Common principles of olfactory coding across olfactory receptor families and species

Inauguraldissertation zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät der Universität zu Köln

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2021

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Tag der letzten mündlichen Prüfung:

19.02.2021

Abstract

Beside vision and hearing, the chemoreception is one of the important senses for organisms in an aquatic environment. To detect food, predators or sexual partners a broad variation of different receptors in the nose of an animal is needed. Therefore, coding strategy of the olfactory receptors is the formation of spatial distribution patterns in order to maintain their continued function in the event of damage to the epithelium. The investigation of individual receptor groups of the olfactory system in fish is now mostly done through phylogenetic studies, as more and more genomes are being deciphered enabled by rapid technical progress. This work focused on the visualization of individual receptors and receptor families of different aquatic animals in the olfactory epithelium and for this, among other things, expression and spatial distribution were investigated. Here it could be shown, that the recently discovered adenosine receptor A2c is expressed in the eel, carp and claw frog in a sparse and distributed pattern within their olfactory epithelium similar to the pattern observed for zebrafish. A sharp contrast is formed by the expression of A2c in lamprey, because of A2c-expressing cells forming a contiguous domain directly adjacent to the sensory region. In addition it was shown, that this expression is consistent, with the expression in neuronal progenitor cells. It could be that A2c switched the function during evolution in the lineage of vertebrates. Also for the first time, the expression pattern of three of six possible V1R receptors, and the absence of two possible V1R and two V2R receptors in adult *Lampetra fluviatilis* was demonstrated. It was shown that the three V1Rs form different expression zones, when looking at the height in the lamella. Here we also completed a project to investigate the spatial distribution of the complete V1R-related Oras receptor family in zebrafish. It was shown, that each of the *ora* genes had its own expression zone. Beside this, two different *in situ* staining methods were used and compared with each other, chromogenic NBT/BCIP staining and fluorescent TSA staining. It turned out that the TSA method is the better choice, as it is much more sensitive, and only through this was it possible to examine the expression pattern of two of the six V1RS, Ora5 and Ora6.

Zusammenfassung

Neben dem Sehen und Hören ist die Chemorezeption einer der wichtigsten Sinne für Organismen in Gewässern. Um Nahrung, Raubtiere oder Sexualpartner zu erkennen, ist eine breite Variation verschiedener Rezeptoren in der Nase des Tieres erforderlich. Die Kodierungsstrategie der Geruchsrezeptoren ist die Bildung räumlicher Verteilungsmuster, um ihre Funktion im Falle einer Schädigung des Epithels aufrechtzuerhalten. Die Untersuchung einzelner Rezeptorgruppen des olfaktorischen Systems in Fischen erfolgt heute hauptsächlich durch phylogenetische Studien, da aufgrund des raschen technischen Fortschritts immer mehr Genome entschlüsselt werden. Diese Arbeit konzentrierte sich auf die Visualisierung einzelner Rezeptoren und Rezeptorfamilien verschiedener Wassertiere im Riechepithel und untersuchte unter anderem deren Expression und räumliche Verteilung. Hier konnte gezeigt werden, dass der kürzlich entdeckte Adenosinrezeptor A2c in Aal, Karpfen und Klauenfrosch in einem spärlich verteilten Muster innerhalb ihres Riechepithels exprimiert wird, welches dem für Zebrafische beobachteten Muster sehr ähnlich ist. Ein scharfer Kontrast dazu, ist das Expressionsmuster von A2c im Neunauge, da die A2c-positiven Zellen dort eine zusammenhängende Domäne direkt neben der sensorischen Region bilden. Zusätzlich aufgezeigt wurde, dass die Lokalisation der A2c Rezeptoren mit der Expressionsregion von neuronalen Vorläuferzellen übereinstimmt. Deshalb könnte es sein, dass A2c die Funktion während der Evolution in der Linie der Wirbeltiere wechselte. Des Weiteren konnte hier zum ersten Mal das Expressionsmuster von drei möglichen V1R-Rezeptoren und das Fehlen dieses bei zwei möglichen V1R- und zwei V2R-Rezeptoren bei adulten Neunaugen (*Lampetra fluviatilis*) bestätigt werden. Bei der Untersuchung der Höhe in der Lamelle, wurde beobachtet, dass die drei V1Rs verschiedene Expressionszonen bilden. Zusätzlich konnte ein Projekt welches die Untersuchung der räumlichen Verteilung der gesamten V1R-verbundenen Ora Familie im Zebrafisch beinhaltete abgeschlossen werden. Es konnte gezeigt werden, dass jedes der *ora*-Gene eine eigene Expressionszone besitzt. Dafür wurden zwei verschiedene *in situ* -Färbemethoden verwendet und miteinander verglichen, die chromogene NBT/BCIP-Färbung und die fluoreszierende TSA-Färbung. Es stellte sich dabei die TSA-Methode als die bessere Wahl heraus, da diese viel sensitiver ist, und nur dadurch das Expressionsmuster von zwei der sechs V1RS, Ora5 und Ora6, untersucht werden konnte.

Table of content

List of Figures

List of Tables

Abbreviations

A2c	adenosine receptor 2c
ACIII	adenylate cyclase 3
AMP	adenosine monophoshate
AP	alkaline phosphatase
ATP	adenosine triphosphate
AOO	accessory olfactory organ
BC	before Christ
cAMP	cyclic adenosine monophosphate
CasR	calcium sensing receptor
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
cm	centimetre
CNS	central nervous system
Cys	cysteine
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
ECDF	empirical cumulative distribution function
ETOH	ethanol
FPR	formyl peptide receptor
FW-Primer	forward primer
GDP	guanosindiphosphat
GPCR	G protein-coupled receptor
GRM	glutamate receptor
GTP	guanosine triphosphate
HCl	hydrochloric acid
HNPP/Fast Red	2-hydroxy-3-naphtoic acid-2'-phenylanilide phosphate/Fast Red
IHC	immunohistochemistry
IL	intracellular loop
ISH	*in situ* hybridisation
KS test	Kolmogorov–Smirnov test
mg	milligram
ml	millilitre
mM	millimolar
NADH	nicotinamide adenine dinucleotide
NBT/BCIP	nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate
MOE	main olfactory epithelia
ng	nanogram
OE	olfactory epithelium
OR	olfactory receptor
OSN	olfactory sensory neuron
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen

PCR	polymerase chain reaction
PFA	paraformaldehyd
PKA	protein kinase A
POD	peroxidase
RNA	ribonucleic acid
RT	room temperature
RV-Primer	reverse primer
SMO	smoothened receptor
SSC	saline-sodium citrate
TAAR	trace amine-associated receptor
TM	transmembrane
Tris	tris(hydroxymethyl)aminomethane
TRPC2	transient receptor potential channel 2
TSA	tyramide signal amplification
VNO	vomeronasal organ
Vglut	vesicular glutamate transporter
WG	whole genome
µg	microgram
µl	microlitre

1. INTRODUCTION

To give an command to their environment, all individuals need to organize and interpret their sensory impressions, which is called perception. In c.350 BC, based on the work of previous great philosophers like Pythagoras, Empedocles, Democritus and Plato, Aristotle was the first one to describe in his script De Anima five senses of perception, which are known until today. These forms of perception are visual perception (vision), auditory perception (hearing), haptic perception (touch), gustatory perception (taste) and olfactory perception (smell). Beyond the Middle Ages, these findings were the cornerstones of the teachings in the Orient and Occident. In the last few hundred years, more senses have been described in nature, such as equilibrioception, thermoception, proprioception, sexual stimulation, nociception and magnetoception.

At the beginning of life in the oceans, it was vital for the first unicellular organisms to find and process energy sources to sustain all the important cellular functions and ensure their own reproduction. The ability to detect chemicals by means of suitable receptors or mechanisms allows all moving organisms access to energy sources and is still today an essential part of the survival of both unicellular and multicellular organisms. This evolutionary oldest process is called chemoception. The basis for this is chemoreceptors, also called chemosensors, which are proteins or protein complexes that bind a chemical substance from outside the cell body and convert it into a cell-like biological signal which the cell can interprete (Wichter and Poll, 2018; Bushdid et al., 2014). Therefore, research on receptors is of great interest not only to science, but also to industry for the development of new products, e.g. flavour industry. Here we deal with basic research on individual olfactory receptors in aquatic animals.

1.1 G protein-coupled receptor

In nature, biological membranes contain different types of transmembrane receptors. These are macromolecule, many of them transmembrane proteins, located in the lipid double layer of the cell, consisting of an extracellular-, transmembrane and intracellular domain. Towards outside of the cell, the function of the extracellular domain is to recognize a

ligand, like atomic ions, hormones or neurotransmitter. The transmembrane domain of receptors forms after the binding of a ligand, either a protein channel, where ions can flow through or undergo a conformational change, which results in effects in the intracellular domain. The function of the intracellular domain of the receptor is relaying the signal. Either the domain has an enzyme activity, like tyrosine kinase, or protein-protein interactions start against an effector protein (Signaling-Molecules-and-Cellular-Receptors; Open stax; CNGX.org) .

Transmembrane receptors can be divided into three different classes: beside the ion channel-linked receptors and the enzyme-linked receptors, the G-protein coupled receptors (GPCRs) form the largest family of membrane proteins and transmembrane receptors in biological systems (Rosenbaum et al., 2009; Pierce et al., 2002).

GPCRs can be activated by small molecules like neurotransmitters, peptide hormones, chemokines, lipids, and also by environmental stimuli such as light, odorants, and flavourings. This is one of the leading reasons why GPCRs are the major targets for pharmaceutical therapeutics (Marrari et al., 2007). GPCRs can be classified, after the GRAFS- or Fredriksson -System (Schiöth and Fredriksson, 2005), into five subfamilies: Rhodopsin receptor family/class A, Secretin receptor family/class B, Adhesion receptor family/class B, Glutamate receptor family/class C and Frizzled (Nordström et al., 2011).

The rhodopsin family/class A can be further divided into groups α, β, γ and δ. One of the largest groups in the Rhodopsin family/class A is the olfactory receptors which are found in group δ. Vomeronasal type 1 and Taste receptors, which was before grouped in former studies into the Frizzled family, are associated with the Rhodopsin family (Nordström et al., 2011). The metabotrobic purine receptors also belong to group δ (Fredrikson et al., 2003). Peptides, proteins (including enzymes), small organic compounds, lipid-like substances and nucleotides are the common type of ligands for the rhodopsin family/class A.

Another small family of the GPCRs, is the Secretin receptor family/class B, which binds peptide hormones in their extracellular hormone-binding domain.

The third family of GPCRs are the Adhesion receptor family/class B, which have long N termini and display a GPCR proteolytic domain. This is one of the reasons why Secretin and Adhesion receptor are grouped in individual families, but because of their sequence similarities they are both members of class B. Another special feature of the Adhesion receptor family is that they bind extracellular matrix molecules.

Members of the Glutamate receptor family/class C, are glutamate receptors (GRMs), the calcium-sensing receptors (CASR), some GABAergic receptors, the sweet and umami

taste receptor (TAS1R1-3), GPRC6A and seven orphan receptors. The Glutamate receptor family classC binds ligands like amino acids, cations, small organic compounds and carbohydrates.

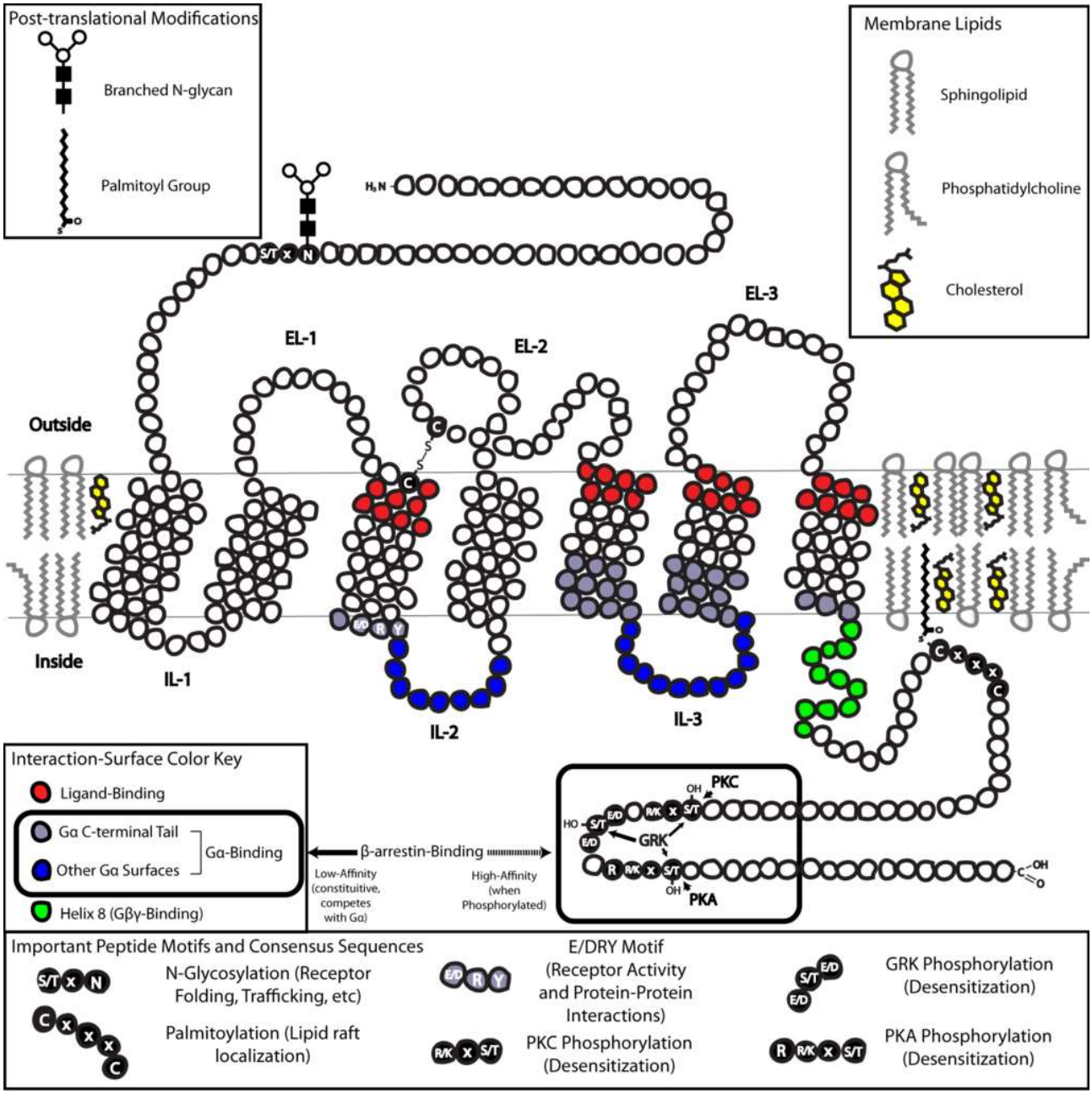

Figure 1.1: Two-dimensional schematic of a typical seven-transmembrane GPCR. A 2-dimensional schematic highlighting select structural features that are important to the biological function of many different GPCRs. Abbreviations, etc: Branched N-glycan Filled black Squares= N-acetylglucosamine Unfilled Circles= Mannose Each circle making up the long chain represents an amino acid. The peptide motifs are represented by filled circles and labeled using commonly accepted single-letter abbreviations for each amino acid. N= Asparagine, x= any amino acid, S/T= Serine or Threonine, C= Cysteine (attached S represents an oxidized sulfhydryl group), E/D= Glutamate or Aspartate, Y=Tyrosine, R/K= Arginine or Lysine GPCR domains: IL-1 to IL-3= Intracellular loops 1-3 EL-1 to EL-3= Extracellular loops 1-3 Other Proteins: G-alpha= Alpha subunit of a heterotrimeric G-protein G-beta/gamma= G-beta/gamma heterodimer of heterotrimeric G-protein PKC= Protein Kinase C PKA= Protein Kinase A GRK= G-protein Coupled Receptor Kinase (GPCR kinase) Final note: The consensus sequence for PKC phosphorylation is written in the legend from C to N terminus, while the other motifs are all shown as N to C. (https://en.wikipedia.org/wiki/G_protein-coupled_receptor 2020)

The superfamily of GPCRs is completed by the Frizzled subfamily. Members of this subfamily are the frizzled receptors and the smoothened receptor (SMO), where frizzled receptors binds proteins and SMO small organic compounds (Lagerström and Schiöth, 2008).

The unique feature of GPCRs, are the seven membrane spanning domains (TM1-TM7) or transmembrane α-helices (7M), which are α-helices connected by three extracellular loops (EL1-EL3) and three intracellular loops (IL1-IL3) (Fig.1.3). Whereby the extracellular parts can be glycosylated. The extracellular regions are responsible for the ligand binding, where the intracellular regions interact with the G-protein, arrestins or other downstream effectors. All GPCRs share a cysteine bridge between the first and the second extracellular loop (Lagerström and Schiöth, 2008). The N-terminus is located extracellular, with different length between the five subfamilies of GPCRs, and can contain a ligand- binding domain, like in the GRMs (Latek et al., 2012). The C-terminus is intracellular and recent studies showed that mutations can have an influence on the proper function of the receptor (Wang et al., 2016). For a faster receptor signalling, all GPCRs shared one or more sides for palmitoylation at the intracellular loops or the C-terminus (Fig.1.3). On these palmitylation sides a covalent modification of cysteine (Cys) residues *via* addition of hydrophobic acyl groups takes place, which has the effect that the receptor is target to cholesterol- and sphingolipid-rich microdomains of the plasma membrane, which are called lipid rafts (Moffet et al., 2000), with the seven transmembranes of the GPCRs, forming a barrel-like structure inside the plasma membrane. All G-proteins consist of three subunits, α, β and γ, and are bound *via* their α-subunit to the IL2 and IL3 domains. GPCRs can be activated by binding a ligand, such as e.g. proteins, proteins, amino acids, amines or small organic compounds, in the N-terminal region or extracellular loops. The transmembrane domains TM3 and TM6 can also form a binding pocket, which can be activated by peptides. Another possibility is that low molecular weight compounds bind to several transmembrane domains, as in olfactory receptors or the GPCRs get activated by heterodimerization or other unknown mechanisms (Lagerström and Schiöth,2008). After activation, the GPCRs undergo a conformational change, which initiates the exchange of guanosine disphosphate (GDP)/guanosine triphosphate (GTP) associated with the Gα subunit. In the inactive state GDP is bound to the Gα subunit and in the active state GTP(Fig.1.4). When the G protein is activated the heterotrimers dissociate into two parts, the active form of the Gα and the Gβ/γ subunit. The Gα subunit can interact and regulate other downstream effector molecules such as protein kinases, phospholipase C or D, calcium or potassium channels

and adenylyl cyclase. The Gβ/γ subunit regulates the potassium channel, adenylyl cyclase, phospholipase C-β (PLC-β), phospholipase A2 (PLA2), phosphoinositide 3-kinase (PI3-kinase), β-adrenergic receptor kinase and can activate the Gα subunit (Tuteja; 2009).

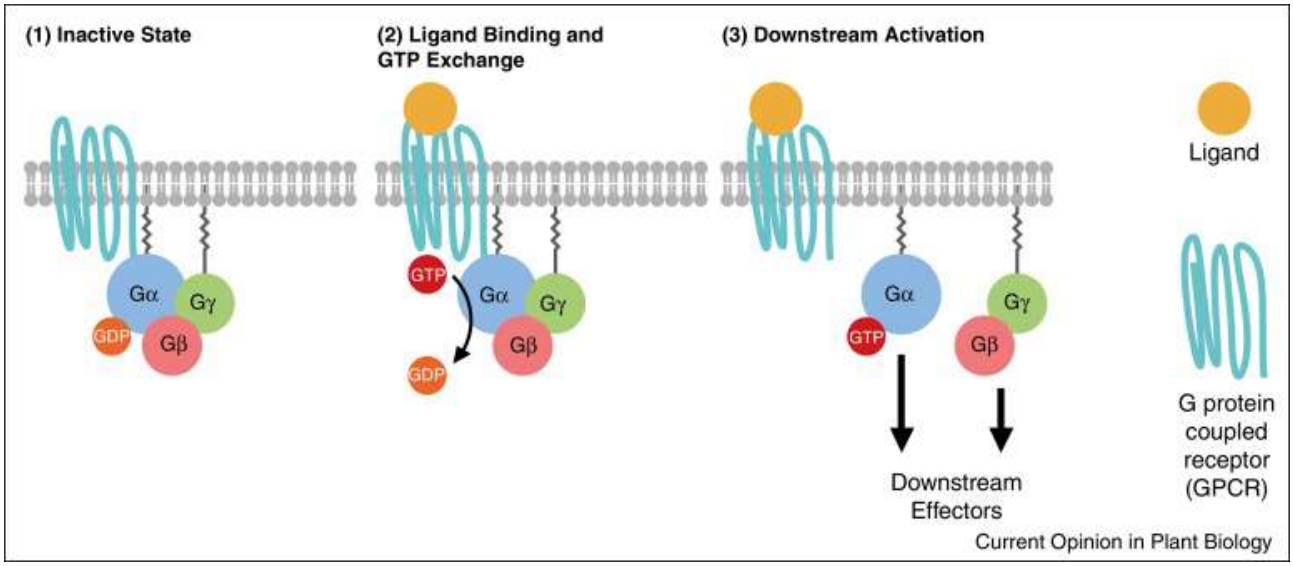

Figure 1.2: Canonical G protein signalling mechanism in animals. (1) Due to the high GTPase activity of Ga and its slow exchange kinetics, the heterotrimeric G protein is bound to GDP by default and in its inactive state. (2) Ligand binding induces a conformational change of the GPCR promoting the exchange of GDP to GTP. (3) GTP-bound Ga and the detached Gbg dimer are capable to activate downstream signaling events. (Stateczny et al. 2016)

In five of the six kingdoms in life, Bacteria, Protozoa, Plantae, Fungi and Animalia, seven transmembrane-containing proteins have been identified. But only in Animalia, Fungi and Protozoa have GPCRs been found (Lagerström and Schiöth,2008).

1.2 Purines and Purinergic receptors

In the present work, the purinergic receptors were of particular interest. Purines consist of a pyrimidine ring which is fused at position five and six of the carbon atoms to an imidazole ring (Fig.1.1). In nature, purines play an essential role in life on earth, because they are part of the two of the four deoxyribonucleotides deoxyadenosine and deoxyguanosine which form the backbone of the DNA together with the other two pyrimidines deoxycytosine and deoxythymine. The purines adenosine and guanine are also major components of two of the four ribonucleotides, where they form, together with the pyrimidines cytosine and uracil, the backbone of the RNA. Purines are also prominent

components in a lot of other significant biomolecules, such as GTP, cyclic AMP, NADH and coenzyme A (Guyton and Arthur C., 2006). Also all living things, all plants, all animals and also humans, in order to remain alive, need the same energy-rich molecule, the purine containing “cellular fuel” adenosine triphosphate (ATP).

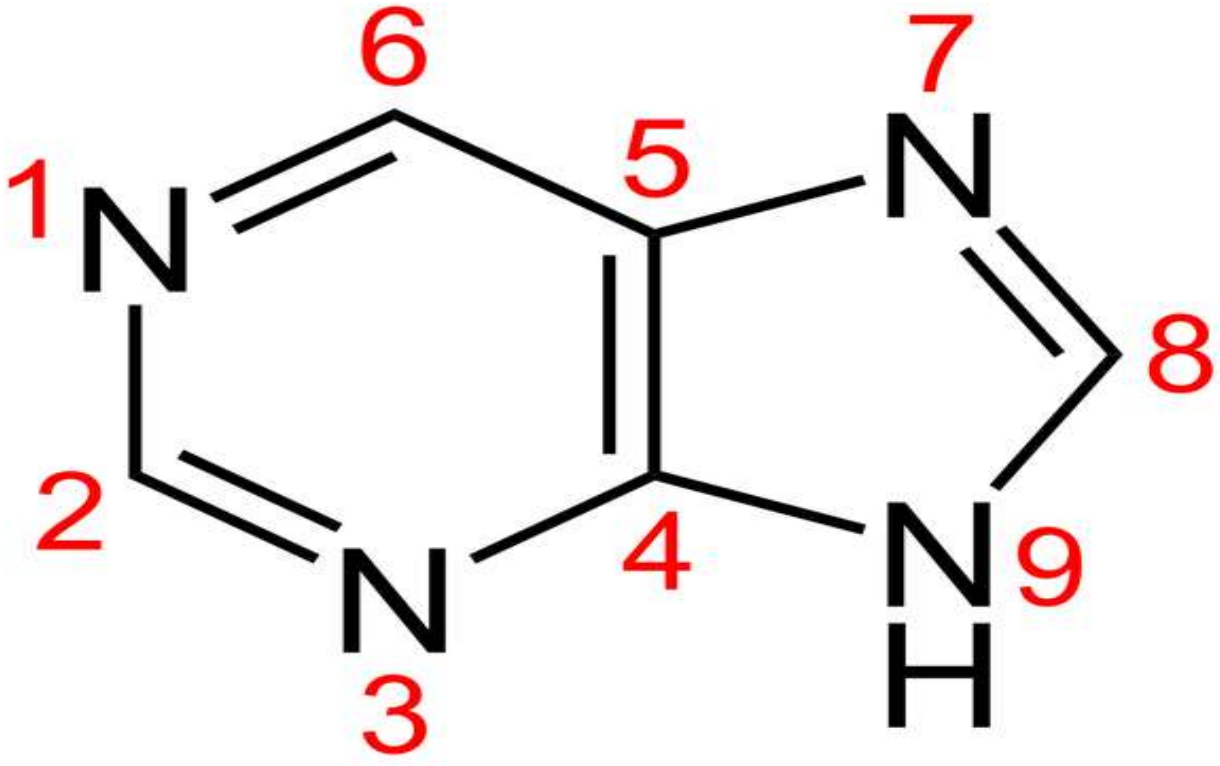

Figure 1.3: Molecular structure of purine. (https://de.wikipedia.org/wiki/Datei:Purin_num2.svg 2008)

In the case of chemical messengers, purines seem to be the most primitive and wide-spread one in the plant and animal kingdoms. In the purinergic signalling system, pyrimidines and purines play a crucial role as signalling molecules. Especially the purines ATP and adenosine are important ligands for a lot of purinergic receptors. In living cells, purines and pyrimidines nucleotides are released by different mechanisms, like active transport, exocytosis and diffusion through membrane channels (Burnstock and Verkhratsky, 2009). But also dying cells can release purines and pyrimidines, especially ATP and other nucleotides can be degraded *via* ectonucliotidases (Zimmermann, 2006) and they can also act as signalling molecules for purinergic receptors.

Purinergic signalling is involved in normal behaviour, which includes locomotion, feeding, cognition and memory and also in neurotransmission and neuromodulation in the central nervous system (CNS); (Burnstock et al., 2011).

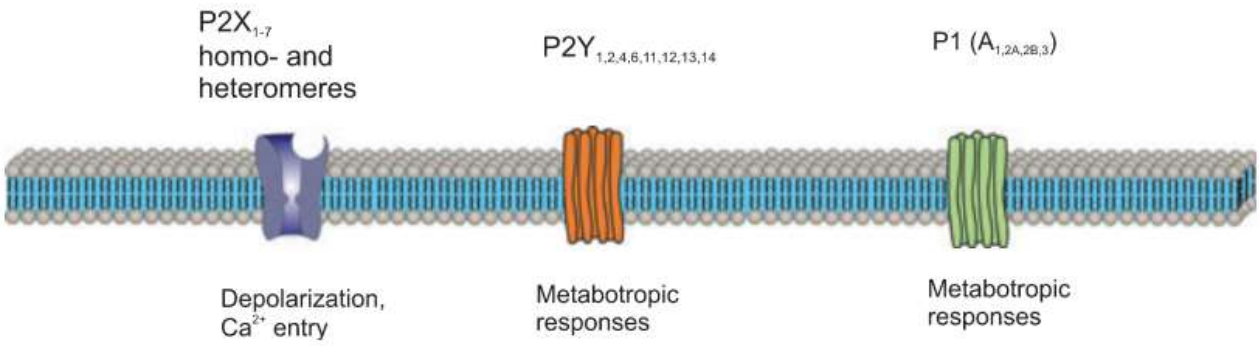

Figure 1.4: Purinergic receptors (Burnstock and Verkhratsky, 2009)

Purines are recognized by two big classes of purinergic receptors, the P1 and P2 class. Both are expressed in various tissues. P1 receptors are activated by adenosine and various synthetic adenosine analogues, whereas P2 receptors are activated by purines and pyrimidines, excluding adenosin.

P1 receptors are metabotropic and are activated by adenosine and various synthetic adenosine analogues. The four members of the P1 receptorclass, A_1, A_{2A}, A_{2B} and A_3 are G proteine-coupled adenosine receptors, generally coupled with adenylate cyclase and have either excitatory (A_1 and A_3) or inhibitory effects (A_{2A} and A_{2B}) on the production of cyclic AMP (cAMP)(Burnstock, G, Verkhratsky, A 2009; Burnstock 2018).

The P2 receptor class is subdivided into the P2X and P2Y class, the former ionotropic and the latter metabotropic. Up to now, seven different ionotropic ATP-gated P2X receptors are known P2X1-P2X7 and all consist of two transmembrane-spanning regions (TM1;TM2), where TM1 is involved in the channel gating and TM2 lines the ion pore. The N-and the C-Terminus are intracellular, where the N-terminus contains a consensus site for protein kinase C and the C-terminus binding motifs for protein kinases (Boué-Grabot E. et al. 2000; Burnstock 2018). In contrast to the P2X class, the receptors P2Y class are nucleotide-sensitive G protein-coupled receptors. At present 14 different ones are known (P2Y1-P2Y14), eight of them in human P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14 and the rest are either mammalian orthologues or do not respond to nucleotides. All P2Y receptors act through the second messenger system of cAMP or inositol triphosphate ($InsP_3$)(Burnstock, 2018). Purinergic receptors are not only present in vertebrates or invertebrates (Burnstock, G,Verkhratsky, A 2009; Burnstock, 2018), they were also recently discovered in plants (Cao Y et al., 2014).

1.3 Olfactory receptors

In 2004 the Nobel Prize in Physiology or Medicine was awarded to Richard Axel and Linda Buck for their discoveries of odorant receptors (ORs) and the organization of the olfactory system. Before they published their paper, the basic principles for remembering and recognizing thousands of odours were not clear. Axel and Buck were able to show that the rat genome contains a large family of GPCRs (~1000), which are about 3% of all genes, coding for olfactory receptors (Buck and Axel, 1991). Olfactory receptors are expressed in olfactory sensory neurons (OSNs) and follow the so-called “one neuron - one receptor” rule. This means that only one of these thousands of specific receptors is expressed in one mature OSN (Serizawa et al., 2004). In immature OSNs various olfactory receptors are co-expressed at the same time, until the OSNs become mature (Tan et al., 2015). OSNs expressing the same set of receptors are connected *via* their axons to the olfactory bulb, where they form glomeruli (McEwen et al. 2008).

In the main olfactory epithelium in mammals, different types of olfactory receptors are expressed. Beside the most well-known OR family, OSNs also express the trace amine-associated receptor (TAARs) and the recently discovered non-GPCR receptor family MS4A. The vomeronasal organ (VNO) or Jacobson's organ, which is also part of the olfactory sensory system and present in many mammals, lizards and snakes, has its own subset of olfactory receptors. It expresses the vomeronasal type one receptors (V1R), the vomeronasal type two receptors (V2Rs) and the formyl peptide receptors (FPRs) (Greer et al.2016). In fish, which lack a VNO, V1Rs and V2Rs are expressed together with TAARs, ORs and the recently discovered novel adenosine receptor A2c in the main olfactory epithelia (MOE)(Wakisaka et al. 2017). Around 430 million years ago the evolutionary separation between the lineage of fish and tetrapodes took place, but the receptor repertoires have a high level of molecular conservation (Kumar et al., 2017).

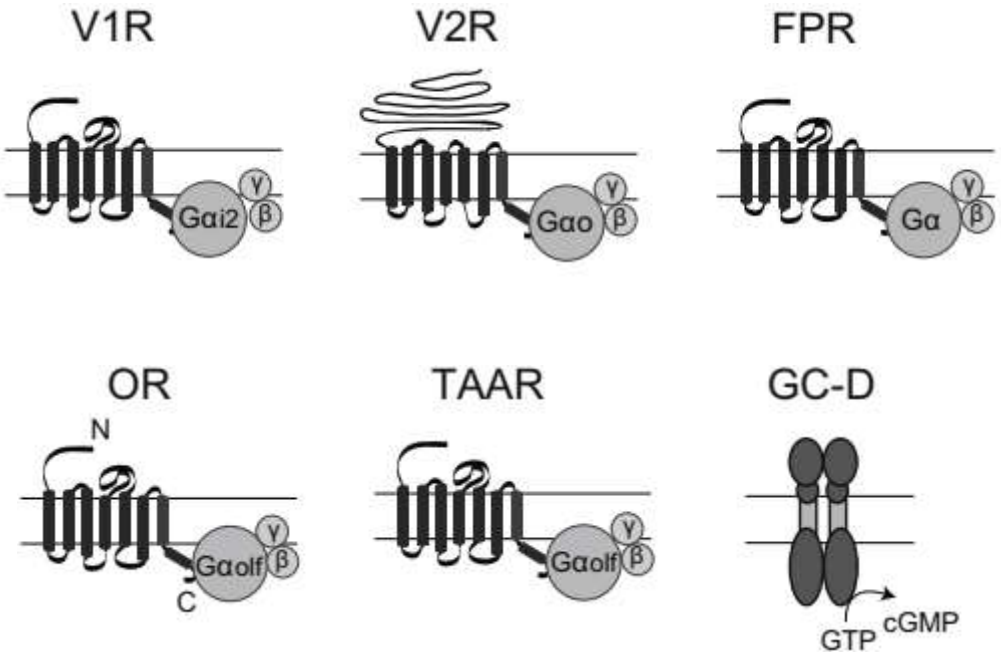

Figure 1.5: Olfactory receptor types modified from (Ihara et al. 2013)

1.3.1 ORs

ORs are the largest GPCR subfamily in all mammals and known to exhibit broad molecular receptive ranges, so that they can detect a large variety of airborne odorants in a redundant manner (Ihara et al., 2013). In humans this superfamily contains around 390 functional genes and 450 pseudogenes, which is many fewer compared to rodents with more than 1000 functional *or* genes. This could reflect the necessity of olfaction for survival of different mammalian species. But the fact that a cow has 970 functional genes and a dog 811, shows that the number of *or* genes is not directly related to lifestyle or environmental requirements (Olender et al., 2008). In mammalian OSNs several subunits of heterotrimeric G proteins have been indentified including $G\alpha_{olf}$, $G\alpha_{s}$ and $G\alpha_{15}$ (Kajiya et al., 2001). The total number of *or* genes in fish is many fewer than in mammals. The zebrafish genome contains about 150 functional ORs, expressed mainly as ciliated types of OSNs (Sato et al., 2005) and mostly following the “one neuron – one receptor” rule of monogenic receptor expression (Sato et al., 2007). Quantitative analysis of some of the ORs *in situ*-hybridization patterns in the zebrafish olfactory epithelium, shows that they form at least three overlapping, but clearly distinct, expression domains (Weth et al. 1996). Vertebrate *or* genes can be grouped into fish-like class I genes and mammalian-like class II genes, in which class I genes are specialized for detecting water-soluble odorants, and class II

genes are for recognizing airborne odorants. In other lower vertebrates like *Xenopus laevis* a member of the family of amphibians, the number of functional *or* genes, with more than 400, is higher than in humans. This can be explained by the fact that amphibians live in both aquatic and terrestrial environments (Niimura and Nei, 2005). Interestingly, some vertebrate ORs are expressed outside the nose in the eye, heart, kidney, skeletal muscle, prostate and sperm (Pronin et al., 2014).

