OLFACTORY NEUROGENESIS FOLLOWING ACUTE INJURY

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ABSTRACT

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For most of the last century, it was believed that neurogenesis is limited to embryonic development and does not occur in the adult nervous system. However, it is now an established fact that neurogenesis also occurs in the post-embryonic central and peripheral nervous system. Yet, adult neurogenesis is a limited process in mammals but more and widespread in lower vertebrates, such as teleost fish and amphibia. The olfactory sensory tissue is an attractive model to study neurogenesis due to its high regenerative capacity in lower and higher vertebrates. Olfactory sensory neurons undergo constant turnover and are replaced by adult-born cells. In addition to ongoing neurogenesis, which maintains the integrity of the tissue, the olfactory epithelium is also capable of mounting strong regenerative responses to acute injury. The differences and commonalities of the mechanisms that mediate these two different modes of neurogenesis are not well understood. Here, studies are described to functionally investigate the response of the olfactory epithelium to acute injury in zebrafish to understand the type, the location, and the properties of stem cells and signaling pathways that are involved in damage-induced neurogenesis. Tissue-wide degeneration of the olfactory epithelium was induced in combination with analysis of cell type-specific and proliferation markers to characterize the subpopulations of cells that respond to the injury and to investigate which molecular signaling pathways are activated. Neurogenesis in the unperturbed tissue is restricted to two distinct regions of proliferative activity, which are located on either end of the sensory tissue. The tissue response to chemical insult is rapid and within 12 h following treatment a significant increase in the number of proliferating cells can be detected. Different from the restricted pattern of maintenance neurogenesis, proliferative activity is distributed throughout the sensory tissue, suggesting that a distinct stem cell population located throughout the basal epithelium is recruited upon injury. Changes in gene expression following induced de- and regeneration was analyzed by transcriptome profiling to describe the molecular responses at different time points following damage.

ÖZET

AKUT HASAR SONRASINDA KOKU NÖROGENEZİ

Geçen yüzyılın büyük kısmında, nörogenezinin sadece embriyonik gelişim ile sınırlı olduğuna, yeni nöronların erişkin sinir sisteminde oluşmadığına inanılırdı. Ancak, şu anda nörogenezinin embriyonik dönem sonrası merkezi ve çevresel sinir sisteminde de meydana geldiği yaygın kabul edilen bir gerçektir. Diğer taraftan, yetişkin nörogenezisi memelilerde sınırlıyken, bu süreç teleost balık ve amfibiler gibi alt omurgalılarda yaygındır. Alt ve üst omurgalılardaki yüksek yenilenme kapasitesi nedeniyle koku duyu dokusu, nörogenezi çalışmaları için cazip bir modeldir. Duyusal koku nöronları sürekli bir hücre devir daimi geçirir ve yeni doğan hücreler tarafından değiştirilirler. Doku bütünlüğünü korumak için yapılan sürekli devam eden nörogeneziye ek olarak, koku epiteli akut yaralanmaya güçlü rejeneratif tepki oluşturma yeteneğine de sahiptir. Bu iki farklı nörogenez modlarına aracılık eden mekanizmaların farklılıkları ve ortak yönleri iyi anlaşılamamıştır. Bu tezde, hasar kaynaklı uyarılan nörogeneziyle ilişkili sinyal yolakları ve kök hücrelerin yeri, türü ve özelliklerini işlevsel olarak araştırmak için zebrabalığı koku dokusunda yapılan çalışmalar tarif edilmiştir. Hasara yanıt veren hücre alt popülasyonunu karakterize etmek ve aktive olan moleküler sinyal yollarını araştırmak için koku epitelinin doku çapında dejenerasyonu başlatıldı. Nörogenezi, müdahale edilmemiş dokuda, duyusal bölgenin her iki ucunda yer alan iki farklı proliferatif aktivite bölgesi ile sınırlandırılmıştır. Kimyasal hasara doku tepkisi hızlıdır ve tedaviden sonraki 12 saat içinde çoğalan hücrelerin sayısında önemli bir artış tespit edilmiştir. Doku onarımı için yapılan sınırlı nörogenezden farklı olarak, proliferatif aktivite doku boyunca dağılmıştır, ki bu da bazal epitel içinde bulunan ve hücre yaralanması sonrası aktifleşen ayrı bir kök hücre olduğunu düşündürmektedir. Proliferatif aktiviteyi görüntülemek için yaralanmadan önce ve sonra çift proliferasyon işaretleyici testi kullanmak, onarım koşullarında gerçekleşen nörogeneziye katkıda bulunan nörojenik bölgelerden gelen progenitör hücrelerin yerlerinin değiştiği olasılığını bertaraf etti. Yaralanma sonrasında oluşan dejenerasyon ve rejenerasyonun sebep olduğu gen ifadesindeki değişiklikler, hasar sonrası farklı zaman noktalarında moleküler tepkileri açıklamak için transkriptom profili ile analiz edilmiştir.

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LIST OF ACRONYMS / ABBREVIATIONS

ACSF	Artificial Cerebrospinal Fluid
CNS	Central Nervous System
CYKII	Cytokeratin Type-2
GBC	Globose Basal Cells
HBC	Horizontal Basal Cells
IHC	Immunohistochemistry
ILC	Interlamellar Curve
LP	Lamina Propria
MS222	ethyl-3-aminobenzoate-methanesulfonate
NGS	Normal Goat Serum
OE	Olfactory Epithelium
OMP	Olfactory Marker Protein
OR	Olfactory Receptor
OSN	Olfactory Sensory Neuron
PFA	Paraformaldehyde
pH3	phospho-histone-H3
PBS-T	Phosphate Buffered Saline with Triton X-100
S/NS	Sensory/Non-sensory
SC	Sustentacular Cells
TRPC2	Trans Receptor Potential Channel 2

1. INTRODUCTION

Certain tissues, such as skin and liver can repair themselves because they maintain a high regenerative capacity through proliferation and differentiation of resident stem cell populations (Rando, 2006). However, unlike those systems, the regenerative capacity of the highly specialized adult central nervous system (CNS) is very limited. This inability to repair itself makes the CNS particularly susceptible to any type of insult that causes deterioration and ultimately loss of neurons by acute ranging from trauma to neurodegenerative diseases that cause progressive deterioration of neurons (Magavi *et al.,* 2000). The injury in a neuronal tissue both break the communication between healthy neurons and trigger secondary events leading to neuronal degeneration and therefore neuronal cell death (Ming *et al*., 2005). Hence, a better understanding of the regulatory mechanisms, molecular pathways, and genetic networks that underlie neurogenesis in general and adult neurogenesis in particular would ultimately help developing new therapeutic applications to repair CNS damage. Thus, being able to activate mechanisms that trigger proliferation of neuronal stem cells holds the key to fight devastating neurological disorders, such as amyotrophic lateral sclerosis, Alzheimer's disease and Rett's syndrome, and CNS injuries resulting from stroke or trauma (Fleisch *et al.,* 2010; Ronnett *et al*., 2003; Ghanbari *et al*., 2004).

1.1. Neurogenesis

Neurogenesis is the process that new neurons are generated and integrated into the existing nervous network in embryonic and adult stages of an organism. As in the formation of new cells of other tissue types, neurons originate from stem cells and progenitors. The neuronal stem cells also exhibit typical stem cell properties such as quiescence and self-renewal via slow divisions. Ependymal and subependymal cells are proposed to be primary neural stem cells, which express nestin, GFAP and Prominin-1 / CD133 (Traiffort *et al*., 1998). As slow divisions are asymmetrical, one of the daughter cells turns out to be transit amplifying cell that would undergo many division cycles and

eventually differentiates into neurons and glial cells. The transit amplifying cells are known to express nestin and EGFR which then differentiates into DCX, Dlx2 and PSA-NCAM-expressing neuroblasts joining to rostral migratory stream to compose interneurons, expressing NeuN and GAD65, in the olfactory bulb (Doetsch *et al*., 2002; Seki and Arai, 1993; Encinas and Enikolopov, 2008). In the dentate gyrus of the hippocampus, on the other hand, neural stem cells are actually quiescent radial glia-like cells that produce neurogenic markers GFAP, nestin and Sox2 (Luskin *et al.*, 1993; Kempermann *et al*., 2004). After they divide asymmetrically, one of the daughters, determined to be restrictedly neurons, still contain nestin and Sox2 but losing the expression of GFAP (Nacher *et al*., 2007). Then, these cells become doublecortin- and adhesion molecule-expressing, PSA-NCAM, neuroblasts, which later on give rise to granule cells in the dentate gyrus (Faigle and Song, 2013).

Neurogenesis is achieved via extrinsic and intrinsic signals which include morphogens, growth factors, neurotransmitters, transcription factors and epigenetic regulators. As morphogenic signaling, Wnt, Notch, Shh and BMP pathways act on either positively or negatively regulation of neurogenesis (Lie *et al*., 2005; Breunig *et al*., 2007; Balordi and Fishell, 2007, Lim *et al*., 2000). Other extrinsic actors such as growth factors FGF, VEGF and BDNF and neurotransmitters glutamate, GABA and dopamine have role in neurogenesis regulating migration, proliferation, quiescence and survival of the neural stem cells (Benraiss *et al*., 2001; Jin *et al*., 2002; Rai *et al*., 2007; Platel *et al*., 2010; Hoglinger *et al*., 2004). Intrinsically, transcription factors such as Pax6, CREB, Ascl1, Dlx1, NeuroD and Sox2, and some epigenetic regulators have actions on neurogenic pathways (Jagasia *et al*., 2009; Hack *et al*., 2005; Jessberger *et al*., 2008; Ferri *et al*., 2004; Gao *et al*., 2009).

1.2. Mammalian adult neurogenesis

Certain invertebrates, such as hydra and planarians, and some non-mammalian vertebrates, such as axolotls and teleost fish, can regenerate entire body parts, organs and

even neuronal tissues, an ability which provides distinct survival advantages. Nerve cells, like other cells, are generated from committed progenitor or stem cell populations through a process that is commonly known as neurogenesis. While in lower organisms neuronal stem cells remain active during the life of the organism, most stem cells in the mammalian CNS terminally differentiate and lose their regenerative ability during post-embryonic development (Sanchez *et al*., 2006). The reduced neuroregenerative ability in mammals is likely to be the result of a progressive evolutionary loss of this ability, although the underlying reason for this loss is still unclear (Tanaka, 2009, Wagner and Misof, 1992). Alternatively, it has also been hypothesized that different classes of lower vertebrates may have regained their neurogenic and regenerative potential independently in the course of evolution (Kaslin *et al*., 2008).

For most of the past century, the dogma that adult mammalian CNS tissue is unable to replace lost or injured neurons (Cajal, 1913) prevailed and dominated scientific thinking about CNS development and function. For this reason, research on therapeutic approaches for CNS-related disorders focused on limiting further damage instead of rebuilding damaged neuronal tissue. However, in the mid-1990s it was reported that the adult mammalian brain contains actively dividing neuronal stem cells that can contribute to the generation of new functional neurons in contrast to the long-held views (Magavi *et al*., 2000; Schwartz *et al*., 1991; Gage, 2000). Yet, in mammals the areas in which these stem cells are located and the parts of the brain to which they contribute are very confined to few brain regions (McKay, 1997; Gage, 2000; Alvarez-Buylla *et al*., 2001; Temple, 2001; Doetsch, 2003; Ming and Song, 2005). The two subregions recognized as the sites of neurogenesis in the adult mammalian brain are the subventricular zone (SVZ) of the lateral ventricles in the forebrain, which gives rise to local interneurons of the olfactory bulb (Alvarez-Buylla and Garcia-Verdugo, 2002; Luskin *et al*., 1993) and the subgranular zone of the dentate gyrus in the hippocampus, giving rise to granular neurons that are axonless inhibitory interneurons (Gould *et al*., 1998; Eriksson *et al*., 1998; Kuhn *et al*., 1996).

1.3. Mammalian olfactory neurogenesis

In addition to the CNS, adult neurogenesis also occurs in the peripheral nervous system. The vertebrate olfactory tissue is an interesting and promising model system to identify factors that regulate proliferation and differentiation of sensory neurons from stem cells and progenitors. This is because the generation of olfactory sensory neurons (OSNs) in the olfactory epithelium (OE) is maintained throughout life in contrast to most other neuronal tissues, such as the CNS described above (Graziadei and Monti Graziadei 1978; Calof *et al*., 2002). OSNs and taste cells of the tongue are the only nerve cells that directly contact the environment and therefore are particularly prone to damage or injury. To maintain the ability to smell or taste, these peripheral sensory neurons need to be generated at comparably high rates in adult organisms.

According to the commonly accepted view, a heterogeneous cell populations, generally described as basal cells (BCs), including both stem cells and different stages of transit amplifying cells contribute to life-long neurogenesis in the OE (Calof *et al*., 2002; Mackay-Sim and Kittel, 1991). The stem cells, which lie close to the basal compartment of the OE are known to go through multiple rounds of slow asymmetric cell divisions, which sustain the stem cell population and generate more rapidly-dividing transit amplifying progenitors (Beites *et al*., 2005; Calof *et al*., 2002)

Two morphologically distinct subpopulations of BCs with different stem cell / progenitor characteristics have been recognized; the horizontal (HBCs) and globose basal cells (GBCs). HBCs are assumed to be totipotent stem cells of the OE, whereas GBCs serve as transit amplifying cells that generate OSN precursors by dividing several times (Calof *et al.*, 1998; Mackay-Sim and Kittel, 1991; Leung *et al*., 2007). However, this simple concept that HBCs generate GBCs, which in turn generate immature OSNs has been challenged by the observation that HBC-like cells only occur late in OE development (Saxena *et al*., 2013). Thus, HBCs and GBCs may be independent stem cell / progenitor populations with different origin and which may contribute to olfactory neurogenesis under

different conditions (Leung *et al*., 2007). The flattened HBCs are located on the basal lamina and express intercellular adhesion molecule-1 (ICAM-1) while the roundish GBCs reside in more apical regions of the OE (Holbrook *et al*., 1995). GBCs are a heterogeneous cell population that includes stem cells and two subpopulations of transit amplifying progenitors: Mash1-expressing progenitors and Ngn1-expressing immediate neuronal precursors (DeHamer *et al*., 1994; Gordon *et al*., 1995; Wu *et al*., 2003).

The recent view suggests that both types of cells, HBCs and GBCs, have critical roles in neurogenesis, but under different conditions (Huard *et al*., 1998; Mackay-Sim and Kittel, 1991; Caggiano *et al*., 1994). Under normal physiological conditions, GBCs appear to be rapidly dividing stem cells with one cell division per day, generating OSNs during regular tissue maintenance, while HBCs constitute a relatively quiescent populations that divide rarely but is capapble of responding to severe traumatic injury and depletion of the pool of GBCs (Mackay-Sim and Kittel, 1991; Leung *et al*., 2007; Caggiano *et al*., 1994). Under those conditions HBCs would generate cells with GBC-like properties.

GBCs and GBC-like cells have been shown to generate two types of transit amplifying progenitors in the OE (Calof *et al*., 2002; Mackay-Sim *et al*., 1991; Graziadei and Metcalf, 1971). The first one is the immediate daughter of the GBC and expresses the proneural gene Ascl-1, whereas the later one is the daughter of Ascl-1-expressing progenitors, which is also known as the immediate neuronal precursor (INP) and expresses another neuronal marker, Neurogenin1 (Ngn1) (Beites *et al*., 2005; DeHamer *et al*., 1994; Gordon *et al*., 1995; Calof *et al*., 2002). The INP then undergoes a series of symmetric cell divisions before differentiating into functionally mature OSNs. Mature OSNs can be recognized by the expression of olfactory marker protein (OMP) and ultimately chemosensory receptors (Pixley, 1992; Graziadei and Metcalf, 1971; Margolis, 1982; Calof *et al*., 1989; 2002).

1.4. Zebrafish olfactory neurogenesis

Although invertebrates probably have the highest regenerative and divisional capacity, they lack higher level brain centers, which is a major drawback for their use as animal model in neurogenesis research. On the other hand, among vertebrates, the teleost zebrafish has a similarly high regenerative potential to restore a variety of body parts including neuronal tissues. Being exposed to the continuous stress of toxic agents makes the OE vulnerable to damage and causing variable rates of OSN loss (Kilgour *et al*., 2000). Since olfaction is crucial for the survival and reproduction of fish species, accurate and rapid regeneration of OSNs has obvious evolutionary advantages (Hamdani and Døving, 2007). Thus, as an evolutionary adaptation, the zebrafish OE has advanced remarkable regenerative ability to continuously replenish dying OSNs by newly generated cells throughout life of the fish to maintain the functional sense of smell (Gheusi and Lledo, 2007; Schmidt, 2007; Gokoffski *et al*., 2010).

Moreover, chemical and physical tissue insults from the outside without significant harm to the animal are easy to perform particularly in zebrafish OE thanks to its peripheral location, which makes it accessible for injury manipulations (Schwob, 2002). However, comparable studies are much harder in mice OE, which is only accessible by surgery. Hence, the zebrafish OE with its remarkable regenerative capacity is a very useful tissue model for studying underlying molecular mechanisms of neuronal regeneration following injury (Iqbal and Byrd-Jacobs, 2010; Byrd and Brunjes 2001).

It has been shown that unlike the mammalian OE, there are two neurogenic proliferation zones at each end of the lamella (Bryd and Brunjes 2001). The proliferation predominantly occurs around at these two discrete regions called ILC and S/NS boundary. The mature OSNs invade the sensory region of the OE by active migration (Bayramli, unpublished).

1.5. Regulation of olfactory neurogenesis

The mechanisms by which apical layers of the OE, where functional OSNs are located, and the basal compartment of the OE, where proliferative BC populations reside communicate still remain unknown. Somehow, the loss of neurons needs to be communicated from the site of injury or degeneration to stem cells directly to replace the lost cells. Similarly, neurogenesis needs to be restrained to prevent the generation of excessive numbers of OSNs and other cells. Two, not necessarily exclusive, signaling mechanisms have been proposed; active signaling by supporting sustentacular cells (SCs) and signaling by concentrations of stimulating and inhibitory growth factors (Hegg *et al*., 2009; Hassenklöver *et al*., 2009).

