THE CONTRIBUTION OF PURINERGIC SIGNALING TO OLFACTORY NEUROGENESIS IN ZEBRAFISH

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ABSTRACT

THE CONTRIBUTION OF PURINERGIC SIGNALING TO OLFACTORY NEUROGENESIS IN ZEBRAFISH

Compared to other sensory epithelia, the olfactory epithelium (OE) has a remarkably high regenerative capacity: continuous neurogenesis compensates for regular turnover of olfactory sensory neurons (OSNs) while reactive neurogenesis fully recovers lost cells and OE function after damage. It is known from the rodent OE that two populations of multipotent basal progenitor cells, the horizontal (HBCs) and globose basal cells (GBCs) contribute to the repopulation of the OE, under injury and maintenance conditions, respectively. However, progenitor cell populations with equivalent function and how they are regulated in the zebrafish OE are less well characterized. A recent and interesting hypothesis is that purinergic signaling may play a critical role in the regulation of neurogenesis. According to this scenario, purine compounds, such as ATP, would be released from dying cells and stimulate proliferation of progenitors either directly, or indirectly through sustentacular glia cells (SCs). In order to examine the role of purinergic signaling, calcium-imaging experiments were conducted on *ex vivo* vibratome sections through the adult zebrafish OE. Morphometric measurements of Ca^{2+} responses upon extracellular ATP stimulation were compared to cell measurements obtained via immunohistochemistry using antibodies against cell type-specific markers of non-neuronal OE cells, such as Krt5, Sox2 and Tp63. ATP stimulation activates two distinct $Sox2^{+}$ cell populations with different response kinetics, one of which shows HBC-like characteristics, whereas the other resembles SCs. To more directly investigate the physiological effect of purinergic stimulation onto neurogenesis, fish were injected intraperitoneally with ATP or ATP and the P2 receptor antagonist suramin and changes in proliferation were examined using BrdU incorporation assays. ATP stimulation resulted in a significant increase in cell proliferation in the OE and the observed increase could be blocked by co-application of suramin, suggesting a direct purinergic effect. It was found that the majority of newborn cells commit to a neural fate and that $krt5^{+}$ HBC-like cells were not the main contributors to the response.

ÖZET

PÜRİNERJİK SİNYALLERİN ZEBRABALIĞI KOKU ALMA DOKUSUNDAKİ NÖROGENEZE KATKISI

Diğer duyu epitelleriyle karşılaştırıldığında koku alma epiteli dikkate değer bir biçimde yüksek rejenerasyon kapasitesine sahiptir: koku duyu nöronlarını sürekli olarak yenilemek için sürekli aktif bir nörogenez vardır ve hasar sonrasında yenileyici nörogenez kayıp hücreleri ve koku işlevini tamamen geri getirebilir. Kemirgen koku epitelinde yapılan daha önceki bulgular göstermiştir ki, koku epiteli iki kök hücre populasyonuna ev sahipliği yapmaktadır. Yatay bazal hücreler ve küresel bazal hücreler dokunun yeniden yapılanmasında sırasıyla hasar sonrası ve süreğen bakımında olmak üzere rol oynarlar. Lâkin, zebrabalığında bu özelliklere sahip hücre topluluklarının olup olmadığı ve bu hücrelerin nasıl kontrol edildiği iyi bilinmemektedir. Yakıın geçmişte ortaya çıkan ilginç bir hipoteze göre, pürinerjik sinyaller nörogenezin kontrolünde kritik bir öneme sahip olabilir. Bu senaryoya göre, ATP gibi pürin molekülleri ölen hücrelerden salgılanarak, direkt olarak kök hücreleri uyararak ya da ölen hücrenin bilgisinin dolaylı olarak destek hücreleri aracılığıyla kök hücrelere iletilmesiyle onları uyarma potaniyeline sahip gibi gözükmektedir. Pürinerjik sinyallerin bu rolünü araştırmak için yetişkin zebrabalığı koku epitelinin *ex vivo* vibratom kesitleri üzerinde kalsiyum görüntüleme gerçekleştirildi. Hücre dışı ATP uygulamasının yol açtığı Ca^{2+} tepkilerinin morfometrik ölçümleri Krt5, Sox2 ve Tp63 gibi nöronal olmayan işaretçilerin immünohistokimyasal analizleriyle karşılaştırmıştır. ATP uygulamasının ayrı tepki profillerine sahip iki ayrı $Sox2^{+}$ hücre popülasyonunu aktive ettiği, bu popülasyonlardan birinin yatay bazal hücre profiline uyduğu, diğerinin ise destek hücrelerinin profiline benzediği bulunmuştur. Daha direkt bir inceleme yöntemi olarak, zebrabalığına intraperitonal ATP ya da ATP ve P2 reseptörü inhibitörü suramin enjeksiyonu gerçekleştirilip BrdU katım yöntemi ile ATP stimülasyonunun zebrabalığı koku epitelindeki hücre bölünmesi sayılarında geri çevrilebilir anlamlı bir artışa sebep olduğu gösterilmiştir. Bu sonuçlar kök hücreler üzerinde pürinerjik sinyallerin direkt bir etkisi olduğunu önermektedir. Yenidoğan hücrelerin çoğunluğunun nöral hücre kaderi doğrultusunda geliştiği ve $krt5^{+}$ hücrelerin bunda ana rol oynayıcı olmadığı tespit edilmiştir.

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ACSF	Artificial Cerebrospinal Fluid
BDNF	Brain Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
BrdU	Bromodeoxyuridine
cAMP	cyclic Adenosine Monophosphate
CNS	Central Nervous System
DAMP	Damage Associated Molecular Pattern
DNA	Deoxyribonucleic Acid
dpf	days post-fertilization
EBF	Early B-cell Factor
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FACS	Fluorescence-activated Cell Sorting
GBC	Globose Basal Cell
GFP	Green Fluorescent Protein
GPCR	G-Protein-coupled Receptor
HBC	Horizontal Basal Cell
HB-EGF	Heparin-binding EGF-like Growth Factor
hpf	hours post-fertilization
IHC	Immunohistochemistry
ILC	Interlamellar Curve
NGF	Nerve Growth Factor

OCT	Optimal Cutting Temperature
OE	Olfactory Epithelium
OSN	Olfactory Sensory Neuron
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline Triton X-100
PFA	Paraformaldehyde
PRR	Pattern Recognition Receptor
RT	Room Temperature
S/NS	Sensory/Non-sensory
SC	Sustentacular Cell
SEM	Standard Error of the Mean
TLR	Toll-like Receptor
UV	Ultraviolet

1. INTRODUCTION

1.1. Neurogenesis - a historical perspective

The process by which new and functional neurons are generated from neural stem or committed progenitor cells is referred to as neurogenesis. Neurogenesis occurs during embryonic development of nearly all animals with the known exceptions of sponges and the placozoan *Trichoplax*, which both lack nerve cells completely (Sherwood *et al*., 2012; Schierwater, 2005). The nervous system of vertebrates originates from a region of the ectoderm, the external layer of cells at the gastrula stage, with the help of an inducer region to specify the neuroectoderm. While the ectoderm continues to generate epidermal cells, the neuroectoderm and surrounding neural crest gives rise to neurons and glial cells (Campos-Ortega, 1995). In the 1920s, it has been shown with grafting experiments in salamander embryos that transplantation of a region in the dorsal mesoderm, which is now known as the Spemann organizer, to the ventral side of a host embryo resulted in the formation of an additional and intact nervous system (Spemann and Mangold, 1924). Cells of the neural plate then undergo a series of specification and differentiation events that organize different neuron pools along the anterior-posterior and dorso-ventral axes (Wang *et al*., 2000). Cell transplantation and lineage tracing experiments in newborn ferret brains revealed that neuronal stratification in the adult brain is programmed at embryonic stages (McConnell, 1988). Thus, neurogenesis during embryonic development is a highly regulated but largely invariant process that sets up the embryonic central and peripheral nervous system.

In the beginning of 20th century, it was largely thought that neurogenesis in the central nervous system (CNS) only occurs during embryonic and early postnatal development of the organism but ceases in adulthood, resulting in the inability to regenerate the adult CNS (Cajal, 1913). However, in the 1960s, it has been shown that adult rat brains, although confined to isolated and circumscribed regions, are able to generate new cells. [^{3}H]-thymidine incorporation assay, which labels newly synthesized DNA radioactively, allowed for the visualization of newborn cells in rat brain sections by autoradiography (Altman,

1969). Then in the late 1980s, researchers identified several types of neural progenitor cells in rat brains using retroviral tracing methods (Price and Thurlow, 1988). However, adult neurogenesis was widely disregarded until the discovery that brains of song birds can generate large numbers of new cells during song learning, a process that can undergo repeated seasonal changes in some species (Nottebohm, 1989). With advances in technology over time, and reports from additional animal species, such as axolotl and zebrafish, adult neurogenesis is now a widely accepted phenomenon (Waxman and Anderson, 1985; Holder *et al*., 1991).

In the adult primate CNS, neurogenesis appears to be largely restricted to the ventricular-subventricular zone of the lateral ventricles (Gould *et al*., 2012). Although the subgranular zone of the hippocampal dentate gyrus is another well-established proliferative region in rodents (Kaplan and Hinds, 1977), its contribution to adult neurogenesis in primates is still under debate and different studies show conflicting results of ongoing and stalled neurogenesis in the human hippocampus with age (Boldrini *et al*., 2018; Sorrels *et al*., 2018). The ability to generate new nerve cells in adult brains, thus adult neurogenesis, appears to be inversely related with evolution (Kizil *et al*., 2013; Kaslin *et al*., 2013) and at the lower end of the phylogenetic tree, an increase in the number of proliferative regions in the adult CNS seems to emerge. There are reports of seasonal changes in the size of forebrain nuclei in the brains of zebra finches that correlate with seasonal changes in singing behavior (Korsia and Bottjer, 1985). Reptiles also have been demonstrated to have numerous neurogenic zones in the CNS (Lopez-Garcia *et al.,* 2002). Lastly, teleost fish bear an impressive number of more than 10 distinct proliferative regions in the adult brain (Grandel *et al.*, 2006). It has been suggested that the abundance of regions with active cell division in the CNSs may be related to the continuous growth of the entire nervous system or sensory organs in reptiles, amphibians and fish throughout the life, since a growing amount of sensory input would need more processing capacity in the CNS (Kaslin *et al*., 2008).

It is also worth mentioning that a regenerative neurogenesis response appears to be more successful at repairing damaged nervous tissue in newborn animals than in adults and to be more efficient in the peripheral nervous system as compared to the CNS. A study

comparing hind limb recovery rates of newborn and 1-month-old rats to the same lesion reveals a more complete healing in terms of the functionality in the neonatally manipulated rats (Stelzner and Cullen, 1991). A similar study suggests recovery of motor functions after spinal cord injury in neonatal cats, which was not seen in adult animals (Bregman and Goldberger, 1982). Axolotls emerge as a special case in this context as they reach sexual maturity before undergoing metamorphosis and basically remain in an extended larval stage throughout their lives and they are unmatched in limb and organ regeneration, including complete and functional neurogenesis (Galton, 1992; Mchedlishvili *et al.*, 2012). Considering these evidences, a parallel may be drawn between the success of neonatal/embryonic regeneration response and the level of these animals within the phylogenetic tree. Likewise, the peripheral nervous system has been demonstrated to be more permissive to axon regrowth and nerve cell regeneration (Allodi *et al*., 2012). A major obstacle in CNS regeneration, besides reestablishing the complexity of specific connections between cells, appears to be scar formation by activated glia cells (reactive astrocytosis) and the overall non-permissiveness of glial surface markers, such as myelin-associated proteins, for axon extension (Fawcett and Asher, 1999). Taking these observations into account, studies in lower vertebrates with high regenerative capacity and in the peripheral nervous system may provide valuable insight into how successful regeneration of nervous tissue may be stimulated by molecular signals. In the studies detailed in this thesis, I will focus on the peripheral olfactory system of the zebrafish, which appears to combine both of these properties, to understand how activity of neuronal progenitor cells can be activated by purinergic signals, which is a candidate signaling molecule that is indicative of dying cells (Chow *et al*., 1997).

1.2. Neurogenesis in the olfactory epithelium

The peripheral olfactory epithelium (OE) is unique among vertebrate sensory organs, including the sensory organs of mammals because the receptor neurons in the OE are in direct physical contact with the environment. This is a prerequisite for both chemical senses, taste and smell, since the detected molecules needs to be recognized by the respective sensory neurons in order to generate a response (Farbman, 1992). This unique situation

renders the OE vulnerable to potential damage, which can be caused by (cyto-)toxic chemicals or infectious agents present in the environment. The OE also provides a potential entry route for pathogens to the central nervous system, since olfactory sensory neurons (OSNs) make direct axonal connection to the olfactory bulb (OB) of the forebrain. This raises susceptibility to infection as infectious agents can reach the CNS through the OE by crossing only a single synapse (Monath *et al*., 1983). Probably for a combination of these reasons OSNs have a short life span of 60 to 90 days in rodents (Moulton, 1974; Mackay-Sim and Kittel, 1991; Tsai and Barnea, 2014) and 30 days in zebrafish (Bayramli *et al.,* 2017). An inherent limit to OSN lifetime would, thus, continuously shed OSNs, including infected OSNs, to prevent the spread of viral infections to the CNS.

To balance the continuous loss of OSNs, the OE exhibits unparalleled regeneration and neurogenesis capacity compared to other sensory epithelia (Tsai and Barnea, 2014; Schwob, 2002). The OE has been shown to be able to regenerate after being subjected to various insults, such as irrigation with $ZnSO_4$ or exposure to other cytotoxic agents. In catfish, severe neuronal necrosis is observed in the OE following intranasal $ZnSO_4$ application, which recovered completely within 60 days (Cancalon 1982). A similar study in *Xenopus* revealed a complete regeneration in 70 days (Smith, 1951). Studies in rhesus monkeys showed that the damaged OE can fully regenerate from a non-ciliated population of basal cells (Graziadei *et al*., 1980) while regeneration in the rabbit OE takes place in a similar manner and a heterogeneous progenitor cell population rebuilds the tissue within 30 days (Mulvanev *et al.,* 1971). In mice, complete regeneration of the $ZnSO_4$ treated OE occurs in 72 days, with epithelial surface alteration of the cell populations in the OE up to 14 days post-treatment (Matulionis, 1975). Neuronal loss following olfactory nerve lesion is shown to be repaired by the tissue resident stem cells (Harding *et al.,* 1978). Treatment of the mouse OE with MeBr gas, which causes a more severe damage resulting in destruction of neurons, sustentacular cells (SCs), gland cell, duct cells and basal cells, has been shown to be repaired by two different cell populations. (Huard *et al*., 1998).

In order to support the exceptionally high cell turnover and regenerative capacity of the OE after damage, the rodent OE has been shown to harbor two morphologically and

functionally distinct stem/progenitor cell populations: horizontal basal cells (HBCs) and a population of globose basal cells (GBCs). HBCs are multipotent progenitor cells, which are located in the basal most layer of the OE and react to physical changes in the epithelium, such as loss of other cell types, which is possibly sensed through signaling molecules on the cell surface or primary cilia (Herrick and Schwob, 2015; Joiner *et al.*, 2015). GBCs, are a heterogeneous cell population of round-shaped cells that form a stratum just apical to HBCs. GBCs, different from HBCs, are continuously dividing cells, active in neurogenesis, and react to neuronal loss (Schwob *et al*., 1994a). Neurogenesis in the murine OE occurs in an apically oriented direction. $Sox2^{+}/Pax6^{+}$ GBCs give rise to $Ascl1^{+}$ transit-amplifying progenitors and $Neurog1^{+}/NeuroD1^{+}$ immediate precursor cells, which directly generate neurons and sustain the need for new OSNs in the OE (Guillemot *et al*., 1993; Cau *et al*., 1997; Krolewski *et al*., 2012). HBCs, on the other hand, are quiescent stem cells which are only activated upon direct damage to the epithelium (Leung *et al*., 2007). Interestingly, selective ablation of neurons does not activate HBCs and the expression of Notch ligands suggests that cell-to-cell signaling between SCs and HBCs may be important to keep HBCs quiescent under normal conditions in the intact OE (Herrick and Schwob, 2015). Loss of SC to HBC contacts, on the other hand, is suspected to activate proliferative activity in HBCs.

Interestingly, HBCs emerge late in the development of the OE, after other cell types, including GBCs are established. In the absence of HBCs, through genetic ablation of ΔNp63 (an N-terminally truncated isoform of p63, which is predominantly expressed in the OE) expression, which is essential for HBC formation, the OE develops normally (Holbrook *et al*., 1995; Packard *et al*., 2011b). However, their multipotent contribution to OE repair is activated after chemical lesion of the OE. Leung *et al.* (2007) demonstrated that HBCs are capable of giving rise to OSNs, SCs and ciliated respiratory epithelial cells. Down-regulation of p63 following injury triggers HBCs to differentiate into GBCs and regenerate the various neuronal and non-neuronal cell types in the OE (Schnittke *et al*., 2015) Paradoxically, FACS analysis of fluorescently labeled GBCs and GBC-derived cells revealed that GBCs are not only able to give rise to terminally differentiated but can also generate HBCs (Chen *et al.*, 2004). Collectively, both HBCs and GBCs appear to satisfy requirements for being the tissue stem cells in the OE, however, their complex interconnected relationships are only vaguely understood.

1.3. The structure of the olfactory epithelium

The OE is a pseudostratified tissue containing several cell types with characteristic morphologies and functions. In the basal most layer of the OE reside the HBC and GBC tissue stem cells. SCs are the resident tissue-specific glia cells of the olfactory system and provide support to the OE. In mammals their cell bodies are positioned apically and form the tight apical border of the OE (Ding *et al*., 1991). In between those cells OSNs are located, which project their axons to the olfactory bulb from the basal side of the OE (Graziadei, 1974; Graziadei and Monti Graziadei, 1979). In addition to these cells, Bowman's glands, which secrete mucus, are situated beneath the OE along with blood vessels embedded in the underlying connective tissue (Beites *et al*., 2005; Au and Roskams, 2003; Ramer *et al*., 2004; Carson *et al*., 2006).

The olfactory organ of the zebrafish appears first as the olfactory placode in the developing 18 hpf embryo. Initial reports on the development of the olfactory placode suggested that development of the OE occurs without migration of cells from neighboring regions (Hansen and Zeiske, 1993). However later studies proposed that some kind of cellular migration leads to formation of the placode as there is little to no observable cell division around the placode itself (Whitlock and Westerfield, 2000). Saxena *et al.* (2013) have reported that Sox10:eGFP^{+} cells, which will give rise to microvillous OSNs, are derived from the neural crest and actively invade the olfactory placode, whereas ciliated OSNs may be products of placode-associated tissues. However, Harden *et al.* (2012) reported that Sox10:eGFP^{+} cells are absent in the later embryonic OE and, similarly, our laboratory also never observed any Sox10^{+} cells in the neuronal layers of the OE. Regardless of the molecular underpinnings of these events, OSN neurogenesis appears to originate mainly from extraplacodal tissue in the early embryo and progenitor cells with neurogenic capacity settle at later time points to generate OSNs from within the OE in later larvae and adults.

