclude clear-cut conclusions as new transgene-positive cells are constantly added to the tissue. The enhanced version of NTR appears to have a stronger effect, especially at moderate drug concentration. Based on a systematic

variation in concentration and exposure parameters, 20 mM MTZ for up to two days of constant exposure might be the best conditions for further experiments.

5. DISCUSSION

The major aim of the study presented in this thesis was to assess the reaction of the zebrafish olfactory system to the selective removal of ciliated neurons, which form a subpopulation of chemosensory neurons in the zebrafish OE. Different from the rodent OE, the zebrafish OE comprises a variety of chemosensory subsystems that can be found in anatomically distinct structures in the rodent nose, such as the vomeronasal organ (Mombaerts, 2004), the septal organ (Tian and Ma, 2004), or the ganglion of Grüneberg (Fuss *et al.*, 2005; Ma, 2010). Accordingly, distinct morphological and molecular subtypes of chemosensory neurons can be found in the single zebrafish OE. Among those, the ciliated and microvillous OSNs are most numerous (Sato *et al.*, 2005), while crypt and kappe neurons form smaller subpopulations (Hansen and Zielinski, 2004; Germana *et al.*, 2007; Ahuja *et al.*, 2014; Parisi *et al.*, 2014).

The zebrafish OE, similar to the OE of other organisms, has a very high regenerative capacity. Because of their direct exposure to the outside world, chemosensory neurons are vulnerable to environmental insult and have a limited life span (Graziadei and Graziadei, 1979; Mackay-Sim and Kittel, 1991; Tsai and Barnea, 2014). Thus, chemosensory neurons need to be replaced continuously to maintain a sense of smell (Leung *et al.*, 2007). However, this maintenance neurogenesis needs to be carefully balanced as both over- and underproduction of neurons would be fatal. However, very little is known about the regulation of olfactory neurogenesis and how the system achieves to produce exactly the right number of new chemosensory neurons (Brann and Firestein, 2014). In addition, acute injury to OE tissue triggers robust and rapid proliferation responses to restore tissue loss (Schwob, 2002; Iqbal and Byrd, 2010). Thus, proliferative activity must be regulated by as of yet ill-defined signals that mediate the need of neurogenesis between the OE tissue and appropriate stem cell / progenitor populations that give rise to new sensory neurons.

In the mouse and frog OE purinergic signaling has been implicated in regulating olfactory neurogenesis (Hassenklöver *et al.*, 2009; Jia *et al.*, 2009). In the most simplistic form of this pathway, dying neurons release their cytoplasmic content including purinergic compounds, such as ATP and derivatives, into the tissue. The purinergic compounds then either directly, or indirectly through a specific type of olfactory glia, the sustentacular cells, trigger proliferation of stem and progenitor cells (Hassenklöver *et al.*, 2009; Jia *et al.*, 2009). Evidence for this model comes from direct stimulation of cell proliferation by purinergic compounds (Jia *et al.*, 2009), loss of ongoing neurogenesis by inhibition of purinergic receptors (Hassenklover *et al.*, 2009), and calcium imaging in OE slices in response to purinergic substances (Hassenklover *et al.*, 2009; Dittrich *et al.*, 2014). However, additional pathways, including FGF and Wnt signaling have also been implicated in regulating olfactory neurogenesis (Wang *et al.*, 2011; Jia *et al.*, 2011).

The teleost OE is unique, because it contains different subtypes of chemosensory neurons in the same OE. Therefore, an interesting question is whether the different cell types are initially generated from the same neuronal lineage and diverge during differentiation or if they are generated from independent stem cell populations. A similarly exciting question is whether the system can discriminate which neuronal subtypes need to be generated at any given time. Thus, does the generation of ciliated and microvillous cells depend on the same signaling mechanism or do different signaling pathways stimulate proliferation of either subtype?

As a first step towards an understanding of differential neurogenesis in the zebrafish OE, we developed a cell type-specific cell ablation paradigm. The system would allow us to test whether removal of either type of neurons promotes cell proliferation and if such a response would be cell type-specific. Thus, by selectively killing ciliated neurons, we can ask whether new neurons are generated in response to the loss and if only ciliated or also other types of neurons are generated.

The approach is based on chemical-induced, NTR-mediated ablation (Pisharta and Parsons, 2009). By expressing the NTR enzyme under control of the cell type-specific OMP promoter (Celik *et al.*, 2002; Sato *et al.*, 2005) in ciliated cells, these neurons can be selectively targeted by the drug MTZ that is converted into a cytotoxic product by NTR.

5.1. How selective is neurogenesis?

Assuming the cell type-specific cell ablation approach presented here would have yielded more robust outcomes, what could have been possible results? The approach is tailored to examine if different types of chemosensory neurons are generated from the same stem cell / precursor population and if the same signals promote proliferation of ciliated, microvillous, crypt and kappe neurons alike. Thus, the central question is, whether neurogenesis under maintenance or repair conditions is selective for the cell type that needs to be replaced.

A first answer comes from early development, where different cell populations from the olfactory placode and neural crest appear to contribute to microvillous and ciliated OSNs (Saxena *et al.*, 2013; Harden *et al.*, 2012). The olfactory placode develops from the convergence of cellular fields from the edge of the neuronal tube and cell division is blocked until the formation of the placode (Harris and Hartenstein, 1991). It is highly probable that different lineages of cells migrating from the neural tube to the placode might give rise to different cell population in the final olfactory epithelium (Whitlock and Westerfield, 2000). Sox10-positive precursors from the neural crest appear to give rise exclusively to microvillous OSNs through invasion of the OE starting from 18 hours after fertilization and ablation of the neural crest cells prevents restoration of the microvillus cell population (Saxena *et al.*, 2013; Whitlock and Westerfield, 2000).

Ciliated OSNs, on the other hand, appear to arise from Sox2-positive precursors that are generated from the early olfactory placodal field (Tompkin *et al.*, 2009). Thus,

developmentally, microvillous and ciliated OSNs seem to arise from different origins. It is unknown, however, whether microvillous cell progenitors invade the OE tissue at later stages and settle within the tissue to form a resident precursor population or if a switch in neurogenic potential occurs during later phases of development.