1.3.2 V1Rs

Another type of the rhodopsin-like GPCR family is the mammalian vomeronasal receptors type 1 (V1Rs), which are classified as rhodopsin-associated (Nordström et al., 2011), which are expressed in the vomeronasal organ, and contribute to pheromone detection. In mammals, the V1R family varies dramatically in size, from zero in two bat species, dolphin and rhesus macaque to 283 in platypus (Jiao et al., 2019). Amphibians seem to have a comparatively small V1R repertoire, e.g. about a dozen genes for *Xenopus tropicalis* (Saraiva and Korsching, 2007; Ji et al., 2009). Mammalian V1Rs can be coupled to various G proteins, such as $G\alpha_{i2}$, $G\alpha_{o}$, $G\alpha_{q/11}$, $G\beta_{2}$ and $G\gamma_{2}$, which are mostly located in the microvilli of OSNs (Fleischer et al., 2009).
Also only 12 V1Rs were identified in *Xenopus tropicallis*, a member of the amphibians (Ji et al., 2009).
In the teleost fish nose V1Rs were identified first to be represented by a single gene (Pfister and Rodriguez, 2005) or a gene pair (Shi and Zhang, 2007). Later four further *v1r*-like genes were identified in different teleost species and all named Ora (olfactory receptor class-A related). These genes form a single clade, which includes the entire mammalian V1Rs, are not highly conserved across teleosts, because two genes, *ora7* and *ora8,* were lost early in the teleost lineages. All eight *ora* genes, except *ora3* and *ora4*, have only one coding exon (Saraiva and Korsching, 2007; Zapilko and Korsching, 2016). Until today only one of the *ora* genes of zebrafish, *ora1*, has been deorphanized. The ligand, 4-hydroxyphenylacetic acid, acts as a reproductive pheromone and stimulates oviposition (Behrents et al., 2014). And only for one *ora* gene, *ora4*, could it be shown that it is expressed in crypt neurons, a specialized sparse population of OSNs (Oka et al., 2012). That means, that for nearly all *ora* genes either the ligand or the OSN, in which the receptor is expressed, remains unclear until today.

1.3.3 V2Rs

Shortly after the discovery of V1Rs in mammals, the mammalian vomeronasal receptors type 2 (V2Rs) were found in the basal layer of the mammalian VNO, expressed in $G\alpha_o$-positive neurons (Fleischer et al., 2009). V2Rs belong together with the taste receptors for sweet/umami, the metabotropic glutamate receptors, and the Ca2+-sensing receptor to class C GPRC. One of their main characteristic features is their large (~70 kDa) N-terminal extracellular domain (Pin et al., 2003). One of the first putative ligands to be described were protein pheromones, which can activate the $G\alpha_o$ subunit (Krieger et al., 1999). In mammals the V2R repertoire shows extreme size variations, from around 200 in rodents to zero in dog and cow, with a high number of pseudogenes. The V2R genes can be grouped in each species into distinct families A-D, where family A is the biggest one (Young and Trask, 2007). Because fish do not posses a VNO, receptors homologous to the V2Rs are expressed in the olfactory epithelia and were named OlfC for olfactory receptor class C. In *Xenopus tropicallis* more than 330 V2Rs were identified and grouped into A1, A2, A3 and C (Ji et al., 2009; Syed et al., 2017). With 60 intact and one pseudogene, zebrafish exhibits the largest of all teleost OlfC repertoires. OlfCs are expressed in microvillous neurons in zebrafish, forming like the ORs distinct spatial expression zones (Ahuja et al., 2018).

1.3.4 TAARs

The trace amine-associated receptors (TAARs), like the ORs and V2Rs, are a family of rhodopsin-like GPCRs and named TAARs, because they are activated by amines, such as β-phenylethylamine, p-tyramine, tryptamine, and octopamine and were first localized in the brain (Borowsky et al., 2001). Some years later the expression of TAARs in a unique subset of neurons in the mouse olfactory epithelium was observed (Liberles and Buck, 2006). In mammals the number of TAARs is very low, compared to ORs, with six TAARs discovered in humans and 15 TAARs in mice (Lindemann et al., 2005). Interestingly, the TAAR family is the only olfactory family which is much larger in teleost fish compared to mammals. Within teleosts, zebrafish exhibits the largest repertoire of *taar* genes with 112, is, co mpared to other teleosts, stickleback, medaka and pufferfish (48, 25 and18), very

high. These differences could be explained by massive recent gene expansions in teleosts (Hussain et al., 2009). Usually TAARs, like the ORs, are expressed in ciliated neurons, co expressed with the $G\alpha_{olf}$ protein and can increase cAMP levels when activated by ligands in heterologous cells (Liberles and Buck, 2006). In Zebrafish several of the *taar* genes has been deophanized (Li et al., 2015). For exampleTAAR13c recognizes the death-associated odour cadaverine and leads to an aversive response for the fish (Hussaine et al., 2013).

1.3.5 FPRs

Formyl peptide receptors (FPRs), member of the GPCRs, are expressed in mammals in diverse tissues by immune cells, in which they detect pathogenic and inflammatory chemical cues. But only in rodents could it be shown, that six of the seven FPRs (Fpr-rs1 -rs3, -rs4, -rs6, and -rs7) are expressed in a punctate and monogenic pattern in vomeronasal sensory neurons. These six FPRs recognize different types of chemicals, mainly kairomones and an activation of these neuronal populations often leads to innate and stereotyped behaviours. Fprs are exclusively expressed in two main populations of vomeronasal sensory neurons in mice, because million years ago an Fpr coding exon landed in front of a vomeronasal receptor promoter. This hijacking leads to an V1R like expression pattern of Fprs in mice (Dietschi et al., 2017).

1.3.6 GUCYs

Another olfactory receptor is the Guanylyl Cyclase, subtype GC-D, which is expressed in a subset of OSNs in the MOE of mammals, which are therefore designated as GC-D neurons. Instead of using the canonical cAMP pathway like the other receptors, after activation, signaltransduction is mediated by cGMP, which serve as a 2^{nd} messenger molecule and can activate e.g. cGMP-sensitive cyclic nucleotide-gated ion channels. The GC-D receptor, also named gucy, is activated by the urinary peptides uroguanylin, guanylin, low concentrations of CO_2 and carbon disulfide (CS2); (Arakawa et al., 2013; Hu et al., 2007; Leinders-Zufall et al., 2007). In humans and several other primates the *gc-d* gene is a

pseudogene, which could be the reason why CO2 is odourless to humans (Young et al., 2007). In Zebrafish recent studies have identified ten *gc-d* genes. Two of them, *gucy2f* and *gucy1b2*, are definitely expressed in the OE. The chemosensory function of zebrafish GC-D receptors expressing OSNs is up to now not fully understood (Saraiva et al., 2015).

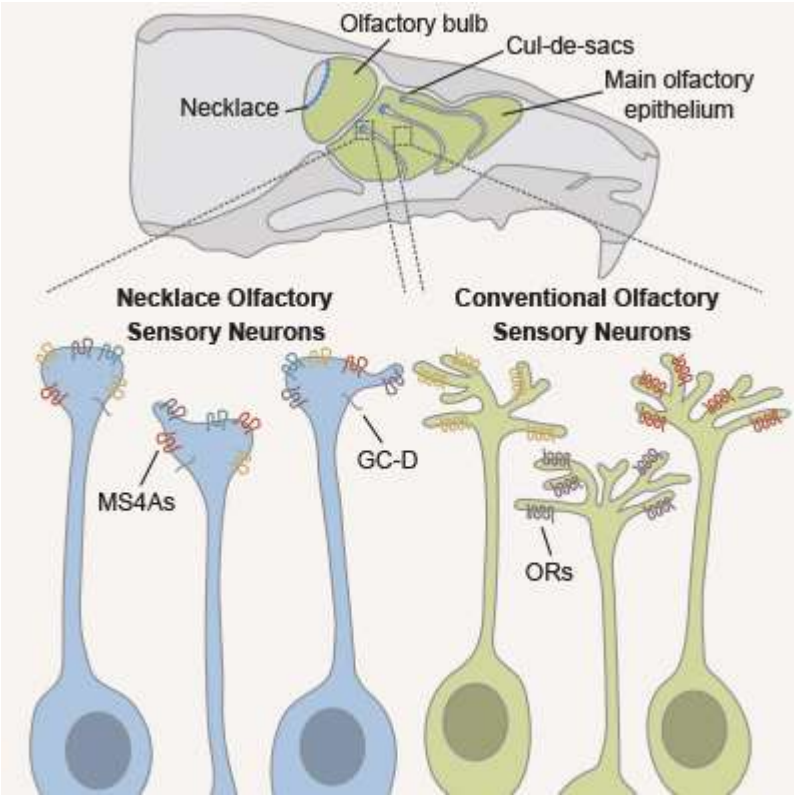

Figure 1.6: Distribution of different OSNs in the olfactory epithelia of mice, modified from (https://greer--lab.com/research)

1.3.7 MS4As

Some four years ago, in 2016, the MS4a receptors, a non-GPCR family of olfactory receptors in mice, were identified. Several of these receptors are co-expressed in so-called necklace OSNs and concentrated in the recesses of the olfactory epithelium, following not the “one neuron - one receptor” rule. These necklace sensory neurons, expressing *ms4as* genes, are activated by fatty acids and the putative mouse pheromone 2,5-DMP. All *ms4a* genes encode a four-pass transmembrane protein, which is co-expressed with guanylate cyclase-D, using cGMP in their signal transduction pathway (Greer et al., 2016). *Ms4a* gene homologues were found in most jawed vertebrates, with a large number of highly conserved *ms4a* genes in zebrafish (Zuccolo et al., 2010).

1.3.8 A2C

Recent studies in zebrafish identified a novel adenosine receptor called A2c. This receptor, a member of the GPCRs, is expressed in the OE, activated only by adenosine and expressed in new pear-shaped OSNs, which are positive for the olfactory marker G_{olf} and ACIII. *A2c* orthologous genes were found in all the fish species, both freshwater and sea water fishes, and amphibians, but not in terrestrial vertebrates or marine mammals. Interestingly, all other adenosine receptor genes are present in all vertebrates, which probably indicates a highly conserved and specific function of A2c in aquatic lower vertebrates (Wakisaka et al., 2017).

1.4 Olfactory systems

1.4.1 The olfactory system of zebrafish (*Danio*)

The olfactory system of zebrafish (*Danio rerio*) is analogous to that of other vertebrates and is a perfect model for studying the olfactory system, because its anatomical components are more accessible compared to some other model vertebrates. Two separate nasal cavities each contain a peripheral olfactory organ, or rosette, which is connected the olfactory bulb. The water enters each of the nasal cavities, through the anterior nostril and leaves it through the posterior nostril (Hara and Zielinski, 2007). The single olfactory organ, also called olfactory epithelium has a bowl-shaped structure and is surrounded by the lumen of the olfactory pit. From the central non-sensory raphe, several lamellae form a bilateral symmetrical structure (Figure 1.7). The olfactory sensory area of the lamellae is located centrally and medially and is connected to the dorsal non-sensory area. The OE is formed mainly by bipolar OSNs, basal cells (BC) and supporting cells (SC). So far, five different types of OSNs have been described in zebrafish: ciliated (cl); microvillous (mv); crypt (cr); kappe (kp) and pear (pr) neurons (Figure 1.7) (Calvo-Ochoa and Byrd-Jacobs, 2019). They differ by their expression of olfactory receptors and corresponding signal

transduction cascades.

Ciliated OSNs, with their elongated cell shape and long apical dendrites containing cilia, are located at more basal positions in the olfactory lamellae. They are known to express ORs and TAARs, together with the olfactory marker protein (OMP). Signal transduction is *via* $G_{\alpha\ olf/s}$, a stimulatory G-protein and ciliated OSNs can be identified with an antibody against $G_{\alpha\ olf/s}$ (Calvo-Ochoa and Byrd-Jacobs, 2019; Hansen and Zielinski, 2005). Microvillous OSNs with their microvilli containing the V2R receptors (Martini et al, 2001) are located in the apical layer of the zebrafish OE. In Zebrafish as in mammals these OSNs can be characterised by a transient receptor potential channel member C2 (TRPC2), which is part of their signal transduction pathway (Calvo-Ochoa and Byrd-Jacobs, 2019; Sansone et al., 2014). The third type of OSNs are the egg-shaped crypt neurons. These OSNs are located in the upper part of the OE and very apical within lamellae and are present in relatively low numbers. In contrast to ciliated and microvillous OSNs, crypt OSNs posses an apical crypt at the top of the cell body, with a rim of microvilli and

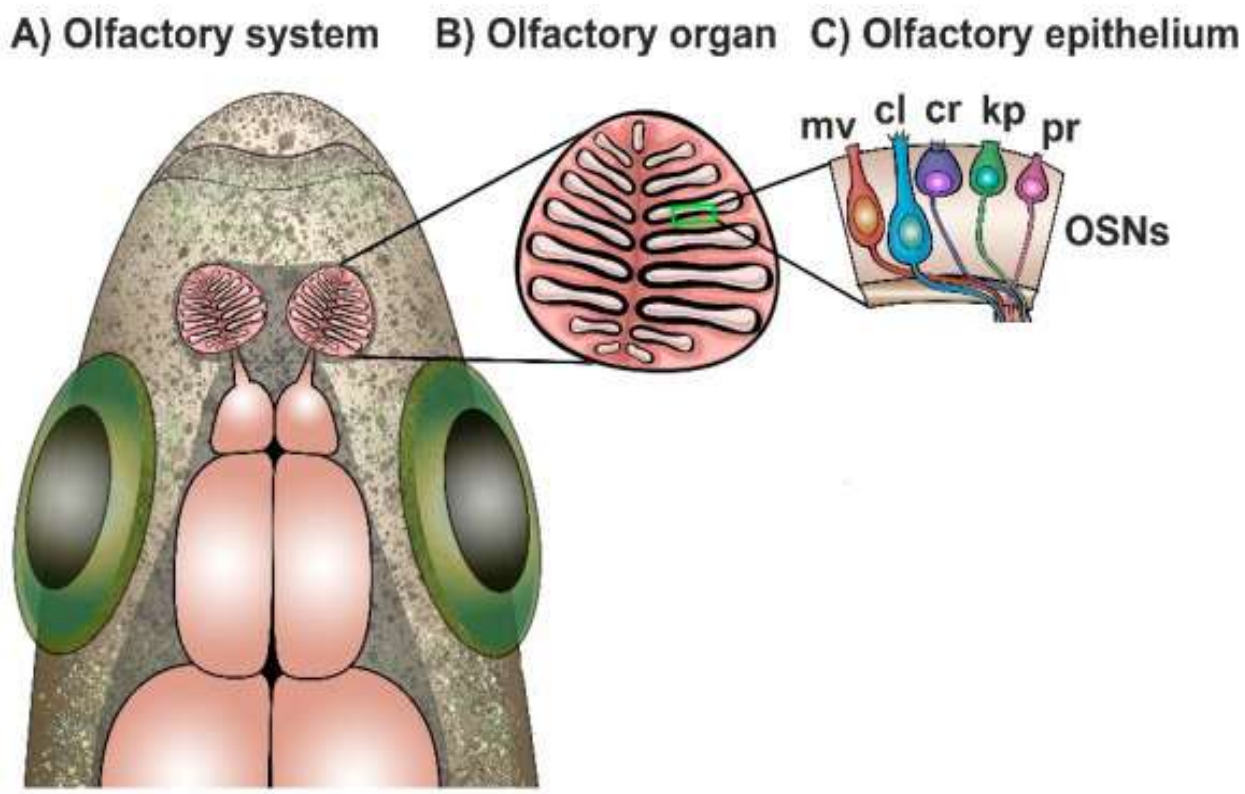

Figure 1.7: Anatomical and morphological organization of the zebrafish olfactory system. (A) Localization of the olfactory system in zebrafish. Dorsal side is shown; rostral side is located upwards; (B) Olfactory organ morphology. Olfactory sensory epithelium arranged in lamellae is shown in black; (C) Olfactory epithelium (OE), composed of the following olfactory sensory neurons (OSNs): microvillous (mv); ciliated (cl); crypt (cr); kappe (kp); and pear (pr) OSNs. (modified from Calvo-Ochoa and Christine A. Byrd-Jacobs, 2019)

several cilia protruding from the bottom of the crypt (Hansen and Zeiske, 1998). Crypt neurons express a single olfactory receptor, Ora4 (Oka et al., 2012). Additionally, crypt neurons can be labeled by an antibody generated against tyrosine receptor kinase A (TrkA) molecule - due to a cross-reactivity to an unknown molecule, TrkA itself is not present (Ahuja et al., 2013).

Not much is known about the recently discovered kappe and pear-shaped OSNs, which like crypt neurons are expressed in the superficial layer of the olfactory lamellae. Kappe OSNs have a short and globose shape with an microvilli bearing cap and are characterized by G_o-like immunoreactivity. Until today no olfactory receptor has been identified for these neurons. The pear- shaped OSNs have an extremely short dendrite and express the novel adenosine receptor A2c as olfactory receptor, which signals via G_{olf} and ACIII. AS a marker for these OSNs, CD73, a membrane-bound nucleotidase, has been discovered (Calvo-Ochoa and Byrd-Jacobs, 2019; Wakisaka et al., 2017).

1.4.2 The olfactory system of the eel (*Anguilla*)

The eel is one of the most interesting species to study, because they are amphyhaline fish and so their olfactory system has to detect odorants both in fresh and salt water. Eels are considered to possess an extremely sensitive sense of smell (Korsching, 2020). They diverged from other teleosts approximately 300 million years ago and their life cycle includes two extremely long-distance migrations across the Atlantic Ocean. Until today no mature spawning eel has been caught in the wild (Churcher et al., 2014). The life cycle of the eel is divided into four stages, the leptocephalus (larval), glass eel, pigmented eel and yellow eel. The eel olfactory organ is formed by a great number of olfactory folds and reaches in the adult stage around 50-100 folds. In its younger stages, like the leptocephalus and glass eel, the number of folds in the OE is considerably lower. The olfactory epithelia in the younger stages of the eel's development are similar to those of the adults in other species of fish (Tesch and Thorpe, 2003). Transcriptome analyses have shown the expression of 112 ORs, 13 TAARs, 6 V1Rs and 38 *v2rs* genes, some of them are expressed in a stage-specific manner (Churcher et al., 2014).Until today expression of olfactory receptors was not analysed by in situ methods such as in situ hybridisation or immunohistochemistry in the olfactory epithelium of eel.

Figure 1.8: Olfactory epithelia *Anguilla anguilla*.

1.4.3 The olfactory system of carp (Cyprinus)

Not many studies have been done on the OE of carp (. The olfactory rosette is closely related to that of zebrafish and eel, with their typical leaf-like structure (Fig. 1.9A). It has been subdivided, as in zebrafish, into five layers, where 1 is apical and 5 basal. Crypt neurons are embedded in lamina 1-2, the microvillous sensory neurons in 3-4 and ciliated sensory neurons in 4-5 (Fig. 1.9B) (Hamdani, 2007). Until today no expression of a single receptor in the olfactory epithelium of carp has been shown by in situ methods such as in situ hybridisation or immunohistochemistry in the olfactory epithelium of carp. An interesting fact is, that prolonged anosmia does not cause significant alterations of taste preferences of free amino acids and sensitivity to them (Kasumyan and Sidorov, 2004), suggesting a relative independence between these two chemosensory systems.

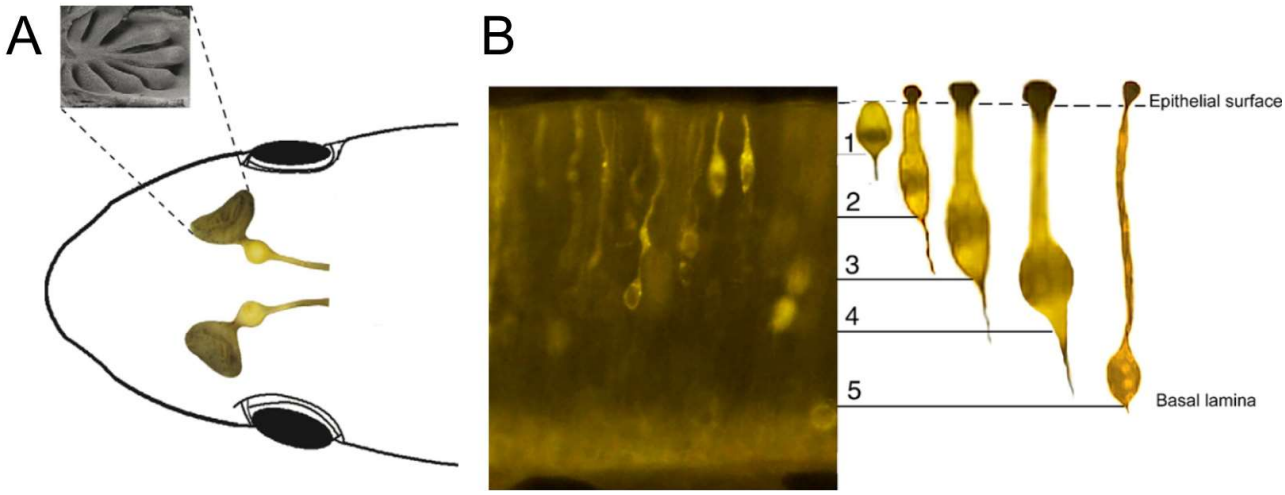

Figure 1.9: The olfactory system of carp with sensory neurons in the olfactory epithelium. (A) Dorsal view of the head of a carp showing the olfactory system. (B) Micrograph of the sensory epithelium of a crucian carp (left) and illustrations of the different types of sensory neurones found after application of the crystal of the stain DiI in the synaptic region of the olfactory bulb. The sensory epithelium is divided into five layers and cell somas are found at different depths. (modified from Hamdani, 2007)

1.4.4 The olfactory system of the clawed frog (*Xenopus*)

Xenopus is a member of the anurans, an order of animals in the class Amphibia that includes frogs and toads. During their lifetime, the development of the olfactory system of *Xenopus* can be divided into two phases, the premetamorphic nose at approximately stage 51-52 and the postmetamorphic nose approximately after stage 65. In the premetamorphic nose, the OE, which is located in the principal cavity, contains ciliated and microvillous OSNs, two populations of supporting cells and basal cells. The epithelium of the accessory system, which is situated in the VNO, contains solely microvillous sensory neurons, one population of supporting cells and basal cells. Several hundreds ORs, V1Rs, TAARs and some V2rs are expressed in the tadpole OE and the majority of the large repertoire of V2rs are expressed in the VNO (Manzini and Schild, 2010; Syed et al., 2015). In the postmetamorphic phase the nasal cavity is divided into principal (OE), middle (additional sensory epithelia) and lower cavity (VNO). The tadpole OE metamorphoses into the principal cavity, whereas the middle cavity is newly formed. The VNO does not change (Dittrich et al, 2016). In adult *Xenopus* the principal cavity is filled with air and serves as an “air nose”, whereas the middle cavity is filled with water and serves as a “water nose”(Manzini and Schild, 2010). The adult principal cavity contains ciliated olfactory cells, microvillous supporting cells and basal cells, while the middle cavity contains microvillous OSNs, ciliated

and microvillous supporting cells, and basal cells.The adult VNO retains the larval cell composition (Dittrich et al, 2016). “Mammalianlike” Class I ORs are expressed in the principal cavity, “fishlike” Class II ORs and V2R subgroups A1 and C are expressed in the middle cavity and V2R subgroups A2 and A3 are expressed in the VNO (Syed et al., 2017).

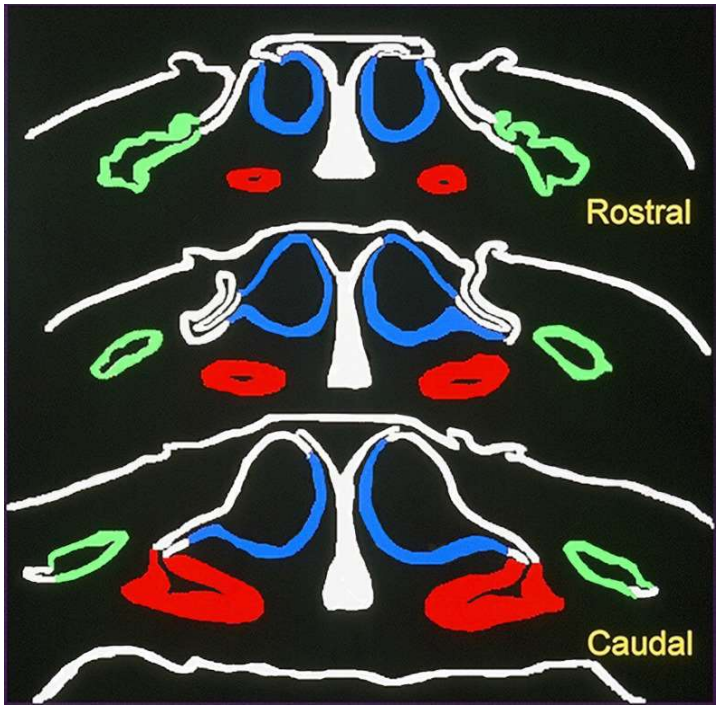

Figure 1.10: Three nasal chambers in *Xenopus laevis.* Blue: upper chamber lined with the olfactory epithelium. Green: middle chamber lined with the middle chamber epithelium. Red: lower chamber lined with the vomeronasal sensory epithelium. (TANIGUCHI and TANIGUCHI, 2014)

1.4.5 The olfactory system of lamprey

Half of the lamprey species are anadromous fishes and carnivorous, feeding on marine mammals or fishes (Nichols and Tscherter, 2011). The first closer anatomical descriptions of the *Lampetra fluviatilis* olfactory epithelia were made in 1967. It was shown that the olfactory organ of the adult *Lampetra fluviatilis* is principally identical to that of the more investigated *Petromyzon marinus* (Kleerkoper and Van Erkel, 1960). The entry for the olfactory system of an adult lamprey is situated anterior to the eyes, at the dorsal surface of the head. There it forms a single median opening of around one mm in diameter, from which a single duct, with the length of around two mm, leads into a single extensive, dorso-ventrally compressed hypophysal sac. With a size of approximately five mm the olfactory capsule is situated posterior to the duct inside the hypophysial sac. The synchronous water influx is maintained due to pressure changes created in the pharynx, which are transmitted

to the closely associated hypophysial sack. A small valve is situated between the olfactory capsule and the duct, which is pressed on the duct during exhalations. This valve is lifted through inhalation and partially closes the duct, so a current of water is directed into the olfactory capsule (Thornhill, 1967). The olfactory capsule is connected on the posterior side *via* the olfactory nerve - which contains the axonal projections of the OSNs with the olfactory bulb. The olfactory capsule of *Lampetra fluviatilis* contains about 16 radial lamellae, which project from the outer shell into the centre. The main olfactory epithelia (MOE) is located on the surface of the lamellae facing the lumen of the olfactory capsule. It has been shown in larval sea lampreys, with the help of a G_{olf} -antibody, that the epithelia in the arc near the outer shell of the olfactory capsule contains a non-sensory area, while the epithelia on the surface of the invaginations is part of the sensory area (Frontini, 2003). Three regions of cell division were discovered in sections of a single olfactory lamella in *Lampetra fluviatilis* (Fig. 1.11), the distal lamellar region, the proximal lamellar region and the basal epithelia region within the sensory area. New mucous secreting cells were primarily generated in the distal lamellar region, and some of them migrate into the sensory area. Most of the cells in the sensory area and a few in the ciliated area at the base of the lamellar are generated by the cells of the proximal lamellar region. The cells which are formed in the basal epithelia region stay in the sensory region (Thornhill, 1970). The whole epithelia is covered with a thick layer of mucous and three different cell types have been described in the sensor area: sensory cells, supporting cells and basal cells. Typical of most sensory cells is their thin and elongated cell body, which swipes through the epithelia from the bottom to the top. The olfactory cilia at the top of the dendrite is five to six µm long and has the typical nine plus two arrangements of its microtubuli. Beside the MOE, the olfactory capsule also includes beside the MOE, an accessory olfactory organ (AOO). This AOO is situated inside the cartilaginous nasal capsule at its postero-inferior portion, forming a complex large median structure, having many follicles which are lined by low columnar ciliated epithelial cells (Scott, 1887; Ren et al., 2009).Retrograde labelling has shown the presence of OSNs also in the AOO (Green at al., 2017). Different shapes of the OSNs are observed in sea lamprey similar to the polymorphisms observed for OSNs in teleost fishes, like ciliated, microvilous and crypt neurons (Laframboise et al., 2007). Unfortunately, the molecular identity of those differently shaped cells in lamprey is not known. Early cloning efforts had identified a family of TAAR receptors in *Lampetra fluviatalis* (Berghard and Dryer, 1998) Later phylogenetic studies identified, 27 ORs, 4 V1Rs, 28 TAARs and the A2c receptor were identified in lamprey (Grus and Zhang, 2009; Libants et al., 2009; Wakisaka

et al., 2017). Yet the expression of only one *or* and one *taar* gene was shown with the help of *in situ* hybridisation (Berghard and Dryer, 1998; Freitag et al., 1999; Libants et al., 2009).

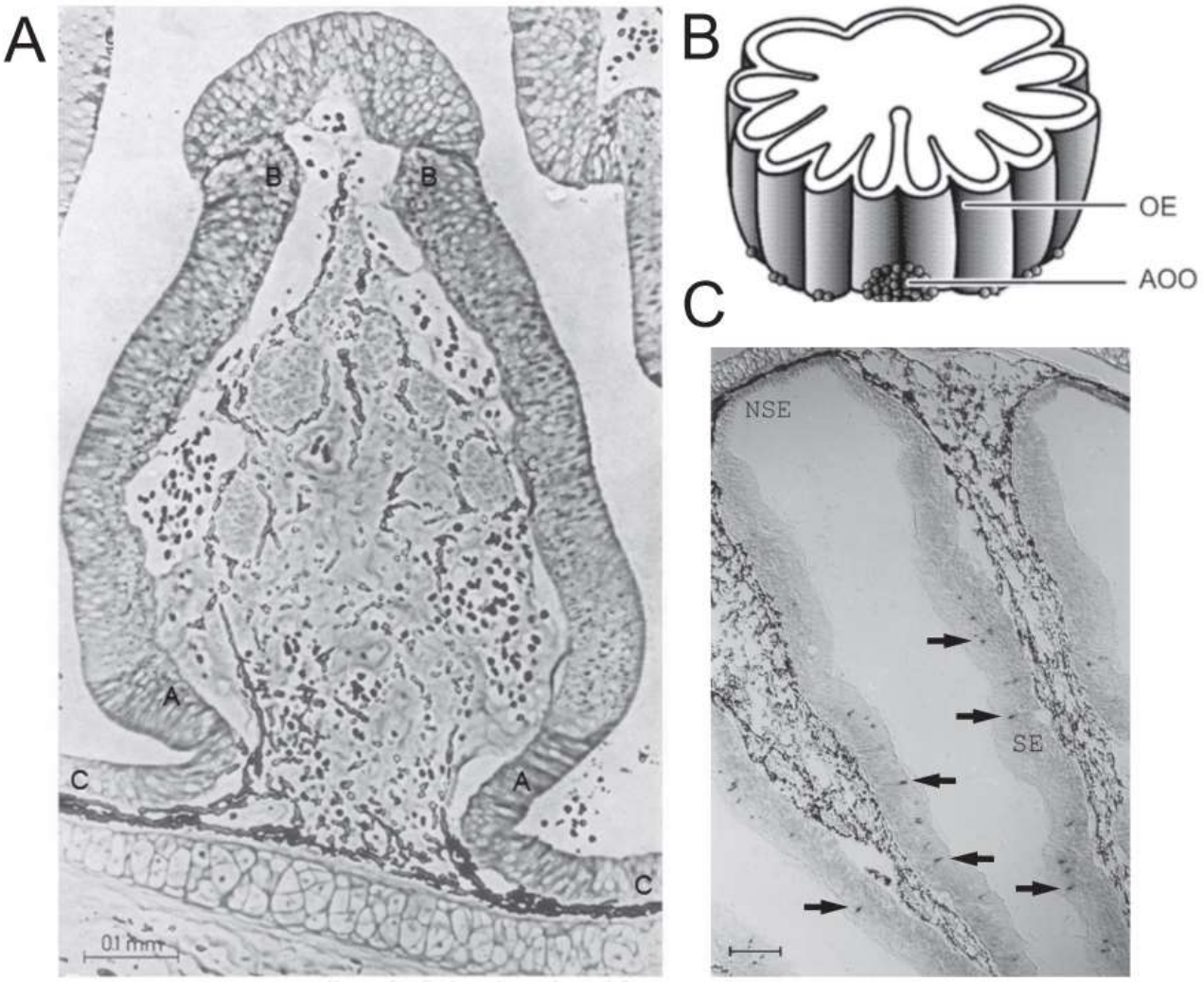

Figure 1.11: Olfactory epithelia of lamprey: (A) Transverse section of a single olfactory lamella from the nasal sac of *Lampetra fluviatilis* showing the regions in which cell division was found. A Proximal lamellar region.B Distal lamellar region. C Basal epithelial region. Osmic acid, phase contrast (modified from Thornhill 1970) **(B)** Olfactory epithelium (OE) lines lamellae, and follicles of the accessory olfactory organ (AOO) are located ventrocaudal to the olfactory epithelium. (modified from Laframboise et al., 2007) **(C)** Low power micrograph of an *in situ* hybridization with digoxigenin-labeled antisense probe of Lfor1 receptor. Hybridization is restricted to the sensory portion of the lamellae. The labelled cells (arrow heads) seem to be randomly distributed. Sensory epithelium (SE); non-sensory epithelium (NSE). Scale bar=100 mm. (modified from Freitag et al., 2009)

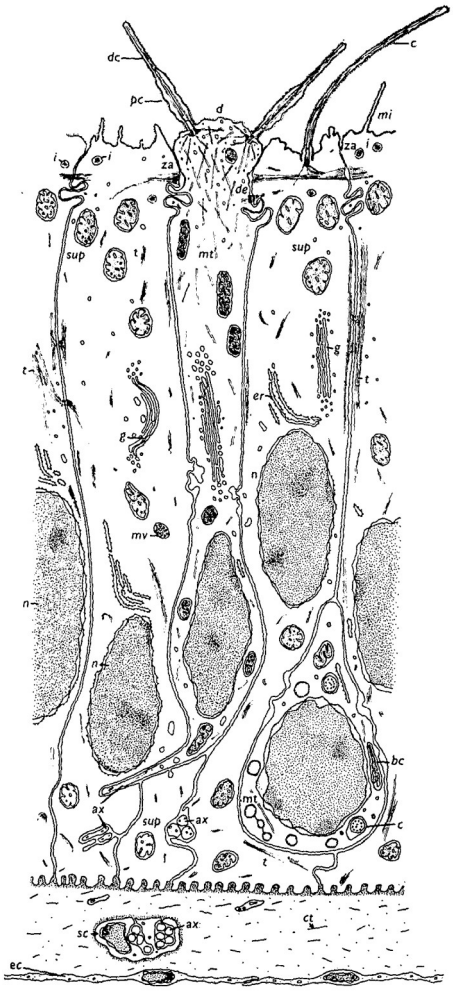

Figure 1.12: Diagrammatic representation of the arrangement of a sensory cell and associated cells within the olfactory epithelium of lamprey {ax, axons; be, basal cell; c, cilium; ct, connective tissue fibres; d, dendrite; dc, distal segment of sensory cilium; de, desmosome; ec, epithelial cell; er, endoplasmic reticulum;g, Golgi apparatus; i, inclusions in supporting cell; mi, microvilli; vit, microtubules; mv, multivesicular body; n, nucleus; pc, proximal segment of sensory cilium; sc, Schwann cell; sup, supporting cell; t, tonofilament; za, zonula adhaerens). (Thornhill, 1967)

2. Material and Methods

2.1 Technical equipment

Typ	Model	Producer
Cryostat	CM1900	Leica
Centrifuge	Micro Star 17	VWR
Centrifuge (cooling)	Z233 MK-2	
Electrophoresis Chamber		Bio-Rad
Electrophoresis Powersupply	Powerpac 300	Bio-Rad
Gel Documentation System	GelDoc XR	Bio-Rad
Heating Plate	Medax	StörkTronic
Hybridiser	HB-1D	Techne
Incubator (Watherbath)	GFL	KMF
Microwave	900 & Grill	Severin
Millipore Water purification system	MilliQSynthesis 10 A	Millipore
pH-Meter	Calimatic 766	Knick
Pipettes	Research Plus	Eppendorf
Precision scale	LA 120 S	Sartorius
Rotary Mixer	R1	Pelco
Scale	universal	Sartorius
Shaker	Bio Shaker 3D	Biosan
Spectrophotometer	NanoDrop One	Thermo Fisher
Thermal mixer	LifeEco	BIOER
Vortex	Vortex Genie 2	Bender & Hobein AG

2.2 Chemicals

2.2.1 Solutions and buffers for long term storage

All solutions were made with deionized water from a Millipore Milli Q Synthesis water purification system. The pH of the solutions were adjusted with the required amount of either 37% HCl (company: VWR) or dissolved sodium hydroxide (company: VWR). Before use, all buffers were sterilized *via* autoclaving. Unless otherwise stated, all buffers were stored at RT.