As development progresses, the initially flat OE starts folding onto itself about 33 dpf and to develop into a rosette-like structure containing radially projecting lamellae. Each

lamella consists of two layers of epithelium connected to each other at their basal sides (Hansen and Zeiske, 1993). The adult zebrafish OE resembles the mammalian OE structurally and molecularly, however with some clear distinctions. The vomeronasal organ, a separate olfactory organ of some vertebrate species, which constitutes specialized microvillous sensory neurons for sensing of pheromones and which is located more anteriorly in the nasal cavity is absent in zebrafish (Silva and Antunes, 2017). Yet, microvillous sensory neurons expressing vomeronasal receptors are integrated into the OE alongside classical ciliated OSNs (Saraiva *et al.*, 2015). Thus, the zebrafish OE combines multiple chemosensory subsystems into a single structure. As of now, four different neuron types, namely microvillous, ciliated, crypt and kappe neurons have been described and functionally characterized to various detail (Sato *et al.*, 2005; Ahuja *et al.*, 2014; Hansen and Finger, 2000). In contrast, non-neuronal cell types residing in the OE have not been described in any detail. Our research group has contributed to the identification of non-neuronal cells and provided evidence for the presence of SCs in the adult zebrafish OE (Bali, 2015). These cells are reactive to an antibody generated against the *Xenopus* Cytokeratin II family. Interestingly, zebrafish SCs have an inverted morphology compared to SCs described in other species with cell bodies positioned basally instead of forming the apical border. Another interesting aspect is that zebrafish SCs can generate Ca^{2+} signals and possibly take a role in cell-to-cell communication in the OE (Bali, 2015). The studies on functional and molecular characterization of other cell types in the adult zebrafish OE are currently ongoing (Kocagöz, unpublished). Primary data for a possible HBC-like basal cell population is presented in this thesis along with the observation that the developing 2.5 dpf olfactory placode already contains $Sox2^{+}$/Cytok II^{+} SCs and OSNs located more apically.

OSNs cover about two-thirds of the inner section of each lamella leaving a non-sensory region in the peripheral OE. Unlike the mouse OE, the zebrafish OE has two distinct zones of neurogenesis under normal/maintenance conditions in the intact tissue: (i) the interlamellar curve (ILC) and (ii) the sensory/non-sensory (S/NS) border of the epithelium. Newborn cells are passively displaced towards the middle of the lamellae over time and are eliminated by an unknown mechanism about 30 d later (Bayramli *et al.*, 2017). Upon chemical insult with the common laboratory detergent Triton-X 100, however, the regenerative capacity of the OE is stimulated and extensive proliferation can be observed

throughout the OE also in regions outside the ILC and S/NS. (Iqbal and Byrd-Jacobs, 2010; Çapar, 2015; Kocagöz, unpublished). It is important to note here that upon damage, progenitor cells all over the OE are activated and the neurogenic activity at the ILC and S/NS become indistinguishable from the rest of the tissue, which is a hallmark of the regenerative phase of neurogenesis. The degree of damage can be observed by the disappearance of immunoreactivity to the pan-neuronal marker HuC/D, which is indicative of neuronal death. Regeneration of the zebrafish OE is rapid and complete within 7 d. Restoration of the tissue goes along with a gradual change in the pattern of proliferation and 7 d post injury the two main proliferative regions in the OE reappear concomitantly with the reestablishment of HuC/D^{+} neurons in the sensory region (Çapar, 2015; Kocagöz, unpublished).

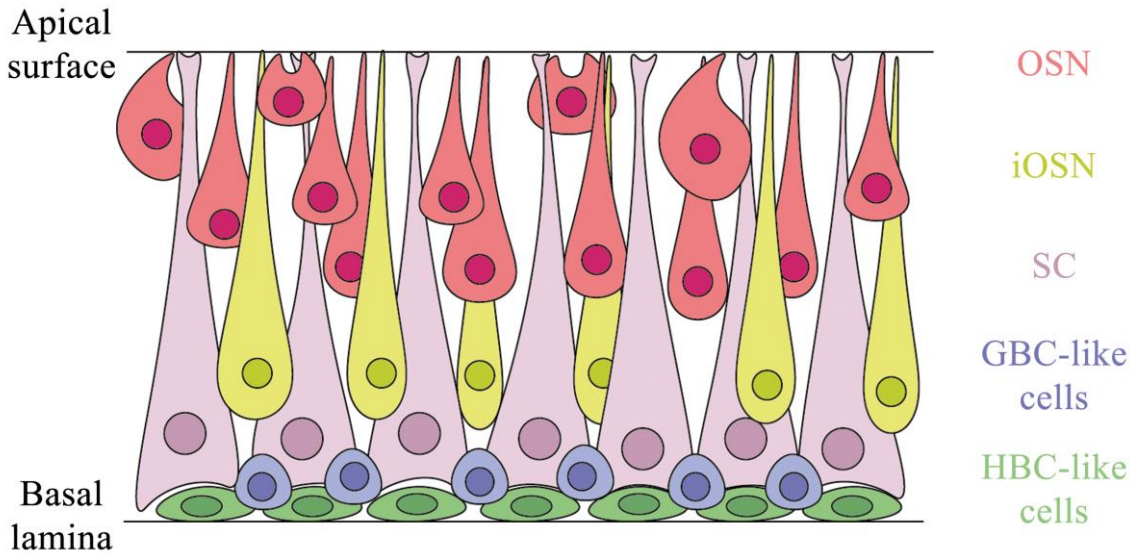

Figure 1.1. Schematical representation of the zebrafish olfactory epithelium.

1.4. Extracellular ATP as a danger associated molecular pattern and the contribution of purinergic signaling to the neurogenesis

Numerous growth factors are shown to affect the cell proliferation in the OE. Leukemia inhibiting factor, basic fibroblast growth factors, epidermal growth factor and transforming growth factor-α have all been shown to increase proliferation in OE (Bauer *et al.*, 2003; DeHamer *et al*., 1994; Carter *et al*., 2004; Farbman and Buchholz, 1996), whereas growth and differentiation factor 11 and transforming growth factor-β have an inhibitory

effect (Wu *et al*., 2003). However, the regulatory effects of nucleotides, which are known to be potent signaling molecules for epithelial cells, on the neurogenesis in the OE comprise a relatively recent topic of investigation (Schwiebert and Zsembery, 2003). Another change in concept may be linked to regenerative neurogenesis and pertains to the direct or indirect function of immune cells (Kizil *et al*., 2015).

It has long been believed that the main goal of the immune system is to discriminate "self" from "non-self". The information of a possible infection by pathogens is sensed by pattern recognition receptors (PRR), such as Toll-like receptors (TLR). Pathogens, over the course of evolution, developed signature molecular patterns and immune systems have co-evolved to recognize those molecules (Janeway, 1989). But this understanding was not sufficient to explain other phenomena, such as the immune response induced after a trauma or the co-existence of mutualist bacteria in the guts. Therefore, Matzinger (1994) called for a change of mind in the field of immunology and introduced the idea that the primary goal of the immune system may be to distinguish between what is dangerous and what is not. With the finding that substances such as the High mobility group 1 (HMGB1) protein and uric acid are capable of eliciting strong immune responses when found outside the cell, the concept of danger associated molecular patterns (DAMP) was formed (Scaffidi *et al*., 2010; Shi *et al.,* 2008). Today it is well established that there is a wide spectrum of DAMPs and their corresponsive PRRs are identified (Schaefer and Anders, 2014; Bianchi, 2007; Idzko *et al*., 2014).

There are three families of purine receptors which differ in their ligands. P1 receptors are mainly sensitive to adenosine and P2 type receptors are activated upon binding to adenine and/or uridine nucleotides. P1 receptors are GPCRs which have important roles in inflammation in numerous tissue types such as nervous and cardiovascular tissues (Fredholm *et al*., 1997). They are shown to contribute to physiological processes like cell migration and proliferation (Burnstock, 2002). P2 type receptors on the other hand consist of two different types of receptors: P2X and P2Y purinoreceptors. P2X receptors are ligand-gated ion channels, which are permeable to Ca^{2+}, Na^{+} and K^{+} ions upon binding to ATP (Kolb and Wakelam, 1983). Similar to P1 receptors, P2Y receptors are also GPCRs but they are

sensitive to nucleotides such as ATP and UTP and they are shown to increase cytoplasmic levels of secondary messenger molecules such as inositol phosphate, Ca^{2+} and cAMP upon binding to their ligands (Waldo and Harden, 2004). A newly characterized subfamily of purine receptors is the P0 family. P0 receptors are also GPCRs and they are found to be sensitive to adenine and there is evidence that they cause an increase in the amount of intracellular cAMP and Ca^{2+} ion concentrations upon binding to adenine (Thimm *et al*., 2013).

ATP, as one of the most potent DAMPs, is shown to be secreted not only from necrotic cells but also from cells under stress as well as immune cells themselves underlining the function of extracellular ATP in inflammatory conditions as reviewed by Eltzschig (2008). Extracellular ATP is sensed by neighboring cells via P2Y and P2X purinergic receptors (Khakh and Burnstock, 2009), which in turn trigger a signaling cascade resulting in an increase in the intracellular Ca^{2+} content (Buvinic *et al*., 2009). This sudden peak in the intracellular Ca^{2+} concentration is shown to trigger expression of a spectrum of transcription factors, including factors that can stimulate cell proliferation (Cruzalegui *et al*., 1999).

There are several studies indicating the importance of purinergic signaling in the OE. P2Y receptors are shown to be expressed in basal progenitor cells and SCs in the mouse OE (Hegg *et al*., 2003). Hassenklöver *et al*. (2009) reported Ca^{2+} responses in *Xenopus* OE upon extracellular ATP application, which diminishes with the introduction of suramin, a P2X and P2Y receptor blocker, indicating that the Ca^{2+} response was mediated by purinergic receptor signaling. They also show a decrease in the number of $BrdU^{+}$ proliferative cells in the OE, following intraperitoneal application of suramin. Jia *et al.* (2009) demonstrated an increase in proliferation in the Swiss Webster mouse OE upon intranasal application of ATP solution, which was also reverted with the addition of suramin to the solution.

In this study, I intended to contribute to the identification of possible progenitor cell populations in the adult zebrafish OE by temporally and spatially analyzing the Ca^{2+}

response generated upon extracellular application of ATP. Furthermore, I show that IHC against krt5 labels a basal cell population of horizontal cells in the adult zebrafish OE, which is not present in the developing olfactory placode of zebrafish embryo up to 7.5 dpf. Morphometrically, this krt5^{+} population overlaps with a population of the cells eliciting Ca^{2+} response to ATP. In addition to these analyses, I propose a regulation of neurogenesis through purinergic signaling in the zebrafish OE. The results show an increase in the proliferation in the OE, especially in the ILC regions but also across the sensory OE. This increase is sensitive to suramin, which indicates that this increase may be a result of a signaling cascade starting with the activation of P2Y and P2X receptors.

2. PURPOSE

The cells and the signals that contribute to OSN neurogenesis are not well understood in the zebrafish OE. Two modes of neurogenesis contribute to maintenance of the tissue under constant loss of OSNs and repair from acute injury. The lineage relationship between the cells that execute maintenance and repair neurogenesis have not been studied in zebrafish and it is currently unknown if, and to which extent, these cell populations respond to common signaling molecules. Among the wide variety of signaling compounds that have been shown to affect OSN neurogenesis, purinergic signaling is interesting because purine compounds are expected to be released by dying cells. Here I wish to investigate in more detail the tissue distribution and characteristics of purine-responsive cells and the effect of purine stimulation on progenitor cell activity.

As a first aim, I wanted to identify and to characterize the cell populations that are sensitive to extracellular purine application by measurements of changes in internal Ca2+ and investigating their morphology and tissue distribution. From the measured parameters I made attempts to establish a link between these cell populations and known markers and positions of candidate progenitor cells in the OE.

As a second aim, I was interested in the effect of purine stimulation on the tissue and examined proliferative responses of progenitor cells in the OE upon intraperitoneal ATP injection and compare them to characteristic changes in the pattern of cell proliferation upon chemical insult by BrdU incorporation assays.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1. Fish

The zebrafish, *Danio rerio*, used in this study were obtained from a local pet shop and kept in the fish room at Boğaziçi University Life Sciences Center (Vivaryum). Only for calcium imaging, wild-type Tüpfel-longfin zebrafish were provided by the zebrafish facility at the University of Göttingen.

3.1.2. Equipment and Supplies

A detailed list of equipment and chemicals used in this study is provided in Appendix A and Appendix B.

3.1.3. Buffers and Solutions

Unless otherwise stated, all buffers and solutions were prepared according to Sambrook and Russell (1989). Zebrafish specific solutions were prepared according to Westerfield (1997).

3.2. Methods

3.2.1. Zebrafish Maintenance and Breeding

PS-WT fish were kept under a 14 hours light / 10 hours dark cycle at a room temperature of 28°C. The fish were kept in separate tanks with volumes of 1-, 3- and 10 l and the number of fish in tanks are arranged according to the size of the container. These tanks were connected to a zebrafish housing system (Stand Alone System, Aquatic Habitats, FL), which provided a continuous flow of filtrated, aerated and UV sterilized water. 100 l of reverse osmosis water was mixed with 2 g sea salt, 7.5 g of sodium bicarbonate and 0.84 g of calcium sulfate for preparing fresh water for the systems. Feeding of the fish was carried twice a day in weekdays with dry flake food (TetraMin, Sera Vipan) in the mornings and live brine shrimp larvae (*Artemia sp.*) in the evenings. On weekends, fish were fed with live *Artemia* once.

In order to obtain embryos, couples of zebrafish were put in breeding chambers, which allowed for the separation of adults from the eggs. The setups were prepared in the evening and the second feeding was skipped in order to promote spawning. The fish were kept in approximately 500 ml of fresh water overnight. After spawning following the first light in the morning, the adult fish were moved back into their tanks and eggs were collected via a Pasteur pipette which sank to the bottom of the breeding chamber. Newborn fish were taken care of petri dishes containing up to 50 embryos with daily renewal of about 75% of E3 embryo medium and removal of dead embryos. After 5 dpf, fish were transferred to the system in 1 l tanks with special filters to avoid escaping and fed with 1 day old live *Artemia* larva and grounded flake food until 10 dpf, after which normal feeding routine was continued.

3.2.2. Dissection of the olfactory epithelium

In order to obtain olfactory epithelia, fish were first euthanized in ice-water. 10 mins after the gills have stopped, the zebrafish was decapitated with a sterile surgical knife. The head was then transferred into 1x ice-cold phosphate buffer saline (PBS, Sigma). Under a stereo microscope (Olympus), using fine forceps, cranial bones were removed starting from posterior side of the head, which exposed the brain and olfactory epithelia. The bones to which the olfactory epithelia were attached were carefully separated from the rest of the head and with the disruption of axonal bundle connecting the OE to OB, the OE was ready for further analysis.

3.2.3. Sectioning of the OE

For immunohistochemistry, cryosections of the OE were prepared. After dissection, the OE was put in optimal cutting temperature medium (OCT, Sakura Technologies). For immediate cryosectioning, the OE frozen -20°C for up to 1 h, if the cyrosectioning was to be performed at a later date, the OE was placed into -80°C. Then the OE was transferred in frozen OCT to the precooled cryostat (Leica) for cryosectioning. 12 µm-thick slices of the OE were cut in the cryostat and transferred to positively charged microscope slides and dried in a ventilated oven for 1h at 65°C. If needed for later use, dried sections were transferred to -80°C.

The OE sections for functional calcium imaging are obtained using a Leica VT1200S vibratome. The dissected OE was embedded into 2% low-melting agarose, which was fixed to the cutting plate with the help of one drop of superglue and immediately moved into the sectioning chamber containing freshly prepared artificial cerebrospinal fluid (ACSF) solution. With the help of the vibratome 140 µm sections of the OE are cut and placed onto special slides filled with ACSF and held in place with a very fine metal mesh.

3.2.4. Immunohistochemistry on OE sections

Sections were rehydrated with 1x cold PBS for 5 mins and fixed with 4% cold paraformaldehyde (PFA, pH 7.4) solution for 10 mins in humidity chambers containing wet tissue papers. Then, slides were transferred in Coplin jars (VWR, The Netherlands) and washed 3 times 10 mins with 0.1% Triton-X 100 (Sigma) in 1x PBS (PBST) at room temperature (RT) on a shaker. The sections were put back into humidity chamber and blocking was performed using 3% bovine serum albumin (BSA) in 1x PBS for 1h at RT. The blocking solution was then removed and sections were incubated with primary antibodies, in freshly prepared blocking solution for 2h at RT or overnight at 4°C in the humidity chamber. The concentrations of the primary antibodies used were as follows: mouse anti-human neuronal protein HuC/D (1:1000, Abcam), rat anti-BrdU (1:250, Abcam), rabbit anti-keratin 5 (1:1000, Abcam), mouse anti-cytokeratin type II (1:1000, Developmental Studies Hybridoma Bank), mouse anti-EBF (1:1000, Santa Cruz Biotechnology), and rabbit anti-Sox2 (1:1000, Abcam). After incubation with primary antibody, the sections were washed with PBST at RT 3 times on shaker and incubated with secondary antibody in PBST either for 2 h at RT or overnight at 4°C in humidity chamber. Anti-rabbit Alexa Fluor 488, anti-mouse Alexa Fluor 488, anti-rabbit Alexa Fluor 555, anti-mouse Alexa Fluor 633 and anti-rat Alexa Fluor 633 (Life Sciences) secondary antibodies were always used in 1:800 dilutions. After incubation with secondary antibody, the slides were washed with PBST 3 times and imaged under a laser-scanning confocal microscope (Leica).

3.2.5. Immunohistochemistry on whole-mount embryos

For whole-mount antibody staining, (dechorionated) embryos were fixed for 3 h at RT or overnight at 4°C with 4% PFA in 2 ml Eppendorfs. After fixation, the embryos were rinsed twice and washed 3 times for 10 mins with PBST on shaker. The embryos were then gradually transferred to methanol (MeOH) with one 5 min wash with 50% MeOH / 50% PBST and 3 times 5 min washes in 100% MeOH. The embryos were then placed at -20°C

overnight in order to aid permeabilization. In MeOH, fixed embryos can be stored up to 6 months at -20°C.

In the first day of staining, the embryos were rehydrated with on 5 min wash using 50% MeOH / 50% PBST and 3 times 5 min washes with PBST. The stock solution of Proteinase K (PK, 20 mg/ml, Finnzymes) was diluted to 1:1000 in PBST and 1x PK solutions were prepared. The embryos were treated with PK with concentrations and durations depending on their ages: 2.5 dpf embryos were treated with 1x PK for 30 mins and 7.5 dpf embryos were treated with 3x PK for 30 mins. The embryos were then rinsed 3 times with PBST and post-fixed in 4% PFA for 20 mins at RT. The embryos were washed again 3 times for 5 mins with PBST. After post-fixation, the embryos were incubated in 3% BSA in PBST for 1 h at RT for blocking. The embryos were then incubated in primary antibodies, overnight at 4°C on shaker. The dilutions of primary antibodies were as follows: rabbit anti-Sox2 (1:100, GeneTex), mouse anti-cytokeratin type II (1:100, Developmental Studies Hybridoma Bank), rabbit anti-keratin 5 (1:100, Abcam) and rabbit anti-Tp63 (1:100, GeneTex).

In the second day, the primary antibody was removed by 3 times rinsing and 6 times 30 min washes with PBST. The embryos were then incubated in secondary antibody with a dilution of 1:200 overnight in PBST at 4°C on shaker. The secondary antibodies used were: Anti-mouse Alexa Fluor 488, anti-rabbit Alexa Fluor 633 and anti-rabbit Alexa Fluor 488 (Life Sciences).

In the third day of the staining, the embryos were rinsed 3 times and washed 6 times for 30 mins with PBST and placed in PBS. The embryos were then embedded into 2% agarose, placed on a cover slide and imaged under a laser-scanning confocal microscope (Leica).