Other lines of evidence for independence of OSN subpopulations come from the rodent OE, where OR gene expression is zonally restricted (Ressler *et al.*, 1993; Vassar *et al.*, 1993; Miyamaichi *et al.*, 2006). Zonal restriction could be a sign of lineage commitment. Indeed, commitment has been observed in two olfactory epithelium-derived cell lines (Illing *et al.*, 2002; Pathak *et al.*, 2007). Those cell lines differentiate into OSNs *in vitro* and generate limited sets of OR genes in a non-clonal fashion. In addition, two types of dorsal OE lineages have been identified in the mouse OE, which are selectively committed to class I and class II OR genes, respectively (Bozza *et al.*, 2009). Thus, lineage commitment of distinct functional subpopulations already exists within ciliated OSNs. However, it is unknown in as much this commitment is cell autonomous, e.g. through random epigenetic switches, or whether commitment would be a secondary effect under the influence of positional cues within the tissue. The proof will await further experimentation, but given the observations outlined above, it will be more likely that morphological OSN subtypes arise from subtype-specific lineages.

In as much could a successful MTZ/NTR approach shed light onto this problem? Selective ablation of a large number of ciliated OSNs could, at least in one scenario, trigger selective proliferation of ciliated neurons but not of the other types. This would argue that specific signals can convey the need to replace a determined subset of cells. Yet, the selectivity could be a consequence of the signal rather than the progenitor cell's potential to generate only one type of neurons. These experiments are not possible in the rodent OE because the different cell types are situated in different anatomical structures (Munger *et al.*, 2009; Breer *et al.*, 2006). Thus again, cell type specificity could be guided by positional cues derived from the tissue. The African clawfrog *Xenopus*, for instance, possess two functionally different OEs in addition to a VNO (Higgs and Burd, 2001). In

the adult, one OE serves detection of airborne chemicals, while the other one detects water-born odorants, similar to the VNO. All tissues derive from a common olfactory placode and undergo substantial remodeling during metamorphosis from larvae to adult, including a change in expression of receptors in a way that the larval waternose will become the airnose in the adult and the adult waternose, or medial cavity, is a new addition (Ivan Manzini, personal communication). Thus, stem cells could switch mode of neurogenesis or a developmental signal could switch activity between different stem cell lineages (Taniguchi *et al.*, 2011; Eisthen *et al.*, 2009; Munger *et al.*, 2009).

5.2. Two modes of neurogenesis

Neurogenesis in the adult zebrafish olfactory epithelium has been demonstrated to occur in two modes: maintenance and injury. During the maintenance of the tissue which happens in response to natural turnover, cell proliferation occurs exclusively in two active zones located in the interlamellar curves between lamellae and at the sensory / nonsensory boundary (Byrd and Brunjes, 2001; Oehlmann *et al.*, 2004; Iqbal and Byrd-Jacobs, 2015; Bayramli, unpublished). It is suggested that constantly proliferating neuronal precursors reside within those regions, differentiate and mature while actively migrating away from their site of neurogenesis to invade the sensory region of the OE (Bayramli, unpublished data).

Different stem cell / precursor populations have been suspected to contribute to tissue maintenance, horizontal and globose basal cells. The contribution of each cell type to maintenance neurogenesis and their interdependence remain largely unexplained. It is suggested that horizontal basal cells are slow cycling stem cells that predominantly contribute to responses to acute injury (Leung *et al.*, 2009), while globose basal cells may contribute predominantly to maintenance or as a transitory amplifying cell in the lineage of horizontal basal cells (Leung *et al.* 2009; Calof *et al.*, 2002; Schwob *et al.*, 2004). Globose basal cells, however, have been shown to be a rather diverse group of cells with different molecular properties and developmental potential (Schwob *et al.*, 2004).

The second mode of neurogenesis can be triggered by acute injury to OE tissue and appears to be different from maintenance neurogenesis. In zebrafish, experimental-induced tissue injury induces a robust proliferation response in regions different from the neurogenic zones at the interlamellar curves and sensory / nonsensory boundary. When the tissue is irrigated with cytotoxic chemicals, such as ZnSO₄ or Triton X-100, strong proliferation is seen all along the lamella including the sensory region of the OE (Iqbal and Byrd-Jacobs, 2015; Capar, 2015). The response to injury appears to be local and can be triggered in individual lamella upon mechanical damage (Capar, 2015).

The nature of the stem cells that respond to injury remains to be explored in more detail in zebrafish. In the rodent system, horizontal basal cells have been implicated in the response to injury (Leung *et al.*, 2009). These normally slow-cycling cells become activated by largely undefined molecular signals to speed up the rate of cell divisions and my replenish the pool of globose basal cells and transit amplifying cells. These two different modes of neurogenesis raise the question as to how the different responses are triggered at the molecular level and how selective they might be in terms of cells that are generated.

Since OSN expressing the same chemosensory receptor target defined glomeruli in the olfactory bulb (Buck, 2000; Braubach *et al.* 2012), axon targeting assays may also be informative about any selective response of the tissue during the neurogenesis. A massive lesion of the OSNs causes mistargeting of the axons in the olfactory bulb, while the lesion of only a specific subpopulation of the OSNs permits more precise targeting (Cheung *et al.*, 2014).. The mistargeting of the newly generated cell axons can be considered as an immediate massive cell proliferation and it may indicate that the regeneration of the cells rather occur randomly regardless to selection of the cell type produced during the huge amount of loss. Similarly, ciliated and microvillous cells target different regions of the olfactory bulb and selective regeneration of OSN subsets should manifest itself at the level of olfactory bulb innervation.

5.3. The effect of metronidazole

Metronidazole-mediated cell ablation method is an increasingly used tool for the cell function and / or cell specific regeneration studies. This system has successfully been used in other systems like cranial motor neurons, spinal inter neurons, spinal motor neurons, and others (Mathias *et al.*, 2014). As its use become more frequent, it has shown that the effect of the method can vary based on cellular dynamics and location of the tissue desired to ablate. For example, dorsal peridermal cells and pineal cells have been reported to be resistant to ablation, most probably as a result of poor drug delivery due to the deep internal position of the cells, while the skin cell ablation could be achieved with near complete efficiency because of its direct exposure to the drug (Davison *et al.*, 2007; Pisharath and Parsons, 2009; Chen *et al.*, 2011). Therefore, it was important to first understand the limitations and the advantages of the system for studies in the zebrafish OE.

In the experiments performed in this study the prodrug MTZ was dissolved in tank water rather than E3 medium in order to avoid any additional effect of NTR influenced by chemicals from the medium. Yet, cell ablation depends on the penetration of the prodrug into the tissue and target cells. Different tissues may have different permeability for MTZ. Even though OSNs are in direct contact with the environment, they are so only with their most apical structures, cilia or microvilli, while most of the cells are hidden inside the OE. Sustentacular cells seal the apical border of the OE tissue and it is not known how well OSN cell bodies are protected from the external environment. It may be that the drug is only able to enter OSNs through their apical protrusions, which may limit effective concentrations of MTZ to build up in OSNs. Penetration of the drug would also depend on the concentration gradient as the driving force for movement of the chemical.