DEPC-H_2O
1 ml $C_6H_{10}O_5$ Diethylpyrocarbonate (Sigma-Aldrich)
was added to 999 ml deionized water and stirred over night. The DEPC reacts with amines and efficiently destroys nucleases, but has to be inactivated itself by subsequent autoclaving.

1 M Tris-HCl; pH 9.5 and pH 7.5
121.1g Tris
was filled up to one litre and stirred until dissolved. pH was adjusted as required.

10x Phosphate-buffered saline (PBS); pH 7.4-7.6
80 g NaCl
2 g KCl
14.2 g Sodium dihydrogen phospate (Na_2HPO_4) (water free)
2 g Kalium dihydrogen
was filled up to one litre and stirred until dissolved. pH was adjusted as required.

1 M Magnesium chloride
20.33 g $MgCl_2$
was filled up to 100 ml and stirred until dissolved.

LB Medium

10 g Tryptone

5 g Yeast Extract

10 g NaCl

was filled up to one litre and stirred until dissolved.

TSA Buffer pH 7.5

0.1 M Imidazole

0.001 % H_2O_2

was filled up with 500ml 1xPBS and stirred until dissolved. pH was adjusted as required.

20X Saline-sodium citrate (SSC) buffer pH 7.0

175.32 g NaCl

88.23 g tri-Sodium citrate dihydrate (0.3 M $C_6H_5Na_3O_7$ $2H_2O$)

was filled up to one litre and stirred until dissolved. pH was adjusted as required.

5 M Sodium chloride

292 g NaCl

was filled up to one litre and stirred until dissolved.

2.2.2 Short term storage solutions and buffers

NBT/BCIP detection buffer 9.5

0.1 M Tris-HCl pH 9.5

0.1 M NaCl

0.05 M $MgCL_2$

HNPP/FastRed reaction buffer pH 8.0

0.1 M Tris-HCl pH 9.5

0.1 M NaCl

0.05 M $MgCl_2$

TNT Buffer
0.1 M Tris-HCl pH 7.5
01.5 M NaCl
0.05% Tween 20

TNB Buffer
0.1 M Tris-HCl pH 7.5
0.15 M NaCl
1 % Blocking reagent
stored at 4º C and stable for approximately one week

TAE Buffer pH 8.0
2 M Tris
1 M CH_3COOH
0.5 M EDTA

Cell lysis buffer for fin clipping
10 mM Tris pH 8.2
2 mM EDTA
0.2 % Triton
200 µg/ml Proteinase K
stored at -20º C

tissue lysis buffer
0.1M Tris pH 7-9
0.2 M NaCl
5 mM EDTA
0.2% SDS
150 µg/ml proteinase K

zebrafish embryo water
118 mg Red sea salt
1.5 mg Methylene blue

hybridisation mastermix
50 % deionized formamide (pH 5)
5x SSC
0.15 µg/µL heparin
5 µg/µL torula-RNA
0.1 % Tween 20

Sulfosuccinimidyl-6-(biotinamido)hexanoate (BIO-NHS)	Sigma-Aldrich
Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (DIG-NHS)	Sigma-Aldrich
Dimethylformamide	Sigma-Aldrich
Ethyl 3-aminobenzoate methanesulfonate salt (MS222)	Sigma-Aldrich
Isopropanol	VWR
peqGREEN	peqlab
Phenol-chloroform-isoamyl alchol mixture	Sigma
RNA Later	Merck
Triethylamine (TEA)	Sigma-Aldrich
Streptavidin Alexa Flour 488	Jackson Immuno (Cat # 016-540-084)
Streptavidin Alexa Flour 633	ThermoFischer (Cat # S21375)
GoTaq Green Mastermix	Promega (Cat # M7122)

2.3 Antibody

Antibodies	host species	supplier
Anti-Acetylated Tubulin	mouse	Sigma (T7451)
Monoclonal Anti-β-Actin	mouse	Sigma (A2228)
Anti-Digoxin- Alexa Flour 488	mouse	Jackson Immuno (Cat # 200-542-156)
Anti-rabbit IgG-Alexa 488	goat	Jackson Immuno (Cat # 111-545-003)
Anti-mouse IgG-Alexa 488	goat	Thermo Fischer (Cat # A28175)
Anti-mouse IgG-Alexa 633	goat	Invitrogen (Cat # A-21052)

Anti-DIG POD	sheep	Roche Diagnostics (Cat. #: 11207733910)
Anti-Flu AP	sheep	Roche Diagnostics (Cat. #: 11426338910)
Anti-DIG AP	sheep	Roche Diagnostics (Cat. #: 11093274910)
Anti HuC/HUD	mouse	Invitrogen (Catalog # A-21271)
Anti PCNA clone PC 10	mouse	Merck (Cat. # MABE288)
c-Fos (E8)	mouse	Santa Cruz (sc-166940)
Phospho S6 Ribosomal Prot.	rabbit	Cell Signalling (# 2215)

2.4 Primer

All primers were designed with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). An ideal length for all primers between 300 and 400 nucleotides, a GC content of minimum 50 % and melting temperatures of around 60 ºC were chosen. All designed primers were manufactured by the Sigma company and after delivery dissolved in nuclease free deionized water and stored at -20 ºC.

Species	**Name**	**Sequence**	**PCR**	**In situ**
Eel	A2C FW A	CTGATCTACGCGCGGATCTT		
Eel	A2C RV A	CATCTCAACCCTGCAGCAGA		
Eel	A2C FW B	AACAGGAAGATGCACACCGT	X	X
Eel	A2C RV B	ATGAGGAAGGACAGGACCCA	X	X
Carp	A2C FW	TCTACGCGCAGATCTTCGTC	X	X
Carp	A2C RV	TTGTTCATCGCCATCACCGA	X	X
Lamprey	A2C FW	TCCTGTTGGAGCTCGTCATC	X	X
Lamprey	A2C RV	GTACCGCACTTGCAGGAAGA	X	X
Lamprey	AC3 FW A	CCAGATCGCACCTCTCTCTC		
Lamprey	AC3 RV A	TAGTAGCAGTCGCCCAGGAT		
Lamprey	AC3 FW B	GCGCAGATCTTCTGCTACCT	X	
Lamprey	AC3 RV B	GAGAGAGAGGTGCGATCTGG	X	
Lamprey	AC3 FW C	CCCTCTTCGACTGCTACGTC	X	
Lamprey	AC3 RV C	ACAGCGTGTAGGGGATGAAC	X	
Lamprey	Casr FW	CAGGTGTTCGAGTCGCAGAT	X	
Lamprey	Casr RV	GAACACCAGCATGCTGAACG	X	
Lamprey	G_{olf} FW A	ATTTTTCGACCACGTGAAGG	X	X
Lamprey	G_{olf} RV A	AGGATGATCGAGATGGTTCG	X	X

Species	Name	Sequence	PCR	In situ
Lamprey	G_{olf} FW B	CGAACCATCTCGATCATCCT	X	X
Lamprey	G_{olf} RV B	AGTGCGGGTAGCAGTAGTGG	X	X
Lamprey	LOR3 FW	CCGCAACGCGTGGTCCTGAT	X	X
Lamprey	LOR3 RV	TCCTAAAGTTGAATAGATCCGTC	X	X
Lamprey	LOR4 FW	TCCTAAAGTTGAATAGATCCGTC		
Lamprey	LOR4 RV	GGACAGGTCCATTGTCCTCGC		
Lamprey	V1R3 FW A	ACGCTGACTCTGAGCATGTT	X	X
Lamprey	V1R3 RV A	AGCGTCTTGATGTTCACCGT	X	X
Lamprey	V1R1 FW A	CACGTGCGTTTTGACCATGT	X	
Lamprey	V1R1 RV A	AACACGCTGACGAGACATGT	X	
Lamprey	V1R1 FW B	ATGTCTCGTCAGCGTGTTGT	X	
Lamprey	V1R1 RV B	TAAGGGTGGCCATGTGGTTG	X	
Lamprey	V1R1 FW C	AATAGCTCCGGATTGCTGCA		
Lamprey	V1R1 RV C	ATAGAGACGCACCGTAACGC		
Lamprey	V1R2 FW A	CTCACACACACACACGCATG		
Lamprey	V1R2 RV A	CTGAGGATCACCGTGAAGGC		
Lamprey	V1R2 FW B	CCTTCACGGTGATCCTCAGC	X	
Lamprey	V1R2 RV B	GATGATGAAGGGGCTGAGGG	X	
Lamprey	V1R2 FW C	GCCTCCAACGTCCTCGAC		
Lamprey	V1R2 RV C	GTAGACGGAGGTGAAGAAGGC		
Lamprey	V1R4 FW A	TCTTCAAGCTCAGCTGCTCC	X	
Lamprey	V1R4 RV A	CATCCCCACCCAGTTGTTGA	X	
Lamprey	V1R4FW B	CTTTCCTCTGCACGAGTCGT	X	
Lamprey	V1R4 RV B	GGAGCAGCTGAGCTTGAAGA	X	
Lamprey	V1R4 FW C	CGAGATCATCCCCTTCCTGC	X	
Lamprey	V1R4 RV C	CACCATCGATGCGAGTCTCA	X	
Lamprey	V1R5 FW A	TACTTCACCGTCCGCTTCAC		
Lamprey	V1R5 RV A	TGACCACCATCAAGACCGTG		
Lamprey	V1R5 FW B	TACTTCACCGTCCGCTTCAC	X	
Lamprey	V1R5 RV B	GATGTACGGGCTCAGGGAAG	X	
Lamprey	V1R5 FW C	CAGTGACTCTGCAGAGCCTC	X	X
Lamprey	V1R5 RV C	GACACGGAACCGATAGCCAC	X	X
Lamprey	V1R6 FW A	GTCTGCACCTCTCGACCAG	X	X
Lamprey	V1R6 RV A	GTAGGCCATGTGGGAGACAG	X	X
Lamprey	V1R6 FW B	CACCGAGAGAGCCATTGTGT	X	X

Species	Name	Sequence	PCR	In situ
Lamprey	V1R6 RV B	AGGATGTCTCCCTCGAGGTC	X	X
Lamprey	TRPC2 FW A	GGTTCAACGAGACGTTCCAG	X	
Lamprey	TRPC2 RV A	GCGATTAGCATGTTGAGCAG	X	
Lamprey	TRPC2 FW B	CAGATCTCCATCGGCAAGAT	X	X
Lamprey	TRPC2 RV B	ATGAGCATGTTGAGCAGCAC	X	X
Lamprey	TRPC2 FW C	GAGTGCAAGGAGGTGTGGAT	X	X
Lamprey	TRPC2 RV C	ATGAGCATGTTGAGCAGCAC	X	X
Lamprey	TRPC2 FW WG1	AAGATGGACATCCGCTCCT		
Lamprey	TRPC2 RV WG1	ACTCGACGAGCAGGAAGAAG		
Lamprey	TRPC2 FW WG2	GTGTCCACACGCAACCAG		
Lamprey	TRPC2 RV WG2	CTGGGGATGAGGTTGAAGG		
Lamprey	V2R1 FW A	TTGGTGTCAGCTTCTCGTCC	X	
Lamprey	V2R1 RV A	ATCACGAACTTGCCCTCGTT	X	
Lamprey	V2R1 FW B	GAGCTGTGGCAGGAGACATT	X	
Lamprey	V2R1 RV B	ATGTCAGACAGATTGGCGCA	X	
Lamprey	V2R1 FW C	TTATGAAGGCTGTGGAGGCC	X	
Lamprey	V2R1 RV C	CGTAGTCATCGTCTGAGCCC	X	
Lamprey	V2R2 FW A	CTGGCGGTGACTGTGATCTT	X	
Lamprey	V2R2 RV A	CAGAGCCACGATGACAACCT	X	
Lamprey	V2R2 FW B	AGCATCGTCGTATTCGCCAT	X	
Lamprey	V2R2 RV B	CAGAATGATTTGGCCCTGCG	X	
Lamprey	V2R2 FW C	TCCTGCACGAACGTCATGAA	X	
Lamprey	V2R2 RV C	ATCCAGGTCCAGCGAAAGTG	X	
Lamprey	V2R2 FW D	CAGTTGCGCCAATGTGTCAA	X	
Lamprey	V2R2 RV D	AGTTCTTCCACATGGGCTCG	X	
Lamprey	PCNA FW A	CGGCTTCGACACCTACAGAT	X	X
Lamprey	PCNA RV A	TCTTTGGTGCAGCAGATGAC	X	X
Lamprey	PCNA FW B	GAGGCCCAAAATCATGAGAA	X	X
Lamprey	PCNA FW B	GGAGTGTAACGGTGGGTGAC	X	X
Lamprey	Vglut FW	TGCCCATCGGAGGACAAC	X	
Lamprey	Vglut RV	CTCGTCCTCGTTGATGAAG	X	
Lamprey	CasR FW	CAGGTGTTCGAGTCGCAGAT	X	
Lamprey	CasR RV	GAACACCAGCATGCTGAACG	X	
Lamprey	ACIII FW A	CCAGATCGCACCTCTCTCTC	X	
Lamprey	ACIII RV A	TAGTAGCAGTCGCCCAGGAT	X	

Species	Name	Sequence	PCR	In situ
Lamprey	ACIII FW B	GCGCAGATCTTCTGCTACCT	X	
Lamprey	ACIII RV B	GAGAGAGAGGTGCGATCTGG	X	
Lamprey	ACIII FW C	CCCTCTTCGACTGCTACGTC	X	
Lamprey	ACIII RV C	ACAGCGTGTAGGGGATGAAC	X	
Stickleback	A2C FW	TCAACTTCTTTGCGTGCGTG	Not tested	Not tested
Stickleback	A2C RV	TACAGGAAAGGGTTGACGGC	Not tested	Not tested
Xenopus	A2C 1 FW	TTAACGTCTCTGTCCGTGGC	X	X
Xenopus	A2C 1 RV	AGAGGGGTTTTATGCCAGCC	X	X
Xenopus	A2C 2a FW	GTGCTGACTGCTGTGCTTTC	X	
Xenopus	A2C 2a RV	GAGGAGTGACAAAGGCACGA	X	
Xenopus	A2C 2b FW	GCTGTGCCTGTAGAACGCTA	X	
Xenopus	A2C 2b RV	TTCTGCGCCTTCTTCTCCTG	X	
Xenopus	A2C 2b FW WG	ATCTCCTCATGTGGGTGGCA	X	
Xenopus	A2C 2b RV WG	ACATTGCCTTCTGTGTTCTCCT	X	
Zebrafish	A2C FW	TTCGTCACGGTTAAGCGTCA	X	X
Zebrafish	A2C RV	ATTCCCCTGCAGCACATGAA	X	X
Zebrafish	Ora1 FW	ATGGACCTGTGTGTCACCAT-CAAAGGCGT	X	X
Zebrafish	Ora1 RV	TCATGGAAGTCCACATG-GCAGAAG	X	X
Zebrafish	Ora2 FW	ATGATTGCGGAGGCTGTG	X	X
Zebrafish	Ora2 RV	TCCACGTTGTTGATGGCGTTC	X	X
Zebrafish	Ora3 FW	ATGGCGCCTCAAAAGAAACC	X	X
Zebrafish	Ora3 RV	AGATGAAGGCAGGGATGGAGT	X	X
Zebrafish	Ora4 FW	TGTGTCTGGCTAACCTGCTG	X	X
Zebrafish	Ora4 RV	AATCACCATCAGCTCCACCG	X	X
Zebrafish	Ora5 FW	TTCTGCGTTACAGGCATCAC	X	X
Zebrafish	Ora5 RV	AAGAAGTGAGGGACGCTGAA	X	X
Zebrafish	Ora6 FW	AGCTGGTGATGGTGGATGTG	X	X
Zebrafish	Ora6 RV	CACCAGCAGGAACACGTACT	X	X
Zebrafish	OMP FW	CAACGAGGACAGAAACGTCA	X	X
Zebrafish	OMP RV	CTTTGGCCAGCTCTGCTATC	X	X
Zebrafish	TRPC2 FW	AGACGAGCTCCTCCGTTACA	X	X
Zebrafish	TRPC2 RV	TGACCGTCTTCTCCAGCTTT	X	X

Table 2.1: PCR primers for all examined genes from all species tested. A cross indicates success in the respective experiment.

2.5 Molecular biology techniques

2.5.1 Synthesis of Digoxigenin and Biotin labelled Tyramides

Tyramides are peroxidase substrates that will form covalent bonds to tyrosine sidechains. Hapten-labeled tyramides thus allow massive signal amplification resulting in very sensitive detection methods.

The easy and fast synthesis of labelled tyramides, for using them in *in situ* hybridisation, was described for the first time by Anton H.N. Hopman (Hopman et al.,1998).

To produce an active ester solution 10 mg of either Sulfosuccinimidyl-6-(biotinamido)hexanoate (BIO-NHS) or Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (DIG-NHS) were dissolved in 1ml of Dimethylformamide (DMF). Because these substances are sensitive to hydrolysis and photolysis, the solutions were prepared immediately before the tyramide synthesis. Similarly, the tyramine-HCl solution was freshly prepared by adding 10 mg of tyramine-HCl to 1 ml DMF. To deprotonate the amino group of the thyramine and to adjust the pH between 7 and 8 10 µl of triethylamine 7.2 M (TEA) was added to the thyramine-HCl solution. For an efficient acylation 100 µl ester solution (DIG-NHS or BIO-NHS) were mixed with 28.9 µl of the tyramine-HCl stock solution and stored for two hours at room temperature in darkness. After this 827 µl absolute ethanol was added to the the resulting tyramine solution to a final concentration of 1 mg/ml. All tyramine solutions were stored at 4 ºC in darkness. Solutions were found to be stable for at least eight months.

2.5.2 Production of cDNA

After dissection, all tissue samples were collected in Eppendorf vials filled with RNA later.

To isolate the RNA the ReliaPrep™ RNA Tissue Miniprep System was used according to the manufacturer`s instructions and the received amount was measured *via* NanoDrop™ photometer. The RNA was stored at -80 ºC or used for making the cDNA. Around 500ng of RNA were used for every round in the SuperScript™ II Reverse Transcriptase System. The cDNA concentrations were determined *via* NanoDrop™ photometer and stored at -20 ºC.

2.5.3 DNA extraction from tissue

At first all inner organs, head and tail were removed from the zebrafish. The rest was refrigerated in either a -80 ºC refrigerator or in liquid nitrogen. After this everything was placed into a mortar covered with aluminium foil and was stored some hours together with the pestles at -80 ºC. The complete tissue was crushed very fast with the help of the pre-chilled pestles. The tissue powder was diluted in 5 ml tissue lysis buffer, containing 0.2 % SDS and dissolved at 55 ºC under light shaken over night. The next day 2 ml of phenol/chloroform/isoamyl alcohol mixture (25:24:1) were added to the tube, mixed gently and centrifuged at 10000 g for 2-3 min. After this 750 µl of the upper phase were collected in new Eppendorf tubes. Then the same amount of isopropanol was added to the tubes and centrifuged at highest speed for 10 min. The supernatant was discarded and 350 µl of 100 % ethanol were added to the vial. The tube was centrifuged at full speed for 10 min and again the supernatant was discarded and the pellet was dried for 10 min. After this 100 µl of Dnase free water was added and kept for 1 h at 55 ºC to dissolve the DNA pellet. The DNA concentration was measured with a NanoDrop Photometer.

2.5.4 Fin clipping and fast DNA extraction for zebrafish

All fishes were anaesthetised with MS222 and a small part of their fin was sliced and placed into a tube with 50 µl cell lysis buffer. All tubes were shaken for 1-3 hours at 55 ºC. After this 300-500 µl DEPC-water was added and the tube was shortly vortex. 8 µl of this mix was used for the PCR.

2.5.5 Production of molecular probes

2.5.5.1 PCR

All primers were designed by using primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) with a size between 300 and 400 base pairs.
Before using the primers for the polymerase chain reaction (PCR), all primers were diluted 1:10 deionized water to get a 10 mM stock solution.
For the following PCR either cDNA, genomic DNA or linearised plasmids with cloned gen inside was used as a template. In most cases the amount of template was between 1-2 µl. The annealing temperature was calculated on the basis of the nucleotide sequence of the primers.

Pipetting and setting for the thermocycler as follows:

Pipetting for PCR tube	Thermocycler	
10 µl GoTaq® Mastermix	1. 5 min	95 °C
1 µl FW-Primer	2. 45 sec	95 °C
1 µl RV-Primer	3. 45 sec	X °C
x µl Template	4. 60 sec	72 °C
filled up to 20 µl wit DEPC-water	5. 5 min	72 °C
	6. ∞	4 °C

After this, 2 µl of the PCR-products were tested with SDS-PAGE on a 1 % agarose gel and the remaining product was purified by using a PCR Purification Kit. The PCR product was eluted with 20-25 µl Tris HCl pH 8.0 and stored at -20 ºC.

2.5.5.2 Ligation of the PCR products into pGEM-T vectors

For long-term storage some of the PCR-products were cloned into pGEM-T vectors. Therefore the PCR-product and and the pGEM-T vector were mixed 3:1 and incubated in a termomixer at 14 ºC overnight.

x µl pGEM-T vector
x µl Insert
1 µl T4-DNA-Ligase
1 µl Ligasebuffer
Filled up with x µl water to 20 µl.

2.5.5.3 The making of the agar plates

500 ml of LB-media was heated up in a microwave and after it reached the liquid phase 500 µl of ampicillin was mixed in. One hour before using, 80 µl IPTG (0.1 M) and 20 µl X-Gal (50 mg/ml) were added onto each pre-made plate.

2.5.5.4 Transforming the plasmid DNA into electrocompetent cells

50 µl of thawed electrocompetent cells were pipetted into pre-chilled cuvettes. Then 5-10 µl of ligation reaction was added to the cuvettes and gently shaken, before the electroporation was started. After the electroporation, 1 ml of LB medium was added and then the mix was transferred into Eppendorf vials and incubated for 30 min at 37 °C. 100 µl of this mix were plated onto the agar plates and the rest was centrifuged at high speed for one minute. After centrifugation the supernatant was discarded and the remaining pellet was resuspended with 100 µl LB medium and also plated on an agar plate. All agar plates were incubated at 37 °C overnight.

2.5.5.5 Picking the colonies

Single positive (white) colonies were picked with a sterile plastic pipette tip and first transferred into a PCR-mix, which was used for a colony PCR and secondly given into a test tube with 3 ml LB Medium containing an ampicillin concentration of 50 µg/ml. All test tubes were inoculated overnight. The products of the colony PCRs were tested on a 1% agarose gel.

2.5.5.6 Plasmid purification

For the purification of the plasmids several commercial kits were used. (Invisorb Spin PlasmidMini Two /Stratec).

2.5.5.7 Sequencing

All plasmids, PCR-products and probes were sequenced by sending 10-15 µl with 2 µl Primer to either GATC Biotech (http://www.gatcbiotech.com) or Eurofins (www.eurofinsgenomics.eu). The sequenced data were downloaded the next day.

2.5.5.8 *In vitro* Transcription

For the in *vitro* transcription an internal lab protocol was used, which was based on the work of (Krieg et al.,1984; Schenborn and Mierendorf, 1985). As a template for the PCR-product, labelled with the T3 or T7 promoter site, either a purified plasmid, genomic DNA or CDNA was used. For the antisense probe the reverse primer was tagged with the T3 or T7-promoter site. If a sense probe was made the forward primer was tagged with the T3 / T7 promoter site. The same PCR scheme was used as described below. After purification, a quality check (1 % agarose gel) and sequencing, the PCR product labelled with the T3 promoter site was used for the in *vitro* transcription. Either Digoxigenin-11-UTPs or Fluor-

escein-12-UTPs were used as nucleotide labelled haptens. However, fluorescein is light sensitive, so all following steps were carried out in darkness.

Pipetting scheme:

X µl Template-DNA (ca. 500 ng)
2 µl Transcriptionbuffer (Roche)
2 µl Digoxigenin– or Fluorescein RNA Labeling Mix (Roche)
2 µl T3-RNA-Polymerase
0,5 µl RNAse Inhibitor
Filled up to 20 µl with RNAse-free water.

The complete mix was incubated 2.5-3 h in a thermocycler at 37 °C. To digest the remaining template DNA, 2 µl of Dnase I was added to the mix and incubated again for 15 min at 37 °C. After this 2.5 µl 4 M LiCl and 2 µl of 0.2 M EDTA were added, to stop all enzymatic reactions. To precipitate the probes, 75 µl of pre-chilled 100 % ETOH was pipetted to the mix and stored for 20-60 min at -80 °C. Afterwards the probes were centrifuged for 20 min with 15000 rpm at 4 °C and then the supernatant was discarded. The next step was to fill the tube, which had the remaining pellet inside, with 100 µl 75% ETOH and centrifuge it again for 2 min at 15000 rpm. After discarding the supernatant, the pellet was dried at the thermocycler at 37 °C until all liquid was gone, then resuspended in 20-25 µl 10 mM Tris/HCl pH 8.0 and stored at -20. To check if the in vitro transcription was working, 2 µl of the the probe was mixed with 8 µl formamide and 1 µl loading buffer. This mix was checked with SDS-PAGE on a 1 % agerose gel.

2.5.5.9 Quantification of digoxigenin- and fluorescein labelled probes

To qualify the amount of probe all probes were measured a minimum of two times with NanoDrop™ . Another method to qualify the labelled probes was to make a Dot Blot. For this 1 µl of each probe was three times diluted with ddH_2O 1:10, 1:100 and 1:1000. All batches were pipetted on a nylon membrane and incubated for 30 min with 1% PBS pH 7.5 blocking solution. After this the membrane was incubated with an anti-digoxigenin-AP or anti-fluorescein-AP-antibody diluted 1:1000 in in 1% PBS pH 7.5 blocking solution and

followed by three-time washing in PBS pH 7.5 for 5 min. To visualise the probes either NBT/BCIP or DAB was used. To stop the reaction, the nylon membrane was transferred into ddH_2O. All steps were done at room temperature.

2.6 Histological methods

2.6.1 Preparation of the olfactory epithelia in zebrafish, carp and eel

Before the preparation of the olfactory epithelia, each kind of fish was anesthetized with different amounts of MS222. After this all experimental animals were decapitated with a sharp razorblade and placed into a petri dish with pre-chilled PBS pH 7.4. The head was fixed at the top with sterile forceps. To simplify the removal of the olfactory epithelia, both eyes were taken out and the gills were pulled into an anterior direction. The next step was to remove the scaly skin at the top of the head and around the nose holes, to set the olfactory epithelia free. Then as much of the cartilage was removed around the olfactory epithelia to set it completely free. For the *in situ* hybridisations, the complete epithelia was placed directly into plastic bottle lids, which were filled with tissue tec-OCT compound and stored at -80 ºC. In case of Immunohistochemistry, the epithelia was given into an Eppendorf vial filled with 4% of PFA diluted into PBS pH 7.4 for 5 min and then placed into a plastic bottle lid, filled with tissue tec-OCT compound and stored at -80 ºC.

2.6.2 Preparation of the olfactory epithelia in lamprey

After the dissection of the olfactory epithelia, it was placed for 17-40 h in 4% PFA, then 3 h in 20 % sucrose and at the end before freezing 15 min in tissue tec-OTC compound.

2.6.3 *In situ* Hybridisation (ISH)

If the tissues were not fixed before cryosectioning, all slides were fixed in 4 % PFA in PBS pH 7.5 for 15 min. After washing for 5 min in 1 M PBS pH 7.5, the tissues were treated with 2 M HCl in DEPC-water to permeabilize the membranes for the probes and to deactivate all enzymatic activity, especially from phosphatases and peroxidases to reduce the background staining. Again a 5 min washing step in 1 M PBS pH 7.5 took place, followed by treatment with 0.25 % acetanhydride and 0.1 M triethanolamine diluted in DEPC-water and adjusted with HCl for the pH, to deactivate Rnase's and to neutralise the positive charges on the slide, to prevent false binding of the negative charged probe. The slides were washed again with 1 M PBS pH 7.5.

In the meantime, each probe was diluted in 200 µl of the hybridisation mastermix in concentrations of 3 ng/µl for the NBT/BCIP and the HNPP/Fast Red or 0.25-0.5 ng/µl for the TSA staining method. The hybridisation mix was heated up for 3 min at 99 °C to denature the probe. The mix was briefly cooled down on ice. Then 20 µl of Denhardt's reagent was added and everything was heated up to 65 °C. After the last washing step the hybridisation mix containing the probe was pipetted on the slide and covered with a piece of parafilm. The slides were stored in a hybridisation chamber covered with plastic foil and containing a mix of 50 % formamide, 25 % 20xSSC and 25 % H_2O, to prevent evaporation. The hybridisation chambers were incubated at 60 °C overnight in a hybridisation oven.

The next day the tissues were rinsed briefly in 5x SSC at 65 °C and then washed for 30 min in 50 % formamide and 2x SSC at 65 °C . After this two more washing steps for 30 min with decreasing salt concentration of 2x SSC and 0.2 SSC at 65 °C were carried out. After two additional washes in 0.2xSSC for 15 min and 1M PBS pH 7.5 for 5 min at RT, the sections were blocked in TNB Buffer for 60 min at room temperature. Either the slides were incubated at room temperature for 1.5 h or at 4 °C over night with the primary antibody. For this sheep anti-digoxigenin 1:1000 or sheep anti-fluorescein antibody 1:250 labelled with an alkaline phosphatase (NBT/BCIP; HNPP Fast Red) or an peroxidase (TSA) diluted in TNB buffer were used.

2.6.3.1 NBT/BCIP staining

All tissues were washed three times 10 min in 1M PBS pH 7.5 to remove the unbound antibodies, followed by two times washing 10 min in NBT/BCIP detection buffer. After this the tissues were incubated over night at 4 °C in 3.7 µl BCIP and 5 µl NBT diluted in 1 ml NBT/BCIP detection buffer. After the signal was developed, the reaction was stopped with ddH_2O.

2.6.3.2 HNPP/Fast Red staining

All tissues were washed three times 10 min in 1 M PBS pH 7.5 to remove the unbound antibody, followed by two times washing 10 min in HNPP/Fast Red detection buffer. For the staining reaction 10 µl HNPP and 10 µl Fast Red were mixed in 1 ml HNPP/Fast Red detection buffer, filtered through a 0.22 µm nylon filter Millex GP (Millipore), added to the slides and incubated at 4 °C overnight. The reaction was stopped with ddH_2O, after the signal was developed.

2.6.3.3 TSA staining

After that the tissues were washed three times 10 min in TNT Buffer. Then biotin- or digoxigenin labeled tyramides were diluted 1:20 in TSA Buffer and added to for 10 min to the tissues, followed by three washing steps in TNT buffer. For the visualising of the signal, either anti-digoxigenin or anti-biotin-antibody, which were tagged with Alexa-Fluor 488 or 633 was used. To get rid of the unbound antibodies, the tissues were washed three times for 15 min in TNT buffer.
Double *in situs* were performed as described in (Twickel et al., 2018).

2.6.3.4 Mounting the tissues

If the tissues were labelled with NBT/BCIP, the slides were dried in a hybridisation oven at 60 °C and a small amount of VectaMount™ was given on each slide, covered with a cover slip and sealed with nail polish and stored at 4 °C. If a fluorescent staining method was used, small amounts of VectaShield, which contains DAPI for a nucleus staining, was directly used for the tissues. The slides were also covered with nail polish and stored at 4 °C.

2.6.4 Immunohistochemistry (IHC)

The slides with the olfactory epithelia were directly stored in -20 °C cold acetone for 15 min, without heating and drying after the cutting. After 3 times of washing for 10min in 1x-PBST 0.1 % at RT, the tissue was blocked with 5 % goat or sheep serum in 1xPBST 0.1 %. The tissues were incubated with the primary antibodies (1:100-1:500) diluted in blocking buffer at 4 °C in a moist chamber overnight. During the incubation of the primary antibody, the slides were covered with parafilm, to achieve a constant moisture on the slide and to avoid evaporation. The next day the tissues were washed 3x for 10 min in PBST 0.1 % at room temperature and then incubated for 2 h with the secondary antibody (1:200) at RT. After 3x washing in 1xPBS pH 7.5, the slides were mounted with small amounts of VectaShield , which contains DAPI for a nucleus staining. The slides were cover also with nail polish and stored at 4 °C. If the IHC staining followed an ISH, the acetone step was skipped and the procedure started with washing steps.

2.7 Detection of odor-induced neuronal acticity in lamprey OE using immediate early gene *c-fos* and SP6 as marker

One day before the stimulation the two lampreys were placed in several tanks with the same size, amount of liquid and protection from external interferences. The odour was diluted to 50 µm in 10 ml of tank water and added *via* a syringe into the tank. After one hour

the animals were sacrificed and OE was dissected and sliced into 10 µm slices and after this an IHC with a c-fos-antibody or SP6-antibody, both immediately early gene markers, followed.