3.2.6. BrdU incorporation assay

The fish were incubated for 12 h in fresh system water containing 30 mg/L BrdU in order to facilitate incorporation of BrdU to the dividing cells. Same steps for immunohistochemistry on OE sections were conducted with an extra step of a 10 min 4N HCl treatment after fixation, which is followed by 3 times 10 min washes with PBST. HCl denatured the DNA on the slides, exposing BrdU for the anti-BrdU antibody to recognize.

3.2.7. Intraperitoneal injection of zebrafish

Fish were anesthetized in MS-222 solution (Sigma) until gill movements slowed down and transferred onto a piece of sponge soaked in cold water. 50 µl of the corresponding solution was injected with an insulin needle anterior to the pelvic fin into the abdomen of the fish, avoiding damage to the internal organs. Fish then were placed back into system water for recovery.

3.2.8. Chemical lesion on the OE with Triton-X 100

Following anesthesia with MS-222 (Sigma), approximately 1 µl of 1% Triton-X 100 solution in 0.1 M PBS with 0.1% phenol red was administrated using a pulled glass capillary tube into one nasal cavity of the adult zebrafish 2 times for 45 s each under a stereo microscope (Olympus). Applied solution was flushed away with artificial system water and the fish was put back in tank filled with system water to recover. Untreated OE was used as an internal control.

3.2.9. Functional calcium imaging

Freshly prepared vibratome sections of the OE were incubated with the Ca^{2+} sensitive dye Fluo4-AM (excitation wavelength 488, Life Technologies) for 35 mins at RT in dark. Following a 5 min wash with 1x ACSF in order to remove excess dye, the sections were placed under a perfusion stream of ACSF on the stage of a LSM 780/Axio Examiner confocal microscope (Zeiss). Images were obtained at an imaging speed of 1 Hz. After first 10 images were acquired, 100 µM ATP was added to the perfusion and changes in the fluorescence were captured.

3.2.10. Data analysis

Morphometric analyses were conducted using the measurement function in FiJi image analysis software. (Schindelin *et al*., 2012). The horizontal and vertical lengths of cells relative to the basal lamina were measured. Positional data of the cells were measured as a distance from the basal lamina and normalized using the thickness of the lamella on that point. $BrdU^+$ cells in the ILCs were counted by hand in LAS X software (Lecia). Absolute numbers of $BrdU^+$ cells on the whole lamella was counted by a custom macro on FiJi.

4. RESULTS

4.1. krt5 antibody labels an HBC-like cell population in adult zebrafish OE

The pseudostratified organization of the mammalian OE is well understood by morphological and molecular characterization of non-neuronal cell types and their functions in the tissue. The basal most layer of the OE is occupied by $p63^+$ and $Krt5^+$ HBCs, which are capable of giving rise to all other cell types in the OE, including OSNs, SCs, and various populations of intermediate neuronal progenitor cells (Fletcher *et al*., 2011; Leung *et al*, 2007). Situated more apically are two different populations of GBCs; the $Mash1^+$ stem cells and their $Ngn1^+$ daughter cells, which produce HuC/D^+ ORNs (Cau *et al*, 2002; Iwema and Schwob, 2003). Along with these cell types, $SUS\text{-}1^+$ SCs provide structural support to the tissue, remove dead cells by phagocytosis, and promote cell division by releasing growth factors (Hempstead and Morgan, 1983; Suzuki *et al*, 1996; Bauer *et al*, 2003). SCs typically span the entire apico-basal dimension of the OE with their apically positioned cell bodies forming the border of the OE and their basal processes interacting with HBCs and GBCs.

Although containing cell types with similar characteristics or morphologies, the exact organization and molecular definition of the zebrafish OE is only poorly characterized. Previously, it has been shown that SCs can be identified in the zebrafish OE according to their immunoreactivity with a Cytokeratin type II antibody (Bali, 2015), which stains a similar population of cells in the *Xenopus laevis* OE (Hassenklöver *et al*, 2008). These newly identified SCs also appear to express the stem cell marker transcription factor Sox2 (Bali, 2015). Yet, due to the filamentous nature of the Cytok II proteins, it is not clear whether SCs are the only $Sox2^+$ cell population in the zebrafish OE.

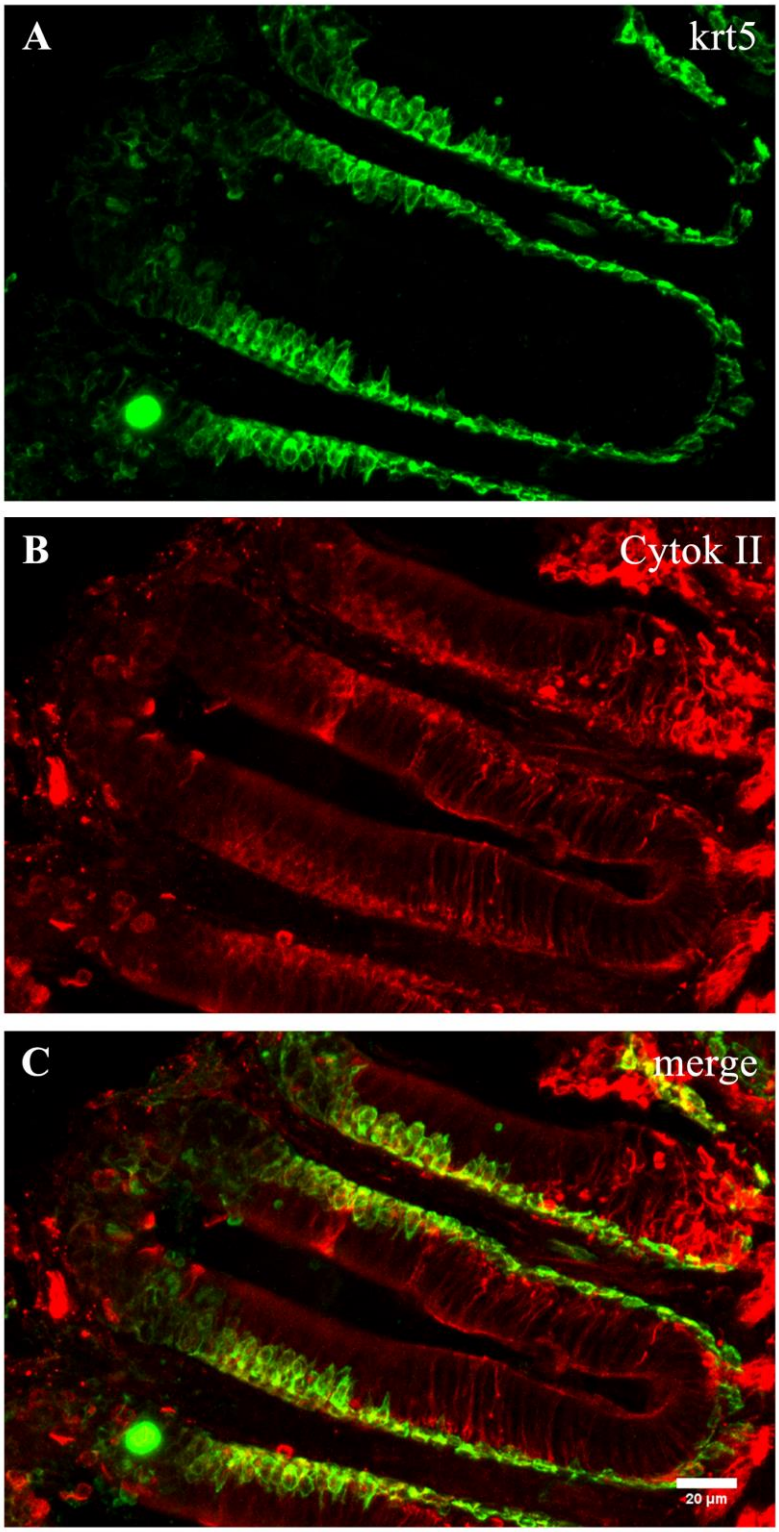

Figure 4.1. krt5 antibody labels a distinct basal cell population with horizontal cell bodies surrounding the epithelium. Confocal stacks of vibratome sections stained with anti-krt5 antibody (A). SCs with cell bodies residing more apically labeled with anti-Cytokeratin II antibody. (B). Overlay of the images of both cell populations (C).

In order to investigate the presence of possible stem/progenitor cell populations with characteristics similar to cells in the murine OE, immunohistochemistry with Cytok II and krt5 antibodies was performed on vibratome sections through the adult zebrafish OE (Figure 4.1). Interestingly, in the sensory region of the OE, anti-krt5 antibody staining labeled a distinct, one-layered cell population. These cells are flat-shaped with horizontally elongated cell bodies and are positioned more basally than Cytok II-immunoreactive SCs. At the transition between the sensory and the surrounding non-sensory epithelium, some of these cells showed more globose profiles and possessed apical extensions, which may indicate that these cells are capable of undergoing asymmetrical cell division. The sensory/non-sensory border and the interlamellar curves of the sensory OE define regions of high proliferative activity (Bayramli *et al.*, 2017) and cells generated at these sites contribute to maintenance of the OSN population. Consistent with the idea that krt5^{+} cells need to undergo morphological changes, including the adoption of clear apico-basal polarity, occasional krt5^{+} cells could be identified at the interlamellar curves but rarely within the sensory OE. In the more peripheral non-sensory region however, krt5^{+} cells had a columnar organization, appeared to protrude more apically, eventually forming multilayered structures with rounder cell bodies. Interestingly, these cells also seemed to be immunoreactive to Cytok II and Krt5, different from the cells in the sensory OE, which are either krt5^{+} or Cytok II^{+} but not both.

Further analyses has shown that the krt5-immunoreactive cell population in the sensory OE also exhibits Sox2 and Tp63 immunoreactivity, which further supports the idea that these cells may be related to HBC-like stem/progenitor cells. Interestingly, these cells show morphological diversity with cells showing more complex morphologies overlapping with sites of active proliferation in the intact and unperturbed OE.

4.2. Analysis of HBC-like cell population in 2.5 and 7.5 dpf zebrafish embryos

After it has been established that a Krt5-immunoreactive cell population with candidate HBC-like progenitor characteristics can be found in the adult zebrafish OE, its presence and origin in developing zebrafish larvae were investigated. In the mouse OE HBCs have been reported be derived from cells outside the olfactory placode, to be generated late, and to invade the OE postnatally (Packard *et al*., 2011). Immunohistochemistry on whole-mount 2.5 dpf zebrafish embryos with antibodies against Sox2 and Cytok II (Figure 4.2) clearly showed the presence of Cytok II$^+$ SCs in the olfactory placode. Their round cell bodies were filled with Sox2$^+$ nuclei, positioned basally, and intracellular filaments spanned the entire apico-basal dimension of the tissue. SCs form a single cell layer in the early olfactory placode. However, HBC-like Sox2$^+$ cells with horizontally elongated cell bodies could not be observed as a distinctive layer beneath SC cell bodies as it seemed to be the case in the adult OE. Nevertheless, there was a curious population of Sox2$^+$ cells with globular nuclei, which formed a triangular cluster directly underneath the developing olfactory placode. Consistent with the absence of a distinct layer Sox2$^+$ cells in the basal OE, immunohistochemistry against Tp63, another marker for HBCs in the murine OE, did not reveal any Tp63$^+$ cells in the olfactory placode of 2.5 dpf whole-mount zebrafish embryos. This observation further supports the idea that HBC-like cells may not be present in the larval OE at 2.5 dpf (Figure 4.3).

Immunohistochemistry on 7.5 dpf zebrafish embryos with anti-krt5 antibody labeled two distinct cell populations in the vicinity of olfactory placode (Figure 4.4). First type of cells which were imumunoreactive to anti-krt5 antibody were located in the olfactory placode with vertically elongated cell bodies and seemed to be positioned more apically than the SCs. The second population was visible as a tightly clustered collection of cells with globular nuclei near the olfactory placode, possibly in the same position which is occupied by Sox2-immunoreactive cells in 2.5 dpf embryos. It is also shown in the literature that certain stem cell populations invade the OE later in the development, which supports the idea that HBC-like cells may integrated into the tissue at a later stage (Aguillon *et al*., 2018). Collectively, the data suggest that the progenitors of HBC-like cells may be in close

proximity to the olfactory placode as early as 2.5 dpf but they do not integrate into the tissue before 7.5 dpf.

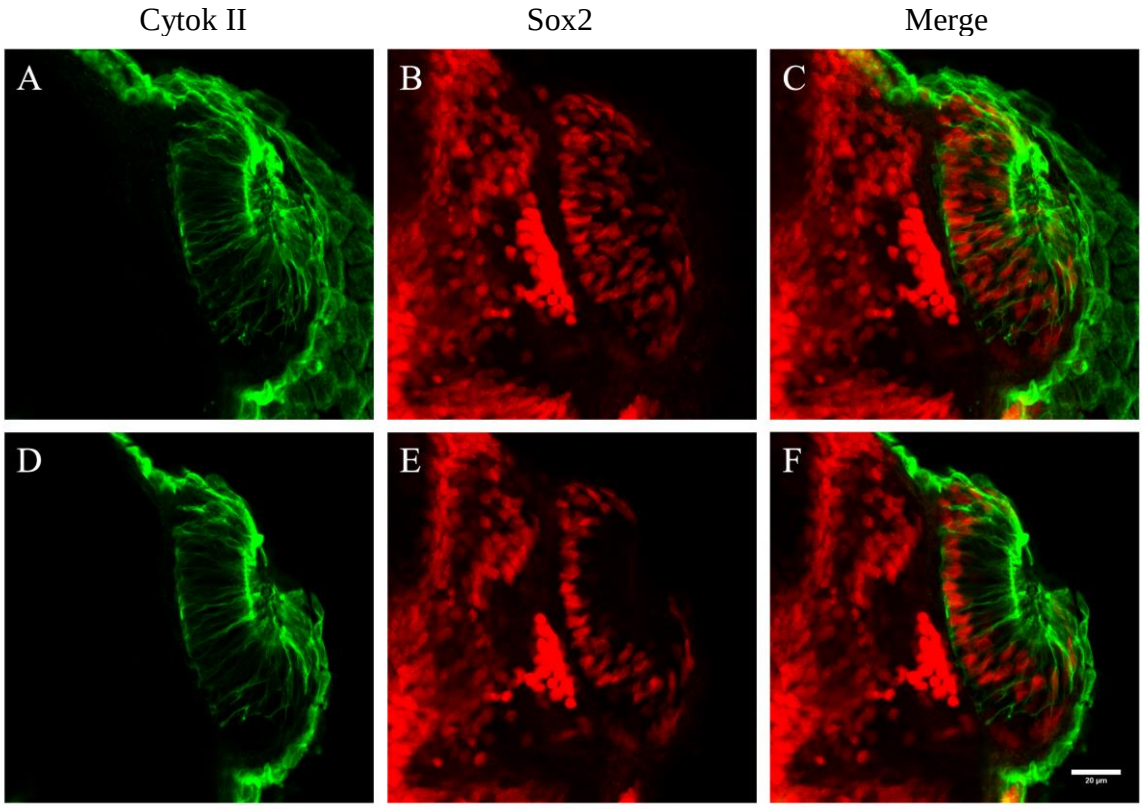

Figure 4.2. SCs and $Sox2^{+}$ cells in and around the developing OE. Top row: Maximum projected confocal stacks, Bottom row: Single confocal sections of IHC on whole-mount 2.5 dpf zebrafish embryos showing SCs labeled with Cytok II (green) (A,D). Nuclear stem cell marker Sox2 (red) (B,E). Overlay of the images (C,F)

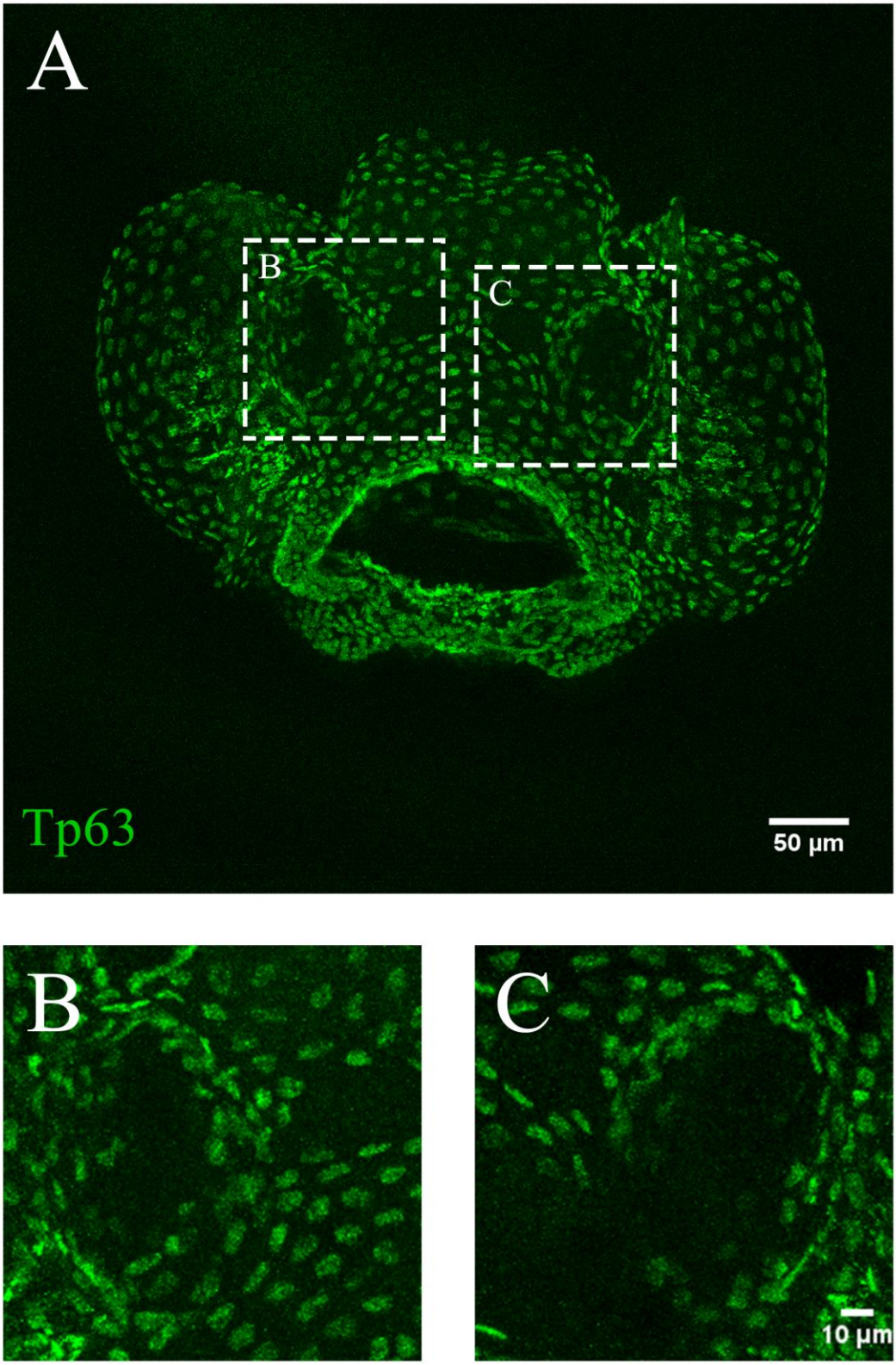

Figure 4.3. Tp63^{+} cells are absent in the OE of 2.5 dpf zebrafish embryo. Maximum-projected confocal stacks of IHC on 2.5 dpf zebrafish embryo with antibody against Tp63 (A). Close-up confocal stack of the right OE (B). Close-up image of a thinner confocal stack of the right OE (C).