In the active signaling model, dying OSNs release their cytoplasmic content, which could provide a signal to trigger physiological responses that are conveyed to basal stem cell populations. In particular purines, such as ATP and its derivatives have been implicated in stimulating neurogenesis (Hassenklöver *et al*., 2009; Zimmermann, 2006; Hegg *et al*., 2009). Purines released from dying OSNs are sensed by SCs, which are the equivalent of radial glia in the OE and traverse the entire apical-basal dimension of the OE and conveyed to BCs. The pharmacological analysis on sustentacular cells via functional calcium imaging revealed that, in the rodent and amphibian sustentacular cells, a calcium wave propagates along the sustentacular cells from apical to basal orientation upon purinergic activation. ATP instillation coupled with BrdU incorporation assay showed that there is an increase in the number of dividing cells along with the expression of growth factors such as FGF2 (Jia *et al*., 2011). The basal end feet of SCs are in close contact with HBCs and GBCs. Sustentacular cells are thought to signal and switch on the basal cells through neurotrophic factors such as NPY and / or through purinergic signaling molecules (Jia and Hegg, 2010). Therefore, these SCs could be part of the communication pathway between the apical and basal OE by releasing stimulating factors on BCs to trigger proliferation.

The second model proposes that the proliferation of OE progenitors is modulated by a balance between positive and negative regulatory factors released from within the OE tissue (Beites *et al*., 2005) Hence, any insult to the OE would cause an imbalance of stimulating and inhibiting factors released from different cell populations, which activates the BCs by diffusion and triggers neurogenesis (Gokoffski *et al*., 2010). There are several stimulating factors known to be released to activate injury-responsive BCs (Murdoch and Roskams, 2007). For instance, EGF has been shown to activate proliferation of HBCs (Lillien *et al.,* 2000) while GBC proliferation can be induced by FGF2 (Calof 1998; Mumm *et al.,* 1996; DeHamer *et al.,* 1994). GDF11 and BMP are known inhibitory signals that could mediate negative feedback of neurogenesis to prevent the overproduction of OSNs and other non-neuronal cell types within the OE (DeHamer *et al*., 1994).

1.6. Organization of the zebrafish olfactory system

The paired peripheral olfactory organs of zebrafish are located in the dorsal part of the head within an olfactory cavity directly connected with the surrounding environment. Inside the olfactory cavity, the OE tissue folds back onto itself and forms individual lamellae radiating from a central raphe to form a characteristic rosette-shaped structure (Laberge and Hara, 2001; Hansen and Zeiske, 1998). Thus, the basal lamina is located in the midline of each lamella.

Functional OSNs are limited to the central sensory parts of each lamellae while respiratory cells form surrounding non-sensory tissue (Byrd and Brunjes, 1995; Weth etal., 1996; Hansen and Zeiske, 1998). A single lamella can be functionally divided into two parts in which almost 70% of the lamellar epithelium is mainly dedicated to OSNs marked by mature neuronal indicator HuC/D and inbetween the OSNs, there exist regularly-spaced apicobasally extending sustentacular cells that are cytokeratin type II and Sox2 positive (Bali, unpublished). The sensory epithelium bends into two parts at central midline raphe region to form neighboring lamellae where it is called interlamellar curve. The interlamellar curve region is one of the proliferative zones of the OE that would suggest a

group of stem / progenitor cells reside where early stage neuronal progenitor marker Ascl-1 is present. The other proliferative zone is observed at the boundary between sensory region and non-sensory region. These two potentially neurogenic proliferative zones are active under maintenance conditions of zebrafish OE.

OSNs penetrate the surface of the epithelium through their dendrites to interact with the odorant molecules through numerous cilia containing sensory transduction proteins (Schild and Restrepo, 1998). These cilia extend into the apical surface covered by mucus which is primarily produced by the olfactory Bowman's glands (Getchell *et al*., 1986; Gold, 1999; Schild and Restrepo, 1998; Menco, 1980). Unlike the mammalian OE, the zebrafish OE contains at least four morphologically distinct OSN subtypes denominated OMP-positive ciliated, TRPC-2-positive microvillous, Trk-A-positive crypt and Golf-positive kappe neurons (Hansen and Zielinski, 2005; Hamdani and Døving, 2006; Oka and Korsching, 2011; Parisi *et al.*, 2014; Gayoso *et al*., 2012).

1.7. Models of olfactory de- and regeneration

The zebrafish OE not only maintains the dynamic OSN population throughout life but also responds to severe acute injury and experimental tissue insult (Carr and Farbman, 1992; Schwob, 2002; Mackay-Sim, 2010; Costanzo and Graziadei, 1983). There are different experimental injury models that are commonly used to investigate neuronal degeneration and following neurogenesis in the OE (Carr *et al*., 2001; Schwob, 2002). The most prevalent ones are nerve transection, inhalation of MeBr gas and the irrigation of the OE with corrosive agents such as $ZnSO_4$, or the detergent Triton X-100. Intranasal irrigation with $ZnSO_4$ on rodent OE resulted in immediate epithelial and axonal degeneration with incomplete recovery after 30 days (Herzog and Otto, 1999; Burd, 1993). Also MeBr inhalation and the detergent Triton X-100 treatment successfully disrupt OSNs and adjacent respiratory epithelium in the rodent OE (Nadi *et al*., 1981; Schwob *et al*., 1999). In catfish, both $ZnSO_4$ and Triton X-100 treatments caused severe disruption of OE, following a recovery after about 2 months (Cancalon, 1982). In addition, treatment of

Triton X-100 on the zebrafish OE, resulted in a severe insult to the tissue and recovery started by 5 days after treatment (Iqbal and Byrd-Jacobs, 2010).

This thesis presents studies in the adult zebrafish OE aiming to better understand the broad range of the molecular mechanisms involved in the regulation of injury-induced regeneration. We questioned how the zebrafish OE can provide robust responses to tissue damage and how it is capable of rapid regeneration by using several traumatic injuries to the adult zebrafish OE. To do so, different models of tissue degeneration were tested and compared with each other and then used to profile the changes in gene expression over the time course of de- and regeneration.

In this study, we performed tissue-wide degeneration of the OE in order to investigate the injury-responsive cells that are activated upon injury. The proliferative response to chemical damage reached its peak within 12 h with a significant increase in the number of mitotic cells. Different from the restricted pattern of maintenance neurogenesis, proliferative activity is distributed throughout the sensory tissue, suggesting that there is an independent progenitor population responding to injury, which is in a quiescent state under unperturbed conditions. The double proliferation marker assay with thymidine analogs showed that progenitor cells residing at the regular neurogenic zones of OE are not displaced in response to tissue insult. Also our HuC/D staining coupled with BrdU incorporation assay suggested that the response to chemical damage is not systemic but locally restricted to the injured area.

2. PURPOSE

In this study, the major aim was to investigate how new OSNs are generated following the damage in the post-embryonic zebrafish olfactory system. To this end, we firstly wanted to investigate the discrimination between different stem cell and precursor subpopulations contributing to different responses under acute injury and tissue maintenance conditions. In particular, we wished to examine the identity and tissue distribution of injury responsive neuronal stem cells that are thought to be different from the stem cell population responsible for the maintenance of the zebrafish olfactory tissue. For initial characterization of the subpopulations of stem cells responding to the tissue damage, several degeneration strategies such as tissue-wide chemical degeneration applying the olfactory irritant agents $ZnSO_4$ and Triton X-100 through the naris and focal physical degeneration by stabbing the OE were established, which then was followed by BrdU incorporation assay.

For further characterization and location of the cell populations responding to damage in the basal OE along the lamella, we performed double marker incorporation assay with the two analogs of thymidine IdU and CldU in order to detect if the stem cells populations located in the ILC and S/NS boundary contribute also to injury response of the tissue.

Finally, we wanted to identify specific molecular signaling pathways playing role in the neurogenesis of adult zebrafish OE via transcriptome analysis. In order to better understand the molecular components in the neurogenesis upon injury, we wanted to analyze regulation patterns of transcripts of various neuronal and olfactory-specific differentiation markers along with stem cell ones.

All in all, we aimed to elucidate the molecular components of the stem cell and precursor populations residing in OE as well as the characterization and the location of the

cells playing role in adult neurogenesis as injury response with the help of IHC against proliferation markers and transcriptome analysis.

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Model organism

Adult zebrafish (3-5 cm) of both sexes (older than 6 months) were procured from a local pet shop. The fish were raised in a dedicated fish room at the Boğaziçi University Life Sciences Center (Vivarium) where they were fed twice a day with either flake food (TetraMin, Sera Vipan) or brine shrimp larvae (*Artemia sp*.). They were housed in 10-liter tanks filled with un-chlorinated aquarium water at temperature of 28 ± 2°C with constant filtration and aeration. Density of five fishes per 1 liter aquarium water was maintained. Animals were kept on under a 14 hours light / 10 hours dark photoperiod.

3.1.2. Equipment and Supplies

A detailed list of chemicals and equipments, including manufacturers and order information are provided in Appendix A and Appendix B.

3.1.3. Buffers and Solutions

The buffers and solutions that were used in molecular biology procedures were prepared according to Sambrook and Russell (2001). Zebrafish-specific solutions and buffers were prepared according to Westerfield (2007).

3.2. Methods

3.2.1. Immunohistochemistry

The dissected zebrafish olfactory epithelium was sectioned on a cryostat (Leica CM3050S) and 14-µm thick horizontal sections were placed onto positively-charged slides. The slides were then baked at 65^{o}C for 2 hours to make the tissue adhere to slide. After incubation in the oven, the sections were washed with 1x phosphate buffered saline (PBS; pH 7.4) and permeabilized with PBS-T (0.1% Triton X-100; Sigma) for 10 min. Sections on the slide was circled with a liquid blocking pen and then the slides were fixed in 4% paraformaldehyde (PFA;Sigma). After fixation the sections were washed in PBS-T 3 times for 5 min on shaker and followed by 1 h incubation with blocking solution in a humid incubation chamber with wet paper towels on the bottom of a plastic container. Non-specific binding was blocked for 2 hours by using 10% normal donkey serum containing PBS-T at room temperature. Then, slides were incubated overnight at 4 °C with the primary antibodies (a volume of 250 µl of solution per slide is sufficient). Primary antibodies were dissolved in fresh blocking solution as follows: mouse anti-human neuronal protein HuC/D (1:500, Abcam), mouse anti-phospho-histone-H3 (1:250, Millipore), goat anti ascl-1 (1:50, Sigma), mouse anti-BrdU (1:250, Becton-Dickinson), rat anti-BrdU (1:250, Abcam). The tissues were then rinsed in PBS-T three times for 5 min and incubated in fluorescent secondary antibodies (anti-mouse Alexa Fluor 488, anti-goat Alexa Fluor 555, anti-rat Alexa 633; Life Sciences) diluted 1:800 in blocking solution for 1.5 hour in room temperature. The secondary antibodies were then removed by several washing steps in PBS. Finally slides were transferred to PBS solution and stored at 4^{o}C. Visualization of samples were performed via an SP5-AOBS laser scanning confocal microscope with the 20X objective (Leica, Germany). Each experiment has been performed by using at least 3 fish.

3.2.2. BrdU incorporation assay

BrdU assays were performed in order to detect proliferating cells within 24 hours time window. Thus, prior to sacrifice the animals, they were incubated in 30mg/L BrdU (Sigma) containing fish water for 24 hours. Following the dissection of the OEs, the tissue was sectioned via cryostat. Then, 14 µm-thick sections were mounted on positively charged slides as described in the section 3.2.1. The same protocol was followed for the staining with BrdU antibody. The only difference is the immersion in 4 N HCl step at room temperature for 10 min. for DNA denaturation to allow primary antibody access to BrdU. This step was carried out after washing steps of 4% PFA. For double co-labeling with BrdU, tissues were incubated with rat anti-BrdU (1:250) and mouse anti-HuC/D primary antibodies (1:500) containing blocking buffer.

3.2.3. Focal lesions in the adult zebrafish OE

Adult fish was anaesthetized in 0.1% MS-222 (Sigma), a commonly used anesthetic for fish, and immobilized on top of a wet sponge. Then fish was placed on the stage of a stereo microscope (Zeiss, Germany). Although the OE is buried in the nasal cavity, the individual lamellae are made visible and accessible by removal of the skin covering the naris. A single lamella was stabbed to impair the tissue by using a fine needle. In order to mark proliferating cells following lesion of the epithelium, fish was put back into tank with 30 mg / liter BrdU within a short time following the onset of anesthesia for 24 hours.

3.2.4. Chemical degeneration of the adult zebrafish OE

Anaesthetized fish were placed in wet sponges. Petroleum jelly was placed between the nasal openings in order to prevent any leakage to the control naris. Degeneration was induced via irrigation by injecting approximately 1 µl of the either 3% ZnSO4 or 1% Triton X-100 and 10% phenol red in PBS solution into right naris through the skin opening of the naris via fine gel loader tips. The solution remained in contact with the OE for 90

sec. Then, the chemical was washed by water efflux with a pasteur pipette and fish were moved to a recovery tank supplied with 30 mg/L BrdU. One of the OEs was kept as untreated control for further comparison. Fish were sacrificed at various time points; 4h, 12 h or 24 h for analysis with IHC.

3.2.5. Detection of proliferating cells with two thymidine analogs

Thymidine analogs IdU (iodo-deoxy-uridine) and CldU (Chloro-deoxy-uridine) were dissolved in water via a magnetic stir plate. CldU was dissolved for 10 min at room temperature whereas IdU requires more than an hour at 37^{o}C on a shaker. Three sets of adult zebrafish that are older than 6 months were incubated in tank water with either IdU or CldU at concentration of 30 mg/L. For Triton X-100 degeneration assays, the fish was first incubated with IdU containing tank water for 24 hours in order to follow the cells generated before chemical injury. Then, fish was washed with unlabeled regular tank water for 24 hours to ensure the depletion of IdU remained in the body of fish. Afterwards, 1% Triton X-100 was introduced for 90 sec to one of the OEs while the other OE served as the unperturbed control. Following the chemical treatment, fish were incubated with the second thymidine analogue CldU for 24 hours later, which labeled the newly divided cells after chemical damage.

For further analysis, fish were sacrificed in ice-cold water and the dissected OEs were embedded into OCT compound. Then 14 μm thick sections were taken by using a Leica CM3050S cryostat. In order to visualize cells incorporating thymidine analogs, antibodies specific for IdU (mouse anti-BrdU, 1:250) and CldU (rat anti-BrdU, 1:250) was used for immunohistochemistry as described above. Firstly, the sections were incubated overnight in 4^{o}C cold room with mouse anti-BrdU, which strongly bind to IdU and weakly bind to CldU. In order to minimize non-specific binding of mouse anti-BrdU to CldU, high stringency wash (freshly prepared low salt TBST buffer: 36mM Tris, 50mM NaCl, 0.5% tween-20; pH 8.0) was performed following one day incubation of IdU. 40 mL of buffer is poured into each 50 mL conical falcon tube. Before adding slides, the solution was

preheated to 37°C. Each of two slides was placed back-to-back so that tissue faced away from one another and inserted into falcon tubes filled with low salt buffer. The tubes were shaked in a bacterial culture incubator for 20 min at 37°C. Slides were rinsed three times in fresh 1x PBS for 5 min before incubation in anti-CldU primary antibody. After incubation, slides were washed three times in fresh 1x PBS for 5 min. Primary antibodies were detected by subclass-specific secondary antibodies labeled with rat Cy2 (for CldU) or mouse Cy5 (for IdU). Then the tissues were imaged via confocal microscopy accordingly.

3.2.6. Preparation of biocytin-backtraced slices

In order to backfill mature OSNs, zebrafish were sacrificed in ice-cold water. Then the jaw was removed from the head of fish and remaining tissue was dissected to uncover olfactory nerve bundle to be transected. The nerve bundle of OSNs was incised with a surgical blade and small crystals of biocytin (Life Technologies, Darmstadt, Germany) were put into the cut nerve. The lesion was then closed with tissue adhesive (Hystoacryl L, Braun, Tuttingen, Germany). About one hour later the OE were dissected and the acute slices were prepared for further IHC experiments.

3.2.7. Transcriptome analysis

The Triton X-100 degeneration experiment has been performed on 50 control and 50 Triton X-100-treated fish, subsequently RNA of OEs were extracted using regular TriZol (Invitrogen) protocols based on the manufacturer’s recommendation. Extracted RNA was submitted to sequencing services at Dresden, Germany for RNA-sequencing analysis.

4. RESULTS

Olfactory sensory neurons have a limited lifetime (Graziadei and Graziadei, 1979; Crews, 1994; Mackay-Sim and Kittel, 1991) and therefore the peripheral olfactory tissue undergoes a constant turnover. In addition to this ongoing neurogenesis that occurs under normal conditions in unperturbed tissue, the olfactory system also has a high regenerative capacity in response to acute injury and severe loss of sensory neurons. This rises the interesting question whether tissue maintenance and response to damage use the same cellular and molecular mechanisms. One possible answer to this question is that the same stem cell and progenitor populations that are responsible for tissue maintenance also responds to any insults to tissue integrity. Alternatively, however, maintenance neurogenesis and damage response could employ different subsets of stem cell and precursor populations as well as different molecular pathways to differentiate between the two conditions. Here experiments are described that aim to provide a better understanding of injury-induced neurogenesis in response to damage in models of tissue-wide induction of degeneration in the olfactory system of the zebrafish.

4.1. Organization of the zebrafish olfactory epithelium

The zebrafish olfactory epithelium (OE) has a characteristic flower-shaped morphology and is generally referred to as the olfactory rosette. The structure is formed by several staggered lamella that extend from a central structure, the midline raphe (Figure 4.1a). Two layers of sensory epithelium on each side of a single lamella share a central basal lamina. Thus, the apical surfaces of each lamella are in direct contact with the external environment while the basal aspects of the lamella reside in the center of the tissue.