To examine the range of drug concentrations that could be used in *in vivo* cell ablation experiments, dose-response viability assays were performed as pilot studies. Surprisingly, even the lowest drug concentration tested, 5 mM, was lethal starting at 4 days after MTZ application. The lethal effect of the drug increased further with higher

concentrations of the drug as reported earlier (Mathias *et al.* 2014). Thus, concentrations between 5 and 20 mM allowed for a 2 day time window of experimentation before any lethal effect of the drug became apparent. However, it remains unknown how long before the death of the embryo detrimental changes are induced in the various tissues. Thus, even though embryos would die from a 20 mM concentration of MTZ at day 4, unspecific effects of the drug might already be well on the way in individual cells or neurons. For this and other reason experiments utilizing higher concentrations of MTZ were conducted at 2 dpf and only for up to 48 h in the presence of the drug.

5.3. Comparison of the effect of wild-type and mutant nitroreductase

Two different variants of nitroreductase were used, one that was directly cloned from *E. coli* genomic DNA and a codon-optimized enhanced isoform. When the performance of both versions is compared differences become visible in the duration over which a noticeable decrease in the number of transgene-expressing cells occurs. When the wild type form was used, cells predominantly died at later periods after the onset of drug exposure, while earlier effects were seen for the *ntr*^{3mut} construct. This observation demonstrates the expected difference in the kinetics of wild-type and enhanced forms of the nitroreductase enzyme (Chen *et al.*, 2011). The delayed onset of an effect in wild type NTR-expressing embryos suggests that the either NTR expression is lower or that the enzymatic rate is slower than *ntr*^{3mut}.

When drug was applied to *ntr*^{3mut} expressing embryos, it can clearly be observed that decrease in the number of cells occurs early in almost all cases, and independent from the MTZ concentration used. Likewise, the number of cells expressing the transgene start to increase shortly after exposure the drug is abolished while rate of the change in cell number was rather stable in wild type NTR expressing embryos.

5.4. Expression of transgenic constructs is highly dynamic during early development

The studies presented here provide an initial feasibility study for the use of NTR-containing constructs in the zebrafish OE. Ideally, such studies should be carried out on adult specimens of stable transgenic lines. Yet, since generation of stable transgenics is a time consuming and effort-prone process, we wanted to understand if the MTZ/NTR approach is feasible at all.

However several problems were encountered with the design of the experiment. The transgenic constructs harboring the NTR were injected into oocytes and developing embryos were analyzed between 2 and 4 days later. A major complication in these experiments is due to the highly dynamic nature of the embryonic OE. Fish show lifelong growth and even though an embryonic OE is formed after 24 h of development, high numbers of OSNs are added to the tissue during the experimental period. The efficacy of NTR was mainly assessed as the loss of neurons over time. Yet, during the time course of the experiment new neurons, including those expressing the NTR constructs are continuously added, complicating the cell count-based approach taken here.

Previously it has been shown that expression of transgenic constructs driven by the zebrafish OMP promoter closely follows endogenous OMP expression, which shows a large increase in cell number over the first 48 hours after fertilization, but decreases to lower levels later (Çelik *et al.*, 2002). However the expression of the endogenous OMP remains throughout the lifetime of the organism also because of continuous regeneration (Graziadei and Graziadei, 1979). In addition, growth of the embryonic olfactory placode continues long after it first appears around 18 hours post fertilization (Hansen and Zeiske, 1993; Miyasaka *et al.*, 2007). Transgene expression in the experiments reported here followed the same dynamics as reported previously and the time window of experimentation that was used here represents the more dynamic window of early olfactory organ development.

Another complication lies in the nature of transgene expression itself. The constructs were designed to include Tol2 sites for efficient integration into the genome (Asakawa *et al.*, 2008). However, even if genome integration is achieved, unless it happens at an early embryonic stage, expression in the OE is still a mosaic. We have observed two modes of expression, low intensity, high cell number and high intensity, low cell number, most likely reflecting early genome integration and transient expression from the injected plasmid DNA, respectively. Transient expression, as the name implies, is not stable and expression might be lost over the time course of the experiment. Thus, any decrease in cell number might be confounded by loss of transgene expression in individual OSNs. Thus, it was imperative to compare the behavior of experimental groups to controls and difference in the dynamics of cell number might be more sensitive indicators of true effects of the manipulation. We employed such a measure with the difference in cell number measurements, which may be a more robust and reliable indicator. Another sensitive measure is changes in apoptotic cells, yet, rates of apoptosis strongly depended on the time interval over which the experiment is performed. Largest changes in cell number and apoptosis, as judged by immunohistochemistry for activated caspase 3, were seen early after onset of treatment and changes appear to level off at later time points. This might be due to the fact that a balance between cell birth, expression of the transgene by new OSNs, cell death, and extinction of expression has been reached. Once the majority of susceptible cells have been killed, only newborn transgene-expressing cells can be ablated, and only small increases, if at all, in the number of caspase-positive cells are expected.

5.5. Variability in expression

For the sake of forthcoming experiments under stable transgenic conditions, the transgenic constructs were flanked by transposable elements in the Tol2 vector system (Urasaki *et al.*, 2009) were co-injected with the transposase mRNA. Therefore, the observation of two phenotypes of transgene expression mentioned above, low intensity, high cell number and high intensity, low cell number was reflects the integration probability of the construct. In the case expression by only a few cells (<20) typically intense signals were observed in individual cells whereas low intensity expression was

observed when large numbers of neurons (>20) expressed the transgene. Intensity of GFP expression is directly linked to expression of NTR because both genes were linked through a sequence for the self-cleavable peptide T2A, which permits a 1:1 expression of two genes from the same construct. Thus, strong differences in expression of NTR may generate a distinction between susceptible and less susceptible cells. It would be expected that high expressors would convert the prodrug more efficiently due to the increased availability of NTR in those cells and therefore should be more susceptible to MTZ. However, a systematic analysis of this observation awaits further experimentation and analysis.