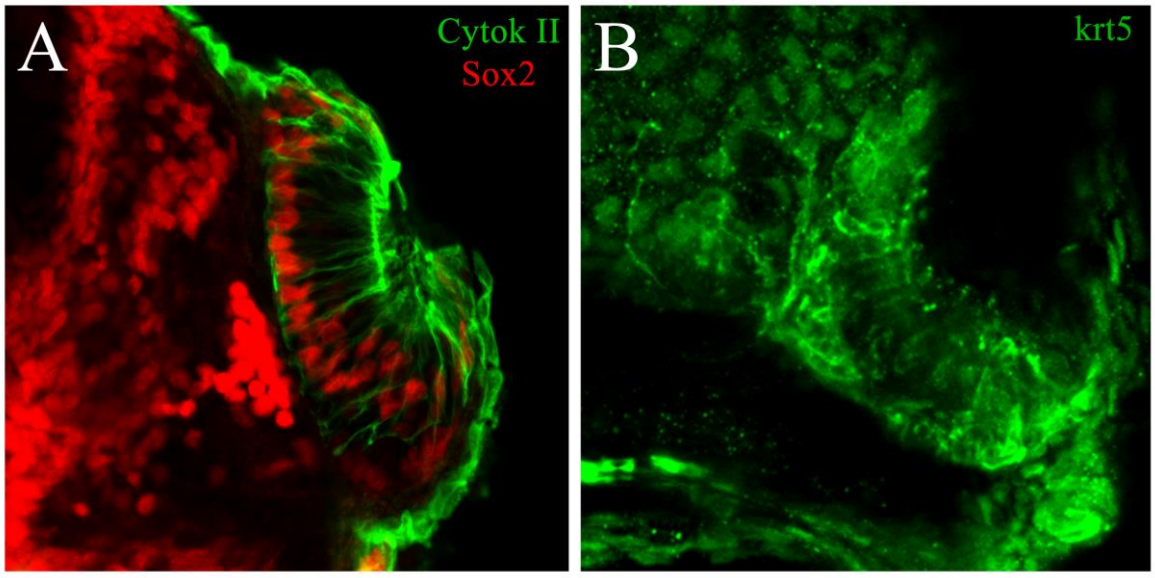

Figure 4.4. A $Sox2^{+}$ population of cells are positioned next to the olfactory placode. SCs are labeled with both Sox2 (red) and Cytok II (green). Another $Sox2^{+}$ population is positioned next to the developing epithelium of 2.5 dpf zebrafish embryo (A). Confocal image of cells labeled with anti-krt5 antibody (green) in 7.5 dpf embryo (B).

4.3. $Ca2^{+}$ Response of the OE to extracellular ATP

A major open question in the regulation of stem/progenitor cell activity in the OE is how the tissue communicates with basal progenitors and how their proliferative activity is adjusted to the momentary need as both over- and underproduction of neurons would be detrimental for its function. DAMPS are intracellular molecules which are released by necrotic cells and have been shown to trigger proliferation and migration of various stem cell populations (Scaffidi *et al.*, 2002). ATP and other purine compounds are concentrated in cells and have been shown to act as DAMPs when found extracellularly as a result of necrosis. Thus, a possible way to identify candidate stem/progenitor cell populations functionally is to analyze their Ca^{2+} responses upon extracellular application of ATP. This can be done by utilizing a membrane-permeable Ca^{2+} indicator dye, such as Fluo4-AM, which undergoes changes in fluorescence relative to the concentration of intracellular free Ca^{2+}. Once inside the cell, the acetoxymethyl moiety of the Fluo4-AM dye gets cleaved by intracellular esterases. This way the dye loses its membrane-permeability and remains inside

living cells and the preparation can be used to record changes in intracellular Ca^{2+} by conventional or confocal microscopy.

To investigate the responses of adult zebrafish OE to extracellular ATP, in vitro Ca^{2+}-imaging was performed on live 50 µm-thick vibratome sections of the tissue, which were kept under a constant perfusion stream of ACSF to aid their survival. 100 µM ATP was applied into the stream of ACSF and time series of images corresponding to the fluorescence of the dye were obtained by a confocal laser scanning microscope. The average change in fluorescence intensity, ΔF/F, was then calculated in ImageJ along with the profile of the response (Figure 4.5). Cooler colors represent no or low changes in fluorescence and warmer colors indicate strong intracellular Ca^{2+} release. Spreading across the whole section from the bottom of the image, Ca^{2+} response started with a rapid and sharp increase in fluorescent intensity followed by a gradual decline of the signal. In principle, two distinct cell populations with different tissue distribution and temporal response profiles could be identified. The stronger and earlier signals were mostly observed to be generated by basal cells with horizontally positioned cell bodies in the center of and between lamellae. In the sensory region of the OE these cells formed a continuous band of cells that was only a single layer of cells in dimension, reminiscent of $Krt5^{+}$ cells. These basal cells showed a more prominent response in the non-sensory areas of the OE, concomitant with a change in the morphology of responding profiles. In the non-sensory region of the OE, responding cells were thicker, multilayered and extended more apically, again reminiscent of the morphology of $Krt5^{+}$ cells in these regions. In addition to basal cells, cells with globular cell bodies or vertically elongated profiles lying more apically also generated Ca^{2+} responses. The columnar signals were most likely generated by SCs spanning the apicobasal length of the epithelium in addition to more globular neuronal populations that have also been shown to respond to purine compounds (Friedrich and Korsching, 1997).

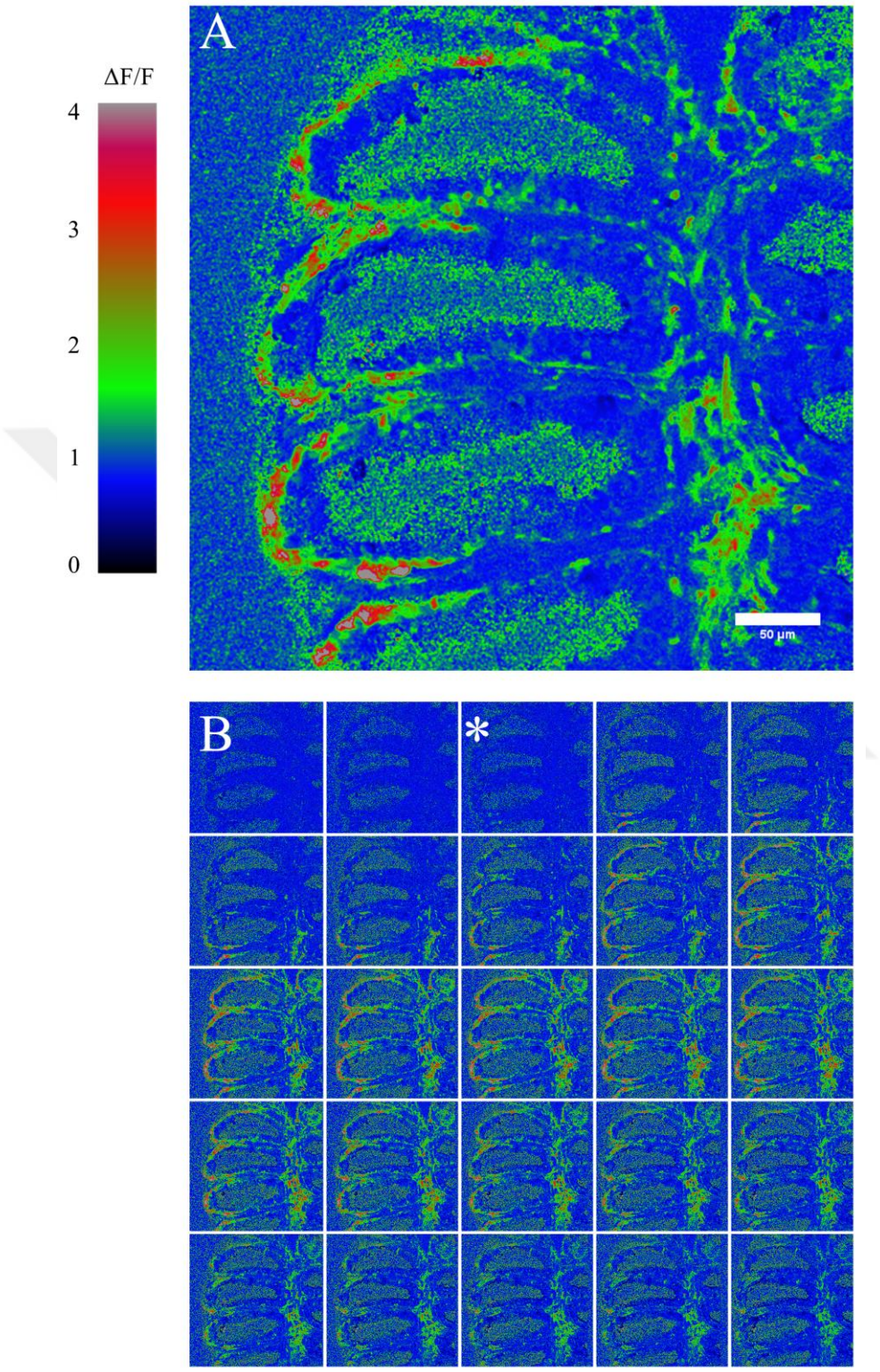

Figure 4.5. Ca^{2+}-response of the adult zebrafish OE following application of ATP. The Ca^{2+}-response is visible throughout the cross section as an increase in the fluorescent intensity with color scale for ΔF/F (A). Temporal representation of the change in the fluorescence. Application of ATP was indicated by asterisk (B).

4.3.1. Spatial analysis of Ca^{2+} response

The structure of murine OE is well-understood with HBCs and GBCs, two populations of progenitor cells, located basally, immature sensory neurons around the middle of the epithelium and mature sensory neurons located apically along with SC bodies. Similar to murine model, cells bearing diverse functions are positioned differently in the pseudo-stratified structure of the zebrafish OE, including a spatial segregation of ciliated and microvillous OSNs (Sato *et al*., 2005). Due to the stratified organization of the OE, analyzing the positions of Ca^{2+} responses may reveal the cell types that generate them.

In order to investigate whether there are spatially distinct populations responding to extracellular ATP in the adult zebrafish OE, the positions of the Ca^{2+} responses were analyzed quantitatively. Normalized positions of cells responding to ATP stimulation (n=50) were obtained by dividing the distance of the responding cell from the basal membrane by the total thickness of the OE at the position of cell. Supporting previous data (Bali, 2015), the dispersion of the normalized positions suggests the presence of two distinct cell populations, one strictly positioned basally and in direct contact with basal lamina and a second population that is dispersed in the basal half of the epithelium. Interestingly, the positions of ATP-responsive cells overlapped with the positions of $Sox2^{+}$ cells (n=181), which includes SCs, HBC-like cells and possibly GBC-like cells (Figure 4.6). This observation suggests that Ca^{2+} responses may be generated by distinct cell populations including candidate progenitor cells in the OE.

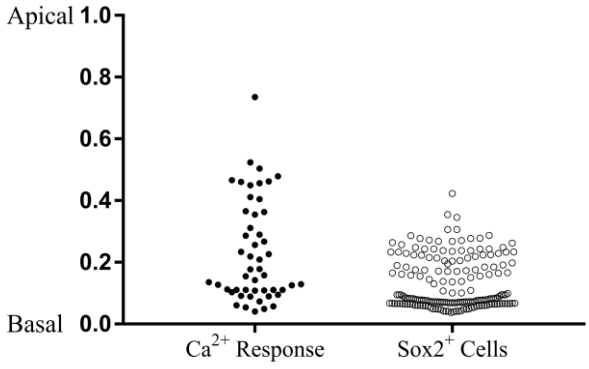

Figure 4.6. Spatial analysis of positions of Ca^{2+} responses (left) and $Sox2^{+}$ cells (right).

4.3.2. Temporal analysis of Ca^{2+} response

Studies in mouse (Jia *et al*., 2009) and *Xenopus* (Hassenkloever *et al*., 2009) have suggested that information about dying cells may be conveyed to the basally located progenitors via the involvement of SCs, which exhibit complex intracellular Ca^{2+} signals upon sensing chemicals such as extracellular ATP with their P2Y receptors. It is even shown that Ca^{2+} signals propagate through the cell body from the apical surface to basal cell layer (Hegg *et al.*, 2009). To investigate whether a similar cell-to-cell signaling could be present in the zebrafish OE, a temporal analysis of Ca^{2+} response was performed.

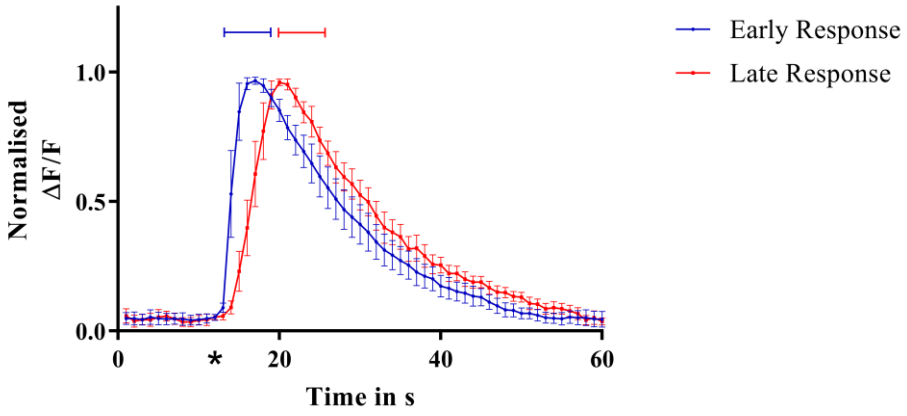

Figure 4.7. Temporal difference of Ca^{2+} response profiles in the same sample (n = 5 cells for each early (blue) and late (red) responses).

In the previous analysis of Ca^{2+} responses upon ATP stimulation it was observed that different cell populations responded at different times after application of the stimulus. Since there was a temporal gap between the peak fluorescence intensities of these populations (Figure 4.7), it was possible to visualize early and late responsive cells separately (Figure 4.8). Here, late responsive cells were colored in red whereas early responsive cells were labeled in blue. Since the signal of early responders persisted throughout the time interval of late responsive cells, those cells appeared magenta. Early responsive cells are mostly located basally and form a single cell layer. On the other hand, late responsive cells had a

broader and more apical dispersion in the epithelium, ranging from just above the layer of HBC-like cells to the apical boundary. Positional analysis using normalized apico-basal positions of the responses revealed two distinct populations of cells (Figure 4.9). A Mann-Whitney test indicated that late responsive cells were distributed more apically (*Mdn* = 0.4865) than the early responsive cells (*Mdn* = 0.1449), $W = 1552.0$, $p < 0.05$.

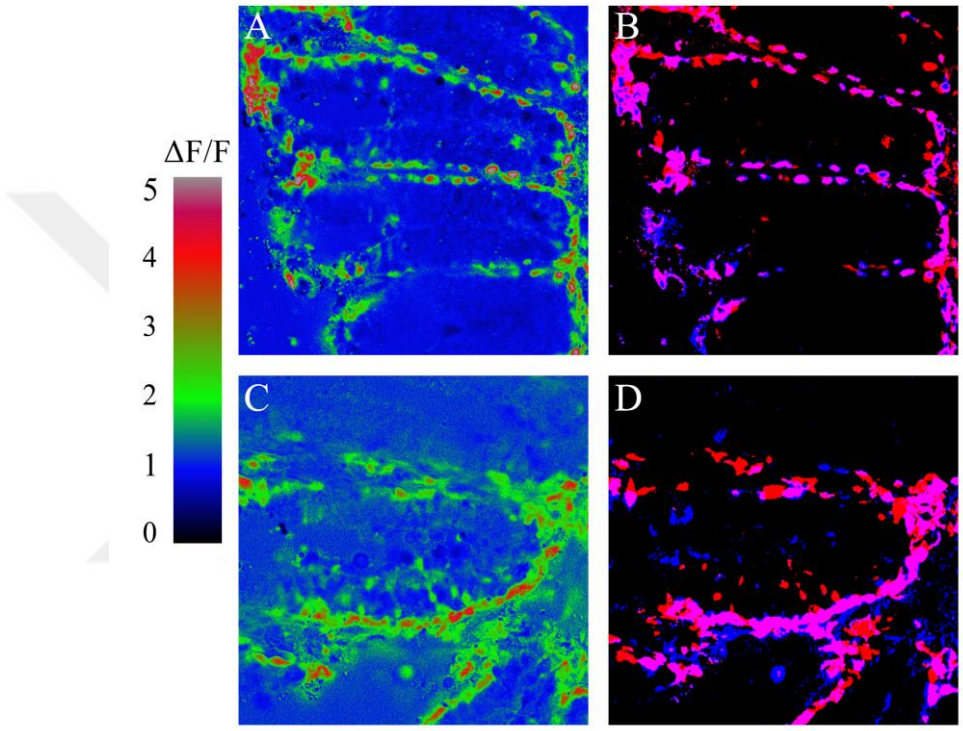

Figure 4.8. Representative images of cells eliciting early (magenta) and late (red) Ca^{2+}-responses. Top and bottom rows indicate two separate samples. ΔF/F over the whole response (A,C). Cells visualized according to their response times, early (magenta) and late (red) (B,D).

Cell shapes and the position of early responding cells strikingly resembled the $krt5^{+}$ HBC-like cells with horizontal cell bodies surrounding the lamella. Late responding cells however covered a broader range of cell shapes and positions. Some responses were observed from the cells positioned apically next to the HBC-like cells, which possibly came from globose basal cells and/or SCs. Some other late responses laid more apically in the

epithelium generated conceivably by SCs and adenosine-sensitive OSNs (Wakisaka *et al.*, 2017)

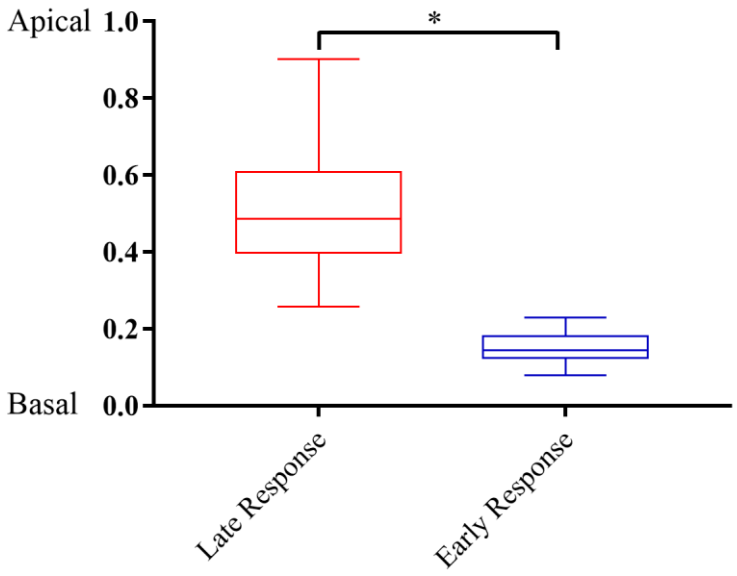

Figure 4.9 Apicobasal positions of two temporally distinct Ca^{2+}-responsive cell populations.

Taken together, these data indicate that at least two cell populations with different spatial and temporal response profiles are sensitive to extracellular ATP application. Cells located more apically elicited a delayed Ca^{2+} response compared to more basally located cells. These observations do not support the hypothesis that information about dying cells can be conveyed to the basal cells via SCs as Ca^{2+} response appeared first in the basal most layer of the epithelium.