The sensory region, in which olfactory sensory neurons (OSNs) reside, extends about 70% of the inner lamellar length, while the remaining outer 30% mostly contain respiratory cells, as can exemplarily be seen in immunohistochemical staining for the mature neuronal

marker HuC/D (Figure 4.1.b). Different from the rodent olfactory system, the single zebrafish OE houses multiple chemosensory subsystems that form unique anatomical structures in rodents, such as the vomeronasal organ (Mombaerts, 2004), the septal organ (Tian and Ma, 2004), or the Grüneberg ganglion (Fuss *et al*, 2005; Ma, 2010).

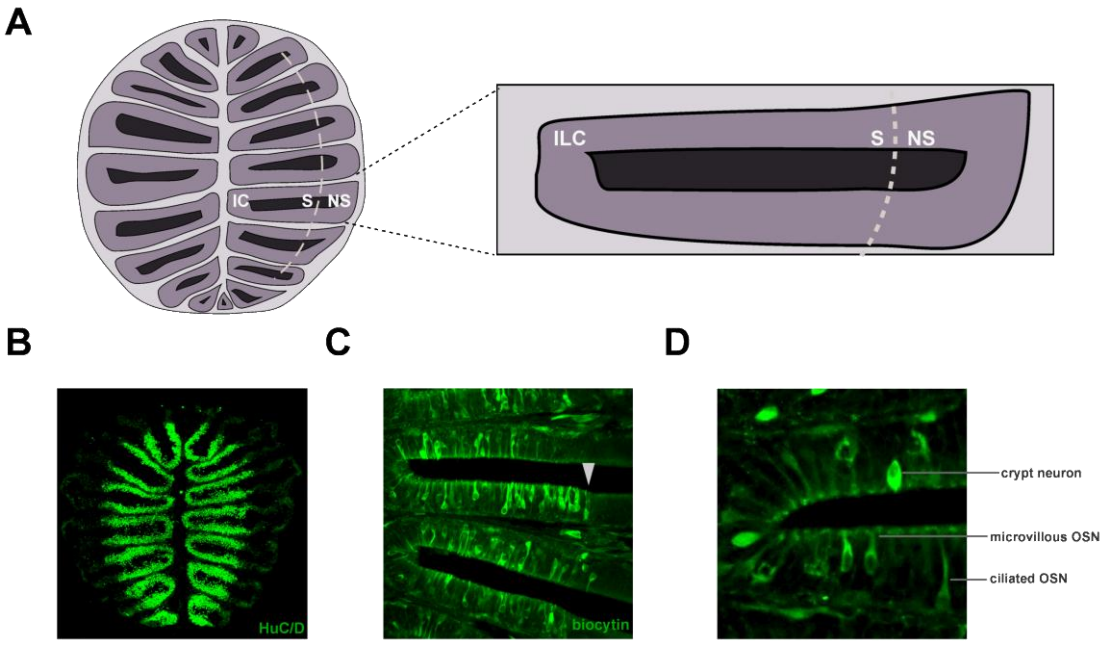

Figure 4.1. Organization of the zebrafish OE. (A) Schematic representation of the OE and single lamella. (B) Horizontal cross section through the zebrafish OE labeled by IHC for the mature neuronal marker HuC/D (green). (C) Detailed view of a single olfactory lamella visualized by retrograde tracing with biocytin. (Arrowhead: boundary between the sensory and non-sensory (S/NS) segments of the lamella) (D) Overview of OSNs types.

Because the single OE combines a variety of chemosensory subsystems, different morphological subtypes of chemosensory neurons can be found in the zebrafish OE. Up to date, four distinct classes of sensory neurons have been described in the zebrafish OE, which are the ciliated, the microvillous, crypt, and kappe neurons (Sato *et al*, 2005; Ahuja *et al*., 2014; Hansen *et al*., 2000). Using biocytin backtracing from the olfactory bulb all four classes of neurons can be visualized and distinguished by their morphological appearance (Figure 4.1c). Cell bodies of the ciliated OSNs are found to be located at more basal position in the OE and have long slender dendrites extending to the apical layer while

microvillous cells bodies sit closer to the apical side compared to the ciliated OSNs and have shorter and thicker dendrites. On the other hand globular-shaped crypt neurons sit directly underneath the apical surface (Figure 4.1d). Ciliated OSNs express OMP and microvillous cells express the transient receptor potential channel TRPC2 as their molecular marker (Sato *et al*, 2005). Another cell type, crypt cells, are characterized by the presence of an indentation (crypt) on the apical top of the cell bodies where chemoreceptors are localize and interact with their ligands (Hansen *et al*., 2000). Finally, kappe neurons were named due to their characteristic hat-like appearance and similar to the microvillous and crypt neurons, their cell bodies are located close to the apical surface of the OE (Ahuja *et al*., 2014). There is no known specific molecule expressed uniquely to kappe and crypt neurons. However, kappe neurons show immunoreactivity, thus can be detected against G_o antibody whereas crypt neurons show immunoreactivity against TrkA and S100 antibodies. (Hansen *et al*., 2000; Ahuja *et al*., 2014).

The visualization of the OSNs by retrograde backtracing with biocytin from the olfactory nerve and olfactory bulb was also performed to help the characterization of basal cells, which are suspected to comprise cell populations with stem cell and / or precursor function (Figure 4.2). Backtracing in combination with immunohistochemistry against a sustentacular cell marker cytokeratin II and nuclear staining would potentially label all mature OSNs and sustentacular cells. With this strategy we hoped to negatively highlight neuronal stem cells and precursors that neither send axons out of the OE, and thus cannot be back-traced, nor stained with cytokeratin II, which represents the glia cell population in the OE. However, due to very short length of the zebrafish olfactory nerve bundle, it was not possible to transect each nerve endings and fill them with biocytin. Thus, we were unable to visualize the entire OSN population and the label-exclusion method did not sufficiently highlight basal cells.

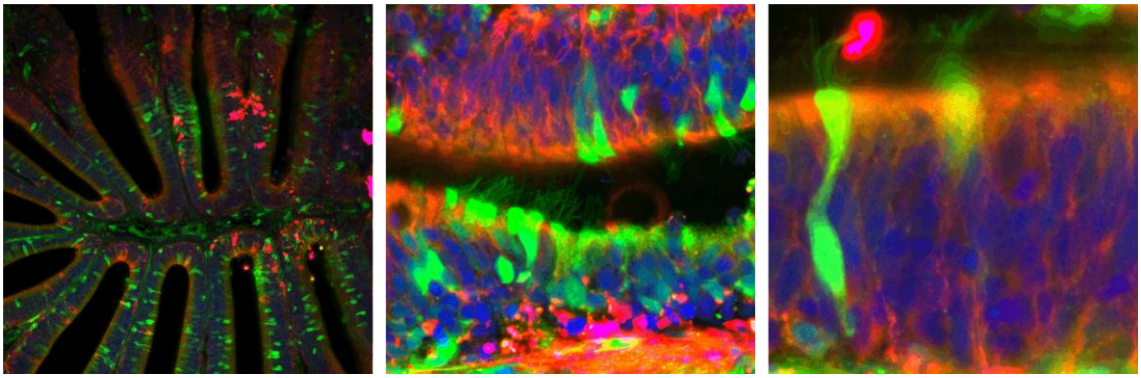

Figure 4. 2. Characterization of cell types in OE via biocytin backtracing. Non-neuronal and non-glial cells were excluded by the backtracing of neurons (green), and staining of sustentacular cells (red). Nuclei were counterstained with PI (blue). OSNs can be visualized in whole olfactory tissue (left), along the lamella (center), and individual OSNs with their cilia structures can be observed (right). Backtracing was not efficient enough to exclude all neurons.

4.2. Ongoing neurogenesis in the adult zebrafish OE

Unlike other nerve cells, OSNs are in direct contact with the surrounding environment and therefore exposed to continuous stress of toxic agents, which makes them vulnerable to damage or injury. For this reason, there is a need for continuous replacement of shortlived OSNs to maintain a sense of smell (Tsai and Barnea, 2014; Mackay-Sim and Kittel, 1991). It has been reported that regions of proliferative activity in the zebrafish OE are localized to distinct spatial subregions (Iqbal and Byrd-Jacobs, 2010). Thus, as a first attempt I wanted to visualize proliferating cells, which may contain newly-born OSNs in the zebrafish OE. In order to do so, adult zebrafish (>1 year of age) were incubated for 24h with the proliferation marker BrdU. BrdU is a synthetic thymidine analogue that is incorporated into newly synthesized DNA in place of thymidine in replicating cells and can be detected by anti-BrdU immunohistochemistry (Figure 4.3). Thus, it allows to visualize neuronal and non-neuronal cells that are born during the 24h BrdU incubation period.

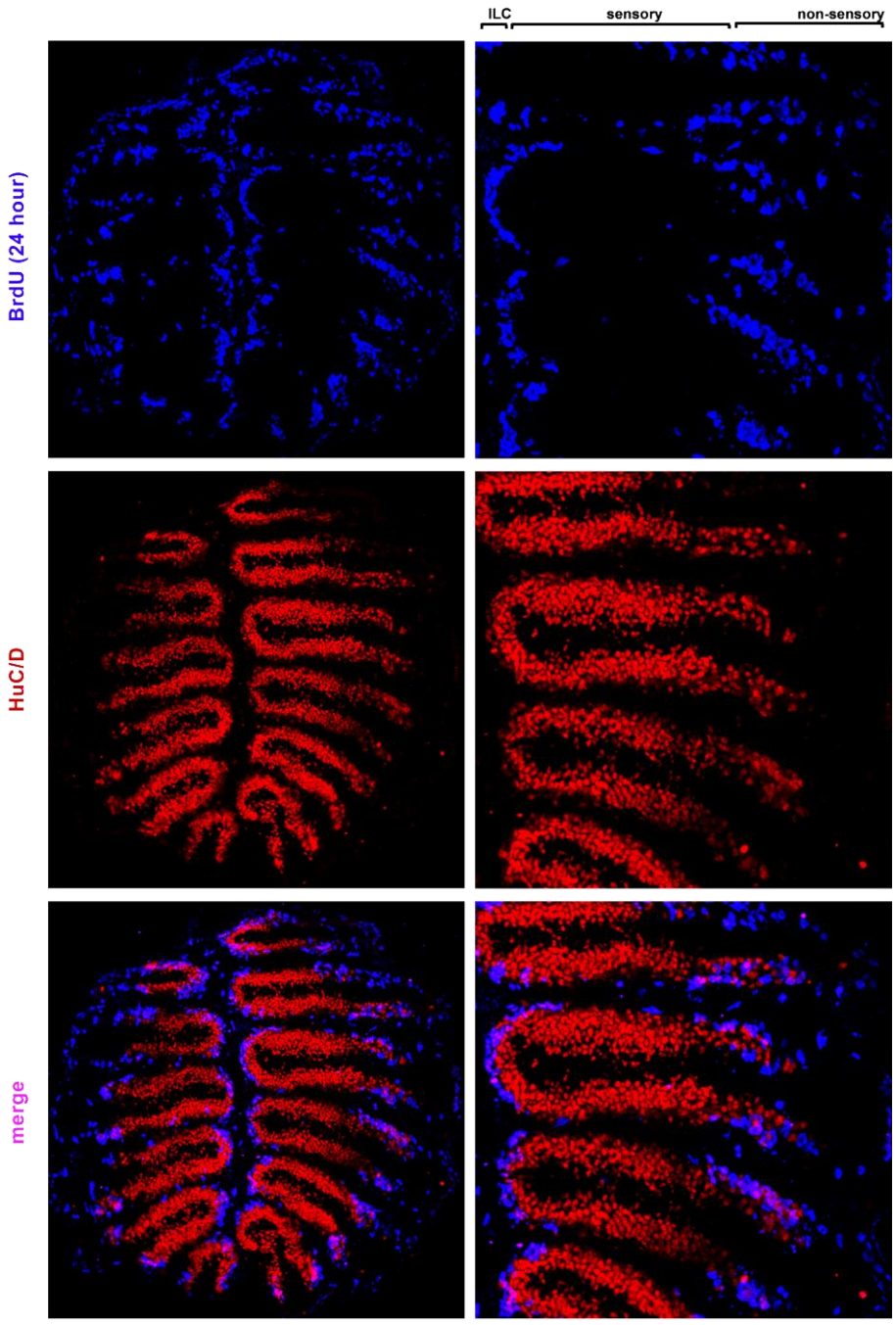

Figure 4.3. Ongoing neurogenesis in the adult zebrafish OE. Cross section through the adult zebrafish OE stained via IHC for the proliferation marker BrdU (blue) and the mature neuronal marker HuC/D (red). The HuC/D staining depicts the sensory region of the OE. BrdU-positive cells divided in 24 h time course are concentrated at the ILC, the S/NS boundary and at the surrounding epithelial tissue.

As expected, BrdU-positive cells are not randomly distributed throughout the zebrafish OE but instead were concentrated at distinct regions each end of the sensory region of the olfactory lamellae. High densities of BrdU-positive cells could be found at the interlamellar curves (ILCs) close to the midline raphe and close to the boundary between the sensory and the non-sensory region (S/NS) of each lamella. In addition, scattered BrdU-positive cells could be detected in the surrounding connective tissue. Importantly, BrdU-positive cells within the sensory region of the OE are scarce as could be demonstrated by immunoreactivity against the neuronal marker HuC/D. Occasional BrdU-/HuC/D double-positive cells could be observed in the ILCs and at the S/NS boundary. This finding suggests that some of the newly generated cells that were born during the 24h BrdU incorporation period completed neuronal differentiation and started to express the mature neuronal marker HuC/D. Related studies have shown that the two proliferation zones at each end of the lamella are indeed neurogenic and that mature OSNs invade the sensory region of the OE by active migration (Bayramli, unpublished).

Assuming that the OE of freely moving fish is not subject to any injury, these proliferation zones represent the pattern of ongoing neurogenesis that maintains the olfactory tissue throughout the life of the animal. Next, I wanted to establish an injury model based on mechanical or chemical damage of OE tissue that is robust and systematically reproducible.

4.3. Tissue response to focal stab lesion to the OE

As a first step to establish a reproducible assay for inducing an injury response in OE tissue, mechanical damage by stabbing OE tissue with fine glass needles was tried out. First, fish were anaesthetized in MS-222 and placed between wet sponges. With the aid of a micropmanipulator, one of the around 16 olfactory lamellae that is easily accessible through the naris was punctured with a dye-coated glass micropipette. Following manipulation, the fish were immediately placed into tank water containing 30 mg/l BrdU for a period of 24h before analysis by IHC against BrdU (Figure 4.4).

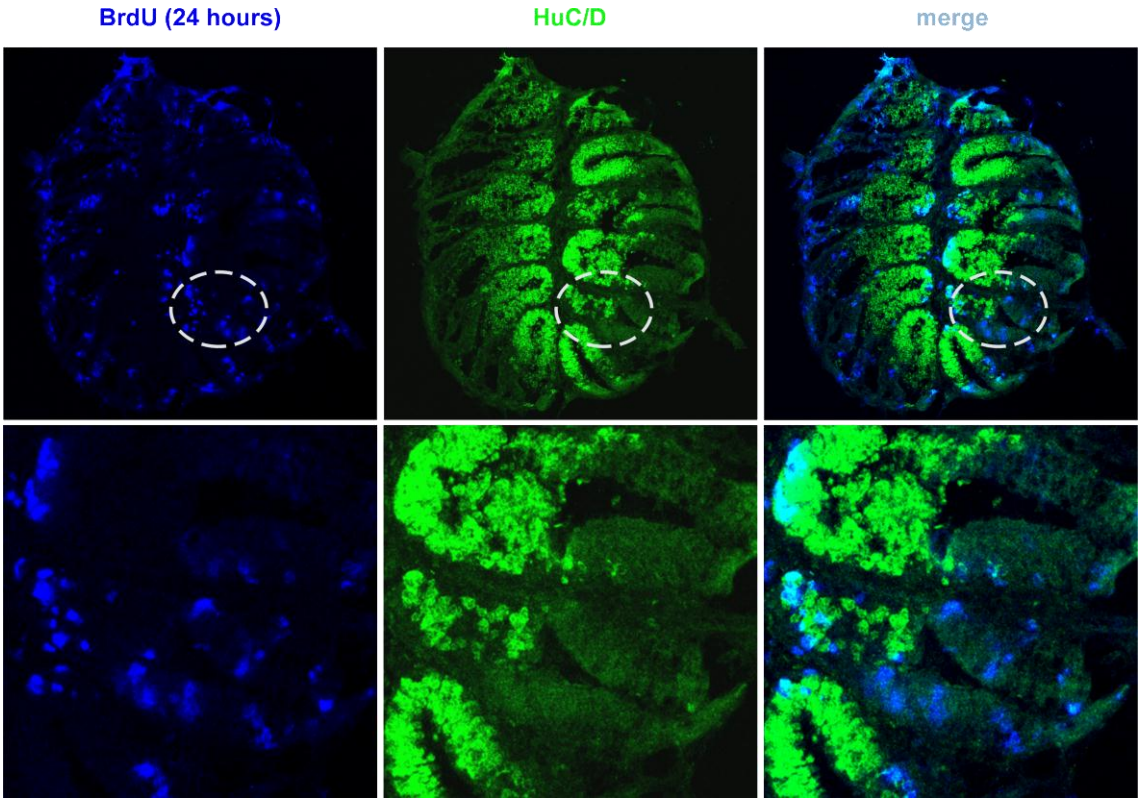

Figure 4.4. Tissue response to focal stab lesion to the OE. Top: entire OE section, bottom: detailed view of four lamellae.. Pattern of the BrdU-positive cells (in blue) proliferated within 24 h is similar with exception of the injured site (dotted circle), an increased number of BrdU-positive cells around the injury site inside the sensory territory of the OE can be observed while the HuC/D staining (in green) is perturbed at the same region.

Clear differences in the pattern of BrdU-positive cells could be observed in punctured OEs. Around the site of injury, a local induction of BrdU-positive cells in the center of the sensory region of the lamella could be detected. This indicates that elevated proliferative activity can be induced by focal tissue damage, whereas other parts of the OE showed the regular restricted pattern of proliferation described above. With the exception of the site of injury, BrdU-positive cells were concentrated around the neurogenic zones at the ILCs and the S/NS boundary. The unusual positions of BrdU-positive cells at the injured site inside the sensory region of the OE indicate that focal damage to the tissue induces cell divisions locally and in cells that are different from progenitors that contribute to maintenance of the OE tissue. Examination of the pattern of HuC/D-positive cells revealed that the density of mature OSNs decreased around the injured site, which

confirms that the tissue was successfully damaged by the manipulation. Although focal stab lesions successfully damaged the tissue and induced a proliferation response, the manipulation with fine glass needles was difficult to reproduce accurately. For this reason, tissue wide chemical degeneration was employed instead of the focal lesions by mechanical force.