2.8 Measurement and analysis of spatial coordinates

The distribution of receptor neurons labelled with a DIG-labeled probe was assessed in complete series of sections of olfactory epithelium. Spatial coordinates were evaluated: radial distance (centre of the lamella to cell position), height within the lamella (basal border of the lamella to cell position; laminar height), and height within the organ (Number of horizontal sections from top to bottom; z axis). A graphical description of the measurements is given in Figure 2.1 No differences in frequency were observed between left and right side of the centre line (the middle raphe). Spatial coordinates were measured in arbitrary units and normalized as described. For example, apical-to-basal position within a lamella (laminar height) was measured as the shortest distance between the centre of the cell and the basal border of the epithelial layer, and normalized to the thickness of the epithelial layer at the position of the cell. Thus the range of values is between 0 (most basal) and 1 (most apical). Unbinned distributions were represented as the corresponding empirical cumulative distribution function (ECDF). In this presentation, data points are sorted by their parameter value (x axis), with their ordinal number (normalized) as y axis. Each data point results in a curve point, thus no information about the distribution is lost in the representation as ECDF, in contrast to the usual histogram representation. To estimate whether two spatial distributions were significantly different, we performed Kolmogorov-Smirnov tests on the unbinned distributions using R with the following command: laply(inputfile.csv, function(x) llply(inputfile.csv, function(y) ks.test(x, y)$p.value)). The Kolmogorov-Smirnov test makes no assumptions about the nature of the distributions investigated, which is essential since the skewness of many distributions showed that these are not Gaussian. Due to the sensitive nature of the test on large distributions ($n > 100$) we selected $p < 0.01$ as cut-off criterion for significant difference, cf.(Ahuja et al., 2018)

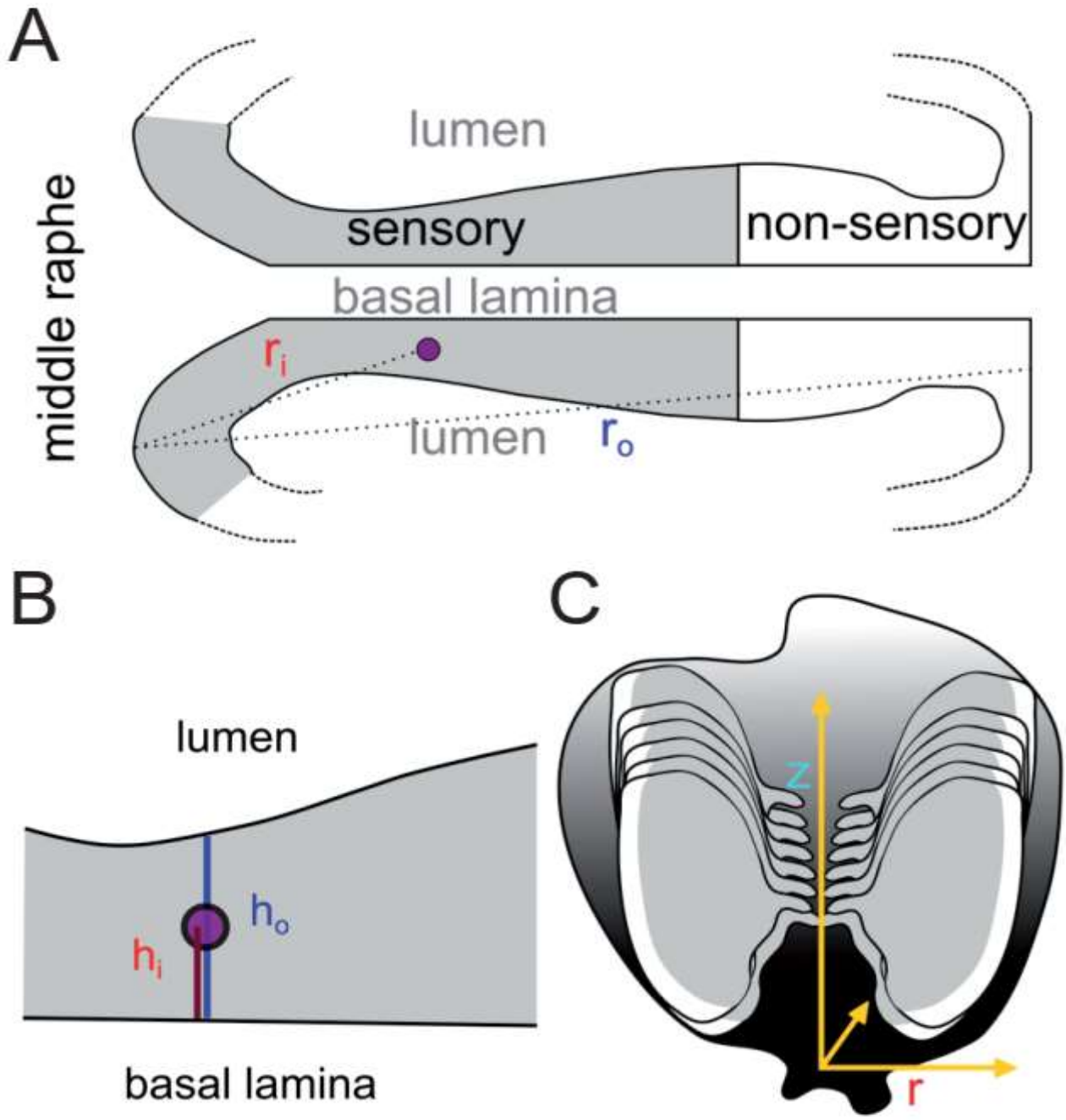

Figure 2.1 Distribution properties. The parameter values for the first, second and third quartile of the distributions, i.e. for the radial**(A)**, laminar height**(B)** and z-axis coordinates**(C)**. Half width of distributions was determined as 3rd quartile-1st quartile difference. (modified from Ahuja et al., 2018)

3. RESULTS

3.1 Shift of A2c expression in jawless fish compared to bony fish

3.1.1 Sparse expression of bony vertebrate *a2c* genes in the nose is consistent with a function as olfactory receptor

3.1.1.1 A2c expression in the olfactory organ of zebrafish - *Danio rerio*

It is known from (Wakisaka et al., 2017), that the zebrafish expresses the recently discovered amine receptor A2c in the olfactory epithelia. To repeat this result and as a starting point in the following research, the published nucleotide sequence of zebrafish was taken from NCBI (https://www.ncbi.nlm.nih.gov/) and a primer pair, for an *in situ* hybridisation, was designed with primer3plus (Fig.3.1).

A2c nucleotide sequence of *Danio rerio*

ATGAACAACTCTATCGCCAACTTTACATTCTCTCCAACTCTTCTCTACATCAACACGACCTTAAGA
AATGCCACGTCCTACGAAAACAACACGCTTACGCAGTTCACCAATGCCTTATCCACCATGTCAACA
CTTACTCAAGCTAAACTCACCCAAACCACAACTAAGACCACCGTTACAACCACCACACCAACAACA
ACAGCAGCACCAGAACCAAACATCCTAGAATCAATAAACCTTCCATACATGATAGCAGAGCTCATC
ATAGCCCTCCTCTCCACCGTTGGCAACCTGCTAGTCTGTGTGGCAGTTGGACTAAACAGGAAGCTC
CAAACAGTCACTAACTACTTTCTAGTATCACTAGCGGTGGCGGATATCTGCGTGGGTGCATTAGCG
ATCCCTTGCGCCATCATGACAGACCTGGGAATCCCGAGGCACAACCTCTACTTGTGTTTACTAATG
CTGTCTGTGCTAATCATGCTAACCCAAAGCTCCATCTTTAGCCTGCTGGCAGTCGCGGTAGAGCGT
TATGTTGCCATTTTTATGCCTTTCCAATACCATCGGCTCATGACACCTCGAAACGCGGTGCTGATT
TTGTGCGTGACTTGGACGCTGGCTTTTCTAATTGGTTTGGTGCCGCTGATGGGTTGGCACAAGCCT
CCGCCTGAATCGGGCTACTGTTTCTTTGTGTTTGTGGTGGATATGACGTACATGGTCTACTTTAAC
TTCTTCGCGTGCGTCTTGGCTCCACTGGTGGTCATGTTTCTCATTTACGCACAGATTTTCGTCACG
GTTAAGCGTCAGATGAGGAGGATCGCTGCCGAACGCGGAGGTGCAGGAAACACAGAGGGAACGGCG
AAGATGAAGAAAGAGATGAAGATGGCAACTTCCCTCTTTTTAGTCCTTTTCCTCTTCACTTCATGC
TGGATTCCGCTTCATATTATTAACTGTTTCCTGCTGCTGTGCCCGTCGTGTCCTGTTCCTCTACCG
CTTTTATTAACGGCTATTATTCTCTCCCATGCTAACTCAGCTGTTAACCCGTTCCTCTATGCATAC
AAGATGAAAACCTTCAGAAACGCCTTCAAGAGCATCTTCATGTGCTGCAGGGGAATCAGTGATGGC
GAGGATGCATGA

Figure 3.1: Zebrafish A2c nuclotide sequence. Nucleotide sequence of *Danio rerio* with primer sequences in yellow.

The olfactory epithelia of either female or male adult zebrafish at the age of 8-18 months, were dissected and cut apical to basal into 10 µm thick slices. An *in situ* hybridisation, with NBT/BCIP chromogenic staining was performed following the standard protocol (2.6.3.1) The expression was exactly as expected, compared to the results of Wakisaka et al., 2017. The A2c-positive cells were mostly localized close to the middle raphe. Distribution of A2c-positive cells appeared random for height within the lamellae, but no quantitative determination was done (Fig.3.2).

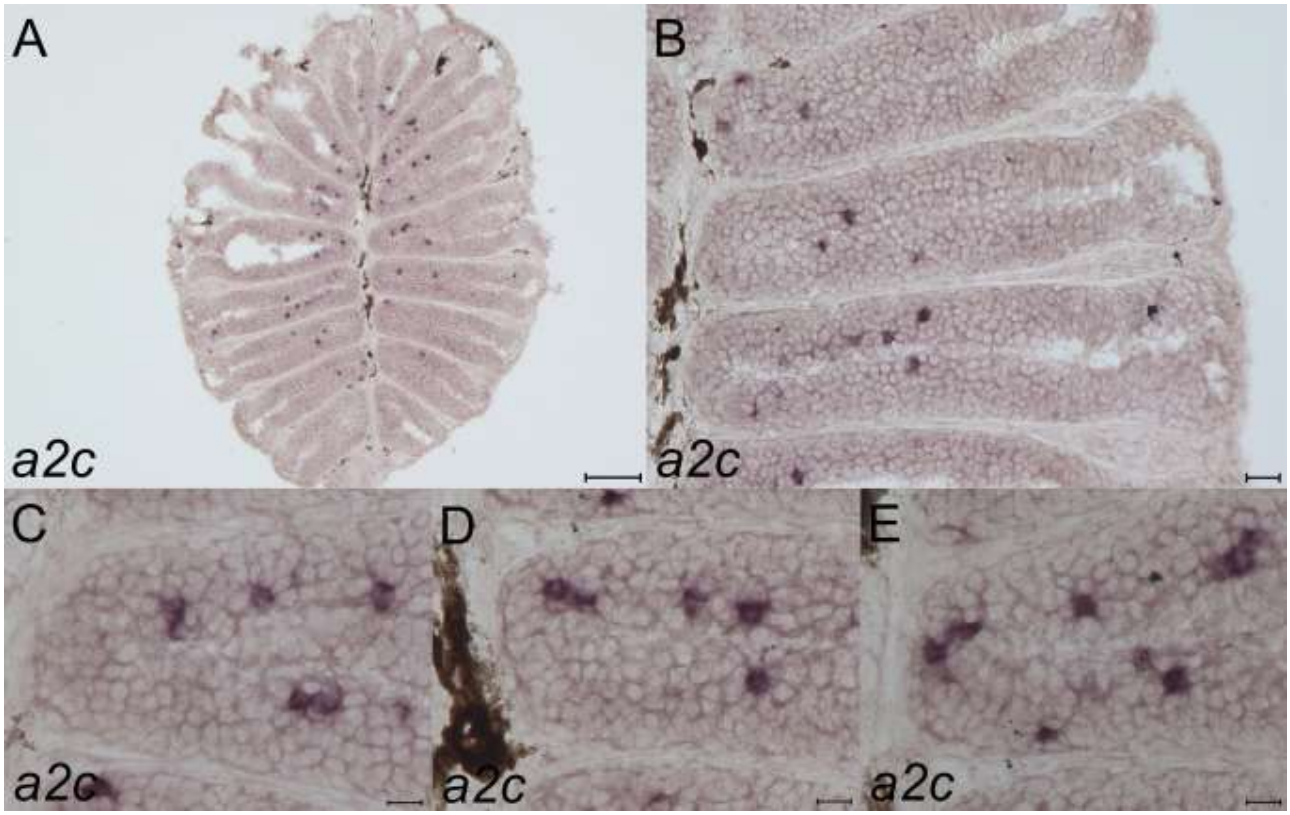

Figure 3.2: A2c *in situ* hybridization signals in the olfactory epithelia of *Danio rerio*. A)-E) NBT BCIP *in situ* hybridisation of A2c in the sensory area of the olfactory epithelia in *Danio rerio*. Scale bar A) 100µm B) 50µm C-E) 10µm. Large pigment cells are found in the middle raphe, distinguishable by their dark brown color from the lilac color of the *in situ* hybridization signal.

3.1.1.2 A2c expression in the olfactory organ of carp – *Cyprinus carpio*

A close relative to zebrafish in the family of teleost fishes is *Cyprinus carpio*, which is a common domesticated food fish and like zebrafish a member of the *cyprinidae*. The A2c amino acid sequence of *Danio rerio* was used to searchthe genome of *Cyprinus carpio with tblastN* NCBI (https://www.ncbi.nlm.nih.gov) (Fig 3.3). The candidate found was identical to the sequence predicted by (Wakisaka et al. 20017) for the carp *a2c* gene. Primers were designed with the help of this sequence and the computer programme primer 3 plus (Fig 3.3).

A

A2c amino acid sequence of *Cyprinus carpio*

MNQPSANFTFLPTLLYTNTTFMNVTSYENNTFLQFATSTMSTLTQGKLTQAASAATTTTTTTTAAP
SILASINLAYMIAELVIALLSTVGNVLVCVAVGLNRKLQTVTNYFLVSLAVADICVGSLAIPCAIM
TDLGIPRHNLYLCLLMLSVLIMLTQSSIFSLLAVAIERYIAIFMPFQYHRLMTPRNAVLVLCFTWT
LAFLIGLVPLMGWHKPPPESGYCFFVLVVDMTYMVYFNFFACVLTPLVVMFLIYAQIFVTVKRQMR
RIAAERGGAANTEGAAKMKKEMKMATSLFLVLFLFTACWIPLHIINCFLLLCPSCPVPLPLLLTAI
ILSHANSAVNPFLYAYKMKTFRNAFKAIFLCCRGIGDGDEQHDDGGGNNAQRS

B

A2c nucleotide sequence of *Cyprinus carpio*

ATGAACCAGCCTTCTGCCAACTTCACATTCCTGCCGACTCTACTCTACACCAACACGACTTTCATG
AATGTCACCTCTTATGAAAATAACACCTTTTTGCAGTTCGCCACATCCACCATGTCGACACTTACG
CAAGGTAAACTAACCCAAGCCGCCTCCGCCGCCACCACTACTACTACTACAACAACAGCAGCGCCA
AGCATCCTAGCATCCATAAACCTGGCTTACATGATAGCCGAGCTTGTCATAGCCCTCCTCTCCACT
GTGGGTAACGTGCTAGTTTGTGTTGCAGTGGGACTAAACAGGAAGCTCCAAACAGTTACCAACTAC
TTTCTGGTGTCATTAGCGGTGGCAGATATTTGCGTTGGTTCACTGGCGATCCCTTGCGCCATCATG
ACAGACCTGGGCATTCCACGGCACAATCTGTATCTGTGTTTGCTAATGTTATCTGTACTCATCATG
TTAACCCAGAGCTCCATTTTTAGCCTGCTGGCGGTCGCCATTGAGCGCTACATTGCCATCTTCATG
CCCTTCCAGTACCACCGGCTCATGACACCTCGCAACGCAGTGCTGGTCTTGTGTTTCACTTGGACG
CTGGCCTTCCTGATTGGTCTGGTGCCGTTGATGGGCTGGCACAAGCCTCCACCCGAGTCTGGCTAC
TGTTTCTTCGTTTTGGTGGTGGATATGACCTACATGGTCTACTTCAACTTCTTCGCCTGTGTTCTG
ACCCCACTGGTGGTGATGTTCCTCATCTACGCGCAGATCTTCGTCACGGTCAAGCGTCAGATGAGG
AGGATTGCAGCAGAGCGGGGCGGCGCGGCCAACACGGAGGGAGCAGCAAAGATGAAGAAAGAGATG
AAGATGGCCACTTCACTCTTCCTGGTCCTCTTCCTTTTCACGGCCTGCTGGATTCCGTTACATATC
ATCAACTGCTTCCTGCTGCTGTGTCCGTCATGTCCCGTGCCTCTGCCGCTGTTACTGACGGCTATC
ATTCTCTCCCATGCAAACTCAGCTGTTAACCCTTTCCTCTACGCCTACAAGATGAAAACCTTCAGA
AATGCGTTCAAGGCCATCTTCCTGTGCTGCAGGGGCATCGGTGATGGCGATGAACAACATGACGAT
GGAGGAGGCAATAACGCTCAAAGATCCTGA

Figure 3.3: Carp A2c amino acid and nucleotide sequence: A) Predicted amino acid sequence of A2c *Cyprinus carpio* based on a search with zebrafish A2c amino acid sequence B) Corresponding nucleotide sequence of *Cyprinus carpio* A2c with primer sequences in orange.

The olfactory epithelia of young carp with a body length of 12-15 cm, were dissected and treated following the standard protocol (2.6.1). Then 10 µm thick cryostat sections were made, and subjected to *in situ* hybridisation with the A2c probe. After the staining with the chromogen NBT/BCIP, a clear staining of A2c-positive cells was observed. The morphology of A2c-positive cells looks like that of OSNs known from other teleosts. Around 2-10 A2c-positive cells were counted per lamella and their position was closer to the middle raphe, compared to zebrafish. In terms of height, the A2c-positive cells are mostly localized in the basal and medial part in the lamella (Fig. 3.4).

These data represent the first expression study of olfactory receptors in carp using *in situ* hybridisation.

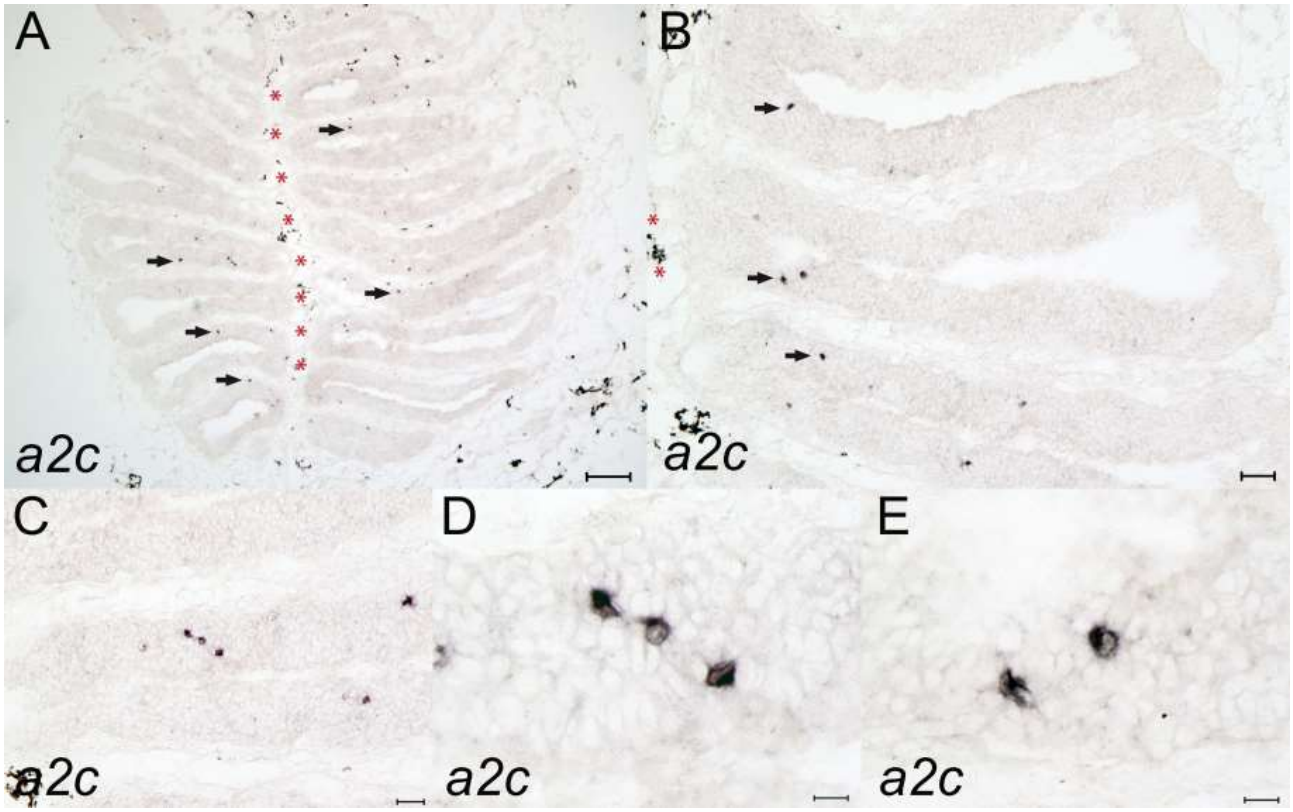

Figure 3.4: A2c *in situ* hybridization signals in the olfactory epithelia of *Cyprinus carpio*. A)-E) NBT BCIP *in situ* hybridisation of *a2c* in the sensory area of the olfactory epithelia in *Cyprinus carpio*. A) and B) Middle raphe with red asterix and some single stained A2c-positive cells with black arrow. Scale bar A) 200µm B) 100µm C) 50 µm D) 20µm E) 10µm.

3.1.1.3 A2c expression in the olfactory epithelia of eel – *Anguilla anguilla*

Another member of the teleost fishes and further from zebrafish, is the eel – *Anguilla anguilla*. Again with the help of the blasted zebrafish A2c amino acid sequence in the genome of *Anguilla anguilla*, a possible *a2c* candidate gene was discovered by the lab (Fig 3.5A). A first primerset A2cA (Fig.3.5B yellow primer set) did not result in amplification from cDNA of eel OE . Therefore a second primerset A2cB was made, which allowed successful amplification (Fig. 3.5B green primer set).

A

A2c amino acid sequence of *Anguilla anguilla*

MNGTHNATLLTTPPDLVLSINVPYMVAELVIAALSTVGNLLVCLAVGLNRKMHTVTNYFLVSLAVA
DACVGGVAIPCAILTDMGIPRHNLYPCLLMLSVLIMLTESSIFSLLAVAVDRYLAIFLPFRYPTLM
TPRNALLVILVTWVLSFLIGLVPLMGWHKTPPESGYCFFVFVVDMTYMVYFNFFGCVLAPLVIMFL
IYARIFATVKRQARRIAADRGQGEAQARRAASMRTEAKTAASLFLVLFLFTVCWIPLHIINCFLLL
CPSCPVPLPLLLTAIILSHANSAVNPFLYAYKMKSFRRSFKAIFLCCRVEMDQDDRIEDGDTTTHK
S

B

A2c nucleotide sequence of *Anguilla anguilla*

ATGAACGGCACTCATAACGCCACGCTGCTGACCACGCCCCCTGACCTGGTGCTCTCCATCAATGTG
CCCTACATGGTGGCCGAGCTGGTGATCGCCGCCCTCTCCACCGTGGGCAACCTGCTGGTCTGCCTG
GCCGTGGGGCTCAACAGGAAGATGCACACCGTCACAAACTACTTCCTGGTGTCGCTGGCCGTGGCG
GACGCGTGCGTGGGCGGGGTGGCCATCCCTTGCGCCATCCTGACGGACATGGGCATCCCCCGGCAC
AACCTGTACCCGTGTTTGCTTATGCTGTCCGTGCTCATCATGCTCACCGAGAGCTCCATCTTCAGC
CTGCTGGCCGTGGCTGTGGATCGCTACCTCGCCATCTTCCTGCCCTTCCGCTACCCTACCCTGATG
ACGCCCCGCAATGCCCTCCTGGTCATCCTGGTCACCTGGGTCCTGTCCTTCCTCATTGGCCTAGTG
CCGCTCATGGGCTGGCACAAGACCCCGCCCGAGTCCGGCTACTGCTTCTTTGTCTTTGTGGTGGAC
ATGACCTACATGGTCTACTTCAACTTCTTCGGCTGCGTGCTGGCGCCGCTGGTGATCATGTTCCTG
ATCTACGCGCGGATCTTCGCCACGGTGAAGCGGCAGGCCAGGCGGATCGCGGCCGACCGGGGGCAG
GGGGAGGCGCAAGCCCGACGGGCCGCCAGCATGCGCACCGAGGCCAAGACCGCCGCCTCGCTCTTC
CTGGTGCTCTTCCTCTTCACCGTCTGCTGGATCCCACTGCACATCATCAACTGCTTCCTGCTGCTG
TGCCCGAGCTGCCCCGTGCCCCTCCCCCTCCTGCTGACCGCCATCATCCTCTCCCATGCCAACTCC
GCCGTCAACCCCTTCCTCTACGCCTACAAGATGAAGTCCTTCCGGCGCTCTTTCAAGGCCATCTTC
CTCTGCTGCAGGGTTGAGATGGACCAGGACGACAGGATAGAAGATGGGGACACAACCACCCACAAG
TCATGA

Figure 3.5: Eel A2c amino acid and nucleotide sequence: A) Predicted amino acid sequence of A2c *Anguilla anguilla* based on a search with zebrafish A2c amino acid sequence B) Corresponding nucleotide sequence of *Anguilla anguilla* A2c with primer sequences in green (A2c B) and yellow (A2c A).

Young eels with a size of 20-30 cm were anaesthetized, and the olfactory epithelia was dissected and cryostat sections with 10 µm thickness were prepared. With the eel A2cB probe and a chromogenic NBT/BCIP staining, A2c-positive olfactory sensory neurons were detected. The localization of A2c-positive cells in terms of radius (distance from from middle raphe to the periphery of the organ) is very close to the middle raphe and the height within the lamella medial to basal (i.e. not close the lumen). Approximately 1-5 A2c-positive olfactory sensory neurons were detected in each lamella (Figure 3.6).

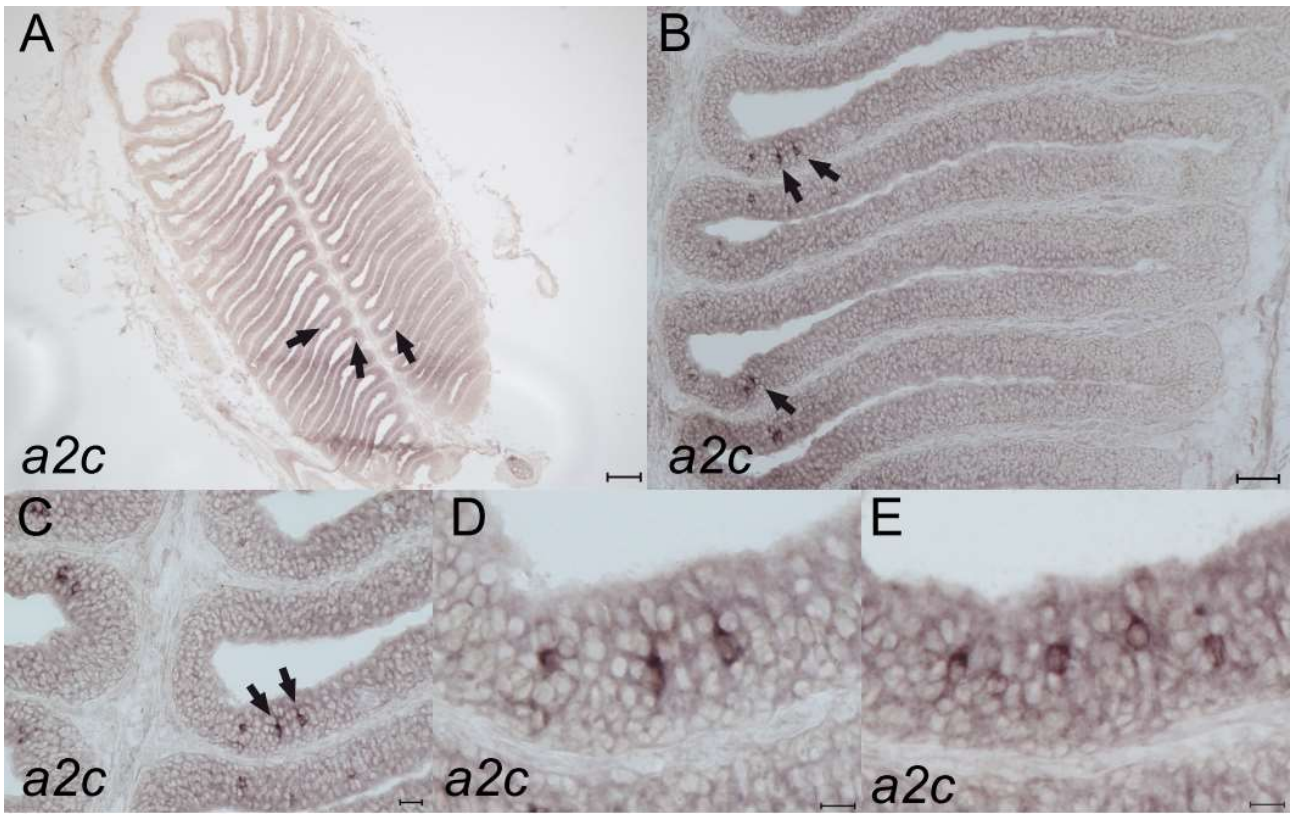

Figure 3.6: A2c *in situ* hybridization signals in the olfactory epithelia of *Anguilla anguilla*. A)-E) NBT BCIP *in situ* hybridisation of *a2c* in the sensory area of the olfactory epithelia in *Anguilla anguilla*. A)-C) single stained A2c-positive cells with black arrow. Note that the plane of section in A) is oblique, with the lower end of the section closer to the base of the organ, whereas the upper end is above the level of the middle raphe. Scale bar A) 200µm B) 50µm C) 20µm D) + E) 10µm.

3.1.1.4 A2c expression in the olfactory epithelia of clawed frog – *Xenopus laevis*

Due to the fact that amphibians are the link between fish and mammals, it was really interesting to observe if A2c is localized in the VNO or OE of *Xenopus laevis*. Recent studies identified one possible *a2c* gene sequence in *Xenopus leavis* and *Xenopus tropicales* (Wakisaka et al., 2017). But *via* blasting the amino acid sequence of zebrafish in NCBI, two possible additional *a2c* gene sequences were found in the genome of *Xenopus laevis* (Fig.3.7 A). For each possible gene a primer set was designed (Tab. 2.1) but only for the A2c.1 primer set (Fig. 3.7 B) did the *in situ* hybridisations show positive results. A second primer set for the *Xenopus laevis a2c2a* and *a2c2b* genes was not made, because of the limiting number of olfactory organs in the lab.

A

A2c amino acid sequences of *Xenopus laevis*

A2c.1

MYMIFSYKYFSNVLFPQVIILTMNASSKVTSNTSGTGLSDTFSLNVPYTAAEMIIAVTATLGNLLV
CMAVVHDKKLRTVTNYFLTSLSVADVCVGAIAIPCAILTSIGYPHNNLELCLLMLSILIMLIICST
LNLLSIAVDRYVAILNPLRYRSIMTPRNALISIIVAWLLSVLSGLLPLMGWHKTPLSPYCLFTQVI
DMTYMFYFFTFTFFILPLTSMLLIYARIFSAVRKHINHGETRATPENMESRQETGLKREIKTATSL
FTIIFLFVLCWAPLHVVNGLILLCSRCSIPPLLVDTGIILSHANSALNPIVYAYKLKSFRDKFKVF
IRCRDNTITPEAH

A2c.2a

MLADMNSTSSPHVGGISGPSFTITNIPYFVTEVLTAVLSVIGNILICLAVIRDRRLRTVTNYFLLS
LAIADILVGAVAVPCAILLDLGVARCSLYCCLFMLCNLMTFSLASIFGLLAVAVERYISIMLPFHY
RAFVTPRTSFLVILGTWFLAAITGLLPLMGWRKPFPLNSECLFSSLISESYMVYLIFFGFVIPPLA
TMLVLYARIFLEIRKQIRQIAEWQVEISRRRRRRIVVKELRMATSLFIVVFCFVICWLPIHLLNV
VHLFFPSCVVPEDVILSAVILSHANSALNPIIYVFRMSSFRRAVEATLSCPCATKAVASRTGSTHV
KPIKENTEGNVRNESLPGK

A2c.2b

MNSTLSPHVGGTSGPSVTLTNIPYFVTEVLTALLSMTGNILICLAVIRDRRLRTVTNYFLLSVAIA
DIVVGAVAVPCAILLEMGVARCNLYCCIFMTFSLASIFGLLAVPVERYTLLCPFHYCAFATPRTSF
LVILGTWFLAAIAGLFPLMGWRKPFPPNSKCESYMVYLIFFGFVIPPLAAMLVLYARIFLEIQKQI
CQIAEEVDISRRRRRRIAVKELHMATSLFIVVFYFDICWLPIHILNVVRLFFPSCVVPEDVILSAV
ILSHANSALIIYVFRMSSFRQAVETLSCFCATRAVVSRTGSTHVKPVKKENTEGNVRNYLESDSQN
AYQ

B

A2c nucleotide sequence of *Xenopus laevis*

A2c.1

ATGTATATGATCTTCTCCTATAAATATTTTAGCAATGTTCTTTTCCCGCAGGTGATCATCTTAACC
ATGAATGCAAGTTCCAAAGTTACATCTAACACAAGTGGCACCGGCTTGAGTGACACCTTTAGCCTG
AATGTTCCCTATACGGCAGCAGAGATGATTATTGCAGTAACTGCCACCCTGGGGAACCTCCTGGTG
TGTATGGCGGTCGTACACGACAAAAAGCTCAGGACAGTGACCAACTATTTCTTAACGTCTCTGTCC
GTGGCGGATGTGTGTGTAGGTGCAATTGCCATCCCTTGTGCCATCCTGACCAGCATTGGGTACCCA
CACAATAACCTAGAACTGTGCCTCCTAATGCTCAGCATCCTAATTATGCTCATCATATGCTCCACC
CTAAACCTCTTATCCATCGCGGTGGACAGATACGTGGCCATATTAAATCCTCTTCGATACAGAAGC
ATTATGACTCCAAGGAACGCGCTCATATCTATCATTGTGGCTTGGCTCTTATCGGTGCTAAGTGGG
CTACTGCCGTTAATGGGCTGGCATAAAACCCCTCTAAGCCCTTACTGCCTGTTCACCCAAGTGATA
GACATGACTTACATGTTCTACTTCTTTACATTCACTTTTTTCATCTTGCCCCTCACATCAATGCTG
CTGATTTATGCACGCATCTTTTCAGCAGTGAGGAAGCATATAAACCATGGCGAAACTAGAGCGACT
CCAGAGAACATGGAGAGCAGGCAAGAGACGGGCTTAAAGAGGGAAATCAAAACAGCCACTTCACTC
TTCACCATCATCTTCCTCTTTGTCCTGTGTTGGGCTCCCCTACATGTTGTAAATGGTCTGATCCTG
CTCTGCTCGCGCTGCTCTATCCCTCCTCTCCTTGTAGACACCGGGATAATATTATCTCATGCCAAT
TCAGCTCTCAATCCAATTGTATATGCTTATAAGCTAAAATCCTTTAGGGACAAGTTTAAGGTGTTT
ATCCGCTGCAGAGACAACACTATAACACCAGAAGCCCATTAA

Figure 3.7: *Xenopus* A2c amino acid and nucleotide sequence: A) Three predicted amino acid sequence of A2c *Xenopus laevis* based on a search with zebrafish A2c amino acid sequence B) Corresponding nucleotide sequence of *Anguilla anguilla* A2c.1 with primer sequences in orange

Larval *Xenopus laevis* olfactory organs, from tadpoles in the stage of 50-54 were provided and prepared by the lab of Prof. Dr. Ivan Manzini (University of Giessen). These prefixed OEs were cut into 10 µm thick slices and dried for 30 min. After this, the standard *in situ* protocol (2.6.3.1) was used to visualize the A2c.1 probe with NBT/BCIP. A2c was detected in sparsely distributed olfactory neurons. A maximum of three to five A2c-positive cells was observed with no special spatial distribution in main olfactory epithelia (Fig. 3.8). Some sections also contained the accessory olfactory organ (VNO), no labeled cells were detected there.