4.4. Morphometric analysis of Ca^{2+} responses and cells with specific markers in the adult zebrafish OE

The positional analysis of Ca^{2+} responses alone was not conclusive about the identification of the responsive cells. In order to gain more insight on the identities of responding cell populations, a morphometric analysis was performed on Ca^{2+}-responsive

cells (n = 277) and cells expressing krt5 (n = 41), Sox2 (n = 172), and Tp63 (n = 100) markers. The vertical and horizontal dimensions of cells were measured and plotted (Figure 4.10). Yet, since Sox2 and Tp63 are nuclear proteins, measurements corresponded to dimensions of cell nuclei, whereas those of krt5, which is a cytoskeletal element, depicted the general shape of the cells. Ca^{2+} responses, however, were cytosolic and therefore reflect the overall dimensions of the responsive cell.

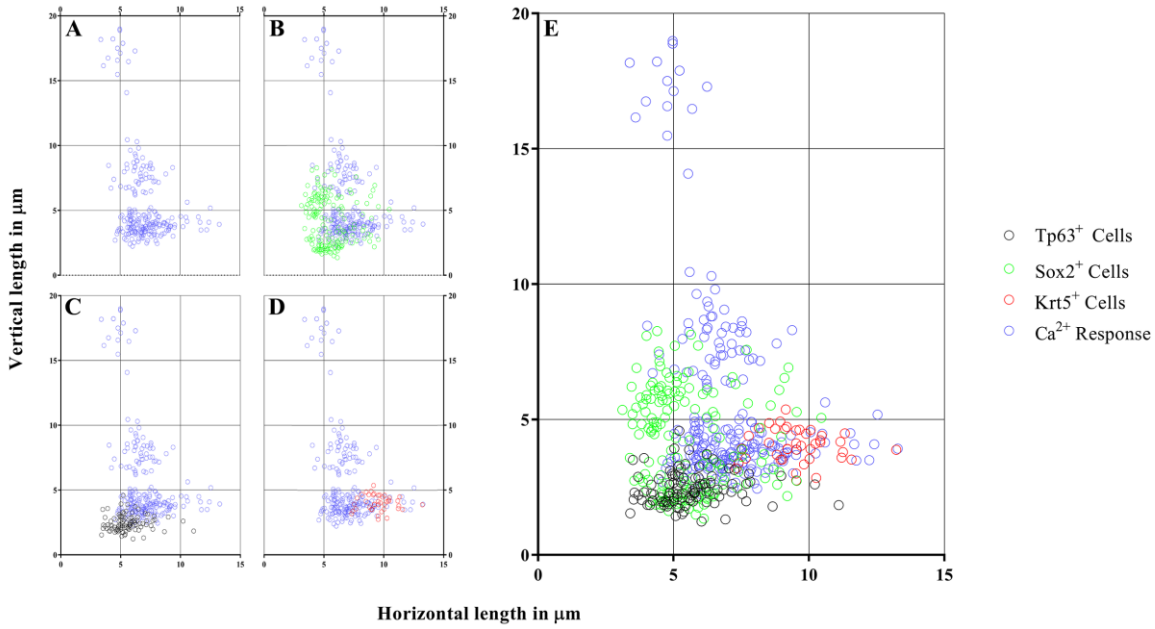

Figure 4.10. Morphometric analysis of Ca^{2+}-responses and stem cell markers. Dimensional measurements of Ca^{2+} response (A), Ca^{2+} response with $Sox2^{+}$ cells (B), with $Tp63^{+}$ cells (C), and with $krt5^{+}$ cells (D). Overlay of the whole morphometric data (E).

According to their dimensional data, Ca^{2+}-responses fell into three main clusters (Figure 4.10A). The top-left cluster with vertically elongated and slim profiles was probably generated by adenosine-sensitive OSNs, which elicit a Ca^{2+} response along their cell bodies. This population made up 5% of the total response. The other two clusters of cells, which were more globular (middle cluster) or flat-shaped (bottom cluster), overlapped with measurements of $Sox2^{+}$ cells (Figure 4.10B). Between them, the middle cluster did not overlap with the other stem cell markers, namely Tp63 (Figure 4.10C) and krt5 (Figure 4.10D). Therefore, the middle cluster of cells might represent the rounder cell bodies of SCs

or a potential, yet unidentified, GBC population. In total, cells in this cluster contributed 18.8% of the total population. The cluster below, however, overlapped with all markers, $Sox2^+$, $Tp63^+$ and $krt5^+$. The measurements of this cluster also indicated horizontally elongated cells which were slim in the apico-basal direction. Thus, this cluster of Ca^{2+}-responsive cells was possibly generated by HBC-like cells and made up 76.2% of the entire population.

Collectively, the data suggests that the majority of Ca^{2+} signals may be generated by $Sox2^+$ cells, which fall into at least two known cell populations in the adult zebrafish OE, one of which resembles HBC-like cells and a second population which may be SCs.

4.5 Effect of purine stimulation on OSN generation

Ca^{2+} imaging of cellular responses to ATP have shown that at least two distinct cell populations respond to extracellular purines (above and Bali, 2015), however, the physiological significance of these responses has not been investigated. As shown earlier in the literature, extracellular ATP can induce cell proliferation in the murine OE through purinergic receptor signaling (Jia *et al*, 2009). In order to analyze whether the apparent Ca^{2+}-influx seen upon extracellular purine introduction can result in a similar change of proliferation in the adult zebrafish OE, BrdU incorporation assay was performed following ATP stimulation. Pilot experiments including *ex vivo* stimulation of intact OE in aerated ACSF solution containing 10 mM ATP and trials using nasal irrigation with 100 mM ATP were not successful (data not shown), probably due to the low penetrance of the apical border for exogenously supplied reagents. Although a direct nasal stimulation was preferable, an alternative strategy was developed by systemic administration of ATP.

The experimental setup consisted of three different experimental groups. The first group of fish only received the PBS vehicle (n = 3) and served as a control and the number of $BrdU^+$ cells derived from this group was compared to two experimental groups. The first experimental group of fish received 10 mM ATP dissolved in 50 µl PBS intraperitoneally (n

= 3) and the second experimental group was injected with 10 mM ATP together with 5.6 mM solutions of the P2Y antagonist suramin dissolved in 50 µl PBS (n = 3). The fish were immediately placed in BrdU water after the treatment and analyzed 12h later by immunohistochemistry against BrdU and HuC/D antibodies (Figure 4.11).

In all three experimental groups the tissue distribution of neurons as indicated by HuC/D immunohistochemistry resembled that of a healthy, undamaged adult zebrafish OE. HuC/D^+ cells selectively occupy the inner two-thirds of the tissue and showed the typical, uninterrupted dense staining throughout the sensory OE. Interestingly, the pattern and number of $BrdU^+$ cells were different between ATP-injected and control fish. Typically, regions of high proliferative activity can be identified at the interlamellar curves and the sensory/non-sensory border of the intact tissue (Bayramli *et al*, 2017), a pattern of cell proliferation that reflects maintenance neurogenesis in the OE. The sensory region of the OE on the other hand is largely devoid of $BrdU^+$ proliferating cells.

The overall pattern of cell proliferation did not change in experimental groups but the absolute numbers of $BrdU^+$ cells was different among groups. ATP-injected fish appeared to have a higher number of proliferating cells at the ILC, the non-sensory OE, and within the sensory OE. This effect was largely reversed by simultaneous application of the purinergic receptor blocker suramin, although a higher than normal proliferation activity remained in the non-sensory OE. To quantify the changes in $BrdU^+$ cell number and their radial distribution across the OE more accurately, a custom macro routine developed in ImageJ was used to obtain cell counts for OE segments between the ILC and the S/NS.

Overall, ATP stimulation increased cell proliferation about two-fold at the ILC and even more pronounced within the sensory OE. This increase in the number of $BrdU^+$ cells was dampened upon simultaneous stimulation with suramin and ATP, suggesting that the observed increase in cell proliferation was the result of purinergic stimulation. The numbers of $BrdU^+$ cells remained close to PBS control in the non-sensory region of the OE (Figure 4.12).

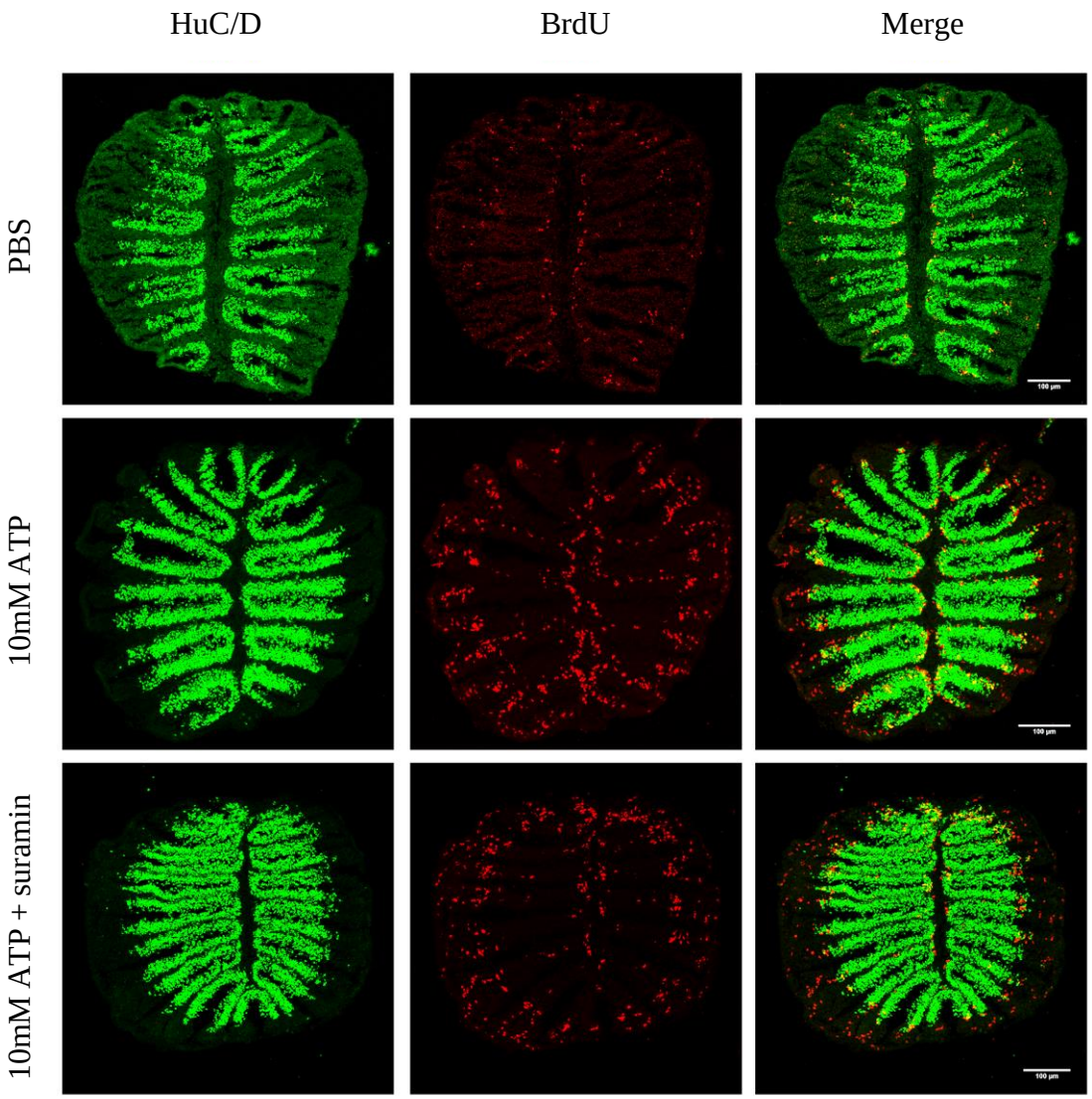

Figure 4.11. Physiological outcome of systemic purine stimulation. First row: PBS control fish show normal proliferation and neuronal patterns. Second row: Intraperitoneal introduction of 10 mM ATP increases proliferation throughout the OE. Third row: The increase in proliferation can be dampened by introduction of suramin along with ATP.

Interestingly, upon ATP stimulation, cells labeled with BrdU appeared in the sensory region, which rarely occurs under normal conditions but is the typical response of the OE to

experimental insult (Çapar, 2015). Cell counts on the lamellae also indicated an increase in the number of $BrdU^+$ cells in the sensory region, as well as ILC and S/NS border. Upon the addition of suramin however, the number and pattern of $BrdU^+$ cells more closely resembled PBS control fish, which supports the idea that extracellular purines may act as a danger signal and that purinergic signaling can initiate/regulate proliferation of the progenitor populations in the adult zebrafish OE (Figure 4.13).

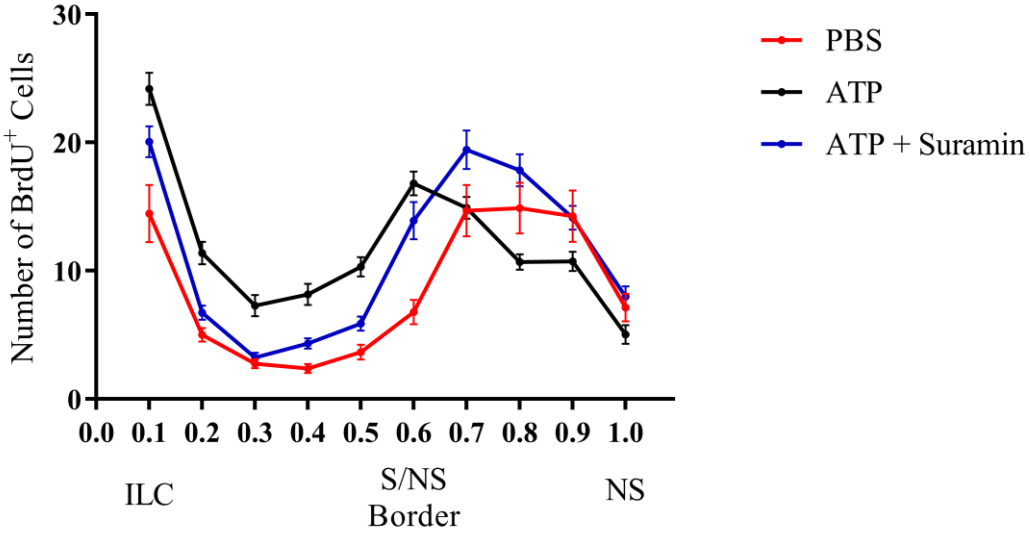

Figure 4.12. The profiles of absolute numbers of $BrdU^+$ cells along the lamella. Proliferation increases with the introduction of ATP especially in the ILC and sensory region. Application of suramin together with ATP has a dampening effect on the proliferation rate.

4.5.1. Determination of suramin dosage

In addition of functioning as a purine receptor blocker, suramin has also been shown to inhibit the binding of numerous growth factors to their receptors and thereby inhibiting the proliferation of certain cell types (Wang and Williams, 1984; Hosang, 1985; Betsholtz *et al*., 1986; Coffey *et al*., 1987). In other studies, however, it has been described that suramin

can also promote proliferation of certain cell types, such as non-small cell lung cancer cells and Chinese hamster ovary cells by stimulation of TGF-α release and directly causing EGF receptor dimerization (Lokshin *et al.*, 1999; Suzuki, 2004; Cardinali *et al.*, 1992). These effects of the suramin are found to be dose dependent, with low doses of suramin promoting proliferation while high doses inhibit proliferation of HeLa cells (Li *et al.*, 2015).

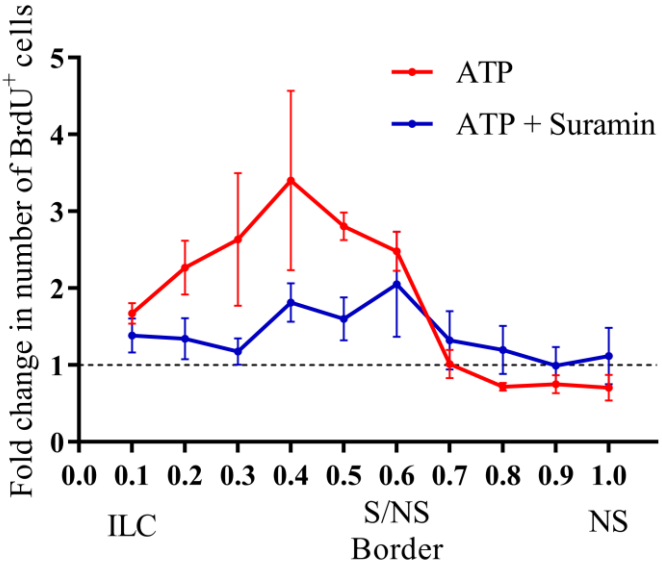

Figure 4.13. Fold change in the $BrdU^+$ cell numbers after ATP and ATP + suramin treatment relative to PBS control, indicating positions on the lamella.

For these reasons, it was crucial to establish a dose-response curve of suramin on ILC proliferation to choose the right dosage during the experiments described above. In order to find a suitable dosage for the experiments, the proliferative response of cells in the ILC to different concentrations of intraperitoneally applied suramin was analyzed (Figure 4.14). Consistent with its described pro-proliferative effect, there was an apparent increase in the number of $BrdU^+$ cell up to a suramin concentration of 2.8 mM suramin. For higher doses the number of $BrdU^+$ cells decreased sharply to near basal levels starting from 5.6 mM suramin. In total there were 5.7 ± 0.3 cells per ILC for PBS-injected fish (n = 32 sections) and 8.5 ± 0.5 cells for 350 µM (n = 25), 12.1 ± 0.9 for 1.4 mM (n = 21), 12.3 ± 0.6 for 2.8 mM (n = 27) and 3.4 ± 0.5 cells 5.6 mM (n = 35) suramin application. Thus, 5.6 mM suramin was the first concentration that did not show a stimulatory effect on ILC proliferation and

was selected as a suitable dose for the upcoming experiments. All injections were conducted with a volume of 50 µl, which then possibly followed by dilution of the introduced dose in the fish to effective concentrations.

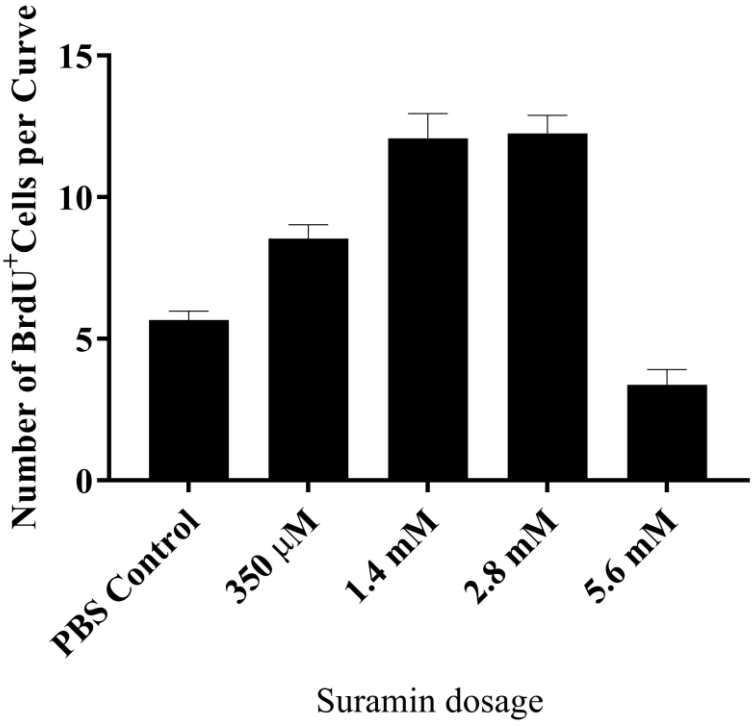

Figure 4.14. Effects of suramin doses on the basal cell proliferation in ILC.