4.4. Tissue response to irrigation with chemicals

Different procedures to damage OE tissue in various fish species have been previously described (Burd, 1993; Beites *et al.*, 2005). The most established ones employed irrigation of the OE tissue with $ZnSO_4$ (Cancalon, 1982) or Triton X-100 (Iqbal and Byrd-Jacobs, 2010). Both approaches were tested and compared to each other in terms of tissue integrity and efficacy.

In order to induce tissue wide ablation of OSNs, first $ZnSO_4$ irrigation was performed on one of the paired OEs and the fish were immediately incubated in BrdU water for 24h to assess induction of cell proliferation. A 90 irrigation of the OE with $ZnSO_4$ resulted in a high number of BrdU-positive cells across the entire OE, including the sensory region of the tissue (Figure 4.5). The distribution of BrdU-positive cells was clearly different from the pattern of BrdU-positive cells in untreated control tissue of the same fish. BrdU-positive cells were predominantly located around the basal lamina and dispersed along the entire length of the lamellae, including the sensory region of the OE.

A similar increase in the number and shift in position of BrdU-positive cells was also observed after irrigation with 1% Triton X-100 for 90 sec. Unlike the pattern observed in the unperturbed tissue, BrdU-positive cells were dispersed throughout the entire OE, including the sensory region in the damaged OE. However, the response to Triton X-100 was not uniform and some regions of the OE did not respond with an increase of proliferation following treatment (Figure 4.6), most likely because the solution did not uniformly reach all regions of the tissue.

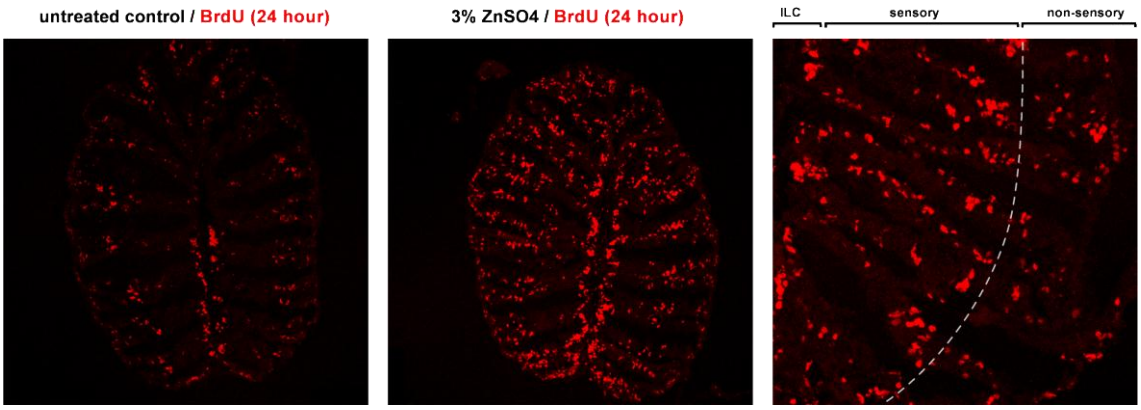

Figure 4.5. Tissue response to irrigation with 3% ZnSO4. Left: untreated control tissue; the proliferation pattern with BrdU-positive cell concentrated at the ILCs and the S/NS border can be observed. Middle: ZnSO4-treated OE. Right: detailed view of four individual lamellae. Induction of the cell proliferation can be observed along the lamellae. The grey dotted line shows the position of the S/NS boundary.

To further examine this possibility, Triton X-100-irrigated OEs were counterstained with the HuC/D marker to gain insight into the extent of tissue damage that was created by the treatment. IHC against the neuronal marker HuC/D revealed that large areas of the OE were devoid of HuC/D staining, although patches of HuC/D-positive cells remained, mostly in the anterior part of the OE, the ILCs and in more ventral regions of the OE. Many times individual OE could be observed in which a damaged lamella, indicated by the absence of HuC/D staining, was situated next to an intact one. The pattern of BrdU-positive cells was complementary to the HuC/D pattern and cell proliferation close to the basal lamina was most obvious in lamellae that were devoid of HuC/D-positive cells. Thus, the Triton X-100 solution may not evenly penetrate all regions of the OE. Interestingly, there was a strong correlation between the absence of HuC/D staining and the presence of BrdU-positive cells in the sensory region of the OE (Figure 4.7). This strongly suggests that the response to tissue injury is local and can be induced across the entire OE. The strength of the proliferation response then depends on the extend of local damage that has been induced in any particular region of the OE.

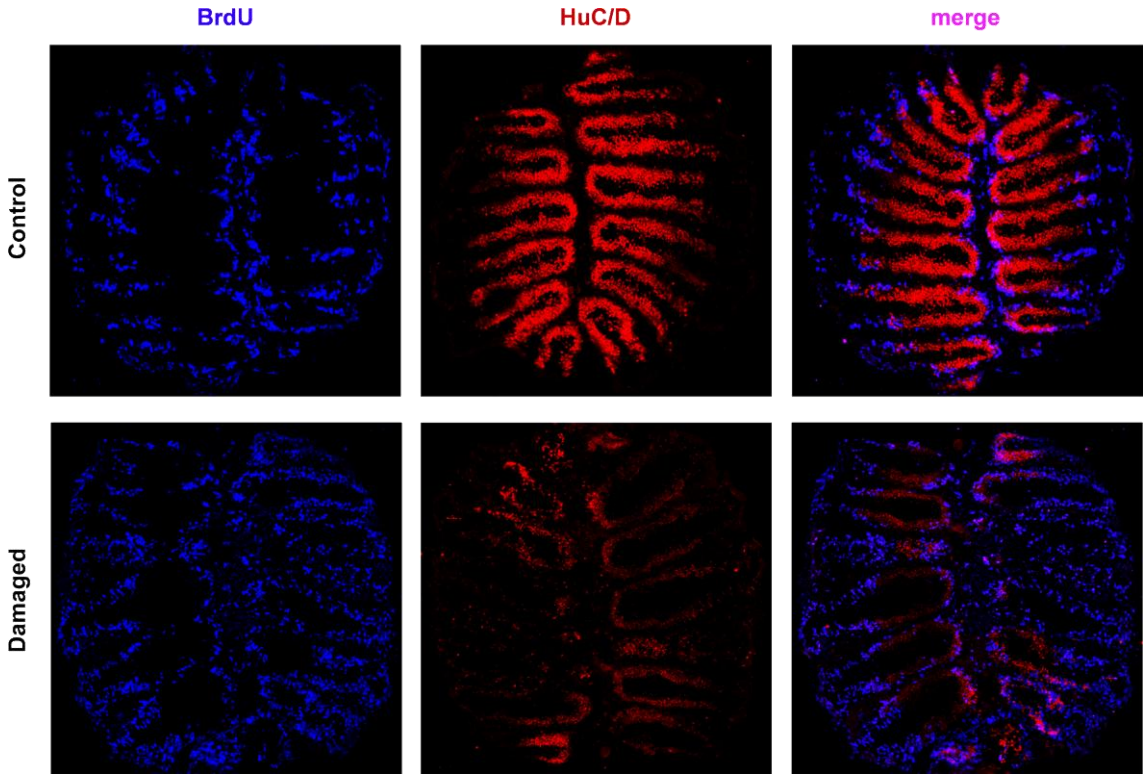

Figure 4.6. Tissue response to irrigation with 1% Triton X-100. The sections were stained by IHC against BrdU (left, blue), the mature neuronal marker HuC/D (middle, red). Untreated control tissue is shown on the top, Triton X-100-irrigated tissue on the bottom. Note strong correlation between the absence of HuC/D staining and presence of BrdU-positive cells in the sensory regions of the OE.

To quantify the change in the distribution of BrdU-positive cells in untreated control tissue and Triton X-100-treated OEs, the position of BrdU-positive cells was analyzed and plotted against the normalized length of the lamellae (Figure 4.8b). In untreated control tissue a bimodal profile can be observed with most BrdU-positive cells being located at the ILC and close to the S/NS boundary. BrdU-positive cells inside the sensory region, however, were sparse. Yet, a clear increase in the frequency of BrdU-positive cells inside the sensory region and a flattening of the profile at the ILC and S/NS boundary could be observed in Triton X-100-treated tissue. This suggests that additional proliferating cells are recruited by the chemical damage. Thus, treatment with 1% Triton X-100 for 90 sec induces a robust and reproducible damage in most regions of the OE and triggers an

accompanying proliferation response in the regions that are different from the proliferation zones that contribute to the maintenance of the tissue.

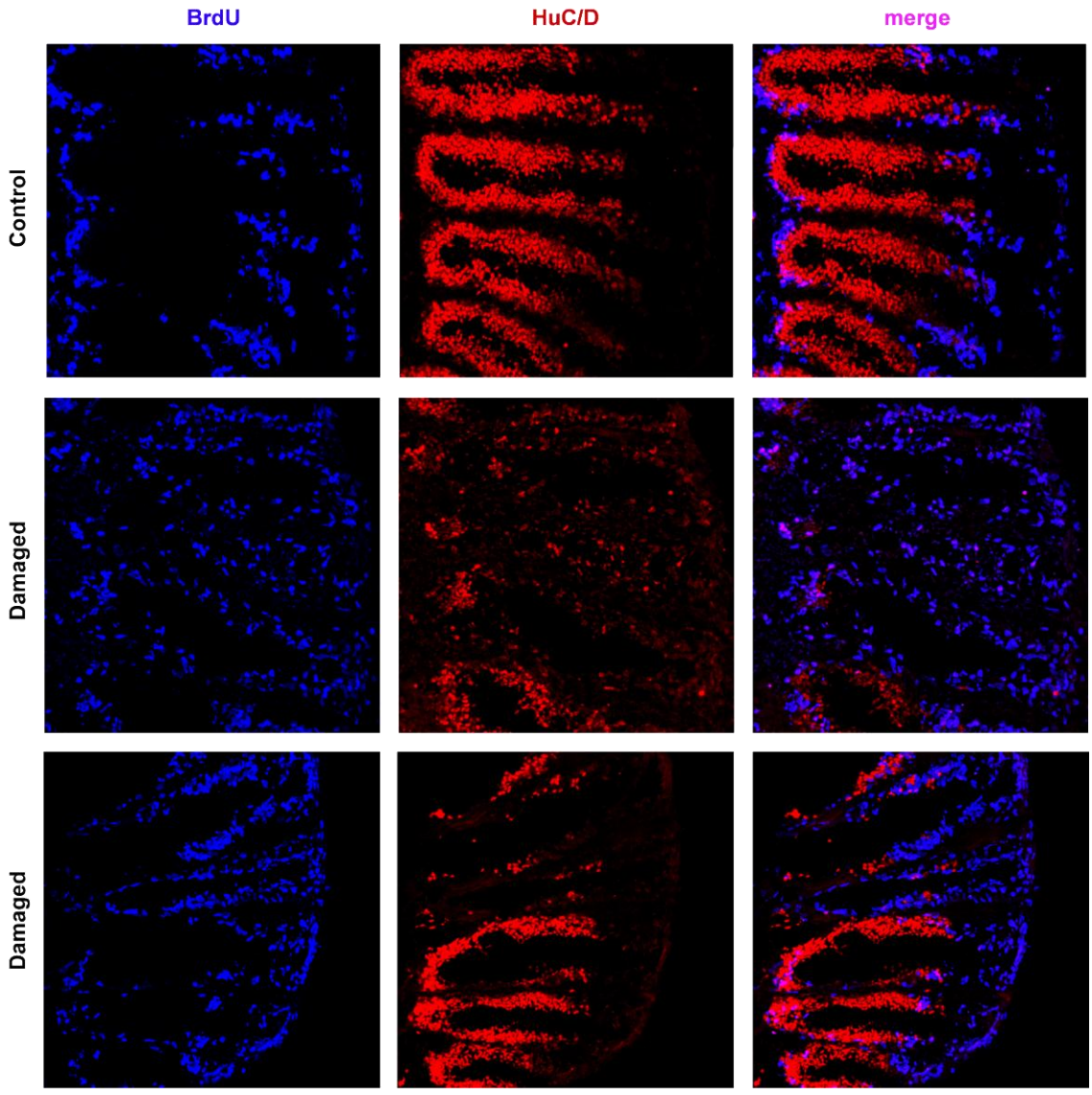

Figure 4.7. Tissue response to irrigation with 1% Triton X-100. Detailed view of four individual lamellae. The sections were stained by IHC against BrdU (left, blue), the mature neuronal marker HuC/D (middle, red). Untreated control tissue is shown on the top, Triton X-100-irrigated tissue on the bottom. Note strong correlation between the absence of HuC/D staining and presence of BrdU-positive cells in the sensory regions of the OE.

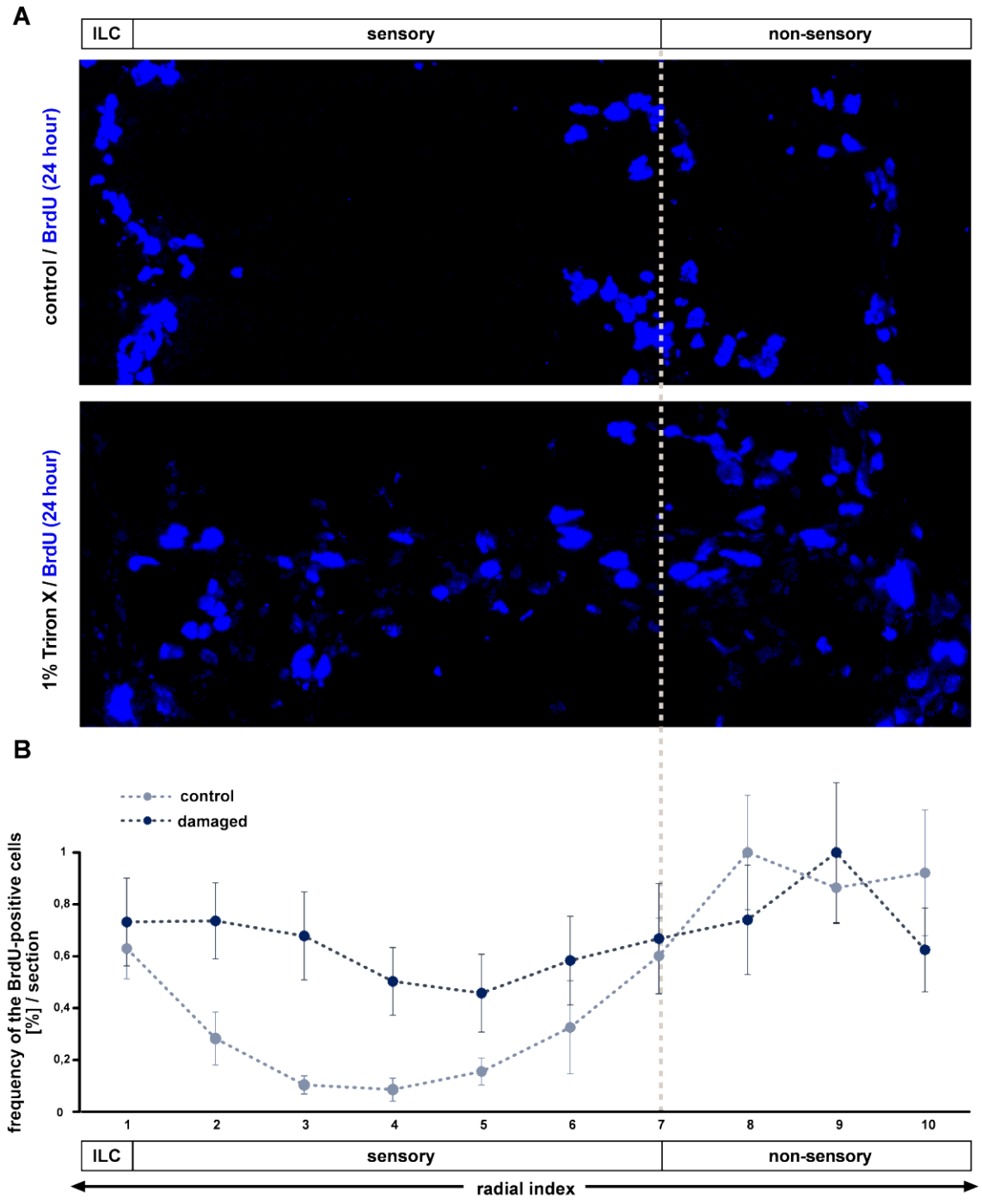

Figure 4.8. The distribution of proliferating BrdU-positive cells along the lamella within 24 h. (A) Untreated control tissue is shown on the top, Triton X-100-irrigated tissue on the middle. The bottom graph shows the distribution of BrdU-positive cells along the lamella in untreated control tissue (light blue) and Triton X-100-treated tissue (dark blue). An increase in the number of BrdU-positive cells inside the sensory region of the OE can be seen in response to injury.

4.5. Time course analysis of the tissue response to Triton X-100 treatment

Although BrdU is a useful proliferation marker, labeled cell represent a cumulative pattern, which is proportional to the incubation period. In order to better understand the time course of the response to chemical damage, the alternative proliferation marker phospho-histone 3 (PH3) that labels only cell that are in the S-phase of the cell cycle was used. Thus, IHC against PH3 allows for the visualization of acute, ongoing cell divisions at the time of analysis. To do so, one of the paired zebrafish OEs from the same animal was treated with Triton X-100 while the other one was not treated and analyzed as unperturbed control, then the OEs were dissected and stained for PH3 immunoreactivity at 4, 12, and 24h after treatment. In order to understand the efficacy of the damage induced in the tissue, alternating sections were stained against proliferation marker PH3 and the mature neuronal marker HuC/D, because cross-reactivity of the secondary antibodies precluded double marker analysis on the same section (Figure 4.9).