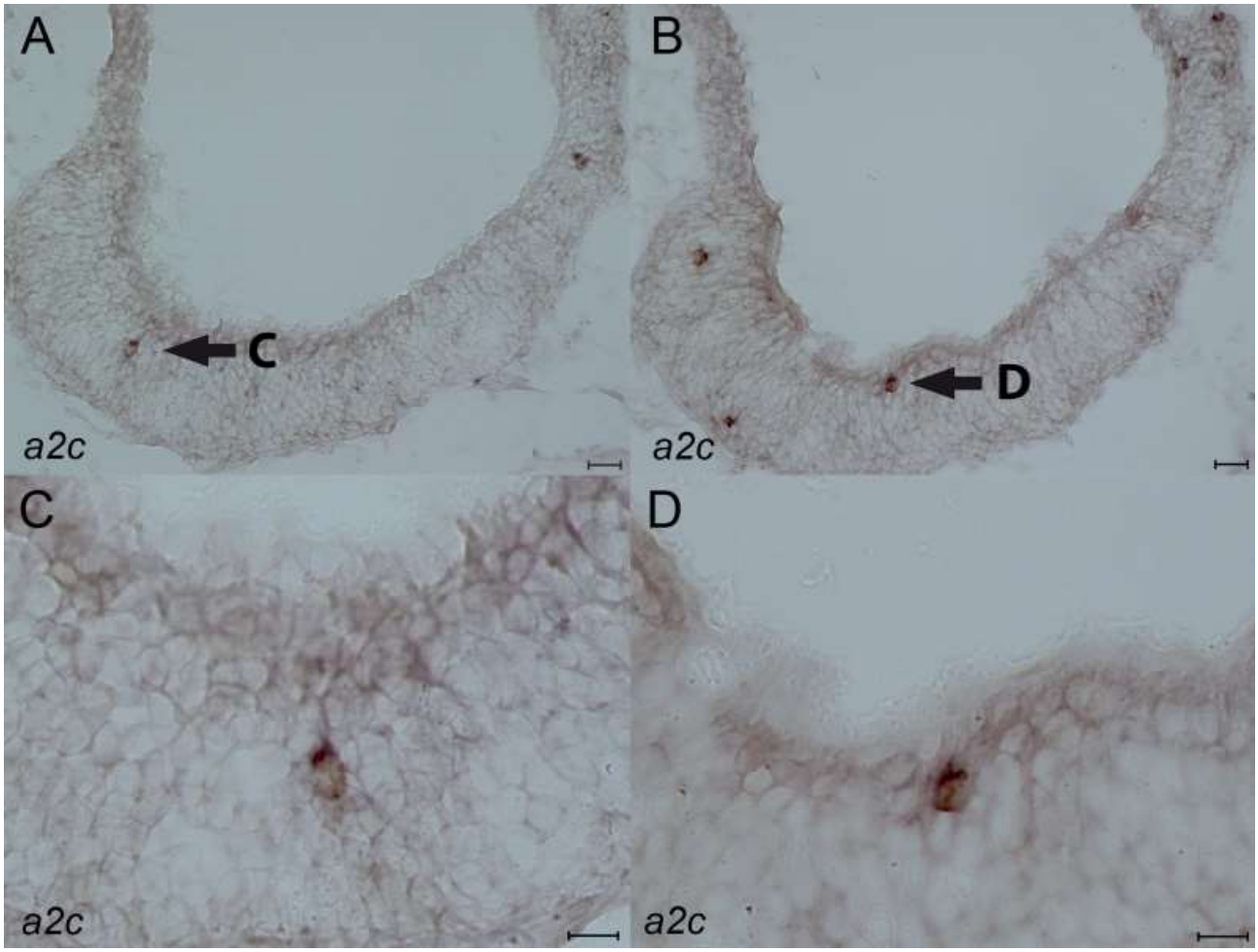

Figure 3.8: A2c *in situ* hybridization signals (NBT/BCIP staining) in the olfactory epithelia of *Xenopus laevis.* A)-E) NBT BCIP *in situ* hybridisation of *a2c* in the sensory area of the olfactory epithelia in *Xenopus laevis* . A) A2c positive cell mark wit arrow in higher magnification in C). B)A2c positive cell mark wit arrow in higher magnification in D). Scale bar A) + B) 20µm C) + D) 10µm

Because of this low number of cells, a more sensitive staining method, a TSA *in situ* hybridisation, was performed. Following the standard protocol (2.6.3.3) for this fluorescent method, the same results, in terms of number, for A2c-positive cells were observed. Also no distinct spatial distribution was identified (Fig. 3.9).

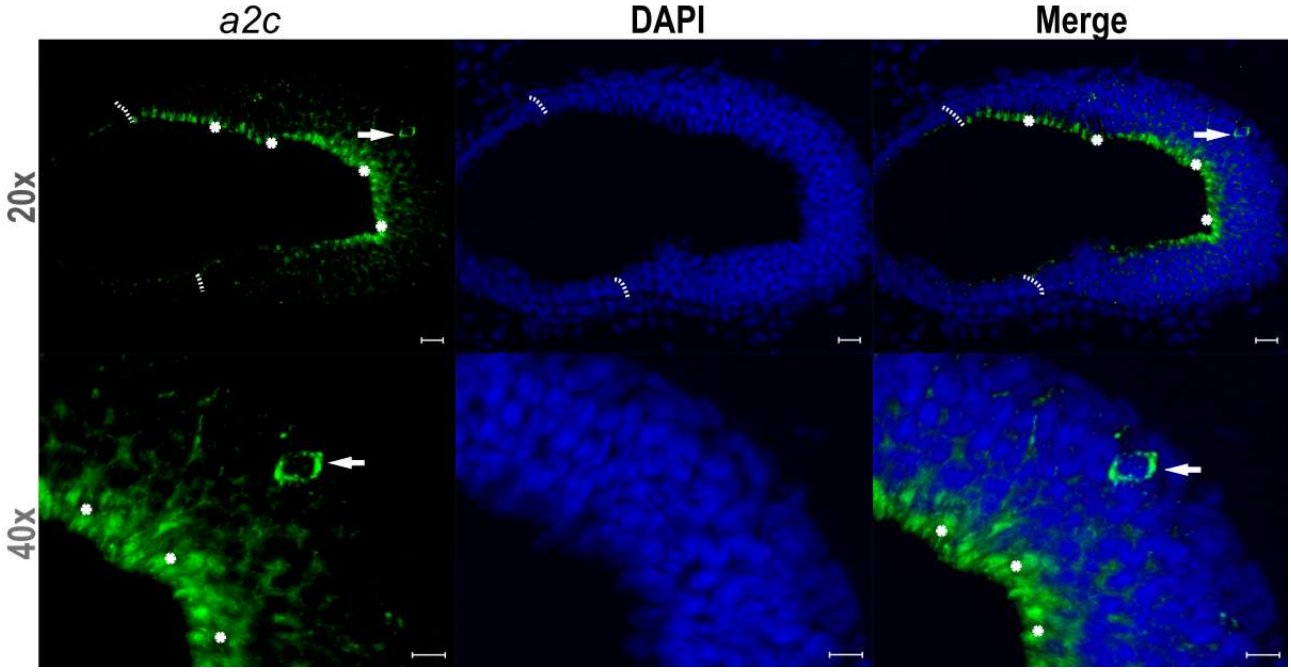

Figure 3.9: A2c *in situ* hybridization signals (TSA staining) in the olfactory epithelia *of Xenopus laevis*. *A2c* was visualized with TSA Alexa Fluor 488. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Dashed line shows extent of sensory epithelium; asterisks, variable unspecific staining at the surface to the lumen; arrow, A2c-expressing cell. Scale bar for 20x) 50µm 120x) 10µm.

3.1.2 A2c expression in the olfactory epithelia of *Lampetra fluviatilis*

Lampreys are living fossils, which have not much changed for 500 million years and that's why they represent one of the earliest diverging vertebrates. A recent study showed the existence of the A2c receptor in the genome of the sea lamprey *Petromyzon marinus* (Wakisaka et al., 2017). To study the spatial distribution of the A2c receptor in lamprey OE, several adult male and female river lampreys *Lampetra fluviatilis* were provided by the lab of Prof. Dr. Ansgar Büschges (University of Cologne). Because no complete genome of *Lampetra fluviatilis* was available the primers were based on conserved parts of the A2c amino acid sequence in the available genomes of *Petromyzon marinus* and *Lethenteron camtschaticum.* With the help of the blasted zebrafish A2c amino acid sequence in the genome of *Petromyzon marinus* and *Lethenteron camtschaticum* a possible a2c candidate

gene was discovered by the lab (Fig3.10). The a*2c* gene is highly conserved between *Petromyzon marinus* and *Lethenteron camtschaticum.* Within the conserved parts of the A2c nucleotide sequence, a primer set for the A2c receptor was chosen, based on the sequence of *Lethenteron camtschaticum*, which is the closer relative to *Lampetra fluviatilis* (Fig 3.10B).

A

A2c amino acid sequence of *Lethenteron camtschaticum*

ERALSRQLKTVKRARLLGCEEPPSEDPPGEVQLVPRTLWEVKMSANNTTLPIEAESPSQVSLNVPY
ILLELVIALLSSVGNLLVCVAVMKNRRLRTVTNYFLVSLAVADVCVGAVAIPCAIMTDLGLPRHNF
LLCVLMLSVLIMLTQSSIFSLLAVAVDRYVAIFSPFRYKVIMTHRNALITIVLTWLVAFLIGLVPA
MGWHKGAPSDSYCYFVAVVDMNYMVYFNFFGCVLTPLLIMFVIYARIFLQVRYQLRRIANEARGSA
GNAQERSMQSLTKEVKTATSLFLVLFLFTVCWIPLHVLNCISLLCPGSNTPYGLLLAAIILSHANS
VVNPFLYAYRMKSYRRAFLSLIMCRGARETDDDTQVPSTIVGIETSRTSLPNMVLQGNPG

A2c amino acid sequence of *Petromyzon marinus*

LPIEAASPSQVSVNVPYILLELVIALLSSVGNLLVCVAVMKNRHLRTVTNYFLVSLAVADVCVGAV
AIPCAIMTDLGLPRHNFLLCVLMLSVLIMLTQSSIFSLLAVAVDRYVAIFNPFRYKVIMTHRNALI
TIVLTWLMAFLIGLVPAMGWHKGSPSDSYCYFVAVVDMNYMVYFNFFGCVLTPLIIMFVIYARIFL
QVRHQLRRIANEARGGAGNAQERSMQSLTKEVKTATSLFLVLFLFVCWIPLHVLNCISLLCPGSNT
PYGLLLAAIILSHANSVVNPFLYAYRMKSYRRAFLSLIMCRDARETDDDTQVPST

B

A2c nucleotide sequence of *Lethenteron camtschaticum*

GAACGAGCTCTCAGCCGTCAGCTCAAAACCGTAAAACGAGCAAGGCTACTCGGCTGTGAGGAACCG
CCGTCAGAAGACCCTCCGGGTGAGGTGCAGCTTGTGCCAAGAACCCTCTGGGAAGTAAAAATGAGC
GCCAATAACACTACGCTGCCGATCGAGGCGGAAAGCCCGAGCCAGGTCTCTCTCAATGTGCCGTAT
ATCCTGTTGGAGCTCGTCATCGCGCTGCTGTCGTCGGTCGGCAACTTGCTGGTGTGCGTCGCCGTA
ATGAAGAACCGCCGCCTACGCACGGTCACCAACTACTTCTTGGTGTCACTGGCGGTGGCCGACGTG
TGCGTGGGTGCTGTTGCCATCCCCTGCGCCATCATGACGGACCTGGGCCTCCCGCGCCACAACTTC
CTGCTGTGCGTGCTCATGCTGTCTGTGCTCATCATGCTCACGCAGAGCTCCATCTTCAGCCTGCTG
GCTGTTGCCGTCGATAGATACGTGGCCATTTTCAGCCCGTTTCGATACAAGGTGAGCGTGCAGCGA
TCGGCATTGGTGATAATGACGCACCGCAACGCTCTAATCACCATCGTGCTCACCTGGCTCGTGGCC
TTCCTCATCGGCCTGGTGCCGGCCATGGGCTGGCACAAGGGTGCGCCCAGCGACAGCTACTGCTAC
TTCGTGGCCGTCGTTGATATGAACTACATGGTGTACTTCAACTTCTTTGGCTGCGTGCTCACGCCT
CTGCTCATCATGTTCGTCATCTACGCGCGCATCTTCCTGCAAGTGCGGTACCAGCTGCGTCGCATC
GCCAACGAGGCCAGGGGCAGCGCCGGCAATGCCCAGCATGCCCCTAACAGTCGATTTCATTGCCAG
GAACGCAGCATGCAGTCGCTGACGAAGGAGGTCAAAACAGCGACCTCGCTCTTCCTGGTGCTCTTC
CTCTTCACAGTCTGCTGGATTCCCCTCCACGTCCTCAACTGCATCAGCCTATTATGCCCAGGCAGC
AACACGCCGTACGGCCTGCTTCTCGCCGCCATCATTCTCTCGCATGCCAACTCAGTGGTCAATCCC
TTCCTGTACGCCTATCGCATGAAGTCGTACCGTCGTGCTTTCCTGTCGCTCATTATGTGCCGGGGC
GCGAGGGAAACCGACGATGACACGCAGGTTCCCAGCACCATCGTGGGCATAGAGACTTCACGCACC

AGCCTGCCCAACATGGTGCTGCAGGGCAACCCAGGG

A2c nucleotide sequence of *Petromyzon marinus*

CTGCCGATTGAGGCGGCAAGCCCGAGCCAGGTCTCTGTCAATGTGCCGTATATCCTGTTGGAGCTC
GTCATCGCACTGCTGTCGTCGGTCGGCAACCTGCTGGTGTGCGTCGCCGTAATGAAGAACCGCCAC
CTACGCACGGTCACCAACTACTTCTTGGTGTCACTGGCGGTGGCGGACGTGTGCGTGGGTGCTGTC
GCCATCCCCTGCGCCATCATGACGGACCTGGGCCTCCCGCGCCACAACTTCCTGCTGTGCGTGCTC
ATGCTGTCTGTGCTCATCATGCTCACGCAGAGCTCCATCTTCAGCCTGCTGGCTGTTGCCGTCGAT
AGATACGTGGCTATTTTCAACCCGTTTCGATACAAGGTGATAATGACGCACCGCAACGCTCTAATC
ACCATCGTGCTCACCTGGCTCATGGCCTTCCTCATCGGCCTGGTGCCGGCCATGGGCTGGCACAAG
GGTTCGCCCAGCGACAGCTACTGCTACTTCGTGGCCGTCGTTGATATGAACTACATGGTGTACTTC
AACTTCTTTGGCTGCGTGCTCACGCCTCTGATCATCATGTTCGTCATCTACGCGCGCATCTTCCTG
CAAGTGCGGCACCAGCTGCGTCGCATCGCCAACGAGGCCAGGGGTGGCGCCGGCAATGCCCAGGAA
CGCAGCATGCAGTCGCTGACGAAGGAGGTCAAAACAGCGACCTCGCTCTTCCTGGTACTCTTCCTC
TTCACAGTCTGCTGGATTCCCCTCCACGTCCTCAACTGCATCAGCCTGTTATGCCCAGGCAGCAAC
ACGCCGTACGGCCTGCTTCTCGCCGCCATCATTCTGTCGCATGCCAACTCAGTGGTCAATCCCTTC
CTGTACGCCTATCGCATGAAGTCGTACCGTCGTGCTTTCCTGTCGCTCATTATGTGCCGGGACGCG
AGGGAAACCGACGATGACACACAGGTTCCCAGCACC

C

```
  1 ERALSRQLKTVKRARLLGCEEPPSEDPPGEVQLVPRTLWEVKMSANNTTL
                                                       |
  1 -------------------------------------------------L
 51 PIEAESPSQVSLNVPYILLELVIALLSSVGNLLVCVAVMKNRRLRTVTNY
    ||||.||||||:||||||||||||||||||||||||||.|||||||
  2 PIEAASPSQVSVNVPYILLELVIALLSSVGNLLVCVAVMKNRHLRTVTNY

101 FLVSLAVADVCVGAVAIPCAIMTDLGLPRHNFLLCVLMLSVLIMLTQSSI
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 52 FLVSLAVADVCVGAVAIPCAIMTDLGLPRHNFLLCVLMLSVLIMLTQSSI

151 FSLLAVAVDRYVAIFSPFRYKVIMTHRNALITIVLTWLVAFLIGLVPAMG
    ||||||||||||||:||||||||||||||||||:||||||||||||
102 FSLLAVAVDRYVAIFNPFRYKVIMTHRNALITIVLTWLMAFLIGLVPAMG

201 WHKGAPSDSYCYFVAVVDMNYMVYFNFFGCVLTPLLIMFVIYARIFLQVR
    ||||:||||||||||||||||||||||||||||:||||||||||||||
152 WHKGSPSDSYCYFVAVVDMNYMVYFNFFGCVLTPLIIMFVIYARIFLQVR

251 YQLRRIANEARGSAGNAQERSMQSLTKEVKTATSLFLVLFLFTVCWIPLH
    :||||||||||||.|||||||||||||||||||||||||||| |||||||
202 HQLRRIANEARGGAGNAQERSMQSLTKEVKTATSLFLVLFLF-VCWIPLH

301 VLNCISLLCPGSNTPYGLLLAAIILSHANSVVNPFLYAYRMKSYRRAFLS
    ||||||||||||||||||||||||||||||||||||||||||||||||||
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251 VLNCISLLCPGSNTPYGLLLAAIILSHANSVVNPFLYAYRMKSYRRAFLS

351 LIMCRGARETDDDTQVPSTIVGIETSRTSLPNMVLQGNPG    390
    |||||.|||||||||||||
301 LIMCRDARETDDDTQVPST--------------------    319
```

Figure 3.10: A2c amino acid and nucleotide sequence of *Lethenteron camtschaticum* and *Petromyzon marinus*: A) Predicted Amino acid sequences of A2c from two different lampreys: *Petromyzon marinus* and *Lethenteron camtschaticum based on a search with zebrafish A2c amino acid sequence* B) Corresponding nucleotide sequence of *Petromyzon marinus* and *Lethenteron camtschaticum*; red: highly conserved regions in both lampreys; green: A2c primers. C) Pairwise comparison between predicted amino acid sequences for A2c in P. marinus and L. camtschaticum shows conserved regions chosen for primer synthesis.Color code as shown in B.

The prefixed olfactory epithelias were cut into 10µm thick slices beginning rostrally. *In situ* hybridization was performed according to the standard protocol (2.6.3), followed by staining with NBT/BCIP. In every lamella a cluster of cells was stained, with a width of approximately 15 to 25 cells,, which appeared to lay outside and flanking the sensory area on both sides . In terms of laminar height, the complete lamellar layer was stained, basal to apical, equivalent to approximately four to six A2c-positive cells on top of each other (Fig.3.11). In some cases a smaller cluster of cells at the tip of the lamellae was also labelled. In this area, all cells from basal to apical were stained, too, but the extent of these clusters along the radial dimension was smaller. The staining was observed in the complete olfactory epithelia, from the base of the organ to the apical opening. Interestingly, no A2c expression was observed in the accessory olfactory organ.

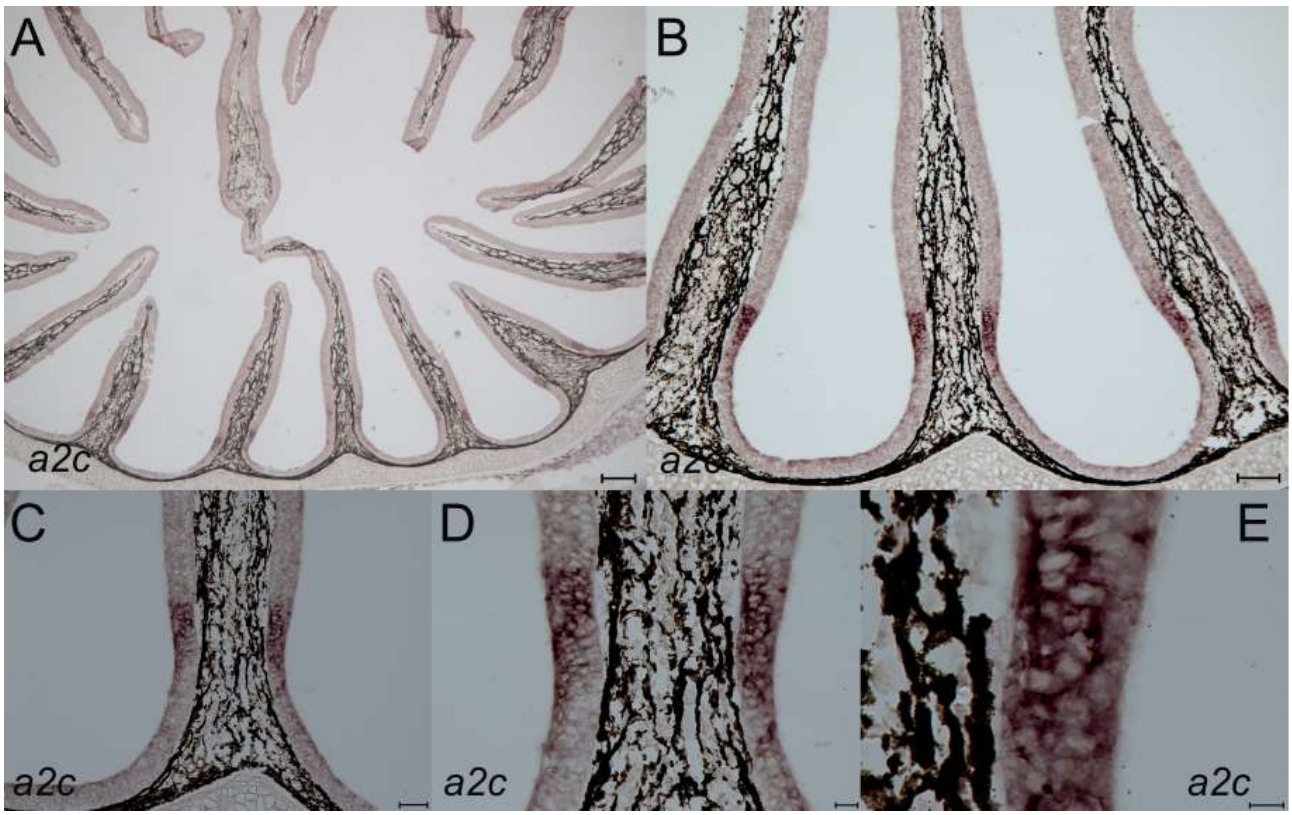

Figure 3.11: A2c *in situ* hybridization signals in the olfactory epithelia of *Lampetra fluviatilis.* A)-E) NBT/BCIP stainings of *a2c* in the lamellae close to the peripheral capsule adjacent to the sensory area. Scale bar A) 200µm B) 100µm C) 50 µm D) 20µm E) 10µm

The unexpected localization of A2c receptors in clusters adjacent to the sensory area and not in single cells within the sensory area as in the other vertebrate species tested suggests a potentially nonsensory function for A2c. Therefore, we wished to examine whether A2c might be present in other organs as well. A PCR was made for A2c in the olfactory organ, the heart and the eye. The PCR result with cDNA from each organ showed that A2c is only localized in the olfactory organ and not in the eye or heart (Fig 3.12) suggesting tissue-specific expression. As a control beta actin was used.

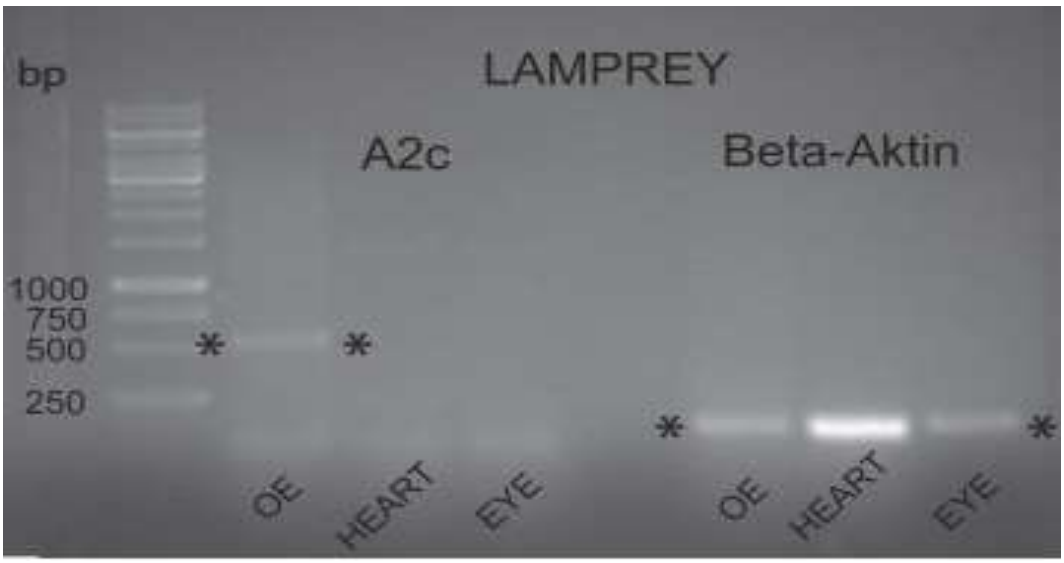

Figure 3.12: A2c is only localized in the olfactory epithelia of *Lampetra fluviatilis*: Localization of A2c and beta actin in different tissues (OE; heart and eye) of *Lampetra fluviatilis.* Expected product size (*asterisks*) is 530 b for A2c and 80 b for beta actin.

3.1.3 Analysis of the radial coordinates of A2c in carp, eel, zebrafish and lamprey yields no evidence for a common expression zone

To compare if A2c is localized in the same zones in the lamella in different fish species, the radial distributions of A2c was measured for zebrafish, eel and carp as described (2.8). Compared to all other fish the measured A2c-positive neurons in Eel (n=299) are localized extremely closer to the middle raphe, followed by zebrafish (n=324) and carp (=244) (Fig. 3.13). Because of the different morphology of the lamprey OE compared to the other tested fishes, the radial distance was measured from the middle of the of the curve between to lamellae, close to the peripheral capsule, to the inner tip of the lamellae. Because the lamprey does not show any individually stained receptor cells, but rather has A2c-positive cells in clusters, the measurements were made in the middle of these clusters. Because the lamellae of the lamprey had to of these clusters, the curve in Fig. 3.13 showed not that typicall sigmoid curve as for the other tested fishes. The Kolmogorov–Smirnov test showed highly significant differences for the radial distributions of all fishes (Tab.6.1).

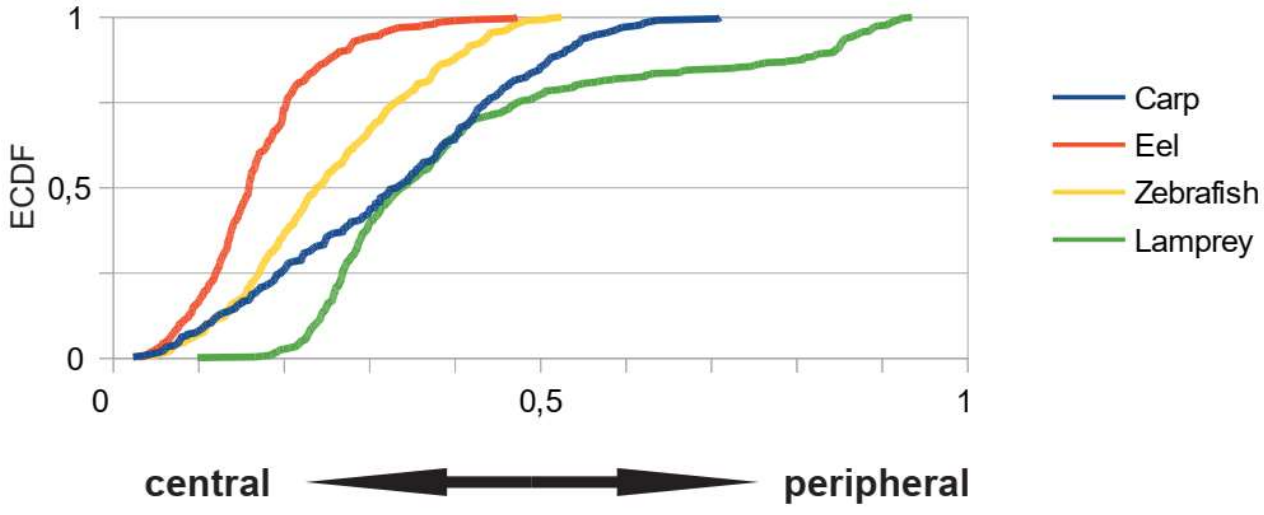

Figure 3.13: Quantitative assessment of radial distributions distributions of A2c in different fish species. Radial distribiutions of A2c-expressing OSNs were quantified for carp (n=240), eel (n=299), zebrafish (n=324) and lamprey(n=444). The radial position order from inner to out: eel, zebrafish, carp and lamprey. Radial position within the section was normalized to maximal radius, i.e. length of the lamella containing the respective labelled cell. (from 0, most central to 1, most peripheral).

3.1.4 Co- labelling of different neuronal markers with A2c in zebrafish, carp and frog

For a closer characterisation of A2c-positive cells, different marker genes can be used. In *Danio rerio* it was recently shown that the A2c-expressing cells are positive for G_{olf} and ACIII, marker for ciliated OSNs but negative for OMP, a marker for ciliated OSNs and TRPC2, a marker for microvillous OSNs (Wakisaka et al., 2017). In this study, additionally HuC, a marker for immature and mature neuronal cells and PCNA (proliferating nuclear cell antigen) a marker for dividing cells, were used for a closer characterisation of the A2c-positive neurons, because of the surprising results in lamprey.

To get robust results in zebrafish, a double labelling with a combination of A2c-labelled probes for the fluorescents *in situ* hybridization and an established PCNA-monoclonal antibody for IHC was used. PCNA-positive cells were extremely basal with respect to laminar height and very close to the middle raphe, with spots of 1-3 cells. No double staining with A2c-positive OSNs was observed, also PCNA and A2c-positive OSNs were at a distinct distance from each other (Fig.3.14). The A2c expression pattern using the TSA florescent labelling was undistinguishable from that using the NBT/BCIP labelling (compare Fig.3.2 and Fig.3.14).

To investigate if HuC, a pan-neuronal marker, is localized together with the A2c receptor in OSNs of carp, zebrafish and frog, a fluorescent double labelling was carried out. In both teleosts, carp and zebrafish, HuC is localized ubiquitously in the sensory area of the lamellae and is co-expressed with A2c (Fig 3.15). In the olfactory epithelia of frog, fluorescent labelled A2c-positive OSNs showing the same localization as those stained with stained with NBT/BCIP (Fig.3.8 and Fig.3.15). For HuC only a surface staining at the apical side of the OE was observed, similar to a commonly observed background staining and no co-expression with A2c was observed (Fig. 3.15). It is conceivable that the monoclonal HuC- antibody used does not recognize frog HuC.