4.5.2. ATP stimulation increases proliferation in the ILC of adult zebrafish OE

The overall change in the proliferation rate and distribution profile of $BrdU^+$ cells in the OE suggests that progenitor cell activity may be triggered by the exposure to extracellular purines. As mentioned above, there are two main proliferation regions in the adult zebrafish OE which contribute to the maintenance of the epithelium under normal conditions: the ILC and the S/NS border (Bayramli *et al*, 2017).

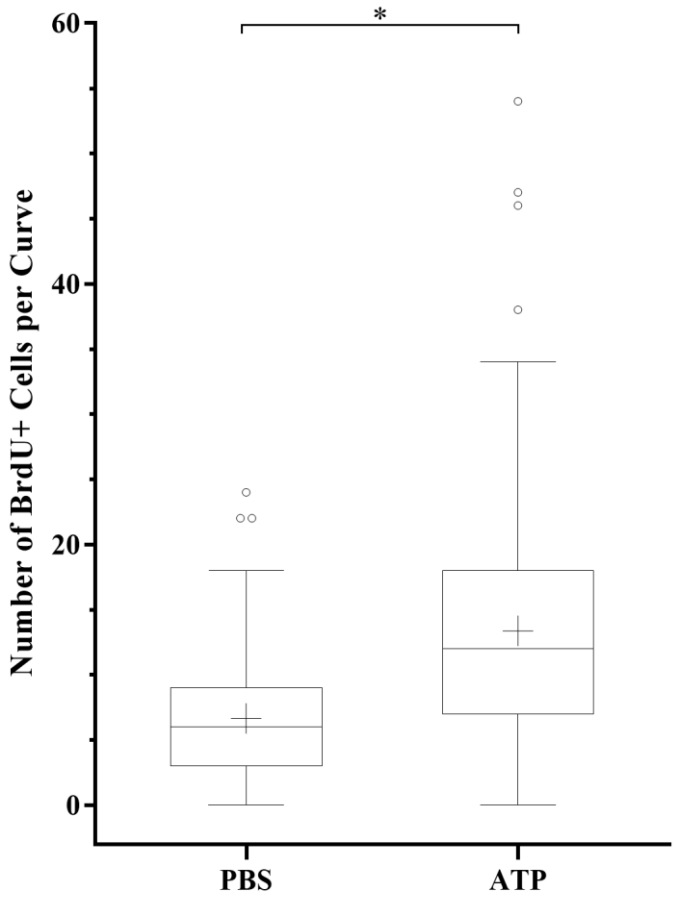

Figure 4.15. Box and whisker plot (Tukey style) representation of the distribution of the numbers of $BrdU^+$ cells per ILC according to the treatments.

To further investigate the responses of candidate progenitor cell populations in the ILC, the local proliferation rate was analyzed in more detail. The number of $BrdU^+$ cells per ILC of single lamellae were counted manually and an average of 6.7 ± 0.3 $BrdU^+$ cells (n = 259 lamellae from 30 sections, 5 sections of each OE from 3 fish) per ILC could be identified in PBS-injected control fish. A doubling of proliferative activity was observed in ATP-injected individuals with 13.4 ± 0.5 $BrdU^+$ cells per ILC (n = 311 lamellae from 32 sections, 5 sections of 4 OE and 6 sections of 2 OE). Thus, there was a significant increase in the mean $BrdU^+$ cell number in the ILC following intraperitoneal introduction of ATP. Obtained counts were subjected to a non-parametric test since the numbers did not fit into a normal distribution. A Mann-Whitney U test indicated that the ILC regions of fish injected with

ATP contained significantly more BrdU-labeled cells (*Mdn* = 12) than PBS control (*Mdn* = 6), $W = 52755.0$, $p < .05$ (Figure 4.15).

These results indicate that stem cells in the adult zebrafish OE may be sensitive to extracellular purines and the purinergic signaling cascade may result in increased proliferation as the extracellular ATP acts as a DAMP. Since the tissue was not physically damaged during this experiment, it is possible to claim that the responding population of progenitor cells may be the one which continuously generate neurons in the ILC, rather than the ones which divide and rebuild the tissue after a destructive chemical insult. However, the increase in proliferation observed throughout the sensory OE may indicate that purinergic signals also have a role in triggering or facilitating damage responses that are executed by a distinct pool of progenitors (Bali, 2015).

4.5.3. ATP stimulation induces proliferation in adult zebrafish OE through purinergic receptors.

Suramin is a purine receptor antagonist, which competitively inhibits the activation of P2X and P2Y receptors (Hoyle *et al*., 1990). In order to investigate whether previous results were a result of purinergic signaling through these receptor subtypes, ATP was introduced intraperitoneally together with suramin. $BrdU^+$ cells in the ILCs were counted and analyzed as before and were compared to the PBS control.

Cell counts in individual ILCs revealed a decrease in the number of $BrdU^+$ cells compared to intraperitoneal ATP introduction alone and the 7.6 ± 0.2 $BrdU^+$ cells per ILC (n = 294 lamellae from 30 sections, 5 sections of each OE from 3 fish; Figure 4.16) were similar to PBS control animals. The observation indicates that the physiological effect of extracellular ATP can be reversed to normal proliferation levels by inhibition of purinergic signaling. A Mann-Whitney U test indicated that the ILC regions of fish injected with ATP alone contained significantly more BrdU-labeled cells (*Mdn* = 12) than ATP + suramin injected fish (*Mdn* = 7), $W = 68758.5$, $p < .05$. The fish injected with ATP + suramin had

comparable rates of proliferation in the ILC with the control fish. Another Mann-Whitney U test indicated that ILC regions of ATP + suramin injected fish have more BrdU-labeled cells (*Mdn* = 7) than control fish injected with PBS (*Mdn* = 6), $W = 66146.0$, $p < .05$. Although being statistically significantly different from each other, the distribution of the numbers of the cells in both ILCs were very similar to each other, with means and medians very close to each other. (Figure 4.16).

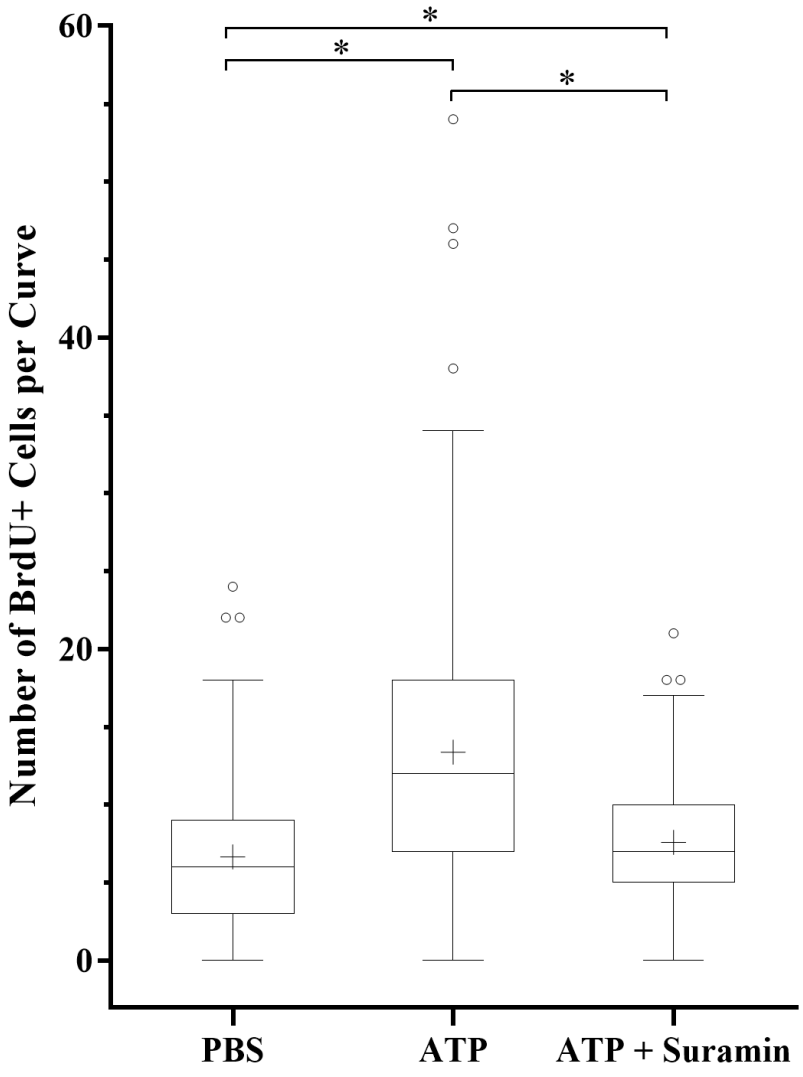

Figure 4.16. Box and whisker plot (Tukey style) representation of the distribution of the numbers of BrdU+ cells per ILC according to the treatments.

4.6. Analysis of newborn cell populations 3 days after ATP injection

In the experiments described above, fish were analyzed by immunohistochemistry with antibodies against HuC/D and BrdU. The analysis was performed 12 h after the treatment and labeled cells labeled were mostly only $BrdU^+$ and HuC/D^-. However, it is shown in the mouse that newly divided cells require at least 24 h to express structural neuronal markers (Huard and Schwob, 1995). In order to investigate if the proliferating cells ultimately contribute to the neuronal population, fish were incubated in BrdU water for 12 h after treatment and analyzed 3 days later to provide sufficient time for newborn cells to commit to a neuronal cell fate. Immunohistochemistry was performed on cross sections of OE using antibodies against Cytok II, EBF and HuC/D each together with krt5 and BrdU.

4.6.1. HBC-like cells stay dormant upon ATP treatment

IHC on cross sections of OE 3 d after ATP stimulation showed BrdU patterns similar to those observed at 12 h after ATP administration. The proliferative zones in the OE seemed active with some of the BrdU signals displaced into more apical regions of the tissue, which may indicate the maturation of newborn cells into sensory neurons. Cytok II pattern remained normal with SCs bearing basket-like cell bodies in the basal region of the epithelium. Unexpectedly, HBC-like $krt5^+$ cells which elicited a stronger and faster Ca^{2+}-response to purine application than other cell populations in the OE and which are positive for multiple stem/progenitor cell markers, were rarely $BrdU^+$, indicating that they did not participate in ATP responses. The majority of observable cell divisions appeared to occur more apically to the $krt5^+$ population (Figure 4.17).

4.6.2. Extracellular ATP application induces neuronal cell generation

The analysis of IHC with antibodies against BrdU, krt5 and EBF, 3 d after intraperitoneal ATP injection provided data which indicated that a population of newly

divided cells commit to neuronal fate. $BrdU^+/EBF^+$ double positive cells were clearly visible in the ILC region of the OE. The double positive cells were located mostly basally. Interestingly, $BrdU^+/EBF^+$ double positive cells were also present in the S/NS border, although at lower numbers.

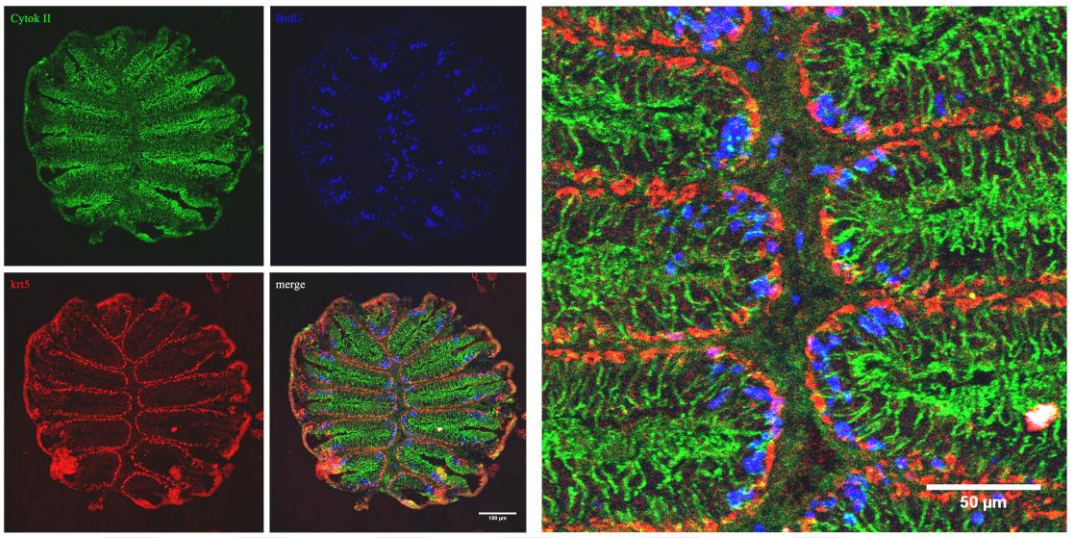

Figure 4.17 HBC-like cells do not divide after ATP stimulation. Left: IHC data of mentioned markers. Right: Close-up on ILC.

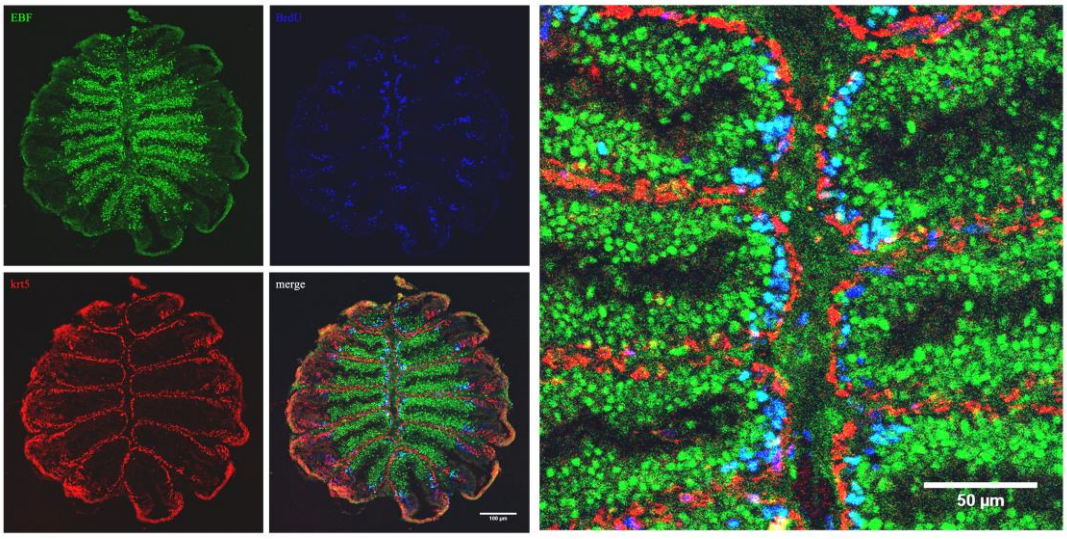

Figure 4.18. A majority of the newly generated cells commit to a neuronal fate. Left: IHC data of mentioned markers. Right: Close-up on ILC.

There were also occasional observations of double positive cells in the middle of the sensory region. The lack of a visible population of $BrdU^+/krt5^+$ double positive cells rules

out the possibility that these newly generated cells which committed to a neuronal fate were generated by the HBC-like cells in the OE (Figure 4.18). IHC with antibodies against HuC/D, krt5 and BrdU also revealed a pattern similar to the EBF one with the majority of $BrdU^+/HuC/D^+$ double positive cells concentrated in the ILC region of the OE, with scarce double positive cells in the S/NS border (Figure 4.19).

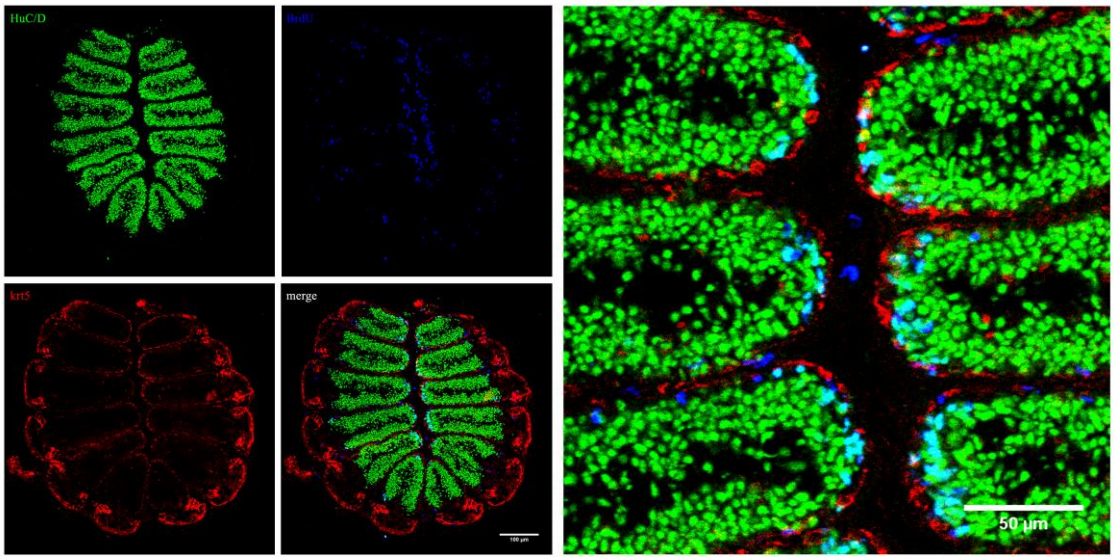

Figure 4.19. A population of newborn cells are HuC/D^+. Left: IHC data of mentioned markers. Right: Close-up on ILC.

To investigate the profiles of dividing cells in the ILC region of the OE after ATP treatment, the number of $BrdU^+$, $BrdU^+/EBF^+$ double positive and $BrdU^+/HuC/D^+$ double positive cells were quantified (Figure 4.20). The cell counts revealed a higher number of $BrdU^+$ cells than analysis after 12 h even after cessation of continued ATP stimulation, which may indicate that the progenitor or intermediate transit amplifying cells may have gone through more than one cell cycle over the 3 days of the experiment. There were a total of 19.8 ± 1.3 (mean ± SEM) cells in EBF samples and 18.7 ± 0.9 cells in HuC/D samples (n = 48 individual ILCs from n = 3 fish each). About half of the $BrdU^+$ cells, 8.2 ± 0.6 (mean ± SEM) cells, were double positive for EBF, a transcription factor critical for olfactory receptor expression, which is considered a marker for mature OSNs in the mouse (Iwema

and Schwob, 2003). The analysis for immunoreactivity against HuC/D gave similar results with 10.5 ± 0.6 (mean ± SEM) cells. Lower numbers of $BrdU^+/EBF^+$ double positive cells compared to $BrdU^+/HuC/D^+$ double positive cells may indicate that some of the newly generated neurons have not yet completed their maturation, since HuC/D is an earlier neuronal marker. Unfortunately, the filamentous, non-cytoplasmic nature of the Cytok II staining did not allow for a reliable quantification of the BrdU/Cytok II double positive cells. Taken together, these data show that stem cells activated by extracellular ATP are capable of generating mature OSNs in the adult zebrafish OE in 3 d time. To be conclusive, however, it will be critical to compare the number of neurons generated after ATP stimulation to unstimulated control fish.

As a whole, these data support that increased cell proliferation due to extracellular purine introduction, result in generation of mature neurons in the adult zebrafish OE. One of the possible progenitor populations, HBC-like $krt5^+$ cells do not seem to involve directly in this cascade of events. Since the morphometric analyses of calcium responses and $Sox2^+$ cells seem to match closely, these results may indicate the presence of another possible stem cell population in the adult zebrafish OE, eventually GBC-like cells, which have yet to be identified.