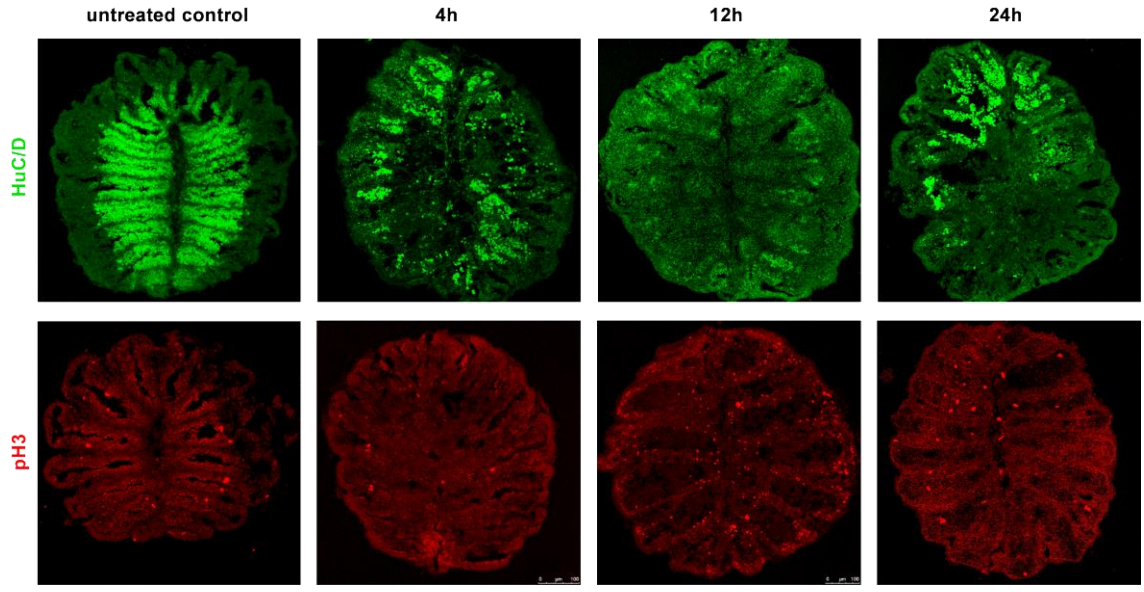

Figure 4.9. Time course analysis of the tissue following Triton X-100 treatment. 4 h (center), 12 h (center), and 24 h (right) following treatment, IHCs against the proliferation marker PH3 (red, bottom) and the mature neuronal marker HuC/D (green, top) demonstrated that degeneration of chemosensory neurons and increased proliferation ocuurs starting from 10h following treatment..

The quantification of PH3-positive cells demonstrated differences in the number and positions of mitotically-active cells at different time points following treatment. Similar to BrdU incubation, cells staining positive for phospho-histone H3 in the untreated control tissue are predominantly located at the ILC and the S/NS boundary. However, the average number of PH3-positive cells is much lower and only around 7.36 ± 1.18 cells could be identified per section (n = 2 sections from 3 fish; Figure 4.10b).

At 4h after treatment, the HuC/D staining pattern in Triton X-100-treated OEs was disrupted. The average area of the sensory region in the treated OE was measured to be 74.24 ± 11.93% of the area in control OEs (Figure 4.10c). Patchy HuC/D staining along the sensory region of the OE could be detected, indicating that 4 hours after Triton X-100 treatment some but not all mature OSNs were lost (Figure 4.9). The number of PH3-positive cells in Triton X-100-treated tissue is similar to the untreated control OE. Similar to the control, mitotically active cells in the experimental sample at 4h after treatment were located predominantly around the ILC and S/NS boundary (in the 6th segments along the radial index shown in Figure 4.10a) but with slight shift towards more central positions. The average number of PH3-positive cells per section doubled from the initial 7.36 ± 1.18 cells to 14.57 ± 2.86 cells after 4h and this increase was statistically significant (Student's two-tailed t-test; p(0/4h) = 0.001).

Yet, the pattern of PH3-positive cells changed dramatically at later time points following Triton X-100-induced damage. Starting from 12h after Triton X-100 treatment, a dramatic loss of mature OSNs could be detected in the treated OE as judged by HuC/D staining. The average area of the sensory region in the treated OE relative to the control OE per section decreased to 29.55 ± 0.06%. Interestingly, at the same time point a massive increase in the number of PH3-positive cells could be observed. The average number of cells per section increased 3-fold from the initial 7.36 ± 1.18 cells per section to 28.11 ± 6.52 cells after 12h ($p_{(0/12h)}$ = 0,000024). The actively dividing cells are scattered along the entire lamellae, including the sensory region of the tissue. However, it should be noted that the cells stained by PH3 immunoreactivity within the sensory region appear smaller than the cells observed in the control OE (Figure 4.9). This finding might reflect the fact that a

subpopulation of cells different from maintenance precursors responded to the chemical damage at 12h after treatment.

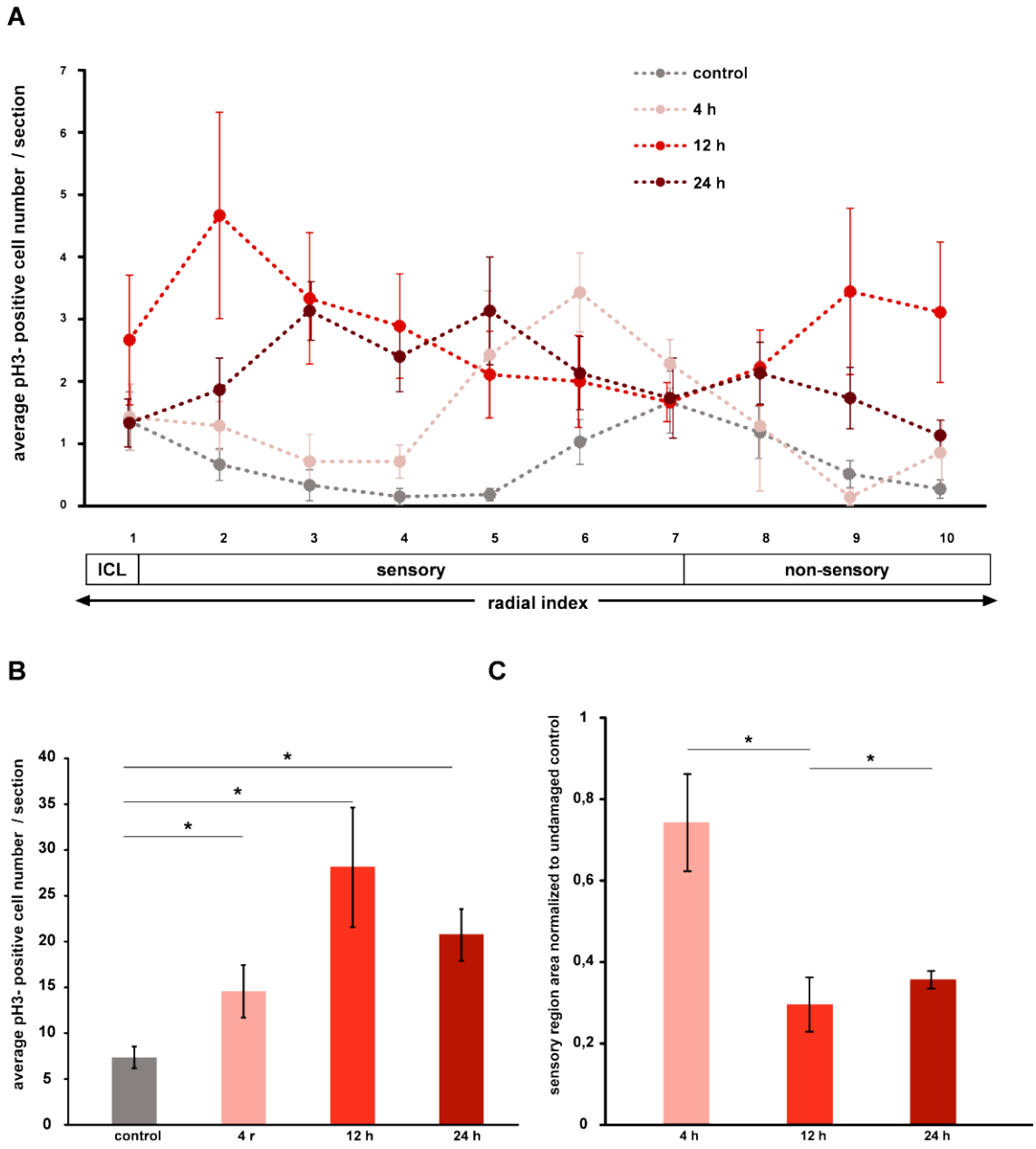

Figure 4.10. Time course analysis of the neuronal response to Triton X-100 damage. (A) Position and average number of mitotic cells were graphed along the length of the lamella. (B) Average number of the pH3-positive cells / section (asterisk: Student's t-test p_{ctrl-4}=0.001; $p_{ctrl-12}$= 0,000025; $p_{ctrl-24}$= 0.00029) (C) Area of the sensory region normalized to control section calculated by the area of HuC/D positive (p_{4-12}=0.019; p_{12-24}= 0.035).

At the later 24h time point patchy HuC/D staining around the anterior extension zone of OE could be observed (Figure 4.9) and the average area of the sensory region in the treated OE normalized to the control OE per section slightly increased from 29.55 ± 0.06% to 35.64% ± 0.02% and this increase was statistically significant ($p_{12\text{-}24h}$ 0.035; Figure 4.10c) while the number of mitotically active cells declined from 28.11 ± 6.52 cells to 20.73 ± 2.82 cells when compared to the 12h time point ($p_{0\text{-}12h}$ = 0,00025, Figure 4.10b). The patchiness in the sensory region can be explained in two ways; either the response of the tissue against injury might be immediate or it could be an indication of incomplete damage due to inefficient drug delivery across the OE and reduced accessibility of deeper tissue parts. The positions of PH3-positive cells in the treated sample at 24h following treatment are dispersed along the entire lamellae, including the sensory region of the tissue, similar to the one at 12h time point (Figure 4.10a).

Parallel with the observations made for the BrdU incorporation assay, the position of proliferating cells shifted from the general neurogenic zones, the ILC and the S/NS boundary, to more central positions in the sensory region at both 12h and 24h following treatment (Figure 4.10a). This finding strongly suggests, yet does not ultimately prove, that a second, dormant stem cell population becomes activated upon acute injury of the tissue. On the other hand, an alternative but less likely possibility is that stem cells or transit amplifying progenitors located at neurogenic zones are shifted towards more central positions due to the chemical-induced disruption of the tissue.

4.6. Double marker incorporation assay

A shift in position of proliferating cells was observed upon tissue wide chemical damage in two independent assays. As outlined above, two alternative mechanisms could account for this observation: either a dormant progenitor / stem cell population becomes activated or the progenitor / stem cells normally located at the ILC and S/NS boundary shift position due to the disruption of the tissue. To discriminate between these two possibilities, adult zebrafish were subsequently incubated with two different proliferation

markers before and after Triton X-100-induced injury. First, fish were incubated with the thymidine analogue IdU for 24 h, followed by a 24 h label-free incubation in regular tank water. We reasoned that the label-free incubation in tank water would sufficiently clear any remaining IdU before the chemical treatment to avoid double labeling of cells induced by the injury. Then, one of the paired OEs was treated with 1% Triton X-100 for 90 sec while the other OE served as the unperturbed control. Following the chemical treatment, fish were immediately incubated with the second thymidine analogue CldU for 24h.

Similar to the observations with BrdU alone, both IdU- and CldU-positive cells were located at the ILCs and at the S/NS boundary in the untreated control tissue (Figure 4.11). However, IdU-positive cells occupied positions that were slightly closer to the central sensory region within the OE. This result is expected since IdU-positive cells were born at least 24 h earlier than CldU-positive cells and newborn OSNs migrate away from the proliferation zone as they mature (Bayramli, unpublished).

However, the staining pattern was drastically different in Triton X-100-treated tissue. The older IdU-labeled cells that went through cell divisions before the chemical damage showed a similar distribution as in the untreated control tissue. IdU-positive cells were predominantly located at the ILCs and S/NS boundary. However, occasional empty spaces could be observed at these positions, probably due to chemical-induced ablation of these cells. More importantly, however, no increase of IdU-positive cells within the sensory region relative to the untreated control tissue could be found. Thus, cells that underwent cell division before chemical injury appear not to respond to the injury and do not show displacement from the ILC and S/NS boundary towards the center of the sensory region.

Interestingly, CldU-positive cells that underwent cell divisions after the chemical damage were scattered along the entire OE including the central sensory region and were not localized to the ILCs and S/NS boundary (Figure 4.12). These observations are in line with a model that favors independent progenitor / stem cell populations for different modes of neurogenesis, such as maintenance and repair conditions. Thus, the scattered pattern of

cell proliferation observed upon chemical damage is not a consequence of displacement of stem cells or transit amplifying progenitors located at the central and peripheral proliferation zones but arise from a second, yet uncharacterized population of dividing cells. Cells located at the ILC and S/NS boundary may, nevertheless, contribute to the damage response, since double positive cells residing in these positions suggest that a few early-born IdU-positive cells also divided after the chemical injury. An open question, however, remains if any of the proliferating cells that were induced by chemical damage have the potential to become neurons.

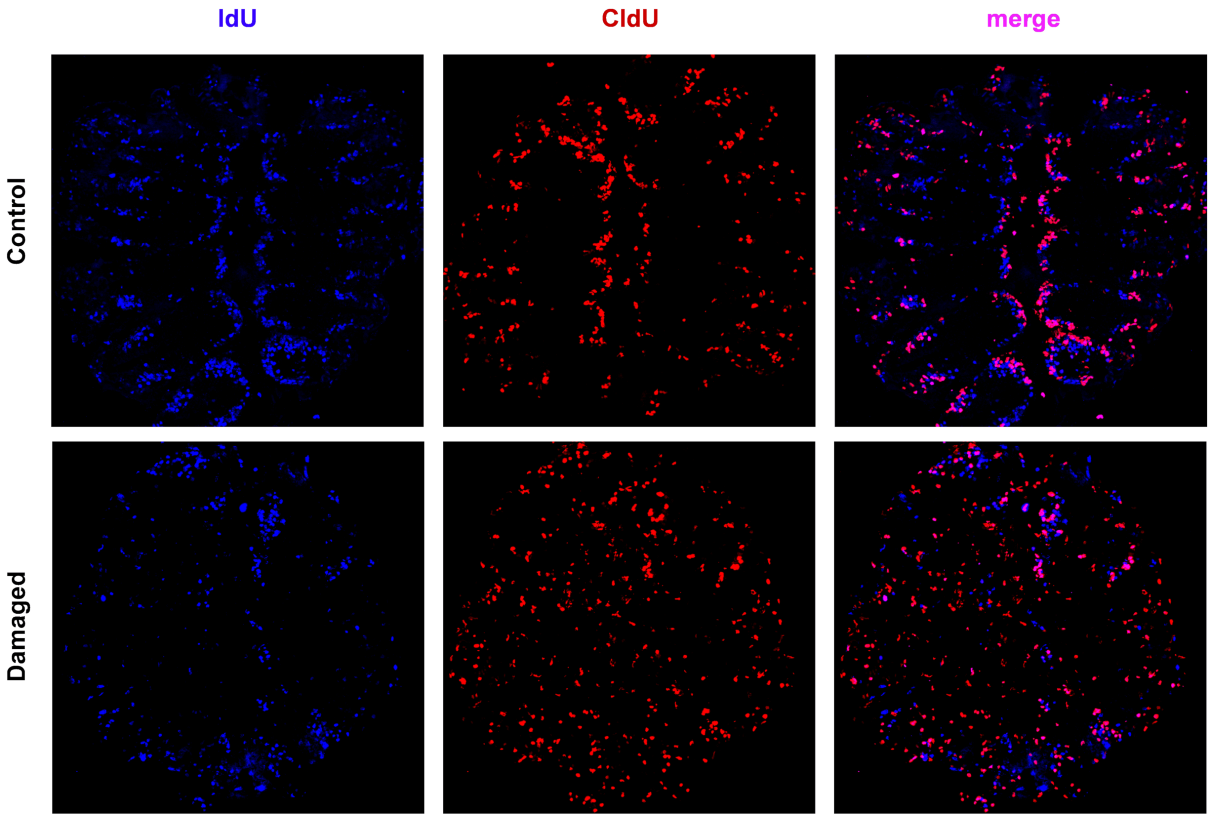

Figure 4.11. Double marker incorporation assay. Fish were incubated in the water with IdU (blue) for 24h, in regular water for 24h and with CldU (red) for the following 24h. Top row: untreated; bottom row: Triton X- 100-treated tissue. In both group, earlier born the IdU-positive cells are found around the proliferation zones whereas the pattern of CldU-positive cells in the Triton X-100-treated tissue scattered along the inner sensory region in damaged tissue.