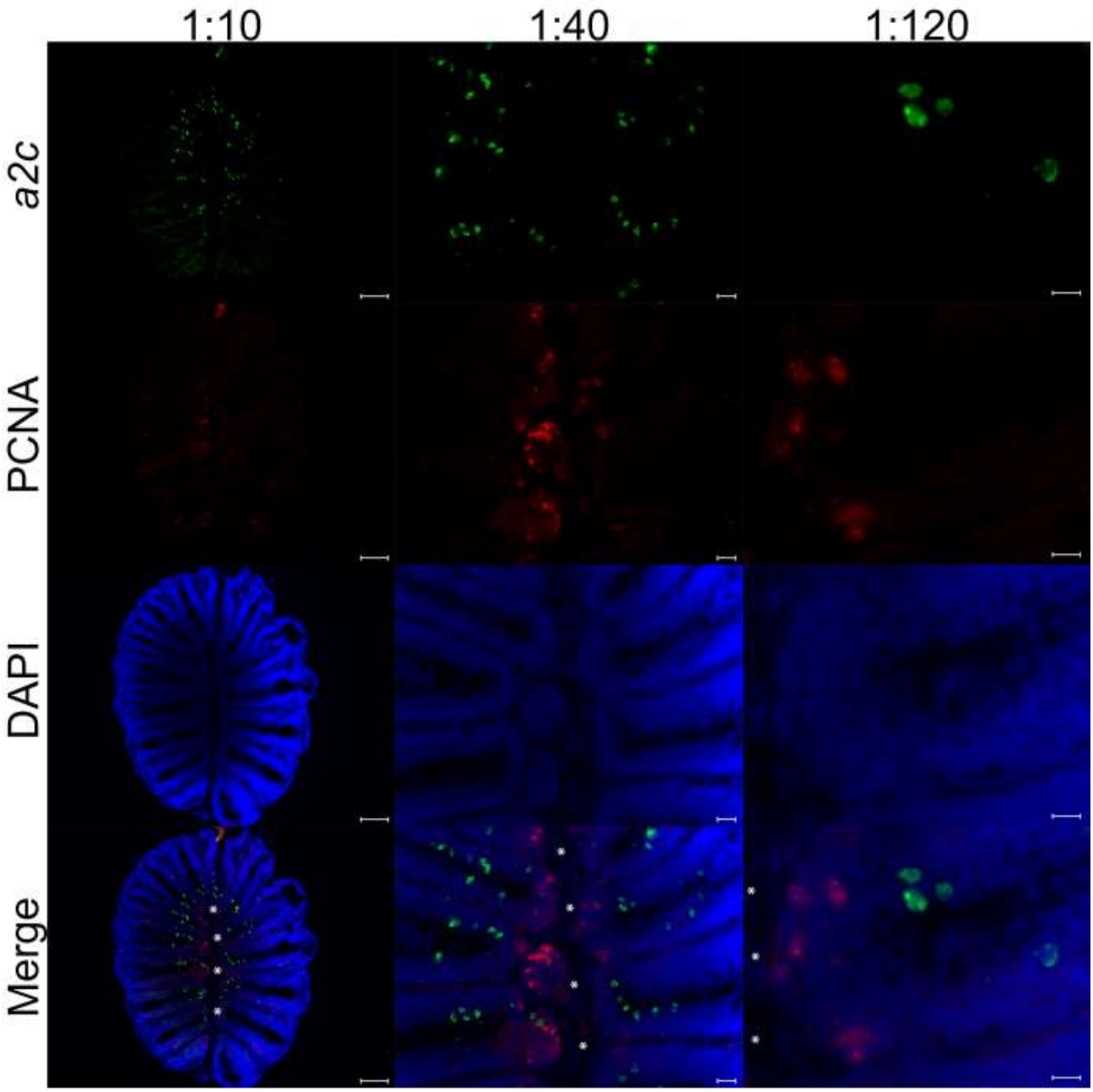

Figure 3.14: A2c *in situ* hybridization signal and the PCNA-antibody staining localize to clearly segregated areas in the olfactory epithelia of *Danio rerio.* Fluorescent double staining of *a2c* (ISH) and PCNA (IHC) in the olfactory epithelia of *Danio rerio*. *A2c* was visualized with TSA Alexa Fluor 488 and PCNA with an anti-mouse Alexa-Fluor 594 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Merge) middle raphe highlighted with white asterix. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µ

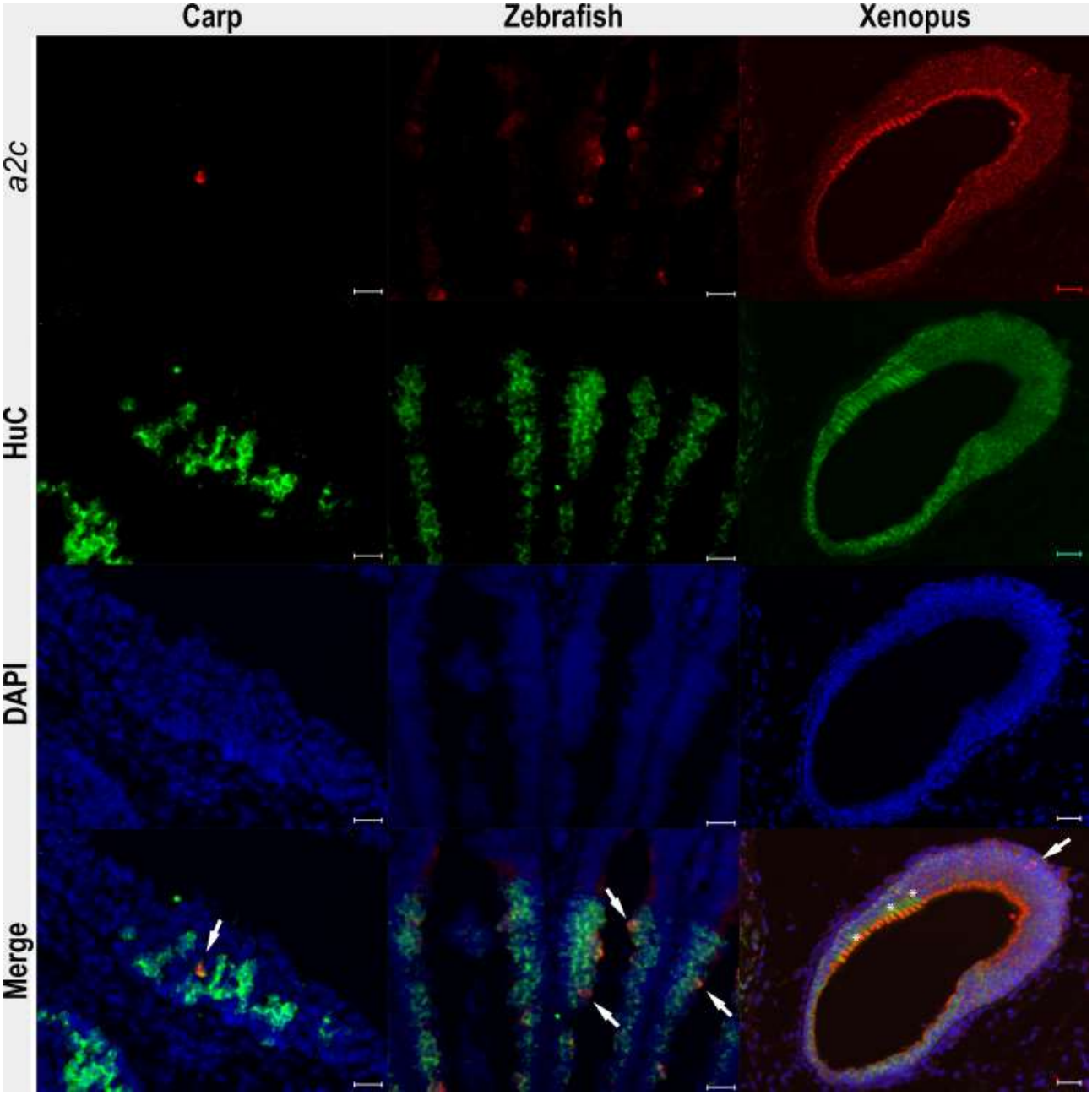

Figure 3.15: A2c *in situ* hybridization and HuC-antibody signal co-expression in the olfactory epithelia of carp, zebrafish and frog. Fluorescent double staining of *a2c* (ISH) and HuC (IHC) in the olfactory epithelia of *Cyprinus carpio*, *Danio rerio* and *Xenopus leavis*. Colocalization of A2c and HuC in zebrafish and Carp and no expression of Huc in frog. of Merge) arrows A2c-positive cells; Asterix variable unspecific staining *A2c* was visualized with TSA Alexa Fluor 488 and HuC with an anti-mouse Alexa-Fluor 594 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for Carp and Zebrafish 20µm; Frog 10µm

3.1.5 Characterisation of A2c-positive cells in *Lampetra fluviatilis* with the help of different cell markers

3.1.5.1 The A2c-positive cells are not co-expressed with the olfactory cell marker G_{olf} in lamprey – *Lampetra fluviatilis*

To further characterize the localization of A2c in the olfactory organ of lamprey, $G_{olf,}$ an established olfactory sensory neuronal marker protein was used. G_{olf} is a cAMP-dependent olfactory GTP-binding protein linked to olfactory receptors, and its immunoreactive expression pattern, using an established antibody, was shown to label the sensory area in *Petromyzon marinus* (Frontinni 2003).

A

G_{olf} amino acid sequence *Lethenteron camtschaticum*

QEFFDHVKALWQDEGVRMCYERSNEYQLIDCAQYFLDRIDTVRQNDYTPTDQDLLRCRVLTSGIFE
TKFQVDKVNFHMFDVGGQRDERRKWIQCFNDVTAIIFVVACSSYNMVLREDNTTNRLKEAINLFKS
IWNNRWLRTISIILFLNKQDLLAEKVLAGKSRIEKYFPEFARYTTPAEVSPELGEDPQVTRAKYFI
RDEFLRISTASDDGRHYCYPHFTCAVDTENIGRVFKDCRDIIQRMHLRQYELL

B

G_{olf} nucleotide sequence *Lethenteron camtschaticum*

CAGGAATTTTTCGACCACGTGAAGGCCCTCTGGCAGGACGAGGGCGTGCGGATGTGTTACGAGCGA
TCCAACGAGTACCAGCTCATCGACTGCGCCCAGTATTTCCTTGACAGAATTGACACGGTGCGGCAG
AACGACTACACGCCCACCGACCAGGACCTGCTGCGATGCCGAGTTCTGACTTCGGGGATCTTTGAA
ACCAAGTTTCAGGTGGACAAGGTCAACTTCCACATGTTTGATGTGGGAGGTCAACGGGACGAGAGG
AGAAAGTGGATCCAGTGTTTCAACGACGTTACAGCGATCATATTCGTAGTCGCGTGCAGCAGCTAC
AACATGGTTCTGCGCGAAGACAACACCACGAACCGCCTCAAGGAAGCCATCAACCTGTTCAAGAGT
ATTTGGAACAACAGGTGGCTCCGAACCATCTCGATCATCCTCTTCCTCAACAAGCAGGACCTCCTG
GCGGAGAAGGTGCTGGCGGGAAAGTCGCGAATCGAGAAGTACTTTCCAGAGTTTGCGCGGTACACC
ACGCCAGCCGAAGTGAGCCCTGAATTGGGCGAAGATCCGCAAGTGACGCGAGCGAAGTATTTCATC
CGGGACGAGTTCCTGCGCATCAGCACGGCAAGCGATGACGGCCGCCACTACTGCTACCCGCACTTC
ACGTGCGCCGTCGACACGGAGAACATCGGGCGCGTCTTCAAGGACTGCCGCGACATCATCCAGCGC
ATGCACCTGCGCCAGTACGAGCTGCTC

Figure 3.16: G_{olf} amino acid and nucleotide sequence of *Lethenteron camtschaticum.* A) Amino acid sequences of G_{olf} from *Lethenteron camtschaticum* based on search with *Danio rerio* G_{olf} amino acid sequence blasted at www.ncbi.nlm.nih.gov B) Nucleotide sequence of G_{olf} from *Lethenteron camtschaticum* ; Primers: G_{olf} A (green); G_{olf} B (blue)

Using the amino acid sequence of G_{olf} of *Danio rerio*, the homologous amino sequence in the genome of *Lethenteron camtschaticum* was identified (Fig 3.16A). Both chosen primer sets (Fig. 3.16B) were positive tested together with the cDNA of *Lampetra fluviatilis* in a PCR reaction. Based on this PCR product a digoxigenin- and fluorescein-labelled probe was made and tested in a single fluorescent and non fluorescent *in situ* hybridization. Both G_{olf} stainings (Fig. 3.17 and Fig 3.18) showed the same distinct sharp and clear expected expression pattern. The G_{olf}-positive cells are localized in the complete sensory area of both sides of the lamellae. The tips of the lamellae and the non-sensory area showing no expression of G_{olf} (Fig. 3.17 and Fig. 3.18). The fluorescent TSA staining, together with the nuclear marker 4',6-diamidino-2-phenylindole (DAPI) reveals also that, in terms of height inside the lamella, only the upper cells, which are arranged like a single cell palisade, are positively stained with G_{olf}. The basal row of cells in the lamellae are G_{olf} negative (Fig 3.18). The next step was to show the localization of A2c and G_{olf} together in the OE of *Lampetra fluviatilis. For this a well-established double in situ hybridization labelling protocol (2.6.3) was used.*

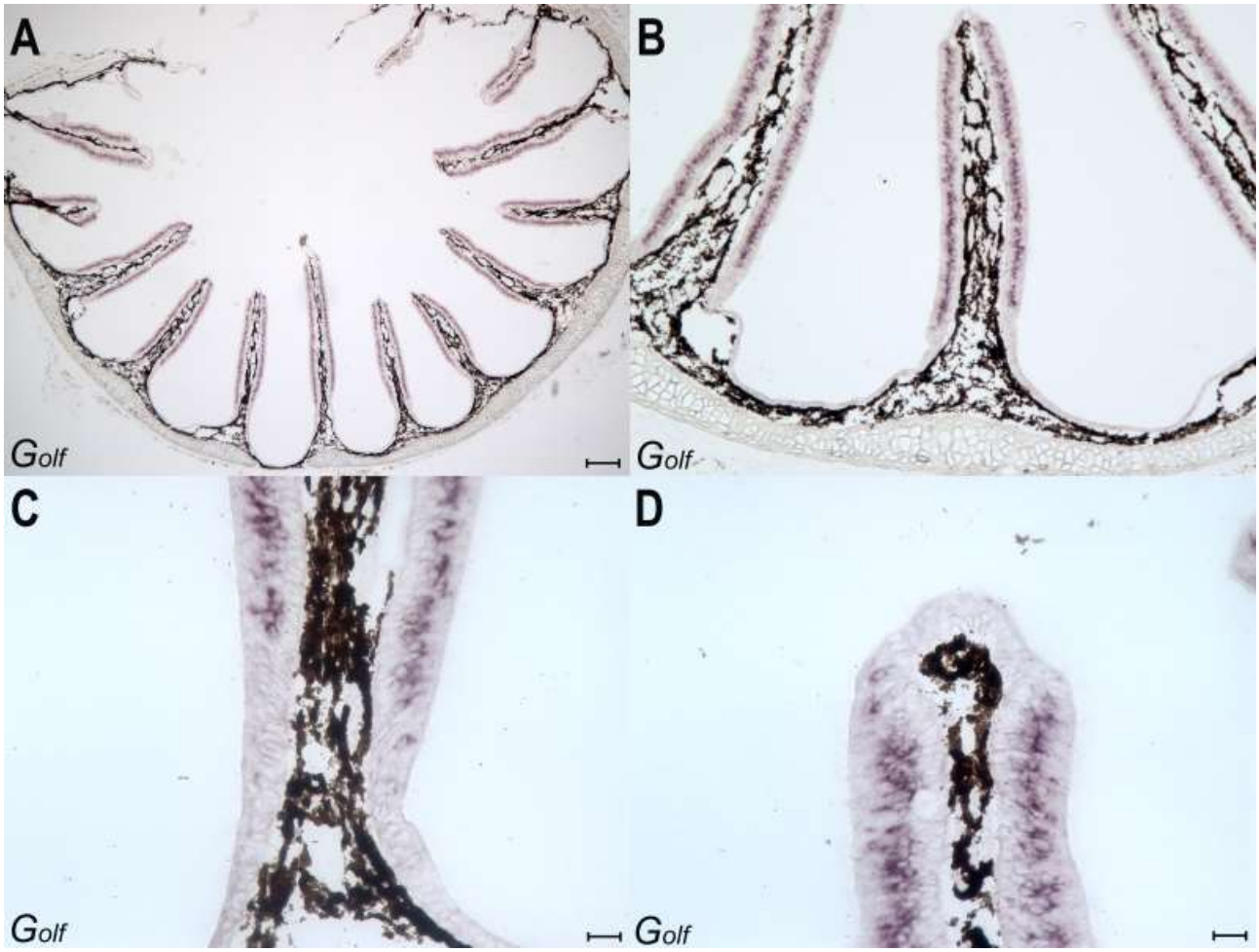

Figure 3.17: G_{olf} *in situ* hybridization (NBT/BCIP staining) signal in the olfactory epithelia of *Lampetra fluviatilis*. A)-E) G_{olf} is expressed in the complete olfactory sensory area of the lamellae. NBT/BCIP staining of a digoxigenin labelled G_{olf} in the middle part of the lamellae excluding the tip and the basal part. Scale bar A) 200µm B) 100µm C) 50µm D) 10µm

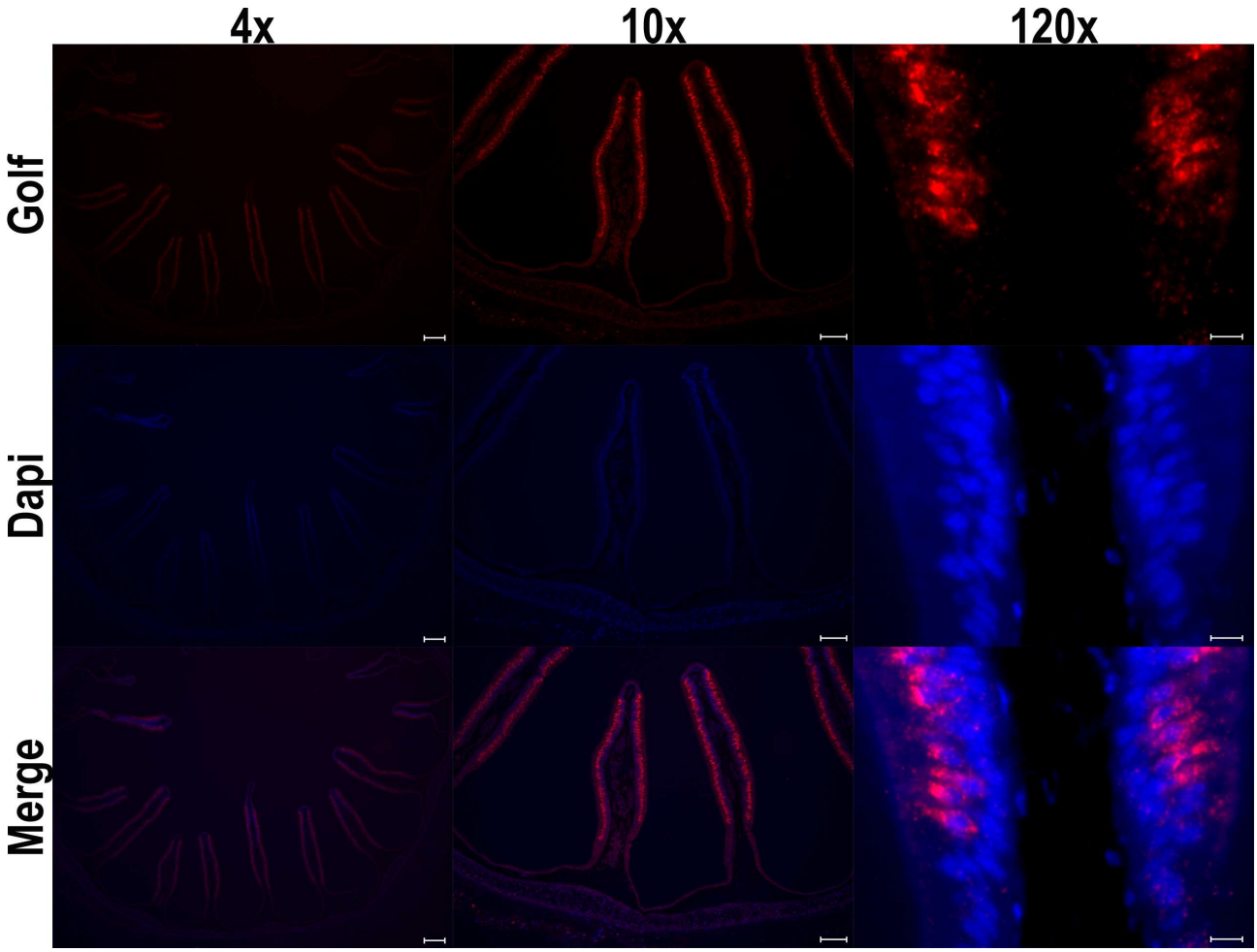

Figure 3.18: G_{olf} *in situ* hybridization (TSA staining) signal in the olfactory epithelia *of Lampetra fluviatilis.* G_{olf} is expressed in the complete olfactory sensory area of the lamellae. G_{olf} was visualized with TSA Alexa Fluor 488. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 4x) 200µm 10x) 100µm 120x) 10µm

During the double labelling the A2c fluorescein-labelled probe was visualized with HNPP Fast red and the G_{olf} digoxigenin-labelled probe was visualized with TSA Alexa-Fluor 488. Both genes showed the same expression pattern as during their single labelling (Fig 3.11 and 3.17). Again G_{olf} is localized in the sensory area of the OE, but A2c is clearly not co-localized with G_{olf} and its localization starts where the G_{olf} localization ends, both expression patterns forming a sharp border between each other. The G_{olf}-positive cells look like OSN cells in terms of their stained shape, whereas in the stained area of A2c-positive cells, no real cell body can be distinguished. The A2c staining looks more like fuzzy strong fog, than clear shaped cells. In both stainings the upper cells in the lamella exhibit signals of both genes, whereas the lower basal cells are not stained with the G_{olf} probe (Fig 3.19),

but fully stained by the A2c probe (Fig 3.11; Fig. 3.21 and Fig. 3.22). The same expression pattern of the rostral caudal sliced OE, was also observed in the dorsal ventral sliced OE. Also a sharp border between both expression zones of A2c and G_{olf} was observed (Fig 3.20).

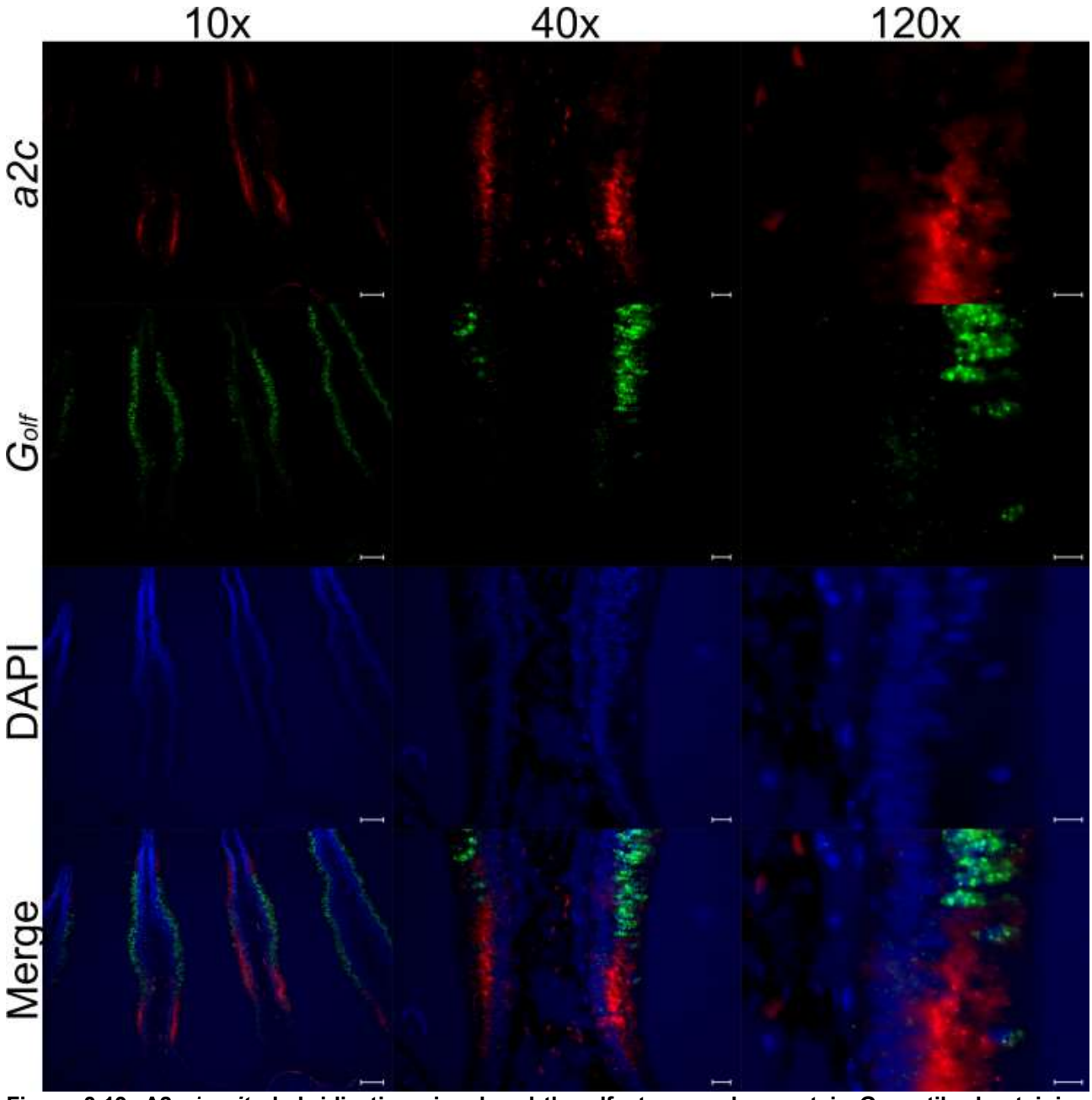

Figure 3.19: A2c *in situ* hybridization signal and the olfactory marker protein G_{olf}-antibody staining localize to clearly segregated areas the olfactory epithelia of *Lampetra fluviatilis*. A2c (red) and G_{olf} (green) are expressed in different zones in the lamellae. Fluorescent double *in situ* staining of *a2c* and G_{olf} in the olfactory epithelia of *Lampetra fluviatilis*. *A2c* was visualized with HNPP Fast Red and G_{olf} with TSA Alexa Fluor 488. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

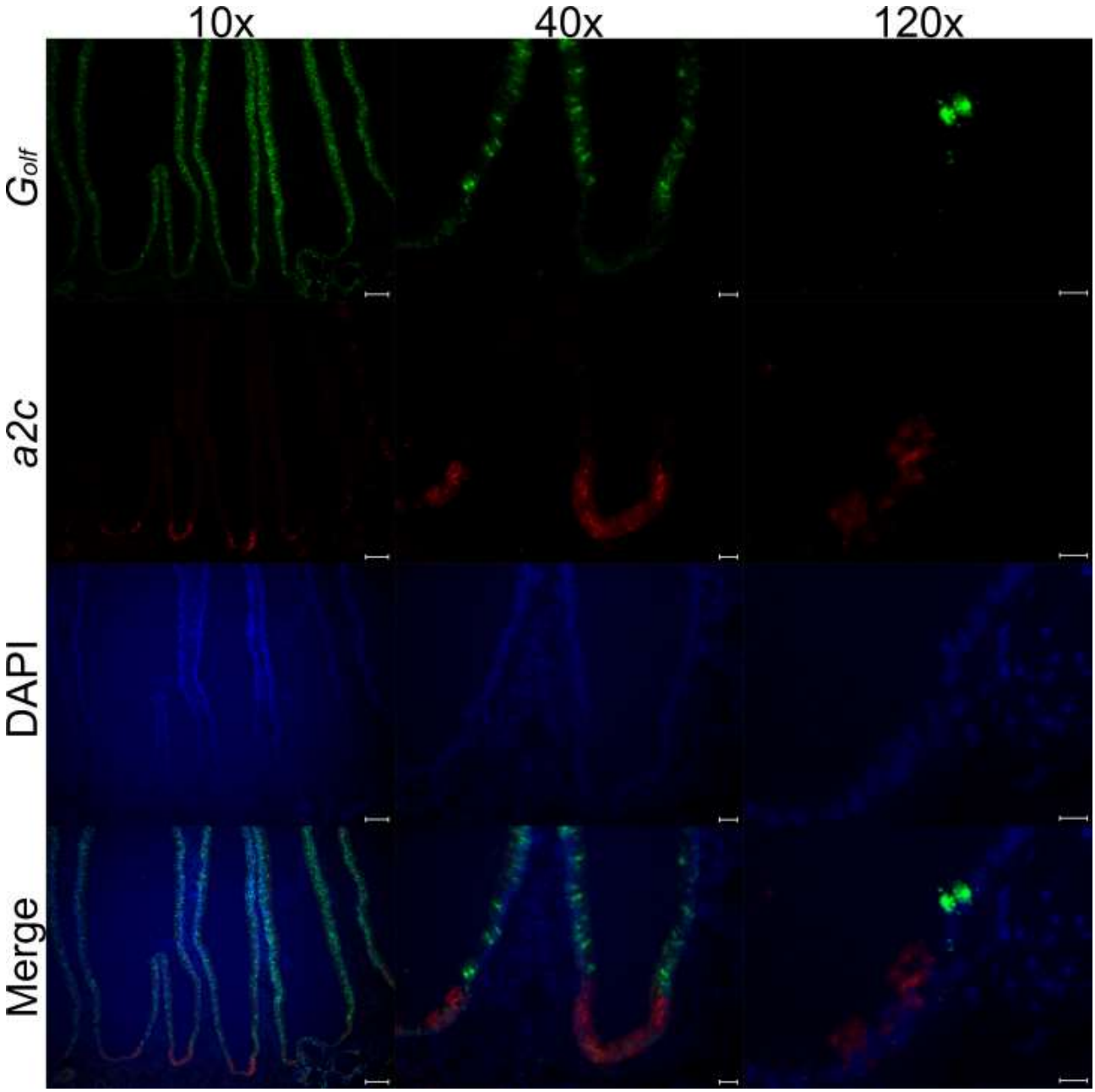

Figure 3.20: A2c *in situ* hybridization signal and the olfactory marker protein G_{olf}-antibody staining localize to clearly segregated areas in the dorsal *ventral cutted olfactory epithelia of Lampetra fluviatilis*. A2c (red) and G_{olf} (green) are expressed in different zones in the lamellae. Fluorescent double *in situ* staining of *a2c* and G_{olf} in the olfactory epithelia of *Lampetra fluviatilis*. *A2c* was visualized with HNPP Fast Red and G_{olf} with TSA Alexa Fluor 488. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

3.1.5.2 A2c-positive cells are not directly co-expressed with acetylated tubulin in the lamprey – *Lampetra fluviatilis*

Acetylated tubulin is used as a common marker of cilia in olfactory organs of vertebrates. Because of the expression pattern of acetylated tubulin antibody in the larval OE of *Petromyzon marinus* (Frontini et al, 2003), in which only the luminal surface of the olfactory sensory area was stained, a double labelling of A2c and acetylated tubulin was done. Therefore a combination of ISH and IHC was carried out, following a standardised protocol. At first the digoxigenin-labelled A2c probe was used in a single ISH visualized with an Alexa Fluor 633, followed by an established acetylated tubulin-antibody visualized with Alexa Fluor 488. The localization of A2c was again a cluster of cells at the border of sensory and non-sensory areas, where all cells interms of height from basal to apical were stained. The acetylated tubulin-antibody labels a fine upper layer in the complete OE, including both the sensory and the non-sensory area, but excluding a small area at the tip of the lamellae. A direct double labelling of A2c-positive cells and acetylated tubulin could not be observed, but in this A2c-positive part of the OE, the same strong acetylated tubulin staining above the A2c-positive cells was noticed (Fig. 3.21).

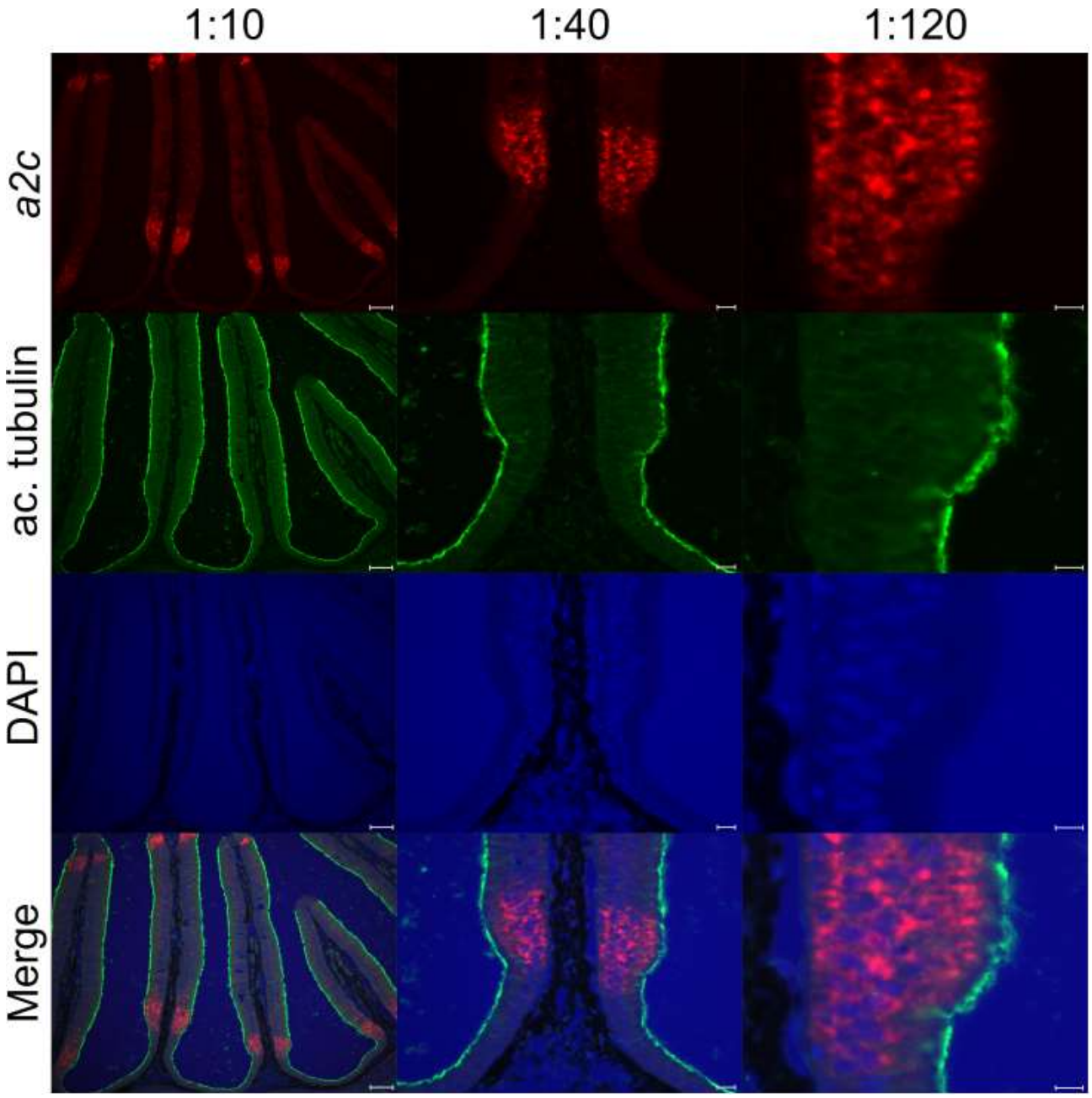

Figure 3.21: A2c *in situ* hybridization signal and acetylated tubulin-antibody staining localize to clearly segregated areas the olfactory *epithelia of Lampetra fluviatilis*. A2c probe (red) is expressed in the same zone as the tubulin-antibody, but co-labeling was observed. Fluorescent double staining of *a2c* (ISH) and acetylated tubulin (IHC) in the olfactory epithelia of *Lampetra fluviatilis*. *A2c* was visualized with TSA Alexa Fluor 633 and acytelated tubulin with an anti-mouse Alexa Fluor 488 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

3.1.5.3 A2c-positive cells are not co-expressed with beta-actin in lamprey - *Lampetra fluviatilis*

Beta actin is a member of the so-called “housekeeping genes”, because it is a major component of the cytoskeleton of each cell. The beta-actin antibody is mostly used as a neuronal marker for visualizing the microfilaments, which are partially made of beta-actin, in neurons in the brain region of vertebrates. To visualize neuronal or cell structures in the A2c-positive tissue of the lamprey OE, a combination of A2c probe (ISH) and beta-actin antibody (IHC) was accomplished. The A2c probe was visualized with the TSA method and a secondary Alexa Fluor-633 antibody and the beta actin-antibody with a secondary Alexa Fluor-488 antibody. The expression pattern of A2c was again in a cluster of cells at the border of sensory and non-sensory areas. The actin expression is not limited to any special area of the OE. The beta actin positive structures seem to be randomly distributed and vary in size and form. All beta actin structures, which are positively labelled in the A2c-expressing area, show no direct double labelling with A2c. Less of the the beta actin structures had a cell-shaped structure and look more like artefacts (Fig 3.22). But in the negative control, where the beta actin antibody was omitted in this experiment, none of these structures were observed.