5.6. Reliability of the assay

The assay used in its current form has severe limitations. To overcome some of these shortcomings, the experiments were set up as follows; injection of the transgenic construct was performed at the one-cell stage and the number of individual embryos expressing the transgene was counted at different time points between 2 and 4 dpf. During this time interval, the expression profile of the transgene is similar to the endogenous expression pattern of the OMP gene (Çelik *et al.*, 2002). However, because of the mosaic expression of the transgene, variability in oocyte quality, variability in injection performance, the bystander effect of the system (Mathias *et al.*, 2014; White & Mumm *et al.*, 2013) the performance of the system is very variable as well. Therefore, three different control groups, (untreated pOMP::NTR (or *ntr*^{3mut})-T2A-GFP, treated and untreated pOMP::GFP) were designed to gain as much control over the system as possible. Negligible side-effects of the drug application or the gene expression were observed and control groups showed parallel changes in cell numbers as expected. The rate change in cell number remained the same even in the application of the highest concentration of the drug, where high lethality of the embryos was observed.

Different measures of a potential effect carried out by counting cell numbers in individual OEs, or calculating the change in cell number relative to the time before

application of the drug. This allowed for different statistical analysis, such as, Student's t-test for raw cell numbers and Kruskal-Wallis rank-sum test for differences in cell number across time points. Ranked data are less susceptible to outliers, and overall the change in cell number analysis performed better.

As for the cell death analysis of the NTR-mediated cell ablation assays it is known that apoptosis takes place at the end of the action mechanism (Knocks *et al.*, 1988; Chen *et al.*, 2011). In general, two common methods were used in similar studies besides the simple observation of signal loss coming from the reporter protein of the transgene for the detection of the cell death: TUNEL assay and immunohistochemistry targeting the activated caspase 3 complex found in apoptotic cells (Pisharath and Parson, 2009). Regarding to the detection of the dying cells, TUNEL assay labels the terminals of the fragmented DNA (Gavrieli *et al.*, 1992) thus it is considered to be a semi-quantitative method that includes other potential cell death mechanisms. However, immunohistochemistry for activated caspase 3 showed strong overlap with transgene-positive cells, including in our study, suggesting that cells die predominantly by apoptosis. Therefore, in our studies, we took advantage of labeling dying cells by activated caspase 3. However the number of apoptotic cells per OE was higher than the number of double-positive cells. This result can be explained by the unspecific effects of the NTR-mediated cell ablation described before in which neighboring cells are influenced by the death of a nearby cell (Mathias *et al.*, 2014; White and Mumm, 2013).

To control for the hidden dynamics in the experiments by switching transgenes on and off and the balance between experiment-induced loss and developmental gain of cells, the photo-convertible reporter protein dendra2 was used to trace the cells which were born before and after the treatment for a more comprehensive analysis. Photoconversion was carried out at the time the MTZ treatment was initiated so that cells that were present before onset of MTZ treatment could be identified by red fluorescence while newly born cells would be revealed by green fluorescence. However, red fluorescent signals were short lived, suggesting that in OSNs the converted form of dendra2 is not as stable as reported

(Chudakov *et al.*, 2007). The underlying reason for this phenomenon might be that the OMP promoter is too strong and that protein turnover is high.

In summary, the experiments described here suggest that metronidazole treatment efficiently and specifically kills transgenic neurons. However the dynamics of the tissue masks the effect. Thus, stable transgenic expression of the construct may overcome this shortcoming in future experiments. Similarly, a cell-based analysis tracking the behavior of the OE in real time through time-lapse microscopy might also be more informative.

APPENDIX A: EQUIPMENT

Table 6. 1. Equipment.

4 °C Room	Birikim Elektrik, Turkey
Autoclaves	Astell Scientific, UK
Centrifuge	Eppendorf, Germany (5417R)
Confocal Microscope	Leica SP5-AOBS, USA
Electronic Balance	Sartorius, Germany (TE412)
Electrophoresis Supplies	Bio-Rad Labs, USA (ReadySub-Cell GT Cells)
Fluorescence Microscope	Leica Microsystems, USA (MZ16FA)
Freezer 1 -20 °C	Arçelik, Turkey
Freezer 2 -80 °C	Thermo Electron Corp., USA (Farma 723)
Gel Documentation	Bio-Rad Labs, USA (GelDoc XR)
Glass Bottles	Isolab, Germany
Incubator 1	Weiss Gallenkamp, UK
Incubator 2	Nuve, Turkey
Incubating Shaker	Thermo Electron Corp., USA
Micropipettors	Eppendorf, Germany (Research)
Microwave Oven	Vestel, Turkey
Microinjector	Eppendorf, Germany (FemtoJet)
Luminometer	Fluoroskan Ascent Fl (Thermo Scientific)
Refrigerator	Arçelik, Turkey
Softwares	Vector NTI (Invitrogen, USA)
Thermal Cyclers	Bio-Rad Labs, USA (C1000)
Vortex	Scientific Industries, USA

APPENDIX B: SUPPLIES

Table 6. 2. List of Supplies.

1 kb DNA Ladder	New England Biolabs, U.S.A. (N3232)
100 bp DNA Ladder	New England Biolabs, U.S.A. (N3231)
Ethanol Absolute	Sigma-Aldrich, U.S.A. (34870)
Ethidium Bromide	Sigma Life Sciences, U.S.A. (E1510-1)
EDTA Disodium Salt	Sigma-Aldrich, U.S.A. (E5134 - 1 kg).
LB Agar	Sigma Life Sciences, U.S.A. (SL08394)
LB Broth	Sigma-Aldrich, U.S.A. (L7658- 1 kg)
Magnesium Chloride, 25 mM	Promega, U.S.A. (A3511)
Magnesium Sulfate	Sigma-Aldrich, U.S.A. (M7506)
NcoI	New England Biolabs, U.S.A. (R0193 L)
pGEM®-T Easy Vector System	Promega, U.S.A. (A1360)
Potassium Chloride	Sigma-Aldrich, U.S.A. (P9541)
SeaKem® Agarose	Cambrex, U.S.A. (50004)
Sodium Acetate	Sigma-Aldrich, U.S.A. (S8625)
Sodium Chloride	Sigma-Aldrich, U.S.A. (S7653 - 1 kg)
Sodium Hydroxide	Sigma-Aldrich, U.S.A. (S8045 - 1 kg)
T4 DNA Ligase	New England Biolabs, U.S.A (M0202L)
Trizma® Base	Sigma-Aldrich, U.S.A. (T6066)
XhoI	New England Biolabs, U.S.A. (R0146 L)
ClaI	New England Biolabs, U.S.A. (R0197S)
Onetaq polymerase	New England Biolabs, U.S.A. (M0480S)
Gibson assembly	New England Biolabs, U.S.A. (E2611S)

APPENDIX C: FIGURE LEGENDS

Figure 4.1. Zebrafish embryos were incubated in different concentrations (5 mM, 10 mM, 15 mM, and 20 mM) of metronidazole starting from 3 dpf and survival of embryos was monitored over different time points as percentage of the initial population. Control embryos (ctrl) were incubated in E3 medium during the experiment (n =10 for each experimental group).