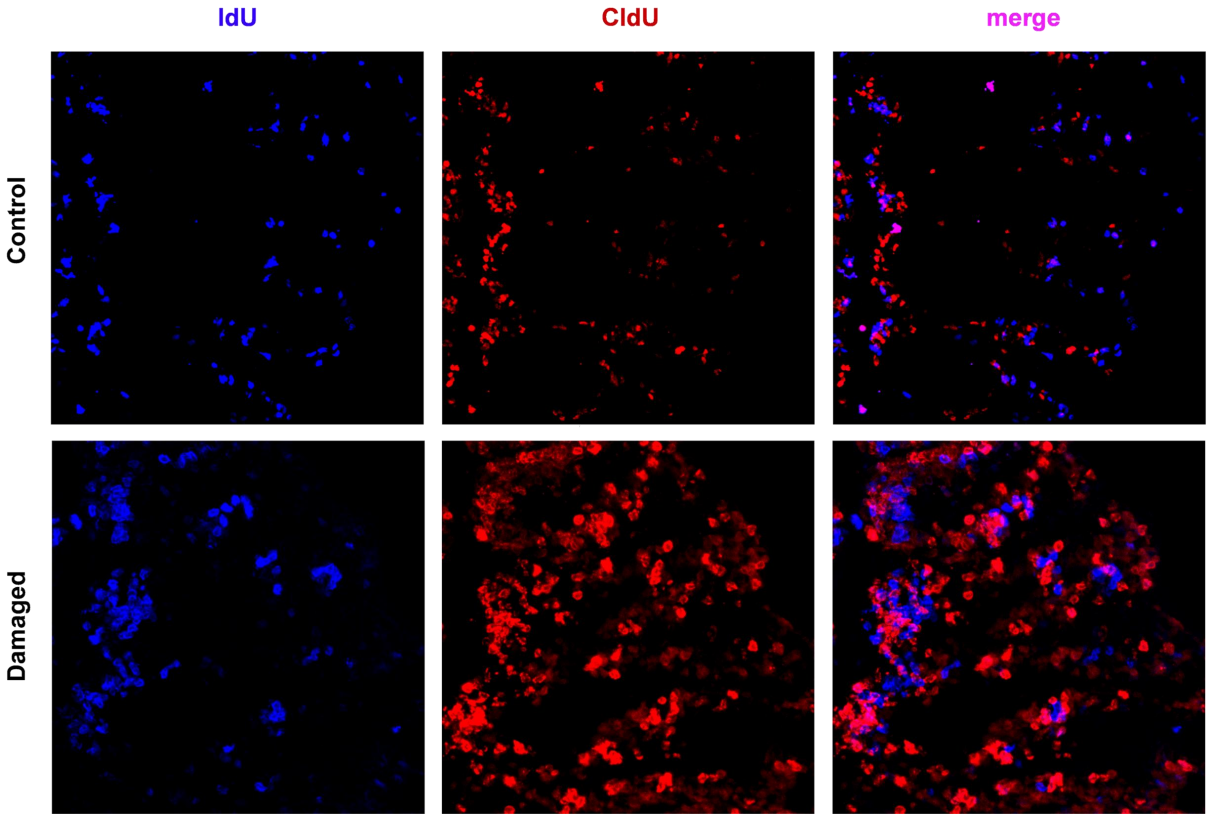

Figure 4.12. Double marker incorporation assay. Detailed view of four individual lamellae. Top row: untreated control tissue; bottom row: Triton X- 100-treated tissue. In both control and damaged tissue, the earlier born IdU-positive cells occupy positions around the proliferation zones whereas the pattern of CldU-positive cells in the Triton X-100-treated tissue scattered along the inner sensory region.

4.7. Expression pattern of the early neuronal differentiation factor Ascl-1

Ascl1 (achaete-scute homolog 1) is a pro-neural transcription factor that plays a critical role during neuronal commitment and differentiation (Krolewski *et al*., 2012). It is one of the earliest neuronal markers and has been shown to be expressed sparsely by cells at the ILC and S/NS boundary (Bayramli, unpublished). Thus, Ascl1 expression may indicate if proliferating cells within the sensory territory of the OE induced by chemical lesion have neurogenic potential. Data from transcriptome profiling of damaged tissue over time shows that Ascl-1 expression is 2-fold upregulated at the RNA level 24h after chemical injury. Thus, it was examined whether the pattern of Ascl1 expression changes

upon Triton X-100 treatment. To visualize Ascl1 expression, IHC using an anti-Ascl1 antibody was employed on treated and untreated control tissue.

Similar to the observation with BrdU incorporation and PH3 immunohistochemistry, Ascl-1 positive cells resides in the ILCs and close to S/NS boundary on the unperturbed control OE (Figure 4.13). This observation is consistent with the ongoing mode of maintenance neurogenesis at these epithelial positions. Surprisingly, however, in OEs analyzed after the 1% Triton X-100 treatment no Ascl1-positive cells could be found. Although a 2-fold increase was expected based on the transcriptome data induction of expression on the RNA level might not faithfully reflect protein expression. It is likely, however, that an increase in Ascl1-positive cells could be observed at later time points.

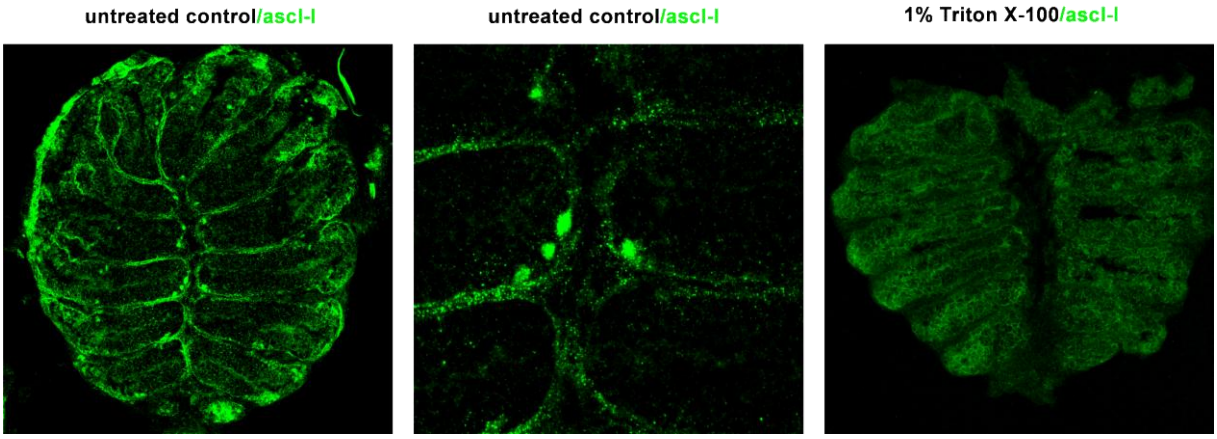

Figure 4.13. Expression pattern of early neuronal differentiation factor Ascl-1. Left: untreated control tissue; middle: detailed view of ILC and right: Triton X- 100-treated tissue. The Ascl-1-positive cells (in green) occupy positions around the proliferation zones at the ILC and the sensory/non-sensory boundary whereas Ascl-1-positive cell in the Triton X-100-treated tissue was detected.

4.8. Transcriptome analysis of zebrafish OE

In order to further understand the molecular components that control adult neurogenesis and to identify genes that may play a role in nervous tissue regeneration, transcriptome analysis of the OE was performed over the time course of de- and

regeneration at 0, 4, 12, 24, 72 and 120 h following Triton X-100 treatment. The up- and downregulation of transcripts in the regenerating OE may allow for the identification of important genes or signaling pathways.

First we investigated the expression levels of the cell type specific genes as confirmation of the OSN death in response to chemical treatment. As expected, guanine nucleotide proteins gng13b, gnao1b, gnb1a, gna1 and cnga4, ompb genes, which are specifically expressed by the ciliated cells (Figure 4.14a) significantly downregulated. Also the expression level of microvillous cell specific trpc2b and PLCB2 genes were downregulated (Figure 4.14b). Expression levels of these cell type specific genes reached their lowest level at either 12h or 24h time points, then they followed an increasing fashion at 72h and 120h. Both data verify the loss of sensory neurons following injury.

The zebrafish olfactory epithelium contains different types of neuronal cells (Figure 4.1d). ORs, TAARs and OMP are expressed by ciliated OSNs (Weth *et al*, 1996; Sato *et al*., 2005) whereas different VNRs, Trpc2 and PLCB2 are found in microvillous cells (Sato *et al*., 2005). In this regard, the transcriptome data analysis indicated that a downregulation followed by a late upregulation in the expression of several OR genes (Figure 4.15a). A significant downregulation of chemoreceptor families of TAARs was also detected. (Figure 4.15b). Additionally, VNRs that were expressed by microvillous and crypt neurons were also prominently downregulated (Figure 4.15c). These results might be interpreted as OR genes are upregulated starting from 5 days after chemical treatment whereas recovery of TAARs and VNRs might require more time to recover. Downregulation in the receptor genes expressed by the mature neurons is expected due to the loss of the sensory neurons following the tissue insult, which was also observed via IHC against HuC/D. On the other hand, contrary to the TAARs and VNRs, early restoration of the OR genes might be due to the different dynamics of the neurogenesis of different OSN types. Ciliated cells expressing various OR receptors might be regenerated more prompt than the microvillous or crypt neurons.

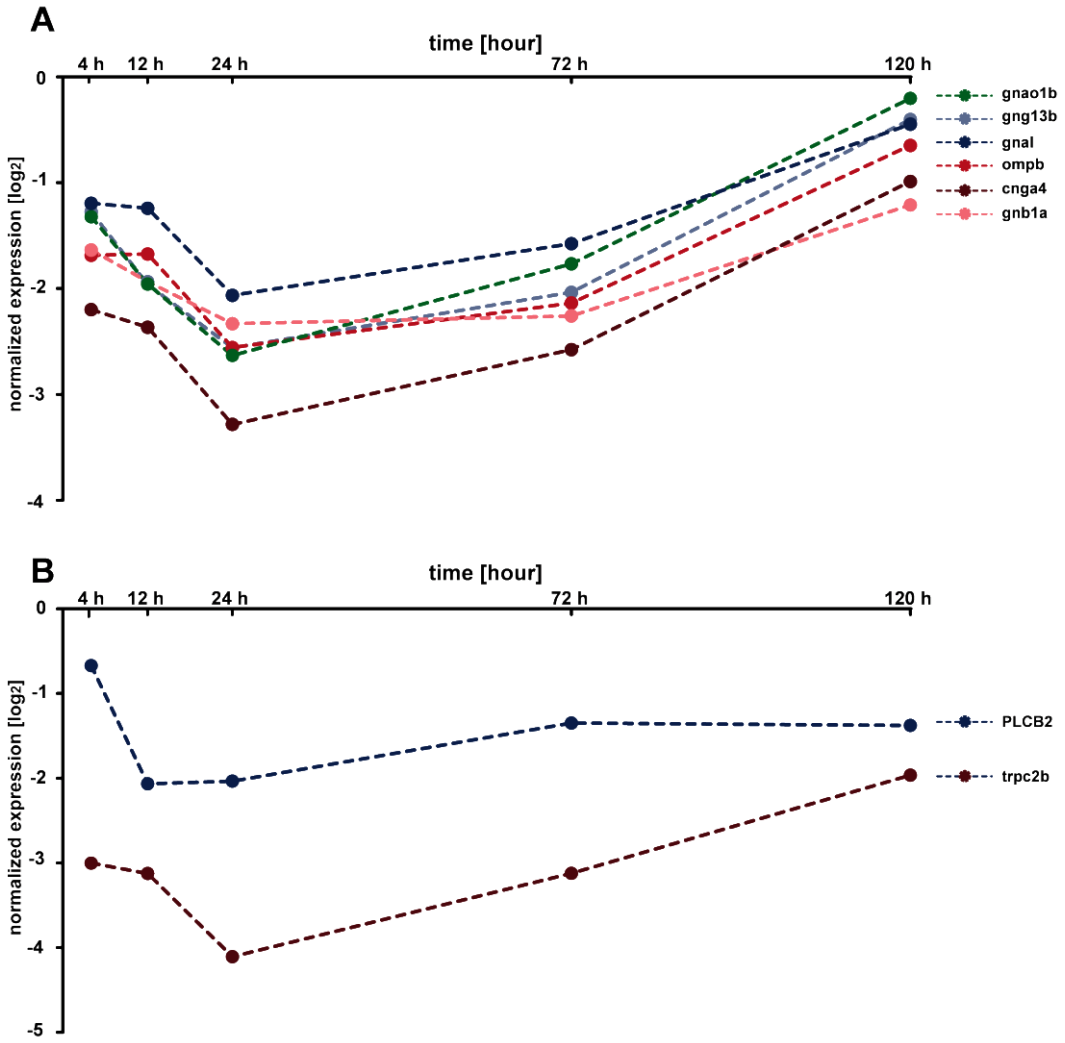

Figure 4.14. Time course expression profiling of cell type specific markers. (A) OSN markers were downregulated at 4, 12, 24, 72 and 120 h after 1% Triton X-100 irrigation of the OE. (B) Expression of the VSN specific genes were downregulated indicating the loss of VSNs following chemical injury (n=50 fish for control group and each time point).

In addition to drastic downregulation of ORs, TAARs and VNRs, transcriptome analysis also revealed that several genes which are playing role in either neural proliferation or differentiation showed dramatic upregulation patterns. An upregulation in the transcriptome of the neuronal markers such as Ascl1, Neurog1, NeuroD1 and tp63 which are well characterized and known to be found in proliferating stem cell and progenitor cells (Figure 4.16). This increase in the expression level is expected and can be interpreted as an indicator of the induced neurogenesis upon injury. Interestingly, the injury

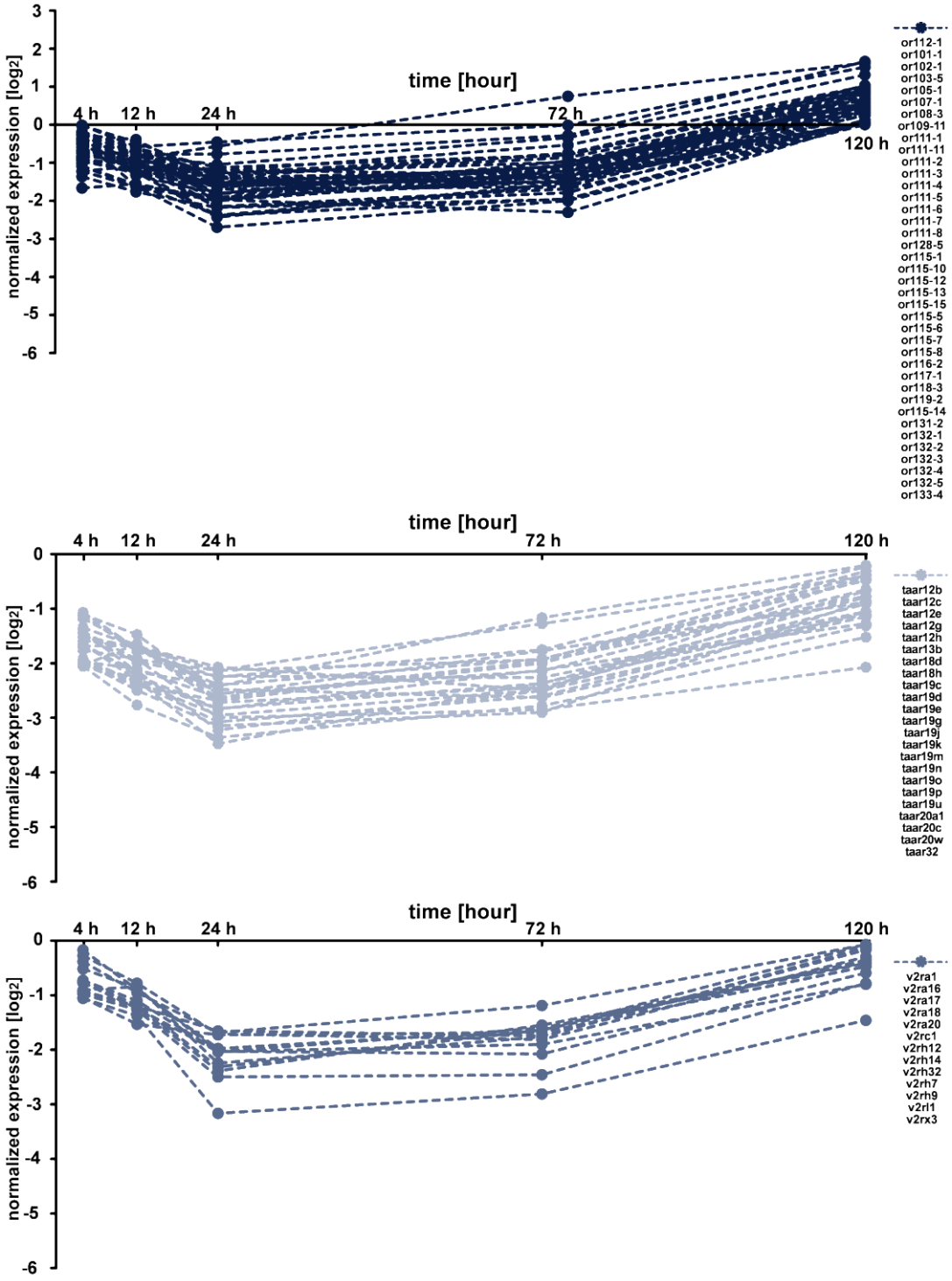

Figure 4.15. Time course expression profiling of ORs (top), TAARs (center), and VNRs (bottom). Odorant receptors shown in the graph were downregulated after 4, 12, 24, 72 and 120 h after 1% Triton X-100 irrigation of the OE. Expression of the OR genes was restored after 5 days from the injury indicating that ciliated OSN population started to be replenished after 5 days following injury (n=50 fish each group).

indicating markers specific for immature OSNs; alcama, alcamb and CHL1 also showed upregulation, which might point out an expansion in the immature OSN pool (Figure 4.17). The expression level of CHL1 is downregulated at later time points, indicating the maturation in a portion of iOSNs.

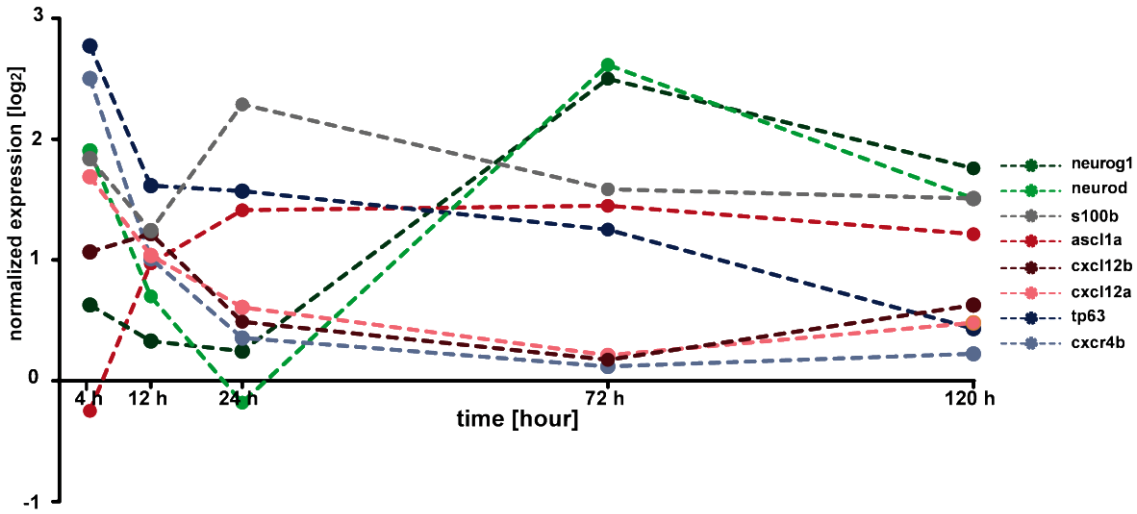

Figure 4.16. Time course expression profiling of neuronal lineage markers. Stem cell and progenitor markers shown in the graph were upregulated after 1% Triton X-100 irrigation of the OE, indicating that the neuronal stem cells and precursors that generate OSNs were activated in response to chemical damage (n=50 fish for control group and each experimental time point).