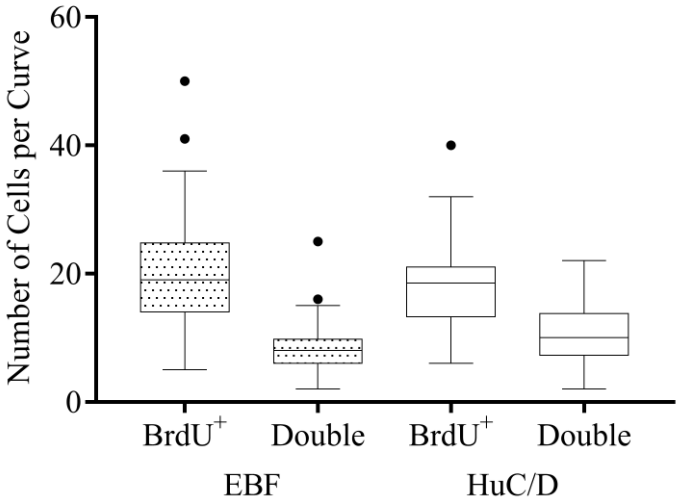

Figure 4.20. Neuronal profiles of the cells generated after ATP stimulation represented in box and whisker plots (Tukey style).

4.7. Morphology change in the krt5^{+} HBC-like cells upon chemical insult

HBC-like cells in the adult zebrafish OE did not seem to be activated upon sensing extracellular purines. But sensing purines released by necrotic cells is not the only known mechanism which activates these cells. Other types of DAMPs, cell-to-cell communication and changes in the ECM are some of the other known mechanisms which may trigger stem cells (Nelson and Chen, 2002; Koohestani *et al*., 2013). One of the ways of inducing such signals in the OE is applying chemical lesion via the use of a common detergent, Triton X-100 (Iqbal and Jacobs, 2010).

In order to investigate the involvement of the HBC-like cells in the rebuilding of the tissue, one OE of a zebrafish was subjected to chemical insult by application of 1% Triton-X in the nasal cavity, while the untreated OE served as a control. The OE was analyzed at the 3rd day following the lesion after incubation in BrdU water during the last 10 hours before analysis. IHC analysis with antibodies against krt5 and BrdU exhibited drastic changes in both the morphology of HBC-like basal cells and proliferation pattern indicated by BrdU (Figure 4.21). Compared to the control, HBC-like cells lost their horizontal, basally positioned cell shapes and invaded the epithelium in the apical direction forming multiple cell layers. A vast majority of the morphologically distinct HBC-like cell population also appeared to be BrdU^{+}, which indicates that they are able to divide and probably involve in the reshaping of the epithelium after chemical insult. Such morphology changes also were observed in HBCs of the mouse OE after chemical insult (Packard *et al*., 2011).

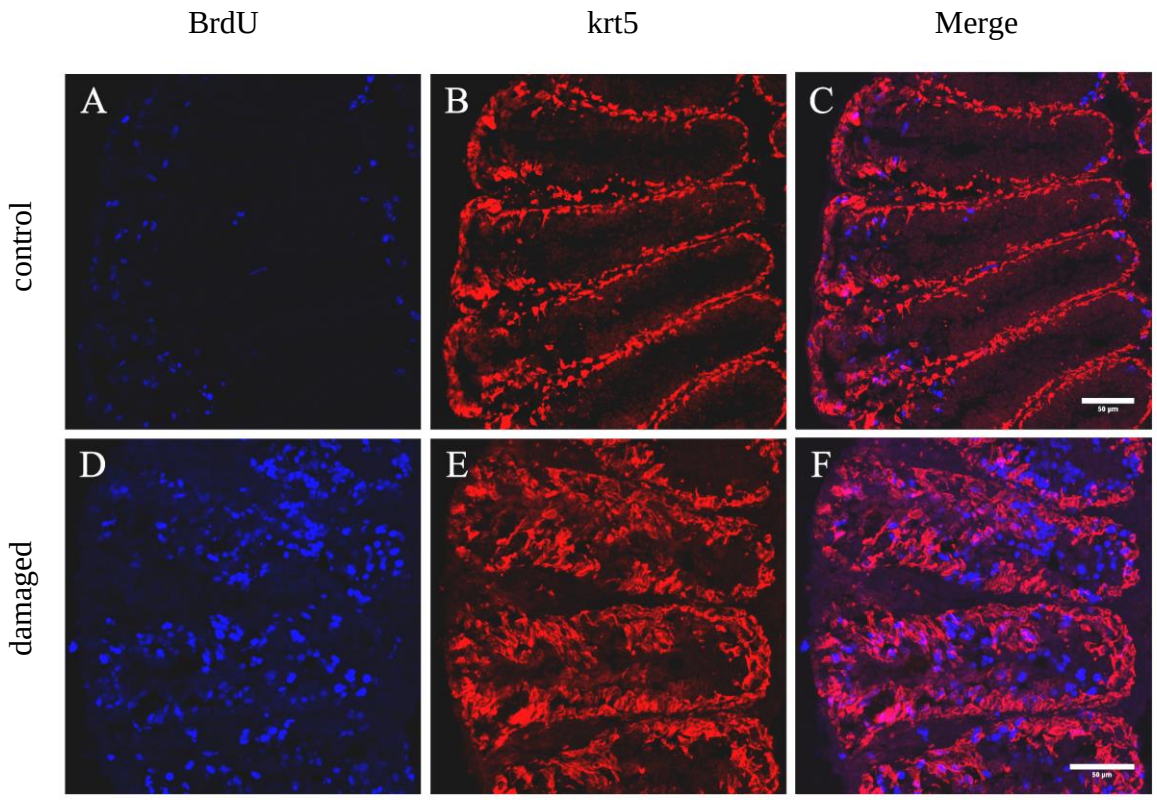

Figure 4.21. HBC-like cells change their morphology and divide upon chemical insult to the OE. Top row: IHC with antibodies against BrdU and krt5 on control OE. Bottom row: IHC with antibodies against BrdU and krt5 on damaged OE.

5. DISCUSSION

The signals that communicate between the intact and injured olfactory tissue and resident stem/progenitor cell populations to match the production of new neurons with the momentary need of the tissue are not well understood. In principle, intact neurons could inhibit cell proliferation or specific signals could be released from dying or damaged cells that activate cell divisions of progenitor or transit-amplifying cell pools. In both cases, the loss of neurons or the overall density of nerve cells would have an active regulatory influence on mitotic activity in the OE. An attractive hypothesis is that injured cells release their content, including some pro-proliferative compounds, such as purines. Purine compounds have been shown to promote cell divisions in the mouse and Xenopus OE (Jia *et al.*, 2009; Hassenklöver *et al.*, 2009).

In this thesis, I describe aspects of the physiological effect of ATP on the adult zebrafish OE. Exogenous stimulation with ATP induces robust Ca^{2+} responses in the OE. Based on morphometric parameters and immunohistochemistry against various non-neuronal cell markers, three distinct cell populations can be discriminated to respond to ATP; a smaller, neuronal population, located apically in the OE and two more basally located populations that are both positive for the general stem cell marker Sox2. One of those shows similarity with SC glia, while the other population most likely comprises cells with HBC-like properties. It has been hypothesized that SC may sense damage signals from dying neurons and in turn stimulate HBC activity. The temporal kinetics of Ca^{2+} response profiles of these cells, however, does not support such an idea, at least not under ex vivo conditions, where HBC-like cells responded earlier than cells with SC profiles.

Consistent with the suspected role of purines in regulating OSN neurogenesis, stimulation of the OE with ATP by intraperitoneal injections resulted in an increase in proliferative activity throughout the OE. The change in activity was most noticeable in the ILCs, which is a region of constitutive cell proliferation and contributes to maintenance neurogenesis (Bayramli *et al*., 2017). However, an increase in the number of cell divisions

was also observed within the sensory OE, which selectively responds with proliferation upon tissue damage (Iqbal and Byrd, 2011; Kocagöz, unpublished). The systemic application of ATP may trigger OSN neurogenesis through secondary effects, yet the effect can be blocked by the selective P2 receptor inhibitor suramin, suggesting that at least one direct ATP-stimulated step is involved. Taken together, the data obtained from this study support, yet do not prove, a role for purines in regulating OSN numbers in the zebrafish OE.

5.1. Analysis of Ca^{2+} response profiles

To investigate the response of the zebrafish OE to exogenous stimulation with ATP, calcium imaging experiments were conducted on 50 µm thick vibratome sections of adult zebrafish OEs using the calcium-sensitive dye Fluo4-AM. Similar to previous observations (Bali, 2015), perfusion of the slice preparation with ATP solution stimulated a Ca^{2+} response throughout the OE. Overall, the response was more prominent in the non-sensory region of the OE and the response was generated mostly by, but not confined to, basal cells, which are situated at the base of each lamella and which are elongated in their horizontal dimension. Additional responses could be observed that spanned the OE in apicobasal direction; globular responses in the middle of the epithelium and responses just apical to the prominent basal cell population. As previously described in *Xenopus* and mouse OE, SCs are sensitive to ATP (Hassenklöver *et al*., 2008; Hegg *et al*., 2009). Considering the morphology and cell-to-cell distances of SCs and Ca^{2+} responses in the zebrafish OE, response profiles spanning the epithelium are most likely generated by SCs (Bali, 2015). The most basal and horizontal responses surrounding the OE instead seem to be generated $krt5^{+}$ HBC-like cells. It should be noted, however, that it was not possible to directly identify the responding cells by IHC on the physiological preparations. Yet, there was a tight correlation by taking into account their positions and profiles in the OE revealed by IHC data.

To further reveal the identity of responding cells, the morphologic features of cells showing Ca^{2+} responses were characterized by measuring the vertical and horizontal lengths of the responses on the images created by a custom macro routine, which calculates $\Delta F/F$

and generates heat maps according to the strength of the response. This analysis revealed at least three different populations of cells with distinct cell shapes. Two of these populations seemed to overlap with $Sox2^+$ cells, according to morphological measurements obtained from IHC with anti-Sox2 antibody. Sox2 is a progenitor cell marker transcription factor and is shown to be expressed by HBCs, GBC and SCs in rat and mouse OE (Guo *et al.*, 2010; Packard *et al*., 2016). In our laboratory, we also observed that SCs and HBC-like cells express Sox2 in adult zebrafish OE (Bali, 2015; Kocagöz, unpublished). However, SCs in the zebrafish OE show an inverted morphology with basal cell bodies, making it somewhat difficult to easily discriminate between juxtaposed $Sox2^+$ cell populations in the basal OE. Due to the limitations in the availability of antibodies that cross-react with zebrafish proteins, it cannot be concluded for sure that SCs and HBC-like cells are the only $Sox2^+$ populations in the basal OE and the possibility of presence of a $Sox2^+$ GBC-like cell population still remains. A major marker for a subpopulation of GBC-like cells is the proneural gene *Ascl1*. While *Ascl1* signals are prominent in the ILC and SNS, no $Ascl1^+$ cells could be observed in the intact sensory OE (Bayramli *et al*., 2017). Yet, Ascl1 signals are induced within the sensory OE after damage, in line with increased neurogenesis for OE repair (Kocagöz, unpublished). Either way, the morphometric measurements of one $Sox2^+$ cell population overlaps with the measurements of $krt5^+$ and $Tp63^+$ cells, which are general HBC markers in mouse OE (Carter *et al*., 2004; Fletcher *et al*., 2011). HBC-like cells express Tp63 under dormant conditions but cease expression upon damage before dividing in the mouse (Packard *et al*., 2011; Schnittke *et al*., 2015) and zebrafish OE (Kocagöz, unpublished). The profiles of other Ca^{2+} response, which do not overlap with the HBC markers but overlap with Sox2 expression, seem to be generated by more globular cells as their vertical and horizontal measurements are closer to each other. The nature of these cells is less clear but some may represent SC cell bodies in the basal OE. Responses of the apical parts of SCs may not have been always detectable in the $\Delta F/F$ images due to limitations in image and temporal resolution as they tend to be much thinner than their corresponding cell bodies, whereas Ca^{2+} response seems to be more prominent in SCs residing in the *Xenopus* OE (Hassenklöver *et al.*, 2008). Thus, it is possible that this second population of Ca^{2+} responsive cells may represent the cell bodies of SCs and occasional GBC-like cells. The third population of the response, which does not overlap with any other measurement and consists of cells elongated in the apicobasal direction, may have been generated by neurons which are sensitive to ATP

or Ca^{2+} response across the cell bodies of SCs (Friedrich and Korsching, 1997; Wakisaka, 2017).

5.2. Purinergic signals increase OSN proliferation

Damage-associated molecular patterns (DAMPs) such as histones, HMGB1, S100 proteins, and purines often are released by necrotic cells and can be sensed by neighboring cells through mechanisms that involve Toll-like receptors (TLRs), NOD-like receptors (NLRs), and purinergic receptors (Tolle and Standiford, 2013). Among the purines, ATP and adenosine are the most abundant molecules released after necrotic cell death and have been shown to stimulate immune cells (Ayna *et al*., 2012) but also to affect regenerative processes in a variety of tissues including the mouse OE (Di Iorio *et al*., 2001; Jia *et al*., 2009; Hegg *et al*., 2008; Hayoz *et al*., 2012). ATP has been shown to activate target cells through P2Y and P2X receptors while P1 type of purinergic receptors are responsive to purine metabolites such as adenosine (Puchałowicz *et al*., 2014). P2Y Receptors are GPCRs, which, upon binding of a ligand, trigger an IP_3-mediated signaling cascade and induces mobilization of intracellular Ca^{2+} via activating IP_3-sensitive ion channels in the endoplasmic reticulum (Salter and Hicks, 1995). The rapid increase in Ca^{2+} levels are thought to induce the expression of a series of various transcription factors, including c-fos and c-Jun, which can promote transitioning of cells resting in G_0 phase to the G_1 phase of the cell cycle (Cruzalegui *et al.*, 1999). On the other hand, P2X receptors are nucleotide-gated ion channels, which allow Ca^{2+} but also Na^+ and K^+ flux across the cell membrane (Evans *et al*., 1996). The influx of Ca^{2+} ions mediated by this receptor may then trigger calcium-induced calcium release from the ER and result in the expression of identical transcription factors to promote mitotic activity (Endo, 2009).

Following intraperitoneal injection of ATP, I observed an increase in the overall cell proliferation in the OE of adult zebrafish 12 h after application. The increase was revertible by co-application of suramin with the injected solution, indicating that active purinergic signaling through P2 type of receptors may be involved in the process. A similar result was also obtained in the mouse OE following intranasal application of ATP (Jia *et al*., 2009) Cell counts in the ILC regions of the OE revealed a nearly two-fold increase in the number of

$BrdU^+$ cells. Unlike the rodent OE where cell divisions occur homogenously throughout the entire basal OE (Schwob, 2002), the zebrafish OE harbors two main proliferative regions under intact conditions, which are the ILC and the SNS (Bayramli *et al*., 2017). It is regularly observed in our laboratory that, except following lesion of the OE, cells in the sensory region of the OE rarely divide. On the other hand, the number of $BrdU^+$ cells in the sensory region greatly increases after chemical insult with Triton-X 100 (Çapar, 2015; Kocagöz, unpublished). Interestingly, upon intraperitoneal ATP injection the number of $BrdU^+$ cells also increased in the sensory region. Thus ATP stimulation of the OE shows hallmarks of the damage response. The increase in the number of $BrdU^+$ cells in ILC and the sensory OE may be explained by calcium-dependent expression of pro-mitotic transcription factors, which contribute to triggering of cell division in resting cells. This is supported by the observation that basal HBC-like cells respond with increased calcium concentrations upon extracellular ATP application.

In the mouse OE it has been shown that intranasal application of ATP solution can increase cell proliferation (Jia *et al*, 2009). Pilot experiments with direct intranasal application of ATP in zebrafish, however, did not induce any changes in the number of cells or the pattern of proliferation in the OE. Different from the mammalian nose, the zebrafish OE is constantly perfused by a stream of water and the observed difference may be caused, in part, by the dilution of the applied ATP solution into the tank water in the course of experiment. Since the fish need to be anaesthetized for nasal irrigation but can be kept outside of the tank water only for a limited time (up to five minutes), the conditions used in my initial trials may have failed to generate the necessary continuous ATP stimulation of progenitor cells in the epithelium. Intraperitoneal injection of ATP, on the other hand appears to provide sufficient purinergic input to these progenitors, however raises the question if the observed effects are direct or secondary through downstream systemic activators.

The OE of the adult zebrafish has a tightly packed structure with processes of SCs and OSNs sealing the apical border of the epithelium possibly with tight junctions (Hansen and Zeiske, 1998). The zebrafish OE also may have a thinner mucus layer on the apical surface of when compared to land-living animals. Sticky mucus may trap chemical

compounds and may have provide prolonged exposure of ATP and thus proliferation in the mouse OE. Ex-vivo preparations of the OE that were kept alive *in vitro* in aerated ACSF solution and which were supplied with ATP also did not show any significant differences in proliferation when compared to control specimens. These experiments suggest that poor penetration of ATP into the dense and tightly sealed epithelium may have prevented the ATP stimulus to reach all the way to the suspected progenitor cells in the basal-most layers of the OE.

Increasing the permeability of the OE border by generating holes in the apical region of the OE through co-application of Triton-X 100 may allow the uptake of ATP into the epithelium. However, Triton-X 100 damages OSNs (Iqbal *et al*., 2010) and in itself triggers a robust proliferation response in the OE. Thus, any attempt to make the OE more permeable would add another variable to the experiment and was not preferred. Intraperitoneal application of ATP seems to eliminate these problems since the ATP is directly introduced into the body cavity of the fish and is distributed by the blood stream and may therefore provide effective stimulation, especially in the basal regions of the epithelium which are closer to blood vessels (Hansen and Zeiske, 1998). The fish were analyzed relatively shortly after ATP injection at 12 h after application to study the acute effects of ATP and to minimize the possibility that it is metabolized in the organism.

The overlap of Ca^{2+} responses that were recorded on OE slice preparations with the distribution of $Sox2^{+}$ cells suggest that a $Sox2^{+}$ cell type may be directly activated upon sensing extracellular ATP or initiate cell-to-cell communication after stimulation. However, the route of systemic ATP application and the relatively long time course of the experiment does not strictly allow the conclusion that ATP stimulates basal cells in the OE directly and the observed pro-proliferative effect could be due to secondary effects of ATP administration. Suramin was able to revert the effect of ATP stimulation. Yet again, this does not rule out that ATP triggers unknown signaling events and the systemic release of other signaling molecules that may be blocked by suramin. Klumpp *et al.* (2006) reported that several human growth factors, such as basic fibroblast growth factor (bFGF), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), can undergo

autophosphorylation in the presence of ATP *in vitro* and which is required for their neuroprotective effects. A similar cross-talk between ATP and growth factors may have resulted in increased proliferation in the OE. Yin *et al.* (2007) also demonstrated EGFR transactivation and HB-EGF shedding upon ATP-γ-S application in human corneal epithelial cells. This is interesting in the light that HB-EGF is a strong activator of cell proliferation in the zebrafish OE (Kocagöz, unpublished). The results observed from intraperitoneal ATP injections may, thus, be a consequence of such complex interaction mechanisms and more direct experiments on HBC-like cells through intraepithelial perfusion of purines may be required.

5.3. Involvement of SCs in neurogeneration

SCs and HBC-like cells have been identified to be $Sox2^{+}$ cell populations in the zebrafish OE. Whether additional $Sox2^{+}$ cell populations are represented in the tissue and which may be involved in OSN generation is currently unknown. It has been hypothesized that SCs sense the presence of dying cells in more apical regions and convey the information to the basal OE, possibly HBC-like cells, with a propagating Ca^{2+} response (Hegg *et al.*, 2009; Hassenklöver *et al.*, 2009). There is conflicting evidence from my observations as to whether such a scenario is also found in the zebrafish OE. Especially the temporal profiles of Ca^{2+} responses that I observed in the *ex vivo* OE preparations do not directly support such a mechanism.