Figure 4.1 (A) pOMP: 1.3 kb zebrafish OMP promoter, NTR: *E. coli* nfsB genes, GFP: green fluorescent protein, T2A: self-cleaving peptide. OMP promoter assures the expression of the construct only in the ciliated cells in OE and T2A yields the bicistronic expression of the NTR and GFP. (B) Two distinct phenotypes with low (left) and high (right) numbers of transgene-positive cells can be observed which most likely represent transient and stable expression of the construct, respectively.

Figure 4.3 Nitroreductase-expressing ciliated cells (arrows) were analyzed at 3 (left, before) and 4 dpf (right, 24 h) before and after a 24 h exposure to 10 mM metronidazole. top row is MTZ-treated embryo and bottom row is untreated embryo. The OE of the embryos were visualized before and 24 h after treatment and the different phenotype of the axons of the treated embryo is indicated with arrowhead in the top right micrograph.

Figure 4.4. (A) Absolute cell numbers of pOMP::NTR-T2A-GFP-expressing OSNs per OE / embryo starting at 3 dpf. Embryos were grown in E3 medium for 24 h (n=5). (B) Average cell numbers of pOMP::NTR-T2A-GFP-expressing OSNs per OE. (C) Difference plots showing the difference in the number of pOMP::NTR-T2A-GFP-expressing OSNs between 0 h and 6 h (left), 0 h and 12 h (middle), and 0 h and 24 h (right). Individual OEs / embryos are shown, the horizontal bar represents median of each data set.

Figure 4.5. (A) Absolute cell numbers of pOMP::NTR-T2A-GFP-expressing OSNs per OE / embryo before and after 24 h continuous treatment with 10 mM MTZ (red: pOMP::NTR-T2A-GFP-injected, MTZ-treated embryos, $n = 12$ embryos; blue: pOMP::NTR-T2A-GFP-injected untreated control embryos, $n = 12$ embryos) (B) Difference plot of pOMP::NTR-T2A-GFP-expressing cell numbers before and after 24 h of 10 mM MTZ treatment (red: pOMP::NTR-T2A-GFP-injected, MTZ-treated embryos; blue: pOMP::NTR-T2A-GFP-injected untreated control embryos; asterisk: Kruskal-Wallis test, $p=0.03$).

Figure 4.6 (A) Absolute cell numbers of pOMP::NTR-T2A-GFP-expressing OSNs per OE / embryo starting at 3 dpf. For treated group (red in graph) embryos were grown in MTZ and for control group in E3 medium for 24 h. (B) Difference plots showing the difference in the number of pOMP::NTR-T2A-GFP-expressing OSNs between 0 h and 6 h (left), 0 h and 12 h (middle), and 0 h and 24 h (right). Individual OEs / embryos are shown, the horizontal bar represents median of each data set (asterisks: Kruskal-Wallis test, $p_{12\text{ h}}=0.034$ and $p_{24\text{ h}}=0.015$). (C) Average cell numbers of pOMP::NTR-T2A-GFP-expressing OSNs per OE. ($n= 6$ embryos for time points and control group).

Figure 4.7. Apoptotic and pOMP::NTR-T2A-GFP expressing cells of 4 dpf embryo, treated with 10 mM for 24 h at 3 dpf. (A) shows transgenic construct pOMP::NTR-T2A-GFP expressing ciliated cells (in green, right) and activated caspase 3 immunoreactive cells (in red, middle) and double positive cells (left) after MTZ treatment in the OE. Arrowhead indicates the double positive cells. (B) shows the actual cell numbers expressing cas3 in each individual OE and (C) is the average cell number expressing cas3, lighter colors means that the cells are double positive for cas3 and GFP ($n=6$ for each experimental group).

Figure 4.8. (A) pOMP: 1.3 kb zebrafish OMP promoter, *ntr* is the triple mutant form of NTR, GFP: green fluorescent protein, T2A: self-cleaving peptide. OMP promoter

assures the expression of the construct only in the ciliated cells in OE and T2A yields the bicistronic expression of the NTR and GFP (B) Absolute cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing OSNs per OE / embryo starting at 3 dpf. For treated group (red in graph) embryos were placed in MTZ for 4 hours and E3 after treatment, for control group in E3 medium for 24 h. (C) Difference plots showing the difference in the number of pOMP::*ntr*^{3mut}-T2A-GFP-expressing OSNs between 0 h and 6 h (left), 0 h and 12 h (middle), and 0 h and 24 h (right) in each graph. Blue represents control group (n=3 embryos) whereas red color represents treated embryos (n=5 embryos). Individual OEs / embryos are shown, the horizontal bar represents median of each data set. (D) Average cell numbers of pOMP::*NTR*-T2A-GFP-expressing OSNs per OE.

Figure 4.10. (A) Absolute cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing (in blue for control group and in red for experiment group) and pOMP::*GFP*-expressing (in green) OSNs per OE / embryo starting at 2 dpf. For treated group (red and green in graph) embryos were placed in 20 mM MTZ for 4 hours and E3 after treatment, for control group in E3 medium for 48 h. (B) Difference plots showing the difference in the numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing and pOMP::*GFP*-expressing OSNs between 0 h and 6 h (left), 0 h and 24 h (middle), and 0 h and 48 h (right) in each graph. Blue represents the control group and red color represents treated in pOMP::*ntr*^{3mut}-T2A-GFP-expressing embryos, green color represents treated pOMP::*GFP*-expressing embryos. Individual OEs / embryos are shown, the horizontal bar represents median of each data set. (C) Average cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing and pOMP::*GFP*-expressing OSNs per OE (n= 12 for treated pOMP::*ntr*^{3mut}-T2A-GFP-expressing group, n= 4 for both pOMP::*ntr*^{3mut}-T2A-GFP-expressing untreated group and pOMP::*GFP*-expressing treated group).

Figure 4.9. (A) Absolute cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing OSNs per OE / embryo starting at 3 dpf. Embryos were grown in E3 medium for 48 h. (B) Average cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing OSNs per OE (C) Difference plots showing the difference in the number of pOMP::*ntr*^{3mut}-T2A-GFP-

expressing OSNs between 0 h and 6 h (left), 0 h and 24 h (middle), and 0 h and 48 h (right). Individual OEs / embryos are shown, the horizontal bar represents median of each data set (n=18 embryos, 4 independent experiments).