Previous studies showed that activation of CXC Chemokine Receptor 4 (CXCR4B) that is expressed at sites of neuronal and progenitor cell migration in the hippocampus supports neurogenesis in the adult dentate gyrus (Kolodziej *et al*., 2008, Lu *et al*., 2002). Transcriptome analysis demonstrated an early upregulation of CXCR4b and more interestingly its only known ligand, stromal cell-derived factor 1 (known as SDF-1 or CXCL12) was also continuously upregulated (Figure 4.16). Thus, our data support the previously suggested hypothesis about the role of CXCR4 in adult neurogenesis (Tran *et al*., 2007). In order to further characterize the role of CXCR4 receptor, CXCL12 might be introduced to the zebrafish OE and test for any induction at the following proliferation levels. In addition, it was previously reported that S100b is associated with injury-induced neurogenesis in the adult hippocampus (Kleindienst *et al*., 2005). As seen in the graph,

upregulation of S100b gene expression reached its peak with 4-fold increase at 24h time point (Figure 4.16).

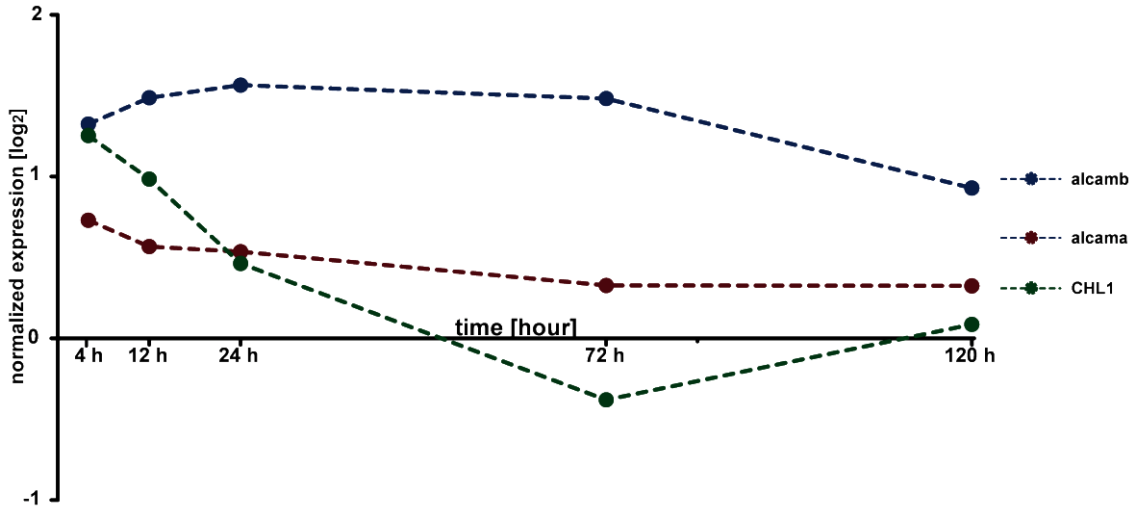

Figure 4. 17. Time course expression profiling of immature OSN markers. These markers shown in the graph were upregulated after 1% Triton X-100 irrigation of the OE, indicating that the iOSN population expanded in response to chemical damage (n=50 fish for control group and each experimental time point).

All in all, in the presented study, we demonstrated that 1% triton X-100 is one of the most suitable and sufficient method for the induction of degeneration and following regeneration of the mature OSNs in zebrafish OE. Different degeneration approaches succeeded to remove OSNs effectively as HuC/D staining shows clearly. Also as double marker assay showed, tissue insult successfully induce cell proliferation, interestingly within sensory region of the lamella, which point out the presence of a second dormant stem cell population activated upon injury. Lastly, our transcriptome data also revealed that most of the ORs, TAARs and VNRs and cell type specific markers are downregulated, which indicates the loss of different OSNs after the damage, while several neural proliferation and differentiation factors are upregulated implying that neural stem cells and precursors are activated in response to injury.

5. DISCUSSION

For most of the last century, it was thought that the adult mammalian CNS is not capable of regeneration. However, beginning from the 1960, studies have shown that new functional neurons can be formed in the adult brain (Altman and Das, 1965). Yet, in mammals this capacity is mainly limited to the subventricular zone of the lateral ventricles in the forebrain and the subgranular zone of the hippocampus (Curtis *et al*., 2012). The peripheral olfactory tissue is an exception to this limitation as lifelong neurogenesis also persists in mammals (Caggiano *et al*., 1994; Schwob *et al.,* 2002). One of the major differences between OSNs and other neurons is their direct contact with the external environment, which makes them vulnerable to toxic substances and increases their risk of damage, injury, and ultimately death. This vulnerability requires the continuous regeneration of cells in the OE in order to maintain a sense of smell that is essential for many aspects of daily life, including food finding, navigation, social interactions, and sexual behavior (Mackay-Sim 2010). In the rodent OE, dying OSNs are continuously replaced from stem cell populations that are located in the basal OE (Vedin *et al.*, 2009; Altman, 1969). This unique feature of lifelong OSN turnover provides an exciting opportunity as understanding the mechanisms that keep the stem cell niche active in the OE may be translatable to other parts of the CNS and may provide an experimental and therapeutic window into CNS repair.

The study presented in this thesis investigates and characterizes induced damage responses and aspects of the mechanism of injury-induced neuroregeneration in the OE. Different experimental setups to damage OE tissue have been tested and their feasibility to study the regenerative property of the tissue have been evaluated. Three different experimental approaches, physically damage by focal stab lesion and chemical damage by irrigation with the corrosive solutions $ZnSO_4$ and Triton X-100, were evaluated with respect to their ability to induce robust injury responses.

5.1. Which one is the better injury model?

All three approaches resulted in the induction of cell proliferation in the OE, albeit with different efficiency and reproducibility. When a single lamella was damaged by mechanically stabbing the tissue, clear differences in the pattern of BrdU-positive cells could be observed. Around the site of injury, a local induction of BrdU-positive cells in the sensory region of the lamella was induced while as opposed to the restricted proliferation pattern at the ILCs and the S/NS boundary in undamaged tissue. This suggests that induction of proliferation is local and not systemic as more distant regions of the OE showed no induction or increase in proliferation. With the exception of the injured lamella, neighboring lamellae and the two neurogenic zones at the ILCs and the S/NS boundary showed no increase in mitotic activity. This is an interesting finding, as the signal that promotes proliferation in the basal OE does not crosstalk to stem and progenitor cells situated at the end of each lamella.

Although the strategy of physical damage by focal stab lesions works in principle, it is difficult to reproduce reliably and very error-prone since targeting only one single lamella without harming other regions of the OE is quite challenging. In turn, chemical damage models turned out to be more feasible and reproducible than focal mechanical damage, provided injury was not excessive. Although a number of chemical substances have been described to effectively lesion OSNs (Peele, 1991; Schwob *et al*., 1995) intranasal infusion of $ZnSO_4$ and Triton X-100 have been most commonly used (Mayer 1993; Burd 1993; Harding, 1978, Iqbal and Byrd-Jacobs, 2010). Following application of either chemical agent, rapid degeneration and subsequent regeneration of OE occurs (Byrd and Brunjes, 2001; Burd, 1993; Matulionis, 1975; Schwob *et al*., 1995). Loss of neurons in the OE appears to occur by necrosis (Schultz 1960), leading to the leakage of cytoplasmic content into the tissue, and ciliated OSNs appear to be affected more than microvilous cells (Iqbal and Byrd-Jacobs, 2010).

The results of the presented experiments replicate and extend previous findings regarding OSN regeneration following intranasal $ZnSO_4$ and Triton X-100 infusions (Burd,

1993; Harding, 1978; Matulionis, 1975; Troitskaya, 1991; Iqbal and Byrd-Jacobs, 2010). However, there are certain limitations to the ability of the OE to regenerate following too extensive chemical damage. For example, $ZnSO_4$ causes not only severe damage to OSNs but also severely deteriorates tissue integrity making it difficult to process the tissue for further investigation by immunohistochemistry. Interestingly, on some tissue sections of $ZnSO_4$-treated OEs, the number of BrdU-positive cells was surprisingly sparse. A possible interpretation of this finding may be that excessive $ZnSO_4$ treatment also destroys neuronal stem and / or progenitor cells thereby preventing proliferation.

Nasal irrigation with aqueous solutions of Triton X-100 causes similar damage to the OE as $ZnSO_4$. Although the regeneration response can be as robust as the induction seen after $ZnSO_4$ treatment, tissue integrity is better preserved (Verhaagen 1990; Burd 1993; Murray 1999). Within 24 h following Triton X-100 treatment, most mature OSNs disappear (Figure 4.8). This is in contrast to previous reports that have claimed that ciliated OSNs are predominantly affected while microvillous cells are largely spared (Iqbal and Byrd-Jacobs, 2010). The neuronal marker HuC/D does not distinguish between both types of OSNs and at 12 to 24h following nasal irrigation HuC/D staining is lost in most of the OE tissue. This difference may be due to differences in the application and incubation times. Here, 90 sec applications of 1% Triton X-100 were used and the agent was renewed after 45 sec, while 0.7% Triton X-100 was applied for 120 sec in the other study. The loss of neurons is followed by an increase in cell proliferation on injured lamellae. As would be expected in a situation in which most cells of the OE are destroyed, many cells proliferate in response to Triton X-100 treatment. Thus, irrigation with Triton X-100 appeared to be the most reliable method to induce neurogenesis responses.

5.2. Systemic or local response?

We performed IHC against the neuronal marker HuC/D and proliferation marker BrdU or mitotic cell marker pH3. These co-labeling experiments revealed a inverse correlation between induced cell proliferation and remaining mature neurons at various time points

following the chemical treatment. Labeling for the neuronal marker anti-HuC/D is sparse in fish examined 24 h following detergent exposure. Around the same time a peak in proliferation is observed as judged by two independent methods, BrdU and pH3 staining.

Similar to the observations made for focal lesions, the response was localized to regions of effective ablation. On some occasions, regions in the sensory part of the lamella lack BrdU-positive cells. Interestingly, these regions always contained residual HuC/D-positive cells, presumably because the detergent did not reach these sections of the tissue efficiently. However, a drastic induction of proliferating cells was always observed in regions of the lamella that were devoid of HuC/D positive cells.

It is reasonable to assume that the signal to induced proliferation results from injured cells residing in the central sensory region of OE. The nature of the signal is not known but different modes of cell-cell interactions could be involved. For instance, altered contact between OSNs and their damaged neighbors could be sensed by cell-cell signaling factors, such as notch components, integrins, or cadherins (Goldman, 2014). The chemical injury may also interfere with signals that maintain stem cell / progenitor quiescence in the uninjured OE or stimulate proliferation more directly. A combination of regeneration inhibiting and stimulating factors may be in balance in the unperturbed OE and injury would disrupt this equilibrium. As OSNs die by necrosis, it is likely that cytoplasmic content is released into the tissue, which could provide a signal for proliferation. All of these mechanisms have in common that they are local or contact dependent and therefore would not spread throughout the tissue to induce a systemic response. For instance, an increase in proliferative activity of cells located at the ILC and S/NS boundary, indicative of a far-reaching diffusible signal, was hardly observed.

Although injured OSNs appear to provide the initial signal that activates injury-responsive cells, it remains unclear whether other cell types may also participate. Particularly, glia cells that are known as sustentacular cells in olfactory tissue might have an active role in

conveying local signals between the tissue and basally-located stem cells, similar to Muller glia in the zebrafish retina (Goldman, 2014).

Unlike the ongoing proliferation, which is restricted to ILCs and the S/NS border during the normal maintenance of the tissue, additional cells that localize to basal positions along the entire lamella are recruited under injury conditions. These findings suggest that alternative pools of stem cells and / or precursor cells respond preferentially to normal loss and damage. The induced response appears to depend on immature cells and progenitors in the basal OE that have to remain undamaged after Triton X-100 application. Triton X-100 treatment kills mature neurons from the apical surface downwards while leaving a pool of immediate precursors on the basal layers intact. This is consistent with the observation that too strong treatment with $ZnSO_4$ eventually prevents the tissue from mounting an injury response if the damage is too massive and impairs stem cells.

Based on the information obtained from three different injury models, it can be concluded that two stem cell populations for maintenance and repair system exist. Previous reports (Leung *et al.*, 2007; Mackay-Sim and Kittel, 1991) suggest that in rodent OE, GBCs are the neurogenic stem cells contributing to maintenance of the tissue while HBCs function as injury-responsive cells and when the GBC population has been depleted (Leung *et al*., 2007). This pattern might be also true for zebrafish OE, however, unlike the localizations of GBCs more apical to HBCs in the murine OE, the HBC-like stem / progenitor cells might reside only in the sensory region, whereas the transit amplifying GBC-like cells might exclusively locate in the ILC and S/NS boundary.

5.3. Time course and tissue distribution of the response

In addition to the hypothesis that under normal conditions, a quiescent stem cell population contributes to the injury response, an alternative explanation may be that neural progenitors residing in central and peripheral neurogenic zones migrate towards the sensory

regions of the lamella. In order to discard this possibility, we examined the OE of fish sacrificed at 4 h, 12 h and 24 h after intranasal irrigation with Triton X-100 and stained with IHC against pH3, which shows mitotic cells.

When the tissue was observed at 4 h after treatment, antibody-labeled cells were found almost exclusively in the ILC and S/NS boundary (Figure 4. 8). Very few pH3-labeled cells could be observed in the sensory regions of the lamella, indicating that GBC-like cells remain in their proper positions. However, our examination of mitotic activity after 12 h and 24 h showed that pH3-positive cells were not concentrated around the ILC and S/NS boundary but instead were scattered through the lamella.

There may even be additional immature neurons not revealed by anti-HuC/D labeling if they have recently formed and are not expressing Hu protein yet (Adolf *et al*., 2006; Grandel *et al*., 2006). Our Triton X-100 treatment disrupts mature OSNs and supporting cells of the apical layers but does not penetrate further during the application of the treatment on the OE, which leaves a pool of immediate precursors residing in the deeper layers of lamellla remain after this treatment and able to quickly differentiate into different neurons.

Previously, several studies in zebrafish (Bryd *et al*., 2001) and mice (Cummings *et al*., 2000) showed that the application of the detergent Triton X-100 to the OE result in a significant reduction in the thickness of the lamellae due to a loss of OSNs and epithelial tissue one day after the treatment. Paralleled to this finding, 24 h after detergent exposure, the treated-OE show the presence of few mature neurons, as indicated by the low levels of staining for HuC/D antibody. Patchy remnants of HuC/D positive cells in the treated OE can either indicate cells that did not get exposed to sufficient amounts of the detergent or tissue that has already recovered and where new mature neurons have formed. However, such a rapid recovery only 24 h after chemical treatment is not very plausible since studies using retrograde DiI labeling from the olfactory bulb previously showed that recovery is reached 5 days after chemical damage (Bryd *et al*., 2001). Studies on degeneration of the mouse OE and

the time course of its recovery showed that newly formed cells appear by 3 weeks post-chemical lesion and that a regular pattern is established by 6 weeks (Cummings *et al*., 2000). It was also shown in the garfish that the main phase of axon regeneration, about 50–70% of the original population, did not occur until 12 days after axonal damage (Cancalon 1983). Another, yet unlikely, possibility for the loss of HuC/D labeling in treated OE may be that OSNs do not die and but lose their antigenicity for HuC/D. Although this concern has not been thoroughly ruled out, it seems unlikely since almost all the regions on the lamella that lacks HuC/D labeling shows an induction in the level of proliferating BrdU-positive cells.

5.4. One, two or more stem cell populations?

The observation of a change in the spatial pattern of cell proliferation following damage suggests that a dormant population of progenitors is recruited by the injury. Yet, it cannot be ruled out that cells located at the ILCs and the S/NS boundary become misplaced following chemical damage due to disruption of tissue integrity. We investigated whether proliferating cells in the basal OE may be progenitor cells translocated from the central and peripheral proliferation zones by double marker incorporation assays with two analogs of thymidine. Fish were first incubated in IdU prior to the damage to visualize GBC-like cells and CldU after damage to label HBC-like cells.

The number of IdU-positive cells in the damaged OE was lower than in control OEs, yet the overall pattern was preserved; label-positive cells concentrated at two neurogenic zones at the ILC and S/NS boundary. A possible explanation for the reduced number of IdU-positive cells in treated OEs may be that chemical treatment also removed some of the IdU-positive cells. More importantly, the overall similar pattern of IdU-positive cells suggests that GBC-like cells at the ILC and S/NS did not get displaced by the damage. Contrary, CldU-positive cells that underwent cell divisions after the damage were scattered along the entire OE including the central sensory region but were not localized to the ILCs and S/NS boundary. These results strongly argue that two independent stem cell populations contribute to OSN neurogenesis.