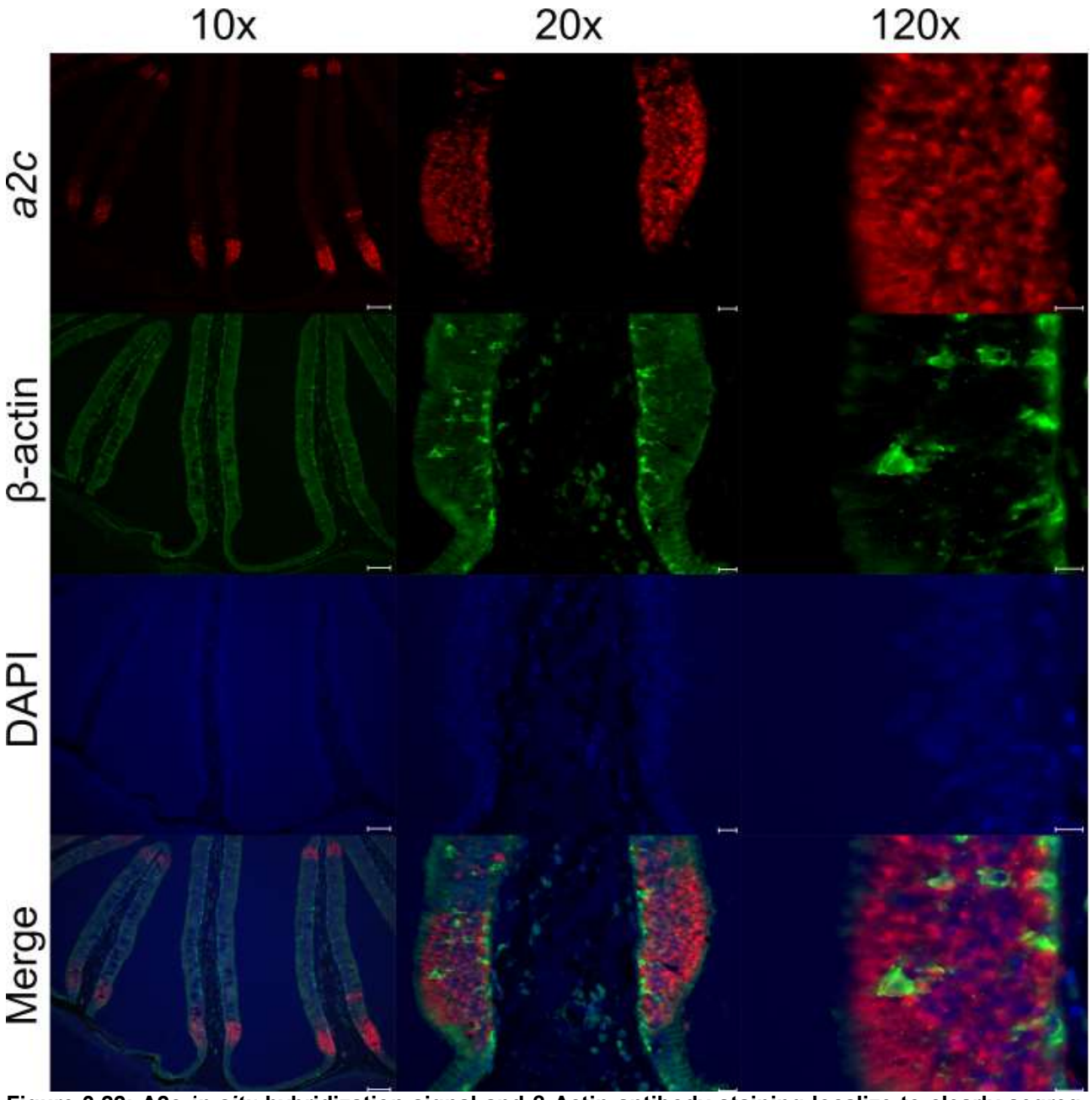

Figure 3.22: A2c *in situ* hybridization signal and β-Actin-antibody staining localize to clearly segregated areas the olfactory *epithelia of Lampetra fluviatilis.* A2c probe (red) is not coexpressed with β-actin -antibody (green) in the lamellae. Fluorescent double staining of *a2c* (ISH) and β-actin (IHC) in the olfactory epithelia of *Lampetra fluviatilis*. *A2c* was visualized with TSA Alexa Fluor 633 and β-actin with an anti-mouse Alexa Fluor 488 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

3.1.5.4 A2c-positive cells are not co-expressed with the olfactory cell marker TRPC2 in lamprey - *Lampetra fluviatilis*

The Transient Receptor Potential Channel 2 (TRPC2) is a marker protein for microvillar cells, which are known to contain V2R receptors, in the olfactory epithelia of vertebrates.

A

TRPC2 amino acid sequence *Lethenteron camtschaticum*

SKVVTLLLDRLDNDKALGHNKMDIRSFSLAIFDHSMDSSRYPPGVTPLTLASQDNLFDIVEVLLKK
GHAIQRPHNISCSCLECQNGRQYDTLKFSLSRINTYRGLAGRPYLCLASGDAILGAFELSRELTRL
ARQEPEFKAEYMELDRQCEEFAVELLAMCQNQREVDSLLNDAPPHGDEELAHGAFEEGIPSLGRLR
LAVSHQQKLFVSHPVCQQVLTSIWCSGMPSWRGSHAAVKAGTCLGIFLGMPLLAIIYWIAPKSRFG
NLLRTPLLKFLMHSASYLWFLFFLLVESIMMDSSSDIVSTRNQTLMDSSFHMVWVAGFLWYECKEV
WIEGLRVYLSDWWNCLDLAMISMYISSFTLRLLVRWSGELACGRDPLSAECAYFTTAERGQWQKDD
PQIIAEVVFAITSALSFTRLAFILPAHESLGTLQISIGKILGDIFRFMFLVMIIGTAFLCGINNLY
VHYTFNKLGRFNETFQYLFWTVFGMEDRRTTDIPNFEIAQFVGRSLYGVYAIIMAIVLLNMLIAMI
TSSFERIEDDADVEWKFARSRLYLSYFREGLTLPVPFNLIPSPKSVYYAI

B

TRPC2 nucleotide sequence *Lethenteron camtschaticum*

TCCAAGGTCGTGACCCTGCTGCTCGACCGCCTGGACAACGACAAGGCGCTGGGCCACAACAAGATG
GACATCCGCTCCTTCTCGCTCGCCATCTTCGACCACTCGATGGACAGCTCGCGCTACCCGCCCGGC
GTCACGCCGCTCACGCTCGCCTCGCAGGACAACCTCTTCGACATCGTCGAGGTGCTGCTCAAGAAG
GGGCACGCCATCCAGCGCCCGCACAACATCTCCTGCAGCTGCCTCGAGTGCCAGAACGGGCGGCAG
TACGACACGCTCAAGTTCTCGCTGTCGCGCATCAACACGTACCGCGGCCTCGCCGGGCGGCCGTAC
CTGTGCTCGCGAGCGGCGACGCCATCCTCGGCGCCTTCGAGTTGAGCCGGGAGCTCACGCGCCTCG
CCAGGCAGGAGCCCGAGTTCAAGGCGGAGTACATGGAGCTGGACCGGCAGTGCGAGGAGTTCGCCG
TGGAGCTGCTCGCCATGTGTCAGAACCAGCGCGAGGTGGACTCGCTGCTCAACGACGCGCCCCCGC
ACGGCGACGAGGAGTTGGCGCACGGCGCCTTCGAGGAGGGCATCCCCAGCCTGGGCCGCCTGCGCC
TCGCCGTGTCGCACCAACAGAAGCTGTTTGTGTCGCACCCGGTGTGCCAGCAGGTCCTCACCTCCA
TCTGGTGCTCGGGCATGCCCAGCTGGAGGGGCAGCCACGCCGCAGTCAAGGCGGGCACCTGCCTGG
GCATCTTCCTGGGCATGCCGCTGCTCGCCATCATCTACTGGATCGCGCCCAAGTCCAGGTTTGGGA
ACCTGCTGCGCACGCCGCTGCTCAAGTTTCTCATGCACTCGGCCTCCTACCTCTGGTTCCTCTTCT
TCCTGCTCGTCGAGTCCATCATGATGGACAGCTCCAGCGACATCGTGTCCACACGCAACCAGACGC
TCATGGACAGCTCCTTCCACATGGTCTGGGTGGCAGGCTTCCTGTGGTACGAGTGCAAGGAGGTGT
GGATCGAAGGCCTGCGCGTGTACCTGTCGGACTGGTGGAACTGTCTGGACCTGGCCATGATCAGCA
TGTACATCTCCTCCTTCACGCTGCGCCTGCTCGTGCGCTGGAGCGGGGAGCTGGCGTGCGGCCGGG
ACCCGCTCTCGGCCGAGTGCGCGTACTTCACCACGGCCGAGCGCGGGCAGTGGCAAAAAGACGACC
CCCAGATCATCGCCGAGGTGGTGTTCGCCATCACGAGCGCGCTCAGCTTCACGCGCCTCGCCTTCA
TCCTGCCCGCGCACGAGTCGCTCGGCACGCTGCAGATCTCCATCGGCAAGATCCTCGGGGATATCT
TCCGGTTCATGTTCCTGGTGATGATCATCGGCACGGCGTTCCTGTGCGGCATCAACAACCTCTACG
TGCACTACACGTTCAACAAGCTGGGCCGGTTCAACGAGACGTTCCAGTACCTCTTCTGGACGGTGT
TCGGCATGGAGGACCGGCGCACCACGGACATCCCCAACTTCGAGATCGCGCAGTTCGTGGGCCGCA
GCCTCTACGGCGTCTATGCCATCATCATGGCCATCGTGCTGCTCAACATGCTCATCGCCATGATCA
CCAGCTCCTTCGAGAGGATCGAGGACGATGCGGACGTGGAGTGGAAGTTTGCGCGCTCGCGCCTCT
ACCTCTCCTACTTCCGAGAGGGCCTCACCCTGCCCGTGCCCTTCAACCTCATCCCCAGCCCCAAGT
CTGTCTACTACGCTATC

Figure 3.23: TRPC2 amino acid and nucleotide sequence of *Lethenteron camtschaticum.* A) Predicted amino acid sequences of TRPC2 *Lethenteron camtschaticum* based on search with *Danio rerio* TRPC2 amino acid sequence B) Corresponding nucleotide sequence of *Lethenteron camtschaticum* TRPC2 with primers: Trpc2 WG1(brown); Trpc2 WG2 (green); Trpc2 A (yellow) ; Trpc2 B (pink), Trpc2 C (blue)

Further studies identified parts of TRPC2 in the genome of *Petromyzon marinus* (Grus and Zhang, 2009). Based on this information and with the TRPC2 amino acid sequence of *Danio rerio*, the homologous sequence in *Lethenteron camtschaticum* could be identified (Fig. 3.23 A), because the compete genome of *Lampetra fluviatilis* was not available at that moment. Two different primer sets TRPC2 WG1 and TRPC2 WG2 were made to get the nearly whole nucleotide sequence of TRPC2 in *Lampetra fluviatilis,* but neither set worked with the cDNA of *Lampetra fluviatilis.* Furthermore*,* three different primer sets for *in situ* hybridisation probes (A-C) were designed, where only TRPC2 B and C showed an staining in the OE (Fig. 3.23 B). At first a single *in situ* hybridisation, following the standard protocol (2.6.3.3) with an NBT/BCIP staining was performed. The staining pattern of both TRPC2 probes showed the same, a staining in the sensory area of the lamella. No staining was observed in the tips of the lamellae and in the non-sensory area. The TRPC2-positive cells are localized more apically in the lamellae (Fig 3.24).

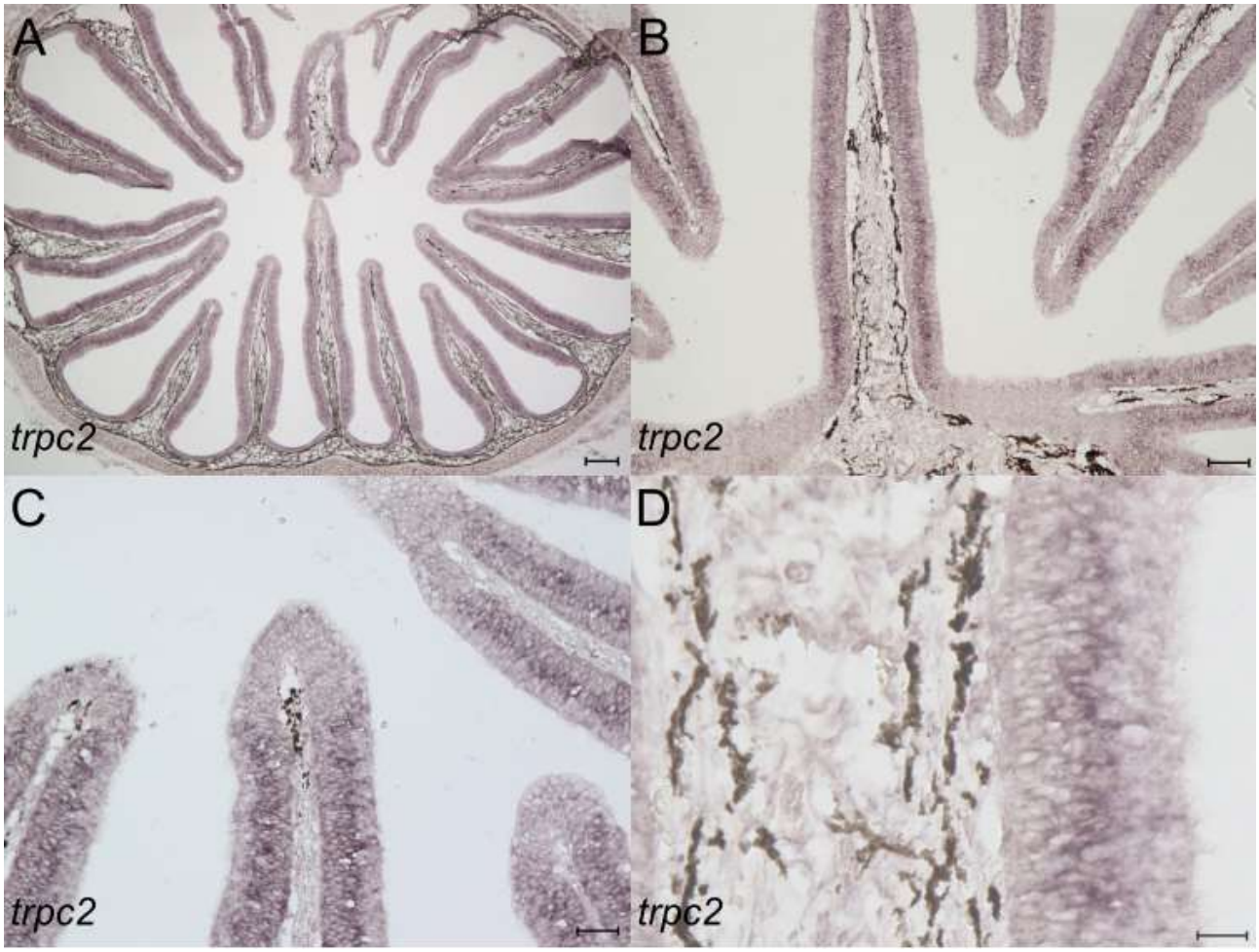

Figure 3.24: TRPC2 (NBT/BCIP staining) is expressed in the olfactory epithelia of *Lampetra fluviatilis*. A)-E) Trpc2 is expressed in a apical cell layer in the lamellae. NBT/BCIP stainings of *trpc2* at the middle part of the lamellae, excluding the tip and the non-sensory area. Scale bar A) 200µm B) 100µm C) 50µm D) 10µm

To investigate the position of the TRPC2-positive cells in *Lampetra fluviatilis*, a fluorescent *in situ* hybridisation was performed with both probes. The expression pattern of TRPC2 does not look the same as in the NBT/BCIP staining. In several fluorescent double *in situ* stainings cells in the non sensory area were also stained (Fig. 3.25 and 3.26). Compared to fluorescent G_{olf}-positive stained cells in lamprey (Fig 3.18), with their thin more palisade-looking cell shape, the TRPC2-positive cells looked more roundish. Together with the DAPI staining of the single TRPC2-positive cells, it becomes apparent that mostly the apical cell layer expresses TRPC2 (Fig 3.25). To investigate a possible co-staining of TRPC2 and A2c, several double *in situ*s were performed, following the established standardized protocol (2.6.3.3.). Therefore, to begin with, a fluorescein-labelled A2c probe was visualized with HNPP/Fast Red, followed by the visualisation of an digoxigenin- labelled TRPC2 probe with a secondary Alexa 488-antibody. Both sliced variants of the OE, rostral-caudal and dorsal-ventral, showed no double labelling of TRPC2 and A2c. The localization of TRPC2 is more above the nucleus, whereas the localization of A2C is around the nucleus. No yellow-coloured cells were observed, whenever Alexa Fluor 488 (green) and Alexa Fluor 633 (red) were localized in the same cell (Fig. 3.26 and Fig .3.27).

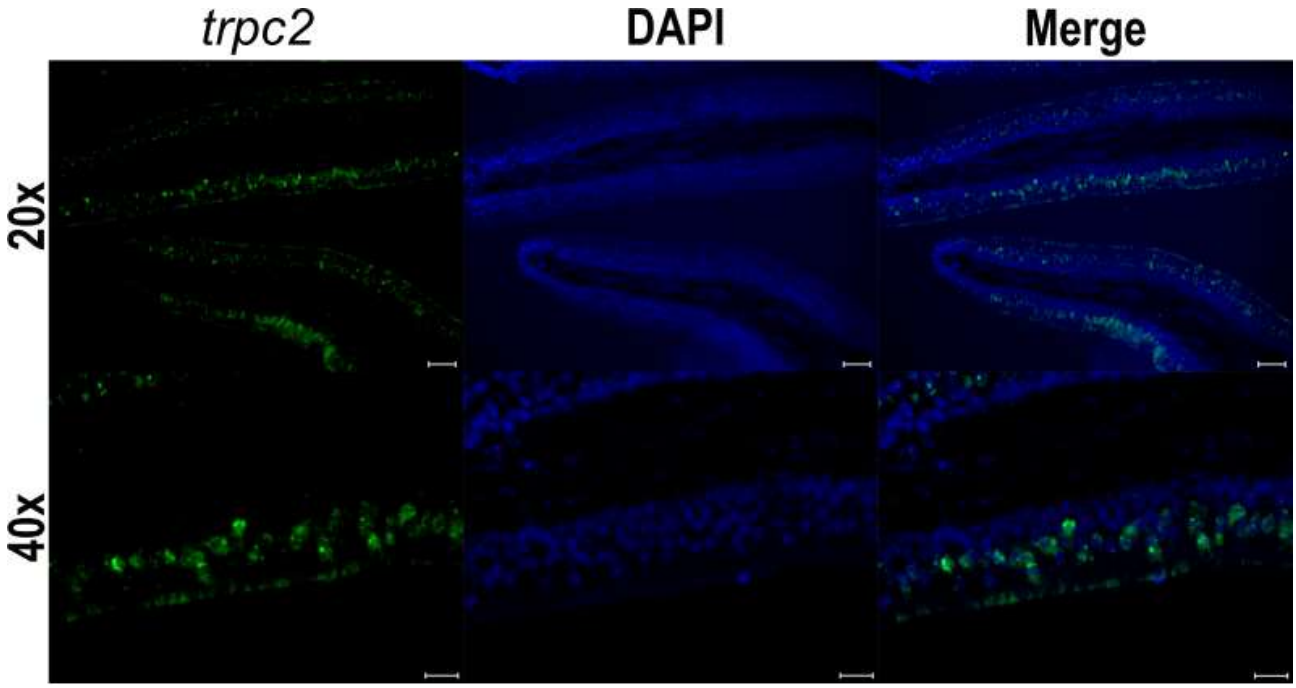

Figure 3.25: TRPC2 expression (TSA staining) in the olfactory epithelia of *Lampetra fluviatilis*. Trpc2 is expressed in apical cells of the lamellae.*Trpc2* was visualized with TSA Alexa Fluor 488. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 20x) 50µm 40x) 20µm

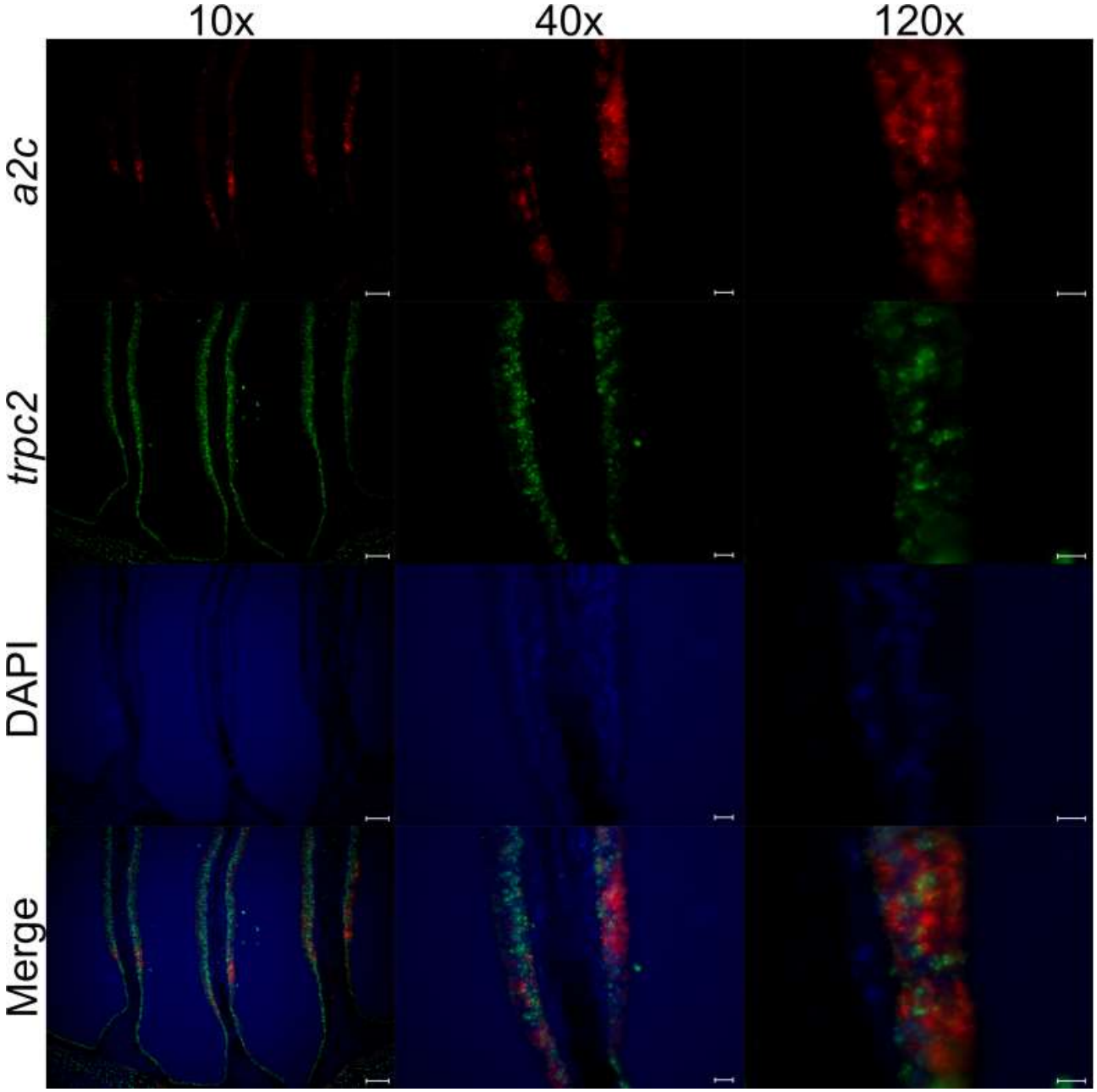

Figure 3.26: The A2c (TSA staining) and TRPC2 (NBT/BCIP staining) double *in situ* hybridization signals are localize in different cells in the olfactory epithelia of *Lampetra fluviatilis*. TRPC2 (green) and A2c (red) are not co-expressed in the same cells. Fluorescent double *in situ* staining of *a2c* and *trpc2* in the olfactory epithelia of *Lampetra fluviatilis*. *A2c* was visualized with HNPP Fast Red and *trpc2* with TSA Alexa Fluor 488. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

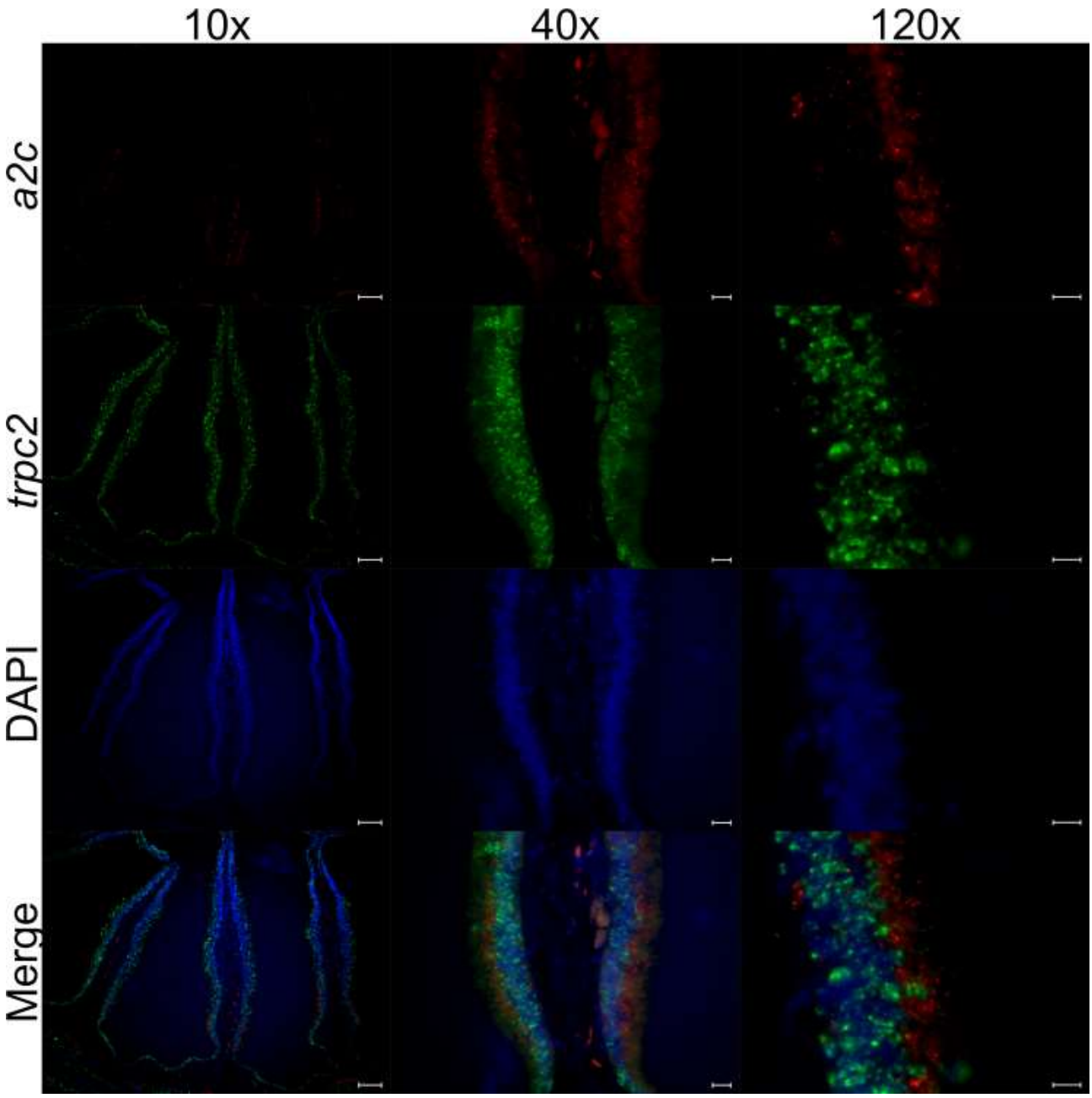

Figure 3.27: The A2c (TSA staining) and TRPC2 (NBT/BCIP staining) double *in situ* hybridization signals localize in different cells in the dorsal ventral cutted olfactory epithelia of *Lampetra fluviatilis.* TRPC2 (green) and A2c (red) are not co-expressed in the same cells. Fluorescent double *in situ* staining of *a2c* and *trpc2* in the olfactory epithelia of *Lampetra fluviatilis. A2c* was visualized with HNPP Fast Red and *trpc2* with TSA Alexa Fluor 488. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

3.1.5.5 A2c-positive cells are not co-expressed with the neuronal marker HuC in lamprey – *Lampetra fluviatilis*

The RNA-binding Protein HuC is a marker of neurons which have left the mitotic cycle. It labels the sensory area of the olfactory epithelia in teleosts (Bayramli et al., 2017). For the experiments an established HuC-antibody was used in an immunohistochemistry after an *in situ* hybridization, in which here A2c was stained with NBT/BCIP. A2c showed the same staining as before, but a high background staining inside the sensory area was observed. The HuC-antibody labels the sensory area in the OE with an localization in the apical cells in terms of laminar height. HuC was not localized in the tips of the lamellae and in the non-sensory area was detected. The expression zones of A2c and HuC formed a sharp border to each other. The HuC-positive cells had a thin, elongated cell body, which is typical for OSNs (Fig 3.28). To get a better and closer look at the border between the expression patterns of HuC and A2c a fluorescent *in situ* hybridization of A2c combined with an immunohistochemistry was performed. After the fluorescent double staining, the sharp border between HuC and A2c was clearly visible (Fig.3.29).

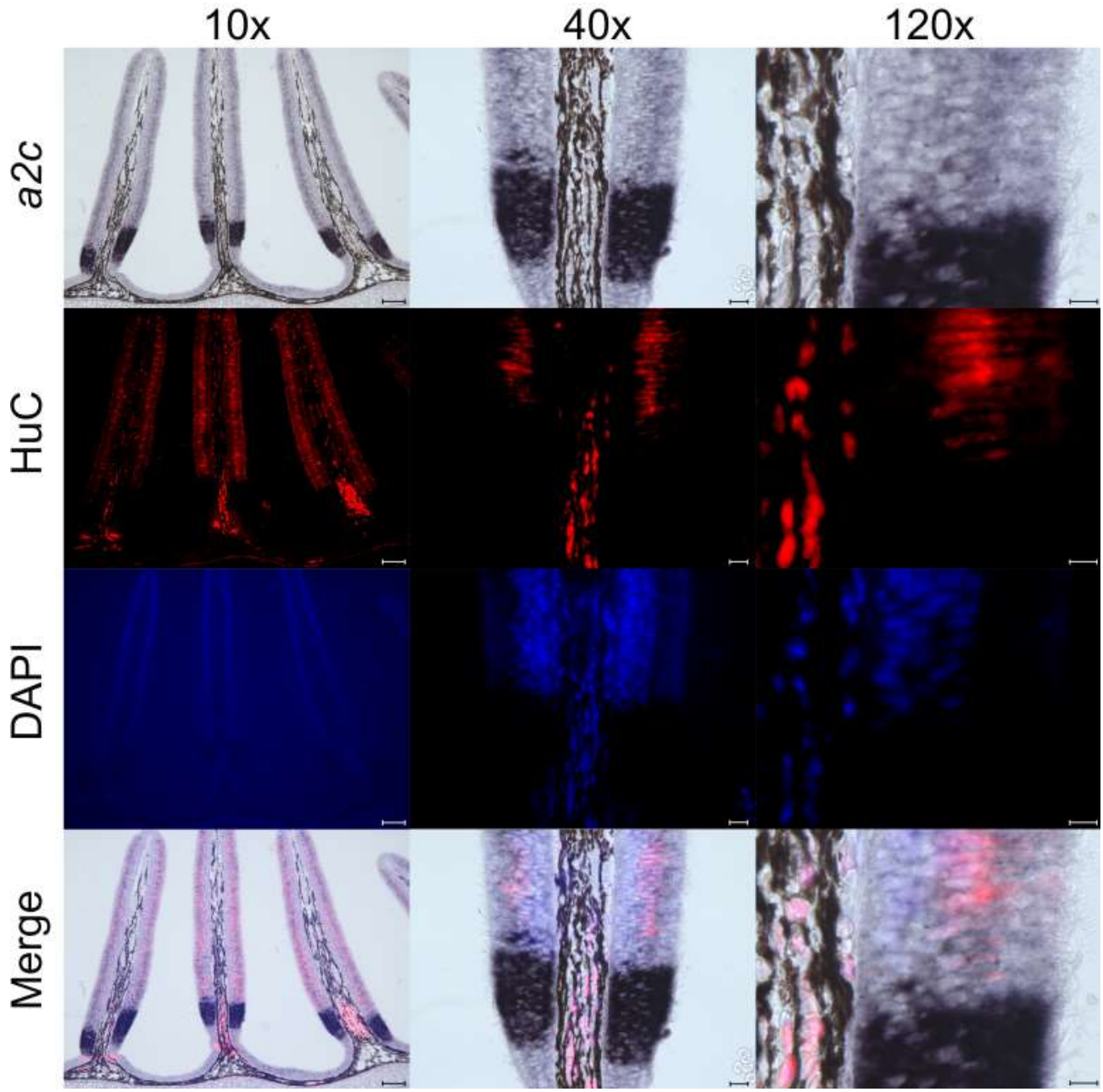

Figure 3.28: A2c *in situ* hybridization signal (NBT/BCIP staining) and HuC-antibody staining localize to clearly segregated areas the olfactory epithelia of *Lampetra fluviatilis*. A2c probe (brown) and Huc-antibody (red) are labelled in different zone of the lamellae**.** Double staining of *a2c* (ISH) and HuC (IHC) in the olfactory epithelia of *Lampetra fluviatilis*. *A2c* was visualized with NBT/BCIP and HuC with an anti-mouse Alexa-Fluor 594 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

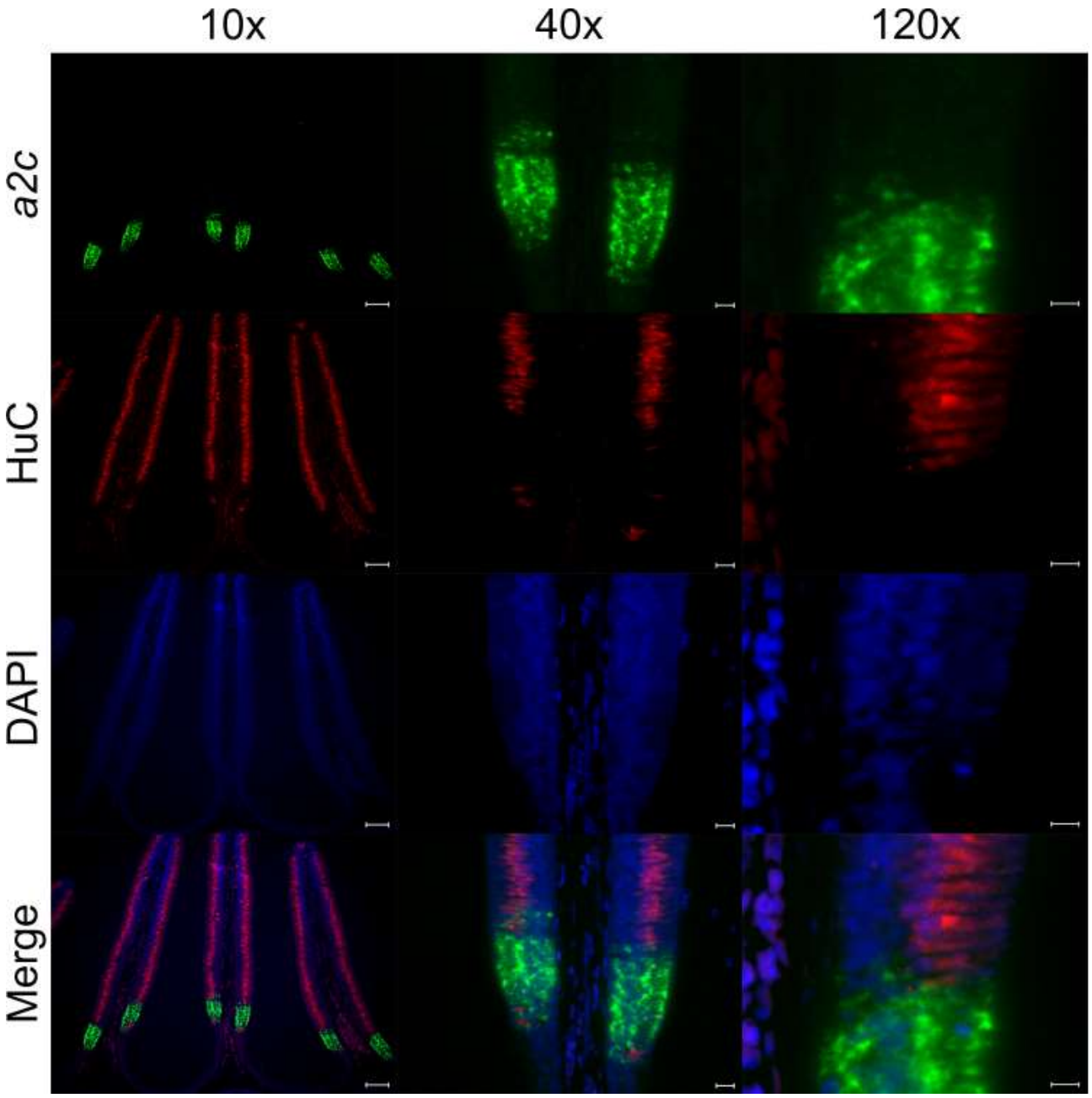

Figure 3.29: A2c *in situ* hybridization signal (TSA staining) and HuC-antibody staining localize to clearly segregated areas the olfactory epithelia of *Lampetra fluviatilis*. A2c probe (green) and Huc-antibody (red) are labelled in different zone of the lamellae**.** Fluorescent double staining of *a2c* (ISH) and HuC (IHC) in the olfactory epithelia of *Lampetra fluviatilis*. *A2c* was visualized with TSA Alexa Fluor 488 and HuC with an anti-mouse Alexa Fluor 594 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

3.1.5.6 A2c-positive cells are not co-expressed with the neuronal marker PCNA in lamprey – *Lampetra fluviatilis*

With PCNA (proliferating cell nuclear antigen), another possible neuronal marker that shows the proliferation zones inside a tissue, was tested together with A2c.