Figure 4.11. (A) Absolute cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing and pOMP::GFP-expressing (in green) OSNs per OE / embryo starting at 2 dpf. For treated group (red and green in graph) embryos were placed in 20 mM MTZ for 24 hours and E3 for the next 24 hours, for control group in E3 medium for 48 hours. (B) Difference plots showing the difference in the number of pOMP::*ntr*^{3mut}-T2A-GFP-expressing and pOMP::GFP-expressing OSNs between 0 h and 6 h (left), 0 h and 24 h (middle), and 0 h and 48 h (right) in each graph. Blue represents the control group and red color represents treated in pOMP::*ntr*^{3mut}-T2A-GFP-expressing embryos, green color represents treated pOMP::GFP-expressing embryos. Individual OEs / embryos are shown, the horizontal bar represents median of each data set. (C) Average cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing and pOMP::GFP-expressing OSNs per OE (n= 6 for treated pOMP::*ntr*^{3mut}-T2A-GFP-expressing group, n=5 for pOMP::*ntr*^{3mut}-T2A-GFP-expressing untreated group and n= 6 for pOMP::GFP-expressing treated group; asterisk: Kruskal-Wallis test, H(1)=5.57, p_{0-4 h}=0.018).

Figure 4.12. (A) Absolute cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing ..(red and blue)and pOMP::GFP-expressing (in light and dark green) OSNs per OE / embryo starting at 2 dpf. For treated group (red and dark green in graph) embryos were placed in 50 mM MTZ continuously and, for control group (in blue and light green) in E3. (B) Difference plots showing the difference in the number of pOMP::*ntr*^{3mut}-T2A-GFP-expressing and pOMP::GFP-expressing OSNs between 0 h and 6 h (left), 0 h and 12 h (middle) in each graph. Blue represents the control group and red color represents treated in pOMP::*ntr*^{3mut}-T2A-GFP-expressing embryos, while dark green color represents treated and light green color represent non-treated pOMP::GFP-expressing embryos Individual OEs / embryos are shown, the horizontal bar represents median of each data set. (C) Average cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing and pOMP::GFP-expressing

OSNs per OE (n= 9 for treated and 7 for non-treated pOMP::*ntr*^{3mut}-T2A-GFP-expressing embryos, and n= 10 for both treated and non-treated group of pOMP::GFP-expressing embryos; asterisk: Kruskal-Wallis test, $H_{0-4}(1)=7.756$, $p_{0-4\text{ h}}=0.0053$; $H_{0-12}(1)=8.431$, $p_{0-12\text{ h}}=0.038$).

Figure 4.13 (A) Absolute cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing and pOMP::GFP-expressing (in green) OSNs per OE / embryo starting at 2 dpf. For treated group (red and green in graph) embryos were placed in 50 mM MTZ for 10 hours and E3 after treatment, for control group in E3 medium for 48 hours. (B) Difference plots showing the difference in the number of pOMP::*ntr*^{3mut}-T2A-GFP-expressing and pOMP::GFP-expressing OSNs between 0 h and 6 h (left), 0 h and 24 h (middle), and 0 h and 48 h (right) in each graph. Blue represents the control group and red color represents treated in pOMP::*ntr*^{3mut}-T2A_GFP-expressing embryos, green color represents treated pOMP::GFP-expressing embryos. Individual OEs / embryos are shown, the horizontal bar represents median of each data set. (C) Average cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing and pOMP::GFP-expressing OSNs per OE (n= 6 for treated and 3 for non-treated pOMP::*ntr*^{3mut}-T2A-GFP-expressing embryos, and n= 4 for treated pOMP::GFP-expressing embryos; asterisk: Kruskal-Wallis test, $p_{0-4\text{ h}}=0.0055$; $p_{0-12\text{ h}}=0.0071$).

Figure 4.14. (A) Absolute cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing and pOMP::GFP-expressing (in green) OSNs per OE / embryo starting at 2 dpf. For treated group (red and green in graph) embryos were placed in 10 mM MTZ continuously, for control group in E3 medium for 48 hours. (B) Difference plots showing the difference in the number of pOMP::*ntr*^{3mut}-T2A-GFP-expressing and pOMP::GFP-expressing OSNs between 0 h and 6 h (left), 0 h and 24 h (middle), and 0 h and 48 h (right) in each graph. Blue represents the control group and red color represents treated in pOMP::*ntr*^{3mut}-T2A_GFP-expressing embryos, green color represents treated pOMP::GFP-expressing embryos. Individual OEs / embryos are shown, the horizontal bar represents median of each data set. (C) Average cell numbers of pOMP::*ntr*^{3mut}-T2A-GFP-expressing and pOMP::GFP-expressing OSNs per OE (n= 5 for treated and 3 for non-treated

pOMP::*ntr*^{3mut}-T2A-GFP-expressing embryos, n=3 for pOMP::*ntr*^{3mut}-T2A-GFP-expressing untreated group and n= 10 for both treated and non-treated group of pOMP::GFP-expressing embryos; asterisk: Kruskal-Wallis test, $H_{0-12}(1)=5.893$, $p_{0-12h}=0.015$).

Figure 4.15. (A) pOMP: 1.3 kb zebrafish OMP promoter, *ntr* is the triple mutant form of NTR, dendra2: photo-switchable fluorescent protein, T2A: self-cleaving peptide. (B) shows pOMP::*ntr*-T2A-dendra2 expressing OSNs in the OE of 3 dpf embryo. Green cells are the dendra2 expressing cells before conversion and the red cells are the activated-dendra2 expressing OSNs. Conversion was operated with a short pulse of UV light (30 sec). The green light emission does not abolish after photo-activation of the dendra2 reporter proteins.

Figure 4.16 (A) Absolute cell numbers of pOMP::*ntr*^{3mut}-T2A-dendra2-expressing non-converted (in green) and converted (in red) OSNs per OE / embryo starting at 2 dpf. Conversion was operated by a short pulse of UV (30 sec) at the 0 point of the experiment. For treated group (darker colors) embryos were placed in 10mM MTZ continuously, for control group in E3 medium for 12 hours (lighter color graph). (B) Difference plots showing the difference in the number of pOMP::*ntr*^{3mut}-T2A-dendra2-expressing converted (in red) and non-converted (in green) OSNs between 0 h and 6 h (left), 0 h and 12 h (right) in each graph. Individual OEs / embryos are shown, the horizontal bar represents median of each data set. (C) Average cell numbers of pOMP::*ntr*^{3mut}-T2A-dendra2-expressing converted (in red) and non-converted (in green) OSNs per OE (n=4 for treated and 3 for non-treated pOMP::*ntr*^{3mut}-T2A-dendra2-expressing embryos, asterisk: Kruskal-Wallis test, $H(7)=29.704$, $p=0.000108$; $df=7$ since there are 8 data set 2 time points for 2 experimental groups – treated and control – and 2 version of each data set as converted and non-converted).