SCs are distributed evenly throughout the OE from the ILC to NS region (Bali, 2015). Therefore, an even increase in cell proliferation would be expected along the entire dimension of the lamella if SCs would mediate between apically detected ATP and basal progenitor cells. Such a behavior is actually observed after chemical lesion and the proliferation pattern along the lamella changes significantly (Çapar, 2015; Kocagöz, unpublished). In the unperturbed OE a bimodal distribution of BrdU+ cells indicative of high rates of proliferation at the ILC and the SNS but not in the sensory OE are seen (Bayramli *et al.*, 2017). Following damage, the profile evens out with a similar rate of proliferation

across the entire dimension of the lamella (Çapar, 2015; Kocagöz, unpublished). In my experiments I observed a relatively even increase in the number of BrdU+ cells across the entire lamella, suggesting that ATP stimulation evenly increased proliferation on top of the base rate of ongoing mitotic activity (Figure 4.12). The entire bimodal profile appears to be shifted upwards by more or less the same number of cells. This observation is in line with the prediction that ATP stimulation equally affects HBC-like progenitors. Interestingly, it does appear to interfere with maintenance neurogenesis. This could imply that different progenitor cells contributed to the BrdU+ cells, the constitutively active progenitors at the ILC and SNS and a second ATP-sensitive population that was recruited upon stimulation.

In contrast, Ca^{2+} responses were observed to appear first in the basal most region of the OE where HBC-like cells are located, and only with a delay in the apical region where SCs, possible GBC-like cells and OSNs reside. This observation suggests a model where HBC-like cells trigger SC activity upon sensing extracellular ATP or that the SC and HBC responses are independent of each other. However, these observations were made on *ex vivo* vibratome slices of the OE, which constantly perfused by a stream of ACSF solution. Under these conditions, the applied ATP solution reaches both apical and basal regions of the OE simultaneously, which does not rule out the possible function of the SCs to convey the information of dead cells from apical to basal regions in the OE *in vivo*.

The observations made here are not easily understood and suggest the presence of distinct cell populations with different behavior and sensitivity to ATP. So far, no molecular markers have been identified to discriminate between these cell populations in the zebrafish OE. Cells with HBC-like characteristics are evenly distributed along the lamella as seen by krt5 and Tp63 IHC, including the ILC. Thus, it is possible that both cell populations are represented at the ILC while only damage-/ATP-sensitive cells are found within the sensory OE.

5.4. Systemic effects of suramin

Proliferation assays showed that the ATP-triggered increase in cell proliferation can be blocked by the systemic administration of suramin, suggesting that increased cell proliferation can be stimulated, either directly or indirectly by ATP through P2 type of receptors. A similar observation was made in the mouse OE (Jia *et al.*, 2009), however, the authors do not comment on potential other effects of the chemical.

Suramin is a hexasulfonated naphthylurea compound, which has a wide variety of pharmaceutical uses. Suramin is reported to be a selective P2X and P2Y purine receptor inhibitor that binds competitively and renders the receptors inactive (Jenkinson and Raid, 2000). This makes suramin a perfect candidate chemical for studying the effects of extracellular ATP on biological systems especially in direct *in vitro* experiments. However, the systemic side effects of the drug complicate its utilization in *in vivo* experiments. To name a few, suramin has been shown to increase the proliferation of renal proximal tubule cells up to a concentration of 100 µM, which is explained by activation of PI3K pathway through an unknown mechanism that does not include activation of EGF receptors (Zhuang and Schnellmann, 2005). Suramin is used in treating African trypanosomiasis in humans as it is shown to non-specifically inhibit a spectrum of enzymes such as 1-α-glycerophosphate oxidase, glycerol-3-phosphate dehydrogenase, RNA polymerase and kinases, thymidine kinase, dihydrofolate reductase, hyaluronidase, urease, hexokinase, fumarase and trypsin, and a higher rate of uptake of the chemical into the parasite is seen as a cure with 99.7% of the drug bound to plasma proteins (Fairlamb and Bowman, 1980). Currently, it is in a trial phase as a drug against autism in humans with unknown side effects, as an inhibitor of P2Y and P2X receptors, possibly decreasing cell danger responses (Naviaux *et al.*, 2017). Another study suggests that suramin can be used as an anti-cancer drug for several cancer types as it antagonizes EGF receptors, hence, it thought to decrease the proliferation of cancer cells (Stein *et al*., 1989), while another study suggests that it increases proliferation in non-small-cell lung cancer cell lines via dimerization of EGFRs (Lokshin *et al*., 1999). Suramin can also inhibit components of complement system, blood clotting and fibrinolysis in human plasma by non-specifically binding to plasma proteins (Eisen and Loveday, 1973).

With such a wide range of dose-dependent and off-target effects, suramin is not the best compound for studying purinergic signaling *in vivo* and needs careful dose-effect determination before being used in an experiment. Application of other candidate purinergic receptor inhibitors possibly with less side-effects, such as PPADS, would be useful in further experiments (Lambrecht *et al.*, 1992). However, assuming that the right concentration was used, application of suramin together with ATP resulted in reversion of the increase of the $BrdU^+$ cell numbers observed following injection of ATP alone. This may be explained by the P2X and P2Y receptor inhibition of the drug. Unable to bind to ATP, the signaling cascade which leads to cell proliferation triggered by these receptors may be blocked specifically resulting in a decrease in the proliferation.

5.5. Who is dividing?

The adult zebrafish OE has two modes of proliferation resulting in neurogenesis (Çapar, 2015; Kocagöz, unpublished). Maintenance proliferation occurs mainly in two well-defined proliferative zones, namely the ILC and S/NS border while regenerative proliferation after acute damage appears to occur within the entire epithelium (Iqbal and Byrd, 2010; Çapar, 2015). Under normal conditions the progenitor cells in ILC and S/NS border generate cells which then passively displace towards the middle, sensory region of the OE (Bayramli *et al.*, 2017). However, after damage, this pattern is changed with proliferating cells occurring in the sensory region. The change in the frequency of $BrdU^+$ cells along the lamellae after ATP application, *i.e.* $BrdU^+$ cells appearing in the sensory region, can be interpreted as a response of the OE that is similar to a response after acute damage. However, this response was relatively small when compared to the severe response after a chemical lesion via Triton-X 100 application into the nasal cavity, probably because lack of other signals such as loss of ECM integrity or cell-to-cell contact which may activate progenitor cells capable of rebuilding the entire epithelium. Interestingly, the two main proliferation regions were still recognizable after ATP application, which does not resemble the proliferation pattern after damage, which is unorganized and random and covers the entire lamella (Çapar, 2015; Kocagöz, unpublished). The application of ATP does induce a strong numerical increase in the frequency of proliferation in the sensory region, but the

already low basal numbers of $BrdU^+$ cells in the region, and the retaining proliferation pattern makes it hard to conclude that ATP causes the exact same effect as damaging the epithelium.

The ILC region of the OE was selected as a basis for most of the experiments as it is the most confined region of the OE where cell divisions occur continuously and unlike the S/NS border, it also bears neuronal cells on both sides. Investigation of ILCs 3 d after ATP application revealed that HBC-like $krt5^+$ cells in the basal OE did not have a major contribution to the generation of neuronal cells as they mostly appear negative for BrdU, whereas most of the $BrdU^+$ cells are positioned in more apical zones. In a few occasions $BrdU^+$ HBC-like cells were found in the sensory region but no $BrdU^+$ cells were found in the apical sensory region implying that HBC-like progenitor cells are able to divide however they may not directly generate neuronal cells upon ATP treatment. This behavior of the HBC-like cells can be interpreted as a precaution for a possible damage by increasing the pool of progenitor cells upon being stimulated by only one type danger signal.

HBCs in the mouse OE are quiescent cells, which are activated upon damage to the tissue (Graziadei and Monti Graziadei, 1979). Interestingly, this mechanism requires the loss/injury of SCs since ablation of neurons alone is not sufficient to activate those (Herrick *et al*., 2017). I also observed very few $BrdU^+/krt5^+$ cells suggesting they also require stronger stimulation in order to start dividing. However, another possibility is that they are fast cycling cells, at least in their damage-/ATP-stimulated state and the originally labeled HBCs may have diluted their BrdU to undetectable amounts upon numerous cell cycles. Another scenario suggests that even a low number of HBC divisions may generate a sufficient pool of transit-amplifying GBC intermediates (Iwai *et al*., 2008).

It was previously shown that SCs in adult zebrafish OE are capable of dividing (Bali, 2015). However, SCs do not seem to contribute to the generation of new neurons after ATP injection, since they appear negative for BrdU labeling. Yet, it is hard to unequivocally make this statement considering the filamentous, non-cytosolic nature of the CykII labeling that is

used to highlight SCs. Studying SCs with a specific nuclear/cytosolic marker would provide better insight into their involvement in this process. Unfortunately, such a marker is currently not available.

Collectively, I observed an increase in the proliferation especially in the ILC region of the adult zebrafish OE, upon extracellular ATP application, possibly without contribution of SCs or HBC-like cells. GBCs in mouse OE are multipotent progenitor cells which are able to generate OSNs (Huard and Schwob, 1995). Krolewski *et al.* (2012) show that GBCs are responsive to the neuronal loss after bulbectomy and contribute in the regeneration of OSNs while HBCs remain quiescent in the process. Retroviral lineage tracings revealed that GBCs are capable of generating OSNs and new GBCs (Schwob *et al*., 1994a). The increase in the proliferation I observed appears parallel the nature of GBCs. Increased amounts of ATP in the tissue may be recognized by GBCs as a signal of dying neurons, resulting in GBC mitotic activity to compensate for the loss. The confinement of the increase in proliferation to the two main proliferative regions in the OE still needs further elaboration and a sub-population of GBCs may exist in the ILC and S/SN border in zebrafish OE. For HBCs however, dying neurons is not a sufficient signal for activation, which may also be the case in adult zebrafish OE (Herrick *et al*., 2017) and selective ablation of SCs in zebrafish OE may provide conclusive results.

These results again point towards the existence of another population of potentially $Sox2^{+}$ progenitor cells, which are neither SCs nor HBC-like cells. The presence of GBC-like cells in the adult zebrafish OE should be investigated in future as they seem to be the candidate, constantly dividing cell population. The $BrdU^{+}$ cells laying directly apical to the basal cells in the OE 3 d after ATP application, provide positional hints about the types of dividing cells, since the zebrafish OE may contain analogous progenitor cell populations to the murine model yet with a distinct and more restricted tissue distribution.

5.6. Morphology of HBC-like cells

Even though there are numerous studies on the neuronal cell types in the zebrafish OE, the progenitor cell populations in the epithelium, which contribute greatly to the maintenance and repair of the tissue, remain under-investigated. Using IHC with antibodies against krt5, a population of cells resembling HBCs in murine OE was observed. The cells labeled with the anti-krt5 antibody, however, display different cell morphologies in the sensory and the non-sensory regions of the adult zebrafish OE. In the sensory region, the population consists of horizontally elongated cells that are directly in contact with basal lamina. In the S/NS border and NS region however, they are vertically more elongated and protrude into apical regions of the OE. This type of morphology difference of $krt5^+$ cells has not been reported in rodents or *Xenopus* and may be due to the different tissue organization of the OE in different animals. Mice have a much larger sensory OE and not much is known about the molecular and cellular details at the sensory/non-sensory border. To further conclude on the significance of this seemingly unique feature of zebrafish $krt5^+$ cells, further investigation of the sensory/respiratory OE border in the mouse is needed.

The glial-like appearance of HBC-like cells in the S/NS border and NS regions may allow them to undergo asymmetric cell division with differential localization of their intracellular contents. Morphological changes in HBCs have been reported after the chemical lesion of the murine OE (Packard *et al*., 2011; Brann *et al.*, 2015). Interestingly, HBC-like cells rarely seem to be $BrdU^+$ in the ILC, sensory region or S/NS border of the adult zebrafish OE under normal conditions. One possible explanation for the quiescence of HBCs under normal conditions is proposed by Joiner *et al.* (2015), which claims that physical contact between SCs and HBCs may be what retains HBCs from dividing by conveying the information that the OE is intact. This explanation is also supported with the fact that chemical insult resulting in ablation of GBC or SCs, but not neurons, leads to activation and proliferation of HBCs (Jia *et al*., 2010; Leung *et al.*, 2007). Gene expression analysis after damage revealed that Notch signaling may take part in the regulation of ΔNp63 levels in HBCs and that ΔNp63 acts as a break on cell divisions. Notch ligands are also expressed in SCs and HBCs, which might explain the activation of HBCs after damage that

causes loss of SCs in the OE (Herrick and Schwob, 2015). A similar mechanism may also be present in the zebrafish OE. Mackay-Sim and Kittel (1990) report two modes of asymmetric cell division, a fast cycle and a slow cycle, in the basal cells of mouse olfactory epithelium, according to changes in numbers of silver grains in the nuclei of cells. Carter *et al.* (2004) suggest that a population of mouse HBCs cultured *in vitro* divided asymmetrically and generated heterogeneous adherent colonies. HBC-like cells in zebrafish OE may also have similar properties. While the morphologic differences along the lamella and possible adherence to the basal lamina are pointing towards such a behavior, further investigation is needed to elaborate this possibility. Either way, the observed dramatic change of HBC morphology after damage and the occasional observation of apically elongated HBCs in the ILC suggest that reorganization and re-establishment of cellular polarity may be required for neurogenic activity of HBCs.

5.7. Contribution of HBC-like cells to the regeneration of the tissue

The exact lineages HBC-like cells, SCs, and the possible population of GBC-like cells are yet to be investigated in the adult zebrafish OE. HBCs have been shown to be capable of generating all neuronal and non-neuronal cell types in the murine OE with the exception of gland calls (Iwai *et al*., 2014). Disputes remain, however, whether GBCs are always HBC derived or if they constitute a cell population by themselves. Some reports even suggest that HBCs are generated from GBCs (Chen *et al*., 2004).

Conducting IHC with antibodies against krt5 and BrdU on OE sections 3 d after chemical lesion with BrdU incubation for last 10 h I observed $BrdU^{+}/krt5^{+}$ cells with cell bodies invading the OE tissue throughout the lamella, even in the apical region of the OE. The cells also seem to form multiple layers which brings up the question, whether these cells are capable of generating all other cell types in the adult zebrafish OE like their murine analogs. Expressing stem cell markers Sox2 and Tp63, HBC-like cells can be argued to be the possible major slow-dividing progenitor cell population in the zebrafish OE. Interestingly, this behavior of HBC-like cells, remaining relatively inactive upon ATP

application but actively contributing to tissue regeneration after heavy damage, closely resembles the nature of HBCs in mouse OE, which require loss of SCs in order to downregulate ΔNp63 and be activated. (Herrick and Schwob, 2015). This issue also needs further investigation such as the analysis of $Sox2^{+}$ cells together with a suitable marker for SCs with the krt5 background in the regenerating OE for a wider time interval or selective *in vivo* ablation of various cell types.

In conclusion, the data presented in this thesis provide important steps towards the morphological and functional characterization of HBC-like cells in the adult zebrafish OE, with the suggestion that they may not be the (sole) ATP-responsive cell population under normal conditions. HBCs exhibit interesting proliferative responses after damage, which opens up a new area to investigate in the teleost OE. Moreover, the data highlights the importance of purinergic signaling in neuro-regeneration and reveals hints on the existence of a yet uncharacterized GBC-like cell population in the zebrafish OE.

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APPENDIX A: EQUIPMENTS

Table A. 1. The table of equipments.

Product Name	**Manufacturer Company**
Aquatic Habitats	Pentair Aquatic Eco-systems, Inc., USA
GELoader tips, 0.5-20 µl	Eppendorf, Germany
Crystal Sea Marinemix	Marine Enterprises International, USA
SZ61 stereomicroscope	Olympus, USA
IKA Color Squid magnetic stirrer	IKA works, Inc., USA
Vortex-Genie 2	Scientific Industries, Inc., USA
Filter tips (10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)	Greiner Bio-One, Germany
Electrical Balance	Sartorius, Germany
-20˚C Freezer	Ugur, Turkey
-20˚C Freezer	Arcelik, Turkey
Microwave oven	Vestel, Turkey
Refrigerator	Arcelik, Turkey
pH-meter, pH315i	WTW, Germany
Universal Incubator	Binder, Germany
Single channel micropipettes (10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)	Eppendorf, Germany
Super Pap Pen	Liquid Blocker, Japan
Serological pipettes (5ml, 10ml, 25ml, 50ml)	Greiner Bio-One, Germany
Glass bottle	Isolab, Germany
Cryostat CM3050S	Leica Biosystems, Germany
Adhesion slides, Superfrost Plus	VWR, Germany
Dishwasher, Melabor G 7783	Miele, Germany
Swiftlock Front loading Autoclave	Astell, UK
Laboratory Drying Oven, KD 200	Nüve, Turkey
Discard Container Autoclave, Midas 55	Priorclave, UK
Analytical Balance, ME54	Mettler Toledo, India
Ice Flaker	Brema, Italy
-86˚C ULT Freezer	ThermoForma, USA
Brine Shrimp Artemia Cysts (*A. franciscana*)	Salt Lake Aquafeed, USA
VWR micro cover glass, 22x50 mm	VWR, USA
Parafilm™	Parafilm, USA
Whetstone	Dan’s Whetstone Company Inc., USA
Forceps, FST	Dumont, Switzerland
Instrument oil, No.29055-00	FST, Switzerland
Laser Scanning Confocal Microscope, SP5	Leica, Germany

APPENDIX B: CONSUMABLES

Table B. 1. The table of consumables.

Product Name	**Manufacturer Company**
Sodium bicarbonate	Proline, U.S.A. (SC12A)
Calcium sulfate	Alfa Aesar, Germany (33301)
Phosphate buffer saline (PBS) tablets	ThermoFisher Scientific, U.S.A. (18912014)
Optimal cutting temperature medium (OCT)	Fisher HealthCare, U.S.A. (4585)
Paraformaldehyde	Sigma-Aldrich, U.S.A. (P6148)
Triton X-100	AppliChem, Germany (A4975)
Mouse anti-HuC/D	Life Technologies, U.S.A. (1661237)
Rat anti-BrdU	Abcam, UK (AB27952)
Rabbit anti-keratin 5	Abcam, UK (AB53121)
Rabbit anti-Sox2	GeneTex, U.S.A. (GTX124477)
Mouse anti-cytokeratin type II	Developmental Studies Hybridoma Bank, U.S.A. (1h5)
Mouse anti-EBF	Santa Cruz Biotechnology, U.S.A. (sc-137065)
Rabbit anti-Tp63	GeneTex, U.S.A. (GTX124660)
Anti rabbit Alexa Fluor 488	Life Technologies, UK (A11008)
Anti mouse Alexa Fluor 488	Life Technologies, UK (A28175)
Anti rabbit Alexa Fluor 555	Life Technologies, UK (A32732)
Anti mouse Alexa Fluor 633	Life Technologies, UK (A21094)
Anti rat Alexa Fluor 633	Life Technologies, UK (A21094)
Absolute methanol	VWR Chemicals, Germany (20847.320)
Proteinase K	Roche, Germany (13996300)
BrdU	AppliChem, Germany (A2139)
MS-222	Sigma-Aldrich, U.S.A. (A5040)
Agarose	SeaKem Cambrex, U.S.A. (50004)
Bovine Serum Albumin (BSA)	New England Biolabs, U.S.A. (B9001)
Suramin	Sigma-Aldrich, U.S.A. (S2671)
ATP	Sigma-Aldrich, U.S.A. (A26209)
HCl	Sigma-Aldrich, U.S.A. (H1758)