Molecular markers for different OE cell populations have been extensively described in the rodent system (Murdoch and Roskams, 2007; Gokoffski *et al*., 2010) but are less well studied in zebrafish. In mice, morphologically flat HBCs that form a single-cell layer directly on top of the basal lamina express a variety of distinct cell adhesion molecules such as ICAM, and integrins and also certain intracellular matrix proteins, such as cytokeratin 5 and 14 (Carter *et al*., 2004; Graziadei and Monti Graziadei, 1979; Holbrook *et al*., 1995). On the other hand morphologically round GBCs, which are primarily situated above HBCs and below the population of immature OSNs, appear to be a more heterogeneous cell population, most likely reflecting different stages of differentiation (Graziadei and Monti Graziadei, 1979). The progenitors in the OSN lineage that reside among the GBC population can be identified by expression of the proneural genes Ascl-1 and Neurogenin-1 (Ngn-1). Acsl-1-positive GBCs are the earliest OSN progenitors identified, which divide and give rise to the immediate neuronal precursor (INP), which is a late-stage transit amplifying progenitor distinguished by expression of the proneural gene Ngn-1 (Wu *et al*., 2003; Gordon 1995; Cau *et al*., 1997). INPs finally divide to give rise to daughter cells that undergo terminal differentiation into postmitotic Ncam-and OMP-expressing OSNs (Calof *et al*., 2002).

HBCs may serve as a reservoir to replenish GBCs, remain largely quiescent during normal neuronal turnover and are slow cycling. Under unperturbed conditions, progenitor GBCs are largely responsible for tissue maintenance. However, after severe loss of mature neurons, HBCs are induced to transiently proliferate and differentiate their progeny to reconstitute the neuroepithelium. It may be that cells of the HBC lineage are molecularly indistinguishable from GBCs. Our data obtained by sequential IdU/CldU labeling therefore support the model in which distinct cell populations mediate normal neuronal turnover and neuronal replacement upon traumatic injury. Sequential administration of thymidine analogues revealed that transit-amplifying GBC-like cells, might not contribute to injury response of the olfactory tissue (Leung *et al*., 2007). Our data suggest that HBCs remain largely quiescent in OSN replenishment and support the studies showing that neuronal

repopulation depends on GBCs (Calof 2002; Carr 1992), whose proliferation is sufficient to support the requirement for replacement.

Previous studies showed that there is a drastic reduction in mitotic basal progenitors in the absence of Ascl1 (Guillemot *et al.,* 1993; Cau *et al*., 1997; 2002). Thus, we set out to visualize neuronal progenitors by IHC against Ascl-1 as one attempt to identify a subpopulation of GBCs. As expected, ascl-1-positive cells were detected both at the ILCs and at the S/NS boundary. Surprisingly, however, in the treated OEs no ascl1-positive cells could be found. This finding might indicate that Acsl-1-positive GBCs might be depleted following the Triton X-100 treatment. The induction in the cell proliferation of the treated OE might stem from injury responsive-HBCs. Another possibility would be that mitotically competent INPs might not be as vulnerable as Acsl-1-positive GBCs, remain and give rise to newly born OSNs.

We also engaged in a back-tracing approach to negatively outline basal cell population by exclusion in order to characterize basal cells that may be stem cells in OE (Hassenklover *et al*., 2009). With this method, OSNs were supposed to be visualized via biocytin back-tracing whereas sustentacular cells were labeled with CYKII antibody. The nuclear counterstaining would reveal the putative stem cells that are not OSNs and that are not sustentacular cells either. However, although it was possible to stain different types of neurons including their detailed morphology, the number of back-traced OSNs was not high enough to exclude all mature OSNs. The zebrafish olfactory nerve is very short and does not allow to make a cut to be filled with the dye.

5.5. What is the signal?

The secreted factors that regulate proliferation and differentiation of stem cells and progenitors in the microenvironment of in the zebrafish OE have not been fully understood. However, some external cues, such as ATP released from the dead OSNs, could be the

possible mediator of the communication between the injured area and neurogenic zones. Previous studies have shown that ATP is released upon injury and can promote proliferation in the OE (Hegg *et al*., 2003; Jia *et al*., 2009, 2010). Activation of fast dividing progenitors, GBCs, and slow dividing injury-responsive neuronal stem cells, HBCs, might be regulated in a dose dependent manner of released ATP or other purines. During the daily maintenance of the olfactory tissue, the low number of dead cells might not release enough purines to activate dormant injury-responsive stem cell population, while following a robust acute injury, the concentration of released purines might reach a critical concentration to finally trigger a response.

It is also reasonable to assume that the activation of the stem cell is controlled by a negative feedback system in which differentiated OSNs produce an inhibitory signal that acts to prevent progenitor cells from dividing and generating new neurons as long as the olfactory tissue is intact. Most likely, some negative regulators such as GDF11 and BMP keep them dormant (Wu *et al*., 2003). Thus, the absence of these inhibitory factors due to the destruction and depletion of GBCs that might be the source of anti-proliferative signal and could induce proliferation and differentiation of these dormant stem cells or precursors following tissue insult (Hegg *et al*., 2009). Indeed, the observation that INPs are rapidly induced to divide following induction of OSN apoptosis by olfactory bulbectomy supports the idea that OSN death overrules an inhibitory signal that normally holds progenitor cell proliferation in check (Wu *et al*., 2003; Gordon *et al.,* 1995; Holcomb *et al.,* 1995). Two different modes of communication, intercellular and intracellular signaling by sustentacular cells may play a role. Sustentacular cells have gap junctions, which help them communicate with each other throughout the lamella (Hegg *et al*., 2009). Via these gap junctions, sustentacular cells might transmit information about the loss of neurons in the sensory region to GBCs residing at the ILCs and at the S/NS boundary. Whereas this mode of communication is speculative, sustentacular cells have been shown to signal between apical and basal regions of OE (Mackay-Sim and Kittel 1991, Hegg *et al*., 2009; Masukawa *et al*., 1983; Hassenklöver *et al*., 2009).

5.6. Is the response neurogenic?

So far, we have only shown that chemical damage induces a proliferation response but did not show that the newly generated cells mature into neurons. However, 5 days after induction the tissue is restored and neurons can be observed (Iqbal and Byrd-Jacobs, 2010; Kocagöz, unpublished). Thus, neurons are produced at high rate. However, it will also be imperative to directly show that the regions along the lamella are neurogenic by examination of neuronal marker expression.

In order to further characterize the injury responsive cell populations, we examined whether the cells generated in these positions would contribute to the neuronal population by analyzing the expression patterns of neuronal markers such as Ascl-1, neurogenin1, her6, notch1-a following chemical damage. However, none of those antibodies except Ascl-1 showed immunoreactivity in the zebrafish OE. Although an increase in ascl-1-expressing neuronal progenitors was expected no ascl-1-positive cells in treated samples could be observed, whereas Ascl-1 positive cells resides in the ILCs and close to S/NS border on the unperturbed control OE. This is in contrast to transcriptome data following injury, which showed a 2-fold increase in ascl1 at 24h. Yet, transcriptome data describe RNA expression levels, which might be different from and precede protein expression. Thus, injury-dependent induction of ascl1 in the OE could not be confirmed.

5.7. Final conclusion

Although there is still a long way to go to fully uncover the mechanisms that control neuroregeneration, lower vertebrates like fish will continue to be important models to solve this complex problem. Using zebrafish, we may be able to learn how they maintain their remarkable plasticity of neural tissue. Understanding the similarities and differences of neurogenic regions in zebrafish and mammals would provide us with insight into regulatory network that defines the neural stem cell state, and will provide us with important knowledge

for facilitating neurogenesis in regions of the adult nervous system where regeneration is limited or absent. Recent progress in characterizing how to boost and guide neurogenesis from the stem-cell pool might eventually lead to a powerful tool for brain repair in human disorders of the CNS and these mechanisms would enable the development of strategies for stimulating neural repair in mammals.

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
Micropipetters	Eppendorf, Germany (Research)
Microwave Oven	Vestel, Turkey
Microinjector	Eppendorf, Germany (FemtoJet)
Luminometer	Fluroskan 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.

Ethanol Absolute	Sigma-Aldrich, U.S.A. (34870)
Paraformaldehyde	Sigma-Aldrich, U.S.A. (P6148)
Triton X-100	Sigma-Aldrich, U.S.A. (T8787)
HCl	Sigma-Aldrich, U.S.A (H1758)
Optimum cutting temperature	Sakura® Finetek, USA (4583)

APPENDIX C: RESULTS FOR INDIVIDUAL EXPERIMENTS

Table 6.3. pH3-positive cell numbers in each time point along the radial index.

Section	1	2	3	4	5	6	7	8	9	10	**Total**
Control											
C1	1	1	0	0	0	0	4	0	0	1	7
C2	1	1	0	0	0	0	0	0	1	0	3
C4	3	1	0	0	1	3	2	0	1	1	12
C5	4	1	5	1	1	5	2	1	2	0	22
C3	1	0	0	0	1	0	1	0	0	0	3
C7	1	0	1	0	0	2	4	3	0	0	11
C6	3	3	0	0	0	0	4	1	0	0	11
C7	2	2	0	0	0	0	0	2	0	0	6
C4	1	0	0	0	0	0	0	2	0	0	3
C5	5	1	0	0	0	0	1	0	0	0	7
C2	1	0	0	0	0	0	0	0	1	0	2
C6	0	0	0	0	0	0	1	0	1	0	2
C8	1	0	0	0	0	0	1	0	1	0	3
C10	0	2	2	0	0	1	1	1	0	0	7
C12	0	0	0	0	0	0	5	1	2	1	9
C18	1	2	0	0	0	0	3	0	2	2	10
C2	0	1	1	0	1	2	0	1	0	1	7
C8	1	0	0	0	0	1	2	2	0	0	6
12 hours											
C12-7	1	0	0	0	0	0	1	6	0	0	8
C12-6	0	0	0	0	0	2	6	1	0	0	9
C12-4	0	0	0	0	0	1	2	0	0	1	4
C12-2	2	3	0	0	1	5	7	1	0	0	19
C6	7	0	0	0	0	0	2	3	2	0	14
C5	2	0	0	2	0	3	1	1	0	0	9
C4	1	0	0	0	0	1	0	6	0	0	8
C9	0	0	0	0	0	1	0	4	1	0	6
C8	0	0	0	0	0	2	1	0	0	0	3
C6	0	1	0	0	0	1	0	2	0	0	4
C5	0	0	0	0	0	0	0	0	3	2	5
C4	6	0	0	0	1	2	0	0	0	0	9
C3	0	3	0	0	0	0	0	0	0	0	3
C2	0	0	1	0	0	2	0	1	0	0	4
C1	0	0	1	2	0	0	4	0	0	0	7
	1	2	3	4	5	6	7	8	9	10	
Average	1.42857	1.28571	0.71429	0.71429	2.42857	3.42857	2.28571	1.28571	0.14286	0.85714	**4hr**
SEM	0.52904	0.38936	0.43866	0.26452	1.02732	0.6343	0.38936	1.04421	0.13226	0.51224	
Average	2.66667	4.66667	3.33333	2.88889	2.11111	2	1.66667	2.22222	3.44444	3.11111	**12hr**
SEM	1.04231	1.65552	1.05409	0.83805	0.6929	0.73703	0.31427	0.60406	1.33436	1.12705	
Average	1.33333	1.86667	3.13333	2.4	3.13333	2.13333	1.73333	2.13333	1.73333	1.13333	**24hr**
SEM	0.3849	0.50655	0.47015	0.56411	0.86341	0.58778	0.64268	0.4977	0.49411	0.24706	
Average	1.36364	0.66667	0.33333	0.15152	0.18182	1.0303	1.66667	1.18182	0.51515	0.27273	**Ctrl**
SEM	0.46912	0.2555	0.24721	0.13104	0.10113	0.3622	0.49861	0.42059	0.21526	0.14822	

Table 6.3. pH3-positive cell numbers in each time point along the radial index (cont.).

Section	1	2	3	4	5	6	7	8	9	10	**Total**
4 hours											
C1	1	1	0	0	0	0	4	0	0	1	7
C2	1	1	0	0	0	0	0	0	1	0	3
C4	3	1	0	0	1	3	2	0	1	1	12
C5	4	1	5	1	1	5	2	1	2	0	22
C3	1	0	0	0	1	0	1	0	0	0	3
C7	1	0	1	0	0	2	4	3	0	0	11
C6	3	3	0	0	0	0	4	1	0	0	11
C7	2	2	0	0	0	0	0	2	0	0	6
C4	1	0	0	0	0	0	0	2	0	0	3
C5	5	1	0	0	0	0	1	0	0	0	7
C2	1	0	0	0	0	0	0	0	1	0	2
C6	0	0	0	0	0	0	1	0	1	0	2
C8	1	0	0	0	0	0	1	0	1	0	3
C10	0	2	2	0	0	1	1	1	0	0	7
C12	0	0	0	0	0	0	5	1	2	1	9
C18	1	2	0	0	0	0	3	0	2	2	10
C2	0	1	1	0	1	2	0	1	0	1	7
C8	1	0	0	0	0	1	2	2	0	0	6
24 hours											
D5	2	0	2	1	0	0	0	0	0	2	7
D7	1	0	3	0	0	0	0	0	0	2	6
D7	5	4	4	0	0	0	0	0	4	1	18
D1	4	2	7	7	10	3	3	2	0	0	38
D2	0	0	5	6	7	5	0	2	0	0	25
D6	0	0	4	4	7	7	9	3	5	3	42
D7	0	0	1	4	8	4	1	6	5	2	31
D1	2	4	0	4	6	5	5	1	2	0	29
D5	2	6	5	4	1	0	1	6	2	1	28
D12	2	4	2	1	2	2	0	0	0	1	14
D20	0	2	3	1	0	0	0	4	0	0	10
D6	0	3	5	2	1	0	0	2	1	0	14
D10	1	3	2	1	1	4	1	1	0	1	15
D2	1	0	1	0	1	1	4	3	4	2	17
D4	0	0	3	1	3	1	2	2	3	2	17

Table 6.4. BrdU-positive cell numbers in each time point along the radial index.

Control										
	10	9	8	7	6	5	4	3	2	1
	64	38	47	43	11	3	0	1	6	39
	67	32	63	24	1	0	0	0	17	37
	84	66	93	31	2	10	1	12	19	15
	58	67	68	32	24	11	8	5	7	14
	8	21	26	17	5	6	4	8	13	41
	13	29	30	15	1	0	6	3	11	21
	1	2	3	4	5	6	7	8	9	10
	42	43	2	3	10	38	44	54	50	46
	48	7	5	4	3	23	42	57	55	26
	36	0	11	18	21	68	59	64	37	84
	12	21	4	1	4	7	17	39	33	25
	35	0	0	0	12	2	4	7	34	22
	12	14	7	3	7	0	8	11	21	18
Total	515	483	559	336	182	87	48	58	158	352
Average	42.9166	40.25	46.5833	28	15.1666	7.25	4	4.83333	13.1666	29.3333
Sd	27.6617	15.7660	25.1159	16.6951	20.4887	5.98672	5.08115	4.01889	11.5980	13.4322
Se	11.2928	6.43646	10.2535	6.81575	8.36448	2.44407	2.07437	1.64070	4.73489	5.48367
Normalized	0.92128	0.86404	1	0.60107	0.32558	0.15563	0.08586	0.10375	0.28264	0.62969
Norm. SE	0.24242	0.13817	0.22011	0.14631	0.17956	0.05246	0.04453	0.03522	0.10164	0.11771
Damaged										
	10	9	8	7	6	5	4	3	2	1
	32	23	23	22	28	9	45	39	22	27
	25	33	33	26	41	39	15	21	63	39
	58	82	44	51	23	22	30	24	30	39
	25	87	69	71	49	35	14	70	22	49
	1	1	11	5	10	11	24	32	23	10
	10	24	8	15	6	9	5	22	28	20
	1	2	3	4	5	6	7	8	9	10
	6	17	18	16	12	16	27	29	51	12
	45	31	21	15	15	6	11	6	17	22
	29	41	29	12	9	34	27	53	54	44
	59	45	38	44	50	49	53	54	61	39
	18	7	5	10	3	0	3	17	20	15
	14	28	10	14	8	21	13	12	32	20
Total	303	485	359	324	283	222	244	329	357	355
Average	25.25	40.416	29.91667	27	23.58333	18.5	20.3333	27.4166	29.75	29.5833
Sd	15.9494	26.6917	20.87825	21.04109	16.914	14.8599	12.9427	16.7899	14.5172	16.7139
Se	6.51135	10.8968	8.523512	8.589987	6.905111	6.0665	5.28386	6.85445	5.92663	6.82344
Normalized	0.62474	1	0.740206	0.668041	0.583505	0.45773	0.50309	0.67835	0.73608	0.73195
Nomr. SE	0.16110	0.26961	0.210891	0.212536	0.170848	0.1501	0.13073	0.16959	0.14663	0.16882

Table 6.5. Percentage of the sensory area measured by the area of HuC/D-positive cells.

percentage of the sensory region [%] / section					**ctrl**	**4**	**12**	**24**	
Section #	**Control**	**4hr**	**12hr**	**24hr**	27.31074	10.68905	5.988812	10.49967	**Average**
1		11.10562	3.237376	10.31177	7.634872	4.64728	4.690601	6.238977	**SD**
2	17.94586	17.61568	2.987492	15.30548	1.30937	2.078327	2.097701	1.158549	**SE**
3	23.53647	10.79332	1.942491	10.22275					
4				9.436975					
5	13.61167	9.255964	12.76518	9.973061					
6	9.101167	4.674687	9.011523						
7				5.115715					
8	33.01878			6.293772					
9				5.437963					
10	19.94298			1.78519					
11	18.01331								
12				9.090509					
13	35.99462			9.255127					
14	38.32934			9.267567					
15	36.54165			10.23319					
16				17.49529					
17	33.2967								
18	40.5055			25.86892					
19	19.28118			3.355699					
20	21.53098			4.106547					
21	36.13171			4.423012					
22				4.456876					
23	38.49879			1.86348					
24	25.82636								
25	28.25989			17.78175					
26	24.5238			22.10595					
27	23.12507			18.11857					
28				12.25208					
29	30.73928			13.13142					
30	32.59383								
31	31.78222			6.390821					
32	26.6628			14.80021					
33	25.65686			19.62725					
34				6.983557					
35	31.38226								
36	34.84049								
37	29.7901								
38	29.55666								
39									
40	30.56689								
41	23.79416								
42	24.77842								
43	16.92203								
44	22.48345								

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