Figure 4.17. The figure shows pOMP::*ntr*-T2A-dendra2 expressing OSNs in the OE of 3 dpf embryo. Green cells are all dendra2 expressing OSNs and the red cells are the activated-dendra2 expressing OSNs. Conversion was operated with a short pulse of UV light (30 sec). The activated faint cells indicated with arrowhead were seemed to lose its signal over time.

APPENDIX C: RESULTS FOR INDIVIDUAL EXPERIMENTS

Table 6. 3. Individual cell numbers of embryos expressing pOMP::NTR-T2A-GFP.

10mM/24h MTZ	treated		control	
nose identitty	before	after	before	after
1_Right (R)	16	11	2	2
1_Left (L)	7	7	4	7
2_R	13	13	26	37
2_L	14	14	4	3
3_R	8	8	7	5
3_L	1	0	15	1
4_R	17	12	9	18
4_L	9	8	16	28
5_R	3	3	18	16
5_L	2	1	3	3
6_R	5	17	2	3
6_L	33	14	0	0
7_R	3	1	10	10
7_L	6	2	11	14
8_R	3	8	25	54
8_L	0	0	10	23
9_L	26	29	15	28
9_R	14	8	11	14
10_R	3	1	12	15
10_L	3	1	8	4
11_R	2	1	18	18
11_L	1	0	7	10
12_R	5	10	11	8
12_L	6	2	9	5
total	200	171	253	326
SD	8,28	7,12	6,81	13,00
SE	2,39	2,05	1,97	3,75
avegrae	8,33	7,13	10,54	13,58

Table 6.3. Individual cell numbers of embryos expressing pOMP::NTR-T2A-GFP (cont.).

10mM Continous MTZ treatment										
6 hr treatment					12hr treatment					
nose identitty	before	after	caspase +	double +	nose identitty	before	after	caspase +	double +	
1_R	20	20	8	4	1_R	1	1	1	0	
1_L	19	15	10	1	1_L	1	0	2	2	
2_R	20	13	8	2	2_R	23	19	4	0	
2_L	23	23	5	1	2_L	3	2	18	0	
3_R	0	1	1		3_R	25	20	9	1	
3_L	8	5	0	0	3_L	27	24	8	2	
4_R	8	2	12	2	4_R	1	2	4	0	
4_L	8	11	13	2	4_L	5	5	7	1	
5_R	4	2	3	2	5_R	7	8	7	2	
5_L	6	6	9	2	5_L	16	18	8	3	
6_R	26	34	23	4	6_R	46	23	7	0	
6_L	14	16	19	7	6_L	28	38	4	2	
avarage			9,25	2,45	avarage			6,58	1,08	
sd			1,40	0,40	sd			0,90	0,22	
avarage	13,00	12,33			avarage	15,25	13,33			
sd	8,41	10,00			sd	14,57	12,06			
semv	1,72	2,04			sem	2,97	2,46			
24 hr treatment	control									
nose identitty	before	after	caspase +	double +	nose identitty	before	6 hr	12 hr	24 hr	caspase e
1_R	8	11	8	0	1_R	18	22	22	21	15
1_L	36	29	5	2	1_L	14	18	16	14	9
2_R	12	14	3	0	2_R	37	33	33		
2_L	6	3	14	4	2_L	31	32	47		
3_R	2	0			3_R	21	18	32		
3_L	3	0			3_L	47	42	28		
4_R	35	25	13	6	4_R	16	25	30	25	5
4_L	28	38			4_L	15	21	15	21	1
5_R	13	9	4	1	5_R	9	14	15	14	5
5_L	19	5	4	1	5_L	8	8	9	9	2
6_R	19	17	1	1	6_R	1	5	2	2	5
6_L	16	15	4	0	6_L	19	29	29	25	1
avarage			6,22	1,67	avarage					5,38
sd			1,51	0,69	sd					1,67
avarage	16,42	13,83			avarage	19,67	22,25	23,17	16,38	
sd	11,58	11,91			sd	12,96	10,70	12,36	8,14	
sem	2,36	2,43			sem	2,64	2,18	2,52	1,66	

Table 6. 4. Individual cell numbers of embryos expressing pOMP::ntr^{3mut}-T2A-GFP.

10mM/ 4 hours treatment on 3dpf embryos								
	treated				control			
nose ID	0	4	24	48	0	4	24	48
1	26	28	36	17				
2	12	12	16	10				
3	8	7	11	9	17	19	10	15
4	4	3	6	4	11	11	17	6
5	16	16	6	3	7	6	6	4
6	3	3	2	1	0	0	0	2
7	17	27	12	15				
8	30	20	27	12				
9	12	8	10	8	6	9	5	3
10	18	10	13	13	5	5	6	4
avarages	14,6	13,4	13,9	9,2	7,7	8,3	7,3	5,7
SD	8,8	9,1	10,3	5,3	5,8	6,4	5,7	4,8
SE	2,8	2,6	3,0	1,5	2,4	2,6	2,3	1,9

50mM/ 10 hours treatment on 2dpf embryos												
	treated				control				GFP_treated			
nose ID	0	4	24	48	0	4	24	48	0	4	24	48
1	12	12	14	18	6	11	15	22	18	21		
2	12	14	15	18	11	20	22	27	18	18		
3					19	22	39	38	13	18		
4					20	23	23	34	21	27		
5	12	5	10	18	4	4	4	4	20	20		
6	2	0	2	2	1	2	4	1	14	15		
7	16	7	16	13					11	12		
8	21	14	13	11					16	18		
9	16	7	11	27								
10	17	11	17	27								
11	10	12	8	11								
12	6	3	8	8								
avarages	12,4	8,5	11,4	15,3	10,2	13,7	17,8	21,0	16,4	18,6		
SD	5,5	4,8	4,6	7,9	7,9	9,3	13,3	15,4	3,5	4,4		
SE	1,8	1,5	1,4	2,5	3,2	3,8	5,4	6,3	1,2	1,6		

Table 6.4. Individual cell numbers of embryos expressing pOMP::ntr^{3mut}-T2A-GFP (cont.).

20mM/ 24 hours treatment on 2dpf embryos												
	treated				control				GFP_treated			
nose ID	0,5	4	24	48	0,5	4	24	48	0,5	4	24	48
1	1	0	0	10	4	4	1	1	4	5	10	2
2	0	0	4	3					8	9	6	6
3	2	5	8	8	3	5	3	3	4	4	5	1
4	5	5	7	13	1	1	4	6	6	4	3	2
5	0	0	0	3	0	0	2	2				
6	0	0	0	2	0	0	8	7				
7					3	5	5	6	6	8	8	8
8					5	6	6	6	6	9	10	10
9	11	3	9	7	0	2	4	3	0	0	3	7
10	7	7	6	5	1	4	5	3	0	2	5	6
11	1	1	2	1					5	7	7	2
12	1	0	3	0					0	1	7	7
13	9	7	10	7								
avarages	3,4	2,5	4,5	5,4	1,9	3,0	4,2	4,1	3,9	4,9	6,4	5,1
SD	4,0	2,9	3,8	4,0	1,9	2,3	2,1	2,1	2,9	3,3	2,5	3,1
SE	1,2	0,9	1,1	1,2	0,6	0,8	0,7	0,7	0,9	1,0	0,8	1,0
10mM/ continious treatment on 2dpf embryos												
	treated				control				GFP_treated			
nose ID	0	4	24	48	0	4	24	48	0	4	24	48
1	0	0	0	3	6	11	15	22	20	21	27	27
2	13	18	23	34	11	20	22	27	25	22	22	22
3	7	9	6	2	19	22	39	38	3	4	16	56
4	2	2	2	3	20	23	23	34	9	13	17	30
5	5	6	5	7	4	4	4	4	13	16	32	57
6	4	6	4	6	1	2	4	1	40	48	50	50
7	0	2	1	0								
8	5	4	4	4								
9												
10												
11	16	22	17	13								
12	10	14	7	3								
avarages	6,2	8,3	6,9	7,5	10,2	13,7	17,8	21,0	18,3	20,7	27,3	40,3
SD	5,4	7,4	7,4	10,0	7,9	9,3	13,3	15,4	13,2	14,9	12,6	15,7
SE	1,7	2,3	2,3	3,2	3,2	3,8	5,4	6,3	5,4	6,1	5,2	6,4

Table 6.4. Individual cell numbers of embryos expressing pOMP::ntr^{3mut}-T2A-GFP (cont.).

20mM/ 4 hours treatment on 2dpf embryos												
	treated				control				GFP_treated			
nose ID	0	4	24	48	0	4	24	48	0	4	24	48
1	7	5	2	9	5	5	2	3	8	8	22	22
2	4	4	8	4	1	2	9	10	7	8	10	10
3	6	5	9	6	7	7	18	31	2	2	2	2
4	9	6	7	9	5	6	9	13	0	0	0	0
5	9	9	5	5	0	0	0	0	3	3	10	4
6	10	10	15	29	1	0	0	0	8	9	6	4
7	10	11	16	22	8	5	15	20	12	16	14	11
8	2	1	3	5	6	6	17	14	7	7	14	21
9	5	3	11	6								
10	4	4	4	4								
11	0	0	0	0								
12	7	7	11	12								
13	0	0	0	1								
14	0	0	0	1								
15	2	1	0	0								
16	5	7	20	20								
17	12	8	11	10								
18	21	16	7	7								
19	1	0	0	1								
20	14	14	12	11								
21	7	7	14	14								
22	12	12	18	18								
23	0	0	0	0								
24	0	0	5	7								
avarages	6,125	5,416667	7,416667	8,375	4,125	3,875	8,75	11,375	5,875	6,625	9,75	9,25
SD	5,359368	4,817691	6,289513	7,643198	3,044316	2,799872	7,478541	10,68961	3,907411	5,012484	7,126417	8,430387
SE	1,093976	0,983407	1,283841	1,560161	1,076328	0,989904	2,644063	3,779349	1,381478	1,772181	2,519566	2,980592

Table 6.4. Individual cell numbers of embryos expressing pOMP::ntr^{3mut}-T2A-GFP (cont.).

50mM/ continious treatment on 2dpf embryos											
	treated			control			GFP_treated		GFP_non		
nose ID	0	4	12	0	4	12	0	4	0	4	12
1	23	21	24	2	2	8	12	12	20	19	15
2	22	17	21	5	6	9	10	18	10	11	11
3	29	29	24	12	21	33	15	15	13	15	17
4	18	18	20	24	24	28	15	15	11	11	9
5	4	3	2	6	6	7	17	12	21	20	28
6	2	2	2	10	14	12	6	6	22	21	28
7	5	4		9	11		23	23	11	11	18
8	16	16		7	18		19	28	18	20	26
9	10	13		8	10		11	13	1	1	
10	15	7		9	12		17	23	0	0	
11	4	6		3	7		10	10	2	4	
12	12	9		3	3		6	6	1	4	
13	7	11		8	13		19	50	17	20	
14	10	13		14	22		37	50	8	16	
15	18	20					15	19	3	5	
16	9	11					16	22	5	5	
17	10	10					5	5	16	16	
18	10	10					6	7	15	21	
19							10	14	0	8	
20							7	9	1	0	
21							9	9	6	11	
22							3	3	3	3	
avarage s	12,4444 4	12,2222 2	15,5	8,57142 9	12,0714 3	16,1666 7	13,0909 1	16,7727 3	9,27272 7	11	19
SD	7,39811	7,01725 2	10,5782 8	5,61199 7	7,06523 8	11,3387 2	7,54610 6	12,6149 3	7,52254 9	7,42582 4	7,52139 8

Table 6. 5. Individual cell numbers of embryos expressing pOMP::*ntr*^{3mut}-T2A-dendra2.

10mM/ continious treatment on 2dpf embryos exprssing dendra2												
	treated/green			treated/red			non/green			non/red		
nose ID	0	4	12	0	4	12	0	4	12	0	4	12
1	3	3	1	3	2	0	3	3	4	3	1	0
2	4	4	0	4	2	0	3	3	2	3	3	0
3	10	7	10	10	4	0	1	2	1	1	0	0
4	18	11	16	18	4	0	3	3	3	3	2	0
5	16	11	10	16	9	0						
6	7	7	6	7	3	0						
7	6	3	4	6	0	0						
8	10	10	8	10	3	0						
avarages	9,25	7	6,875	9,25	3,375	0	2,5	2,75	2,5	2,5	1,5	0
SD	5,418223	3,422614	5,276295	5,418223	2,615203	0	1	0,5	1,290994	1	1,290994	0
SE	1,564106	0,988024	1,523135	1,564106	0,754944	0	0,288675	0,144338	0,372678	0,288675	0,372678	0

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