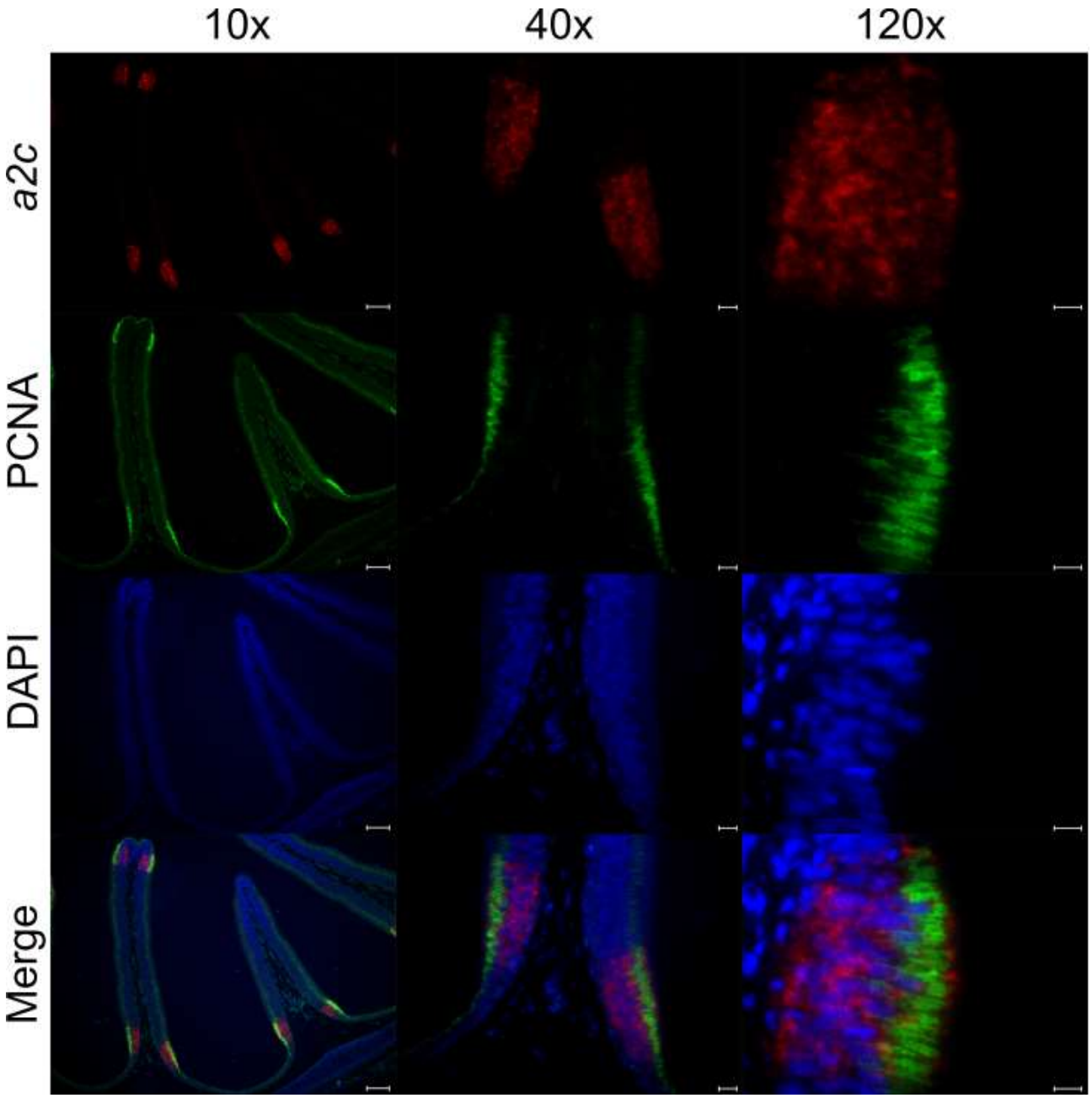

Figure 3.30: Signals for A2c *in situ* hybridization and PCNA-antibody are co-localized in the olfactory epithelia of *Lampetra fluviatilis.* A2c probe (red)and PCNA-antibody (green) are labelled in the same zone of the lamellae. Fluorescent double staining of *a2c* (ISH) and PCNA (IHC) in the olfactory epithelia of *Lampetra fluviatilis*. *A2c* was visualized with TSA Alexa Fluor 633 and PCNA with an anti-mouse Alexa Fluor 488 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei.Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

The PCNA protein is a DNA clamp that acts during eukaryotic replication as an co-factor of the DNA polymerase (Strzalka and Ziemienowicz, 2011). A PCNA-antibody, which is established in other vertebrates, was double-labelled with an A2c probe in a combination of *in situ* hybridization and immunohistochemistry. Both sliced variants of the OE, rostral-caudal and dorsal-ventral, showed no direct double-labelling of A2c and PCNA, but the PCNA-positive signals are exactly above the A2c stained cells at the same position in the OE.

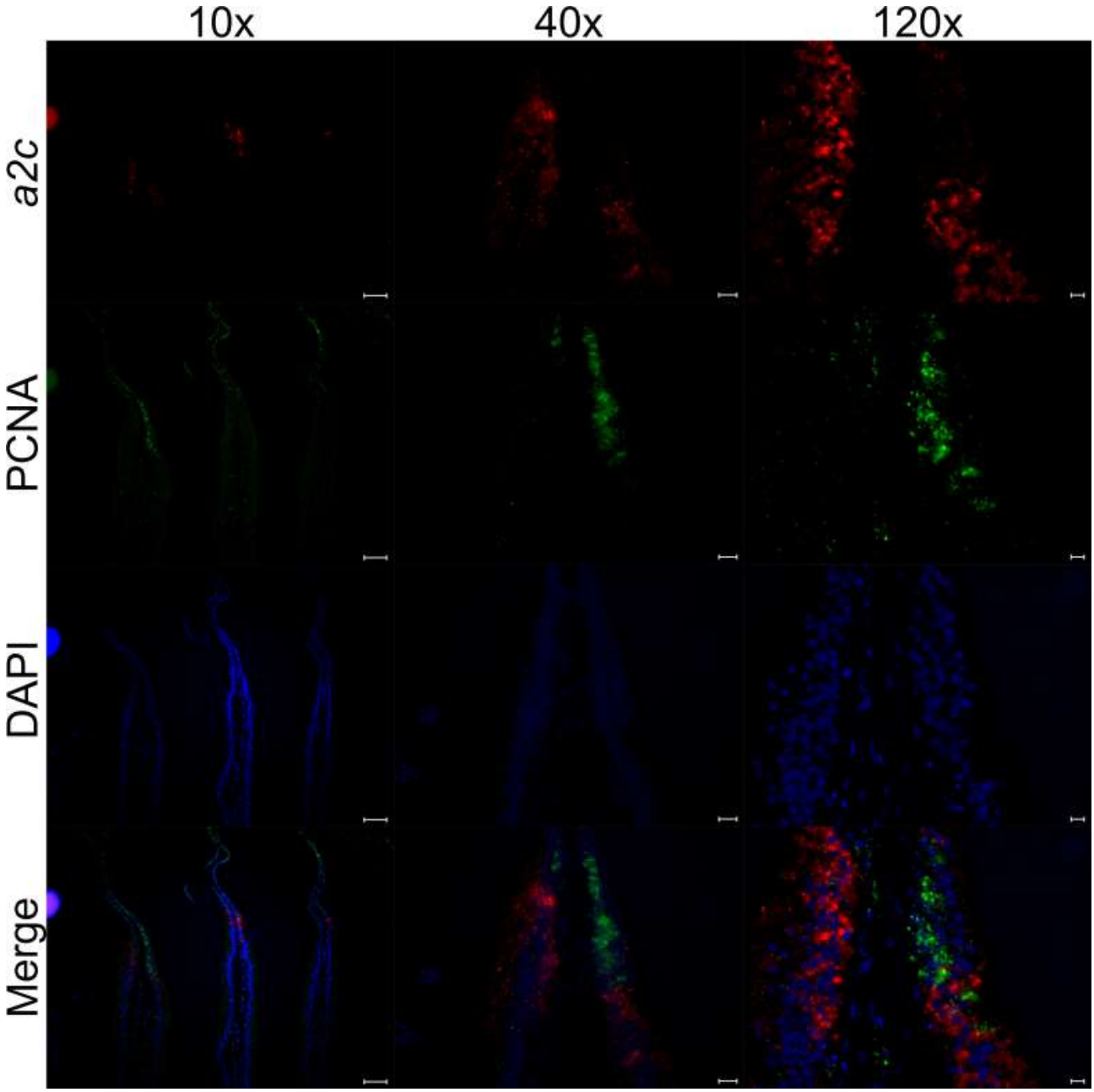

Figure 3.31: Signals for A2c *in situ* hybridization and PCNA-antibody are co-localized in dorsal ventral sliced olfactory epithelia of *Lampetra fluviatilis*. A2c ***probe and PCNA antibody are not directly co-labelled.*** Fluorescent double *in situ* staining of *a2c* and PCNA in the dorsal ventral sliced olfactory epithelia of *Lampetra fluviatilis*. *A2c* was visualized with HNPP Fast Red and PCNA with TSA Alexa Fluor 488. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

As A2c shows a positive signal at the tip of the lamellae, the PCNA-antibody also stained the structures above this position. Compared to the strongly stained zones, a faint staining of all cells in the complete sensory area was also observed, at the same apical position, in terms of laminar height, as in the A2c-positive areas. The PCNA-positive stained parts of the cells are above the nucleus at the tips of the cell, forming a brush like structure above the A2c signal. The PCNA- positive stained cells had an mostly elongated morphology.

3.1.5.7 Double labelling of neuronal marker in lamprey – *Lampetra fluviatilis*

3.1.5.7.1 G_{olf} and HuC

Combined *in situ* hybridisations and immunohistochemistries were done, to confirm the common position of different markers. Because of the sharp border of G_{olf} and HuC-positive cells compared to the expression pattern of A2c, it was interesting to see if both label the exact same area and/or cell populations. A combination of a fluorescent immunostaining of G_{olf} probe and an HuC-antibody, reveals that both are entirely co- localized in the upper cell layer in the sensory area of the OE in *Lampetra fluviatilis.* The non-sensory area at the tip and bottom of the single lamella, showed no positive-labelled cells (Fig. 3.32).

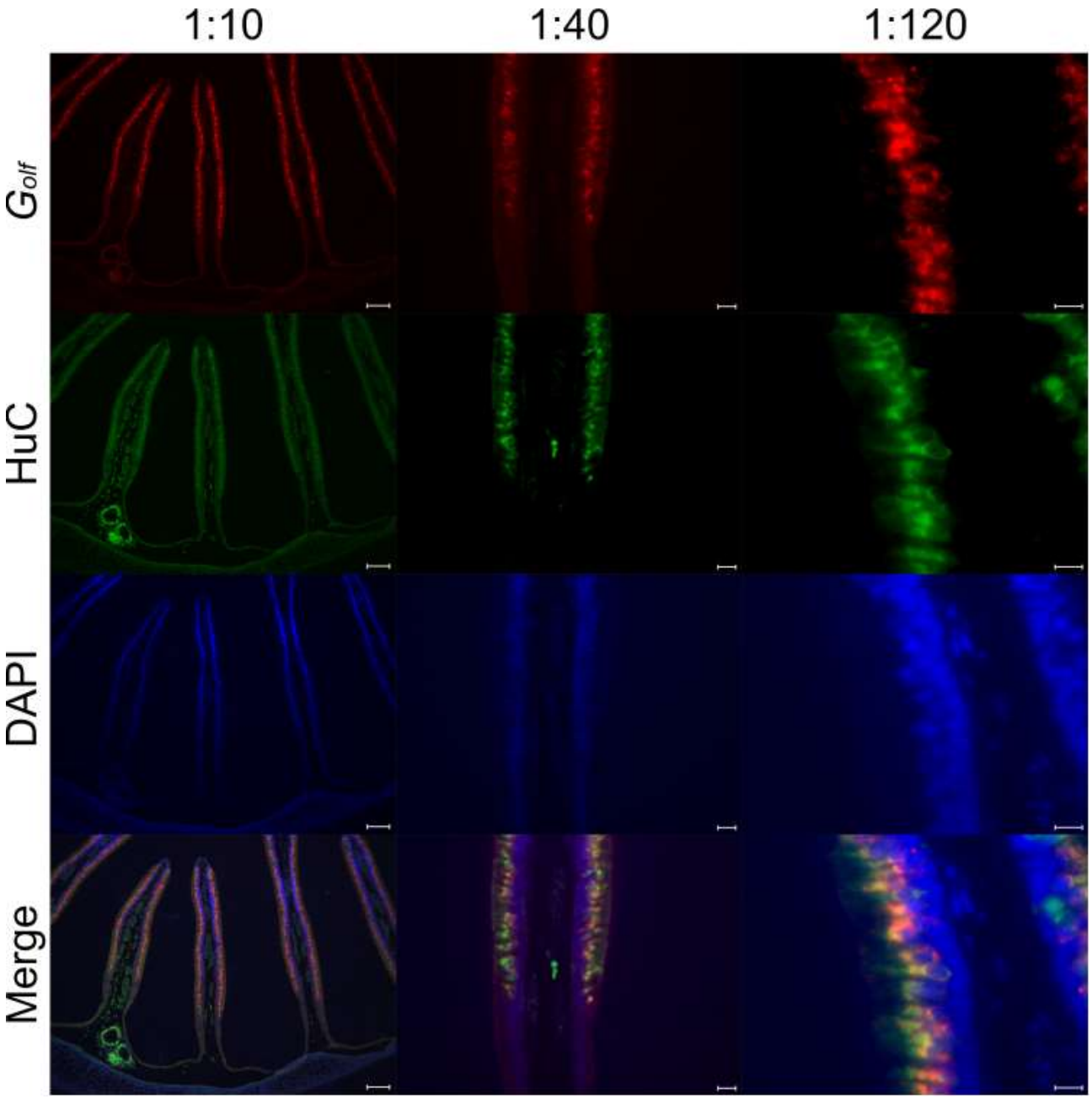

Figure 3.32: G_{olf} is co-expressed at cellular level with the neuronal marker HuC in the olfactory epithelia of *Lampetra fluviatilis.* Golf (red) and Huc (green) are coexpressed in the same cells in the olfactory sensory zone. Fluorescent double staining of G_{olf} (ISH) and HuC (IHC) in the olfactory epithelia of *Lampetra fluviatilis*. G_{olf} was visualized with TSA Alexa Fluor 488 and HuC with an anti-mouse Alexa Fluor 594 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

3.1.5.7.2 G_{olf} and PCNA

Because PCNA was localized together with A2c in the same area of the OE in *Lampetra fluviatilis,* it was interesting to find out if there is a localization of PCNA with one of the markers in the sensory area in the OE. Therefore, a fluorescent double-labelling of a G_{olf}-

positive probe together with a PCNA-antibody was performed. Both markers labelled the upper cell layer in the lamellae, but with a flowing border of their expression zones in the area of the crossing between sensory to non-sensory areas. In the small area both markers were also localized, but no double-labelling was observed (Fig 3.33).

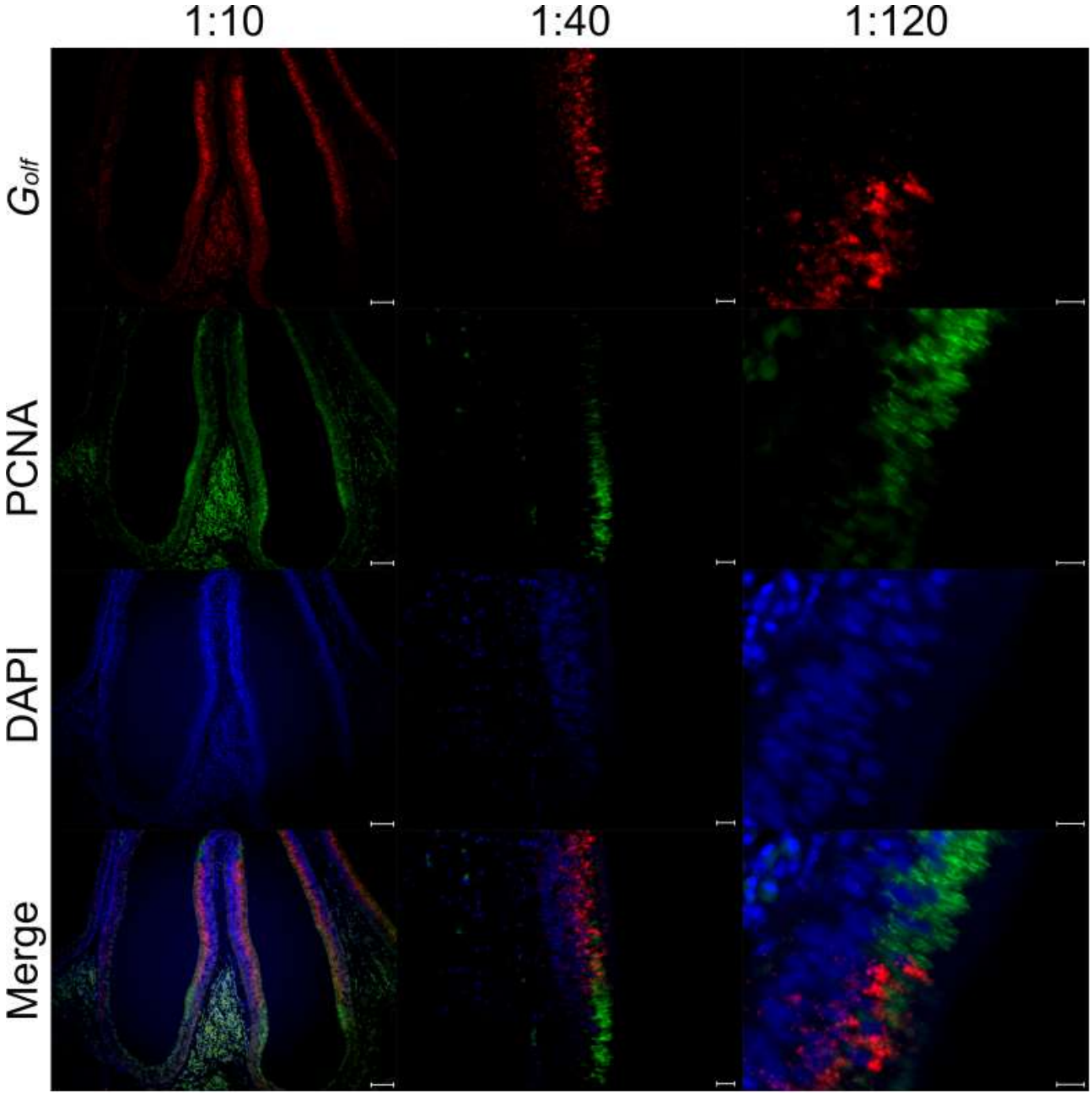

Figure 3.33: G_{olf} *in situ* hybridization signal and PCNA-antibody staining localize to clearly segregated areas the olfactory epithelia of *Lampetra fluviatilis*. G_{olf} (green) and PCNA (red) are expressed in different zones in the lamellae. Fluorescent double staining of G_{olf} (ISH) and PCNA (IHC) in the olfactory epithelia of *Lampetra fluviatilis*. G_{olf} was visualized with TSA Alexa Fluor 633 and PCNA with an anti-mouse Alexa Fluor 488 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

3.1.5.7.3 G_{olf} and TRPC2

For a closer characterisation of the expression pattern of TRCP2, a marker for microvillious cells in vertebrates, a fluorescent double-labelling with G_{olf}, a marker for ciliated olfactory neurons, was done.

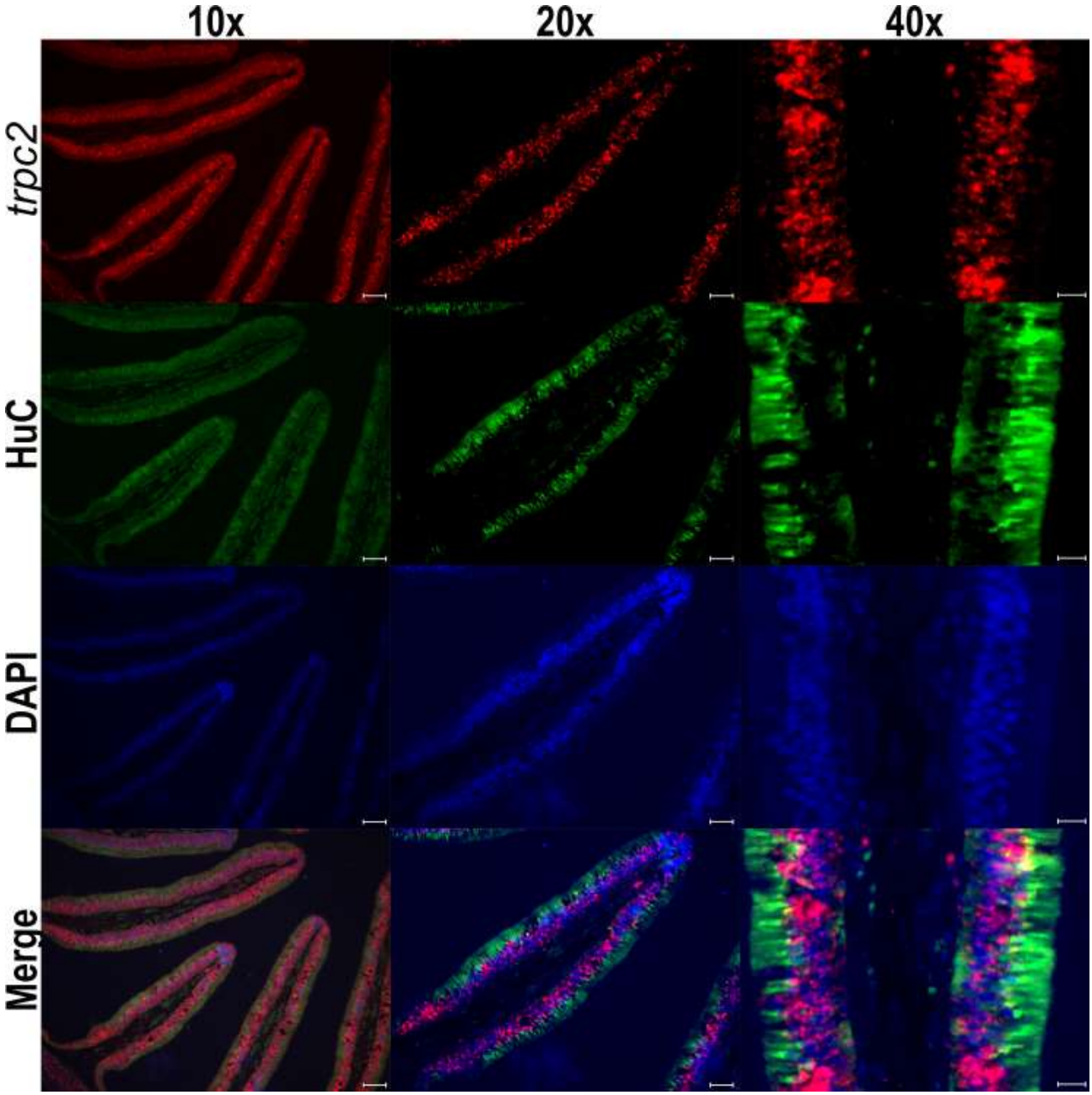

Figure 3.34: Trpc2 *in situ* hybridization signal and HuC-antibody staining partially co localize the olfactory *epithelia of Lampetra fluviatilis* Fluorescent double staining of *trpc2* (ISH) and HuC (IHC) in the olfactory epithelia of *Lampetra fluviatilis*. TRPC2 (red) is partially colabeled with HuC (green). *Trpc2* was visualized with TSA Alexa Fluor 633 and HuC with an anti-mouse Alexa Fluor 488 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

The red TRPC2-positive cells, which were stained *via in situ* hybridisation, and the green HuC-positive cells, which were stained with an HuC-antibody, showed partial overlapping. Only some of the apically-localized TRPC2-positive cells showed a double-labelling with HuC. The TRPC2 signal is also localized in the tips of the lamellae and less signals were detected in the non-sensory area between two lamellae (Fig. 3.34).

3.1.5.7.4 PCNA-antibody and PCNA *in situ* hybridisation

For a closer characterization of the PCNA-antibody, a double *in situ* hybridisation together with a PCNA probe were performed. The amino acid sequence and the resulting nucleotide sequence (Fig 3.35) were taken from ensemble.org.

A

PCNA amino acid sequences of *Petromyzon marinus*

MFEARILQGNILKKVLEALKDLITEACWDISSTGVSLQSMDSSHVSLVQLTMRSDGFDTYRCDRNL
AMGINLTSMSKILKCAGNDDMITLRAEDNADILTLVFEAQNHEKVSDYEMKLMDLDVEQLGIPEQD
YSAVVKMPSGEFSRICRDLSQIGDSVVICCTKDGVKFSASGELGTGNIKLSQTSGADKEEEAVTIE
MSEAVQLTFALRYLNFFTKATPLSPTVTLRMSADVPLVVEYGIADMGHIKYYLAPKIEDEEAA

B

PCNA nucleotide sequences of *Petromyzon marinus*

ATGTTCGAGGCACGCATCCTGCAGGGAAACATCCTGAAGAAGGTTCTCGAGGCGCTCAAGGATCTT
ATAACGGAGGCGTGTTGGGACATCAGTTCGACCGGCGTGAGCCTGCAGTCCATGGACTCGTCGCAC
GTGTCCCTGGTGCAGCTCACCATGCGCAGCGACGGCTTCGACACCTACAGATGCGACCGCAACCTC
GCCATGGGCATCAACCTCACCAGCATGTCCAAAATCCTCAAGTGTGCCGGCAACGACGACATGATC
ACCCTGAGGGCCGAGGACAATGCAGACATACTCACCCTGGTGTTCGAGGCCCAAAATCATGAGAAG
GTGTCTGATTATGAGATGAAGTTGATGGACCTGGATGTAGAGCAACTGGGAATTCCCGAACAGGAT
TACAGCGCCGTTGTGAAGATGCCATCTGGGGAATTTTCTCGGATATGCCGAGATCTGAGCCAGATT
GGTGACTCCGTTGTCATCTGCTGCACCAAAGACGGTGTAAAGTTTTCAGCCAGTGGCGAGTTGGGC
ACTGGCAACATCAAACTTTCACAGACCAGCGGTGCTGACAAGGAAGAGGAGGCTGTAACGATAGAA
ATGAGTGAAGCCGTGCAGCTGACATTCGCCCTTCGCTACCTGAACTTCTTCACCAAGGCCACCCCA
CTGTCACCCACCGTTACACTCCGGATGTCTGCCGATGTCCCGCTTGTTGTGGAATATGGCATCGCA
GACATGGGCCACATCAAGTACTATCTGGCACCCAAGATTGAGGATGAAGAGGCTGCATAA –

Figure 3.35: PCNA amino acid and nucleotide sequence of *Petromyzon marinus.* A) Predicted amino acid sequence of G_{olf} from *Petromyzon marinus* based on search with *Danio rerio* G_{olf} amino acid sequence B) Corresponding nucleotide sequence of *Petromyzon marinus* PCNA *with p*rimers: PCNA A (yellow); PCNA B (green)

Because only the PCNA sequence of *Petromyzon marinus* (ENSPMAT00000008605.1) was available, the chosen primers (Fig. 3.35 B) were used for *Lampetra fluviatilis* and their resulting PCR product was checked *via* sequencing. After the combination of *in situ* hybridisation and immunohistochemistry of PCNA, both methods showed a different expression pattern with fluorescent and chromogenic stained probe. The PCNA cells stained positive *via in situ* hybridisation are only localized to a small amount of two to three cells at the tips of a lamella. The PCNA-antibody is localized in an upper row of cells in the complete sensory area of the lamellae, with stronger localization near the boarders between the sensory and non-sensory area. No double labelling was observed between PCNA probe and PCNA-antibody, but they are partially localized in the same part of the tissue, the antibody apical and the *in situ* probe more basal (Fig. 3.36 and 3.37).

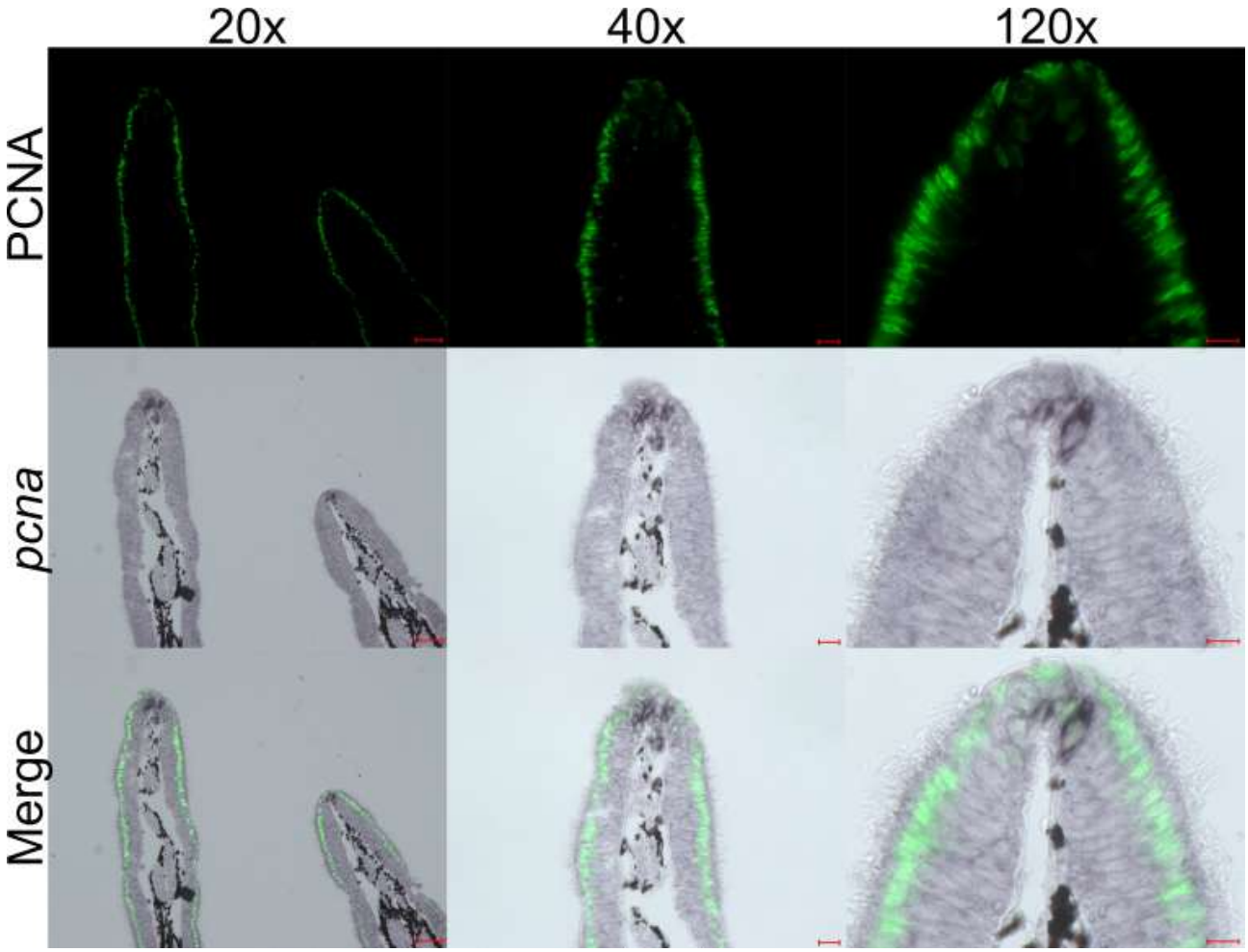

Figure 3.36: PCNA-antibody and PCNA *in situ* hybridization (NBT/BCIP staining) signals are localized in different cells in the olfactory epithelia of *Lampetra fluviatilis*. staining of *pcna* probe (brown) and PCNA antibody (red) reveals that they localized in the same area, but not co-labelled in the olfactory epithelia of *Lampetra fluviatilis*. *Pcna* probe was visualized with NBT/BCIP and PCNA antibody with an anti--mouse Alexa Fluor 488 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

To obtain another impression of the expression pattern of PCNA probe and antibody, the double-staining was repeated in in the dorsal- ventral cut OE of *Lampetra fluviatilis*. Here also, no double-labelling was observed, but both are partially localized in the same area of the lamellae, the PCNA-antibody in an apical and PCNA probe in an basal row of cells (Fig. 3.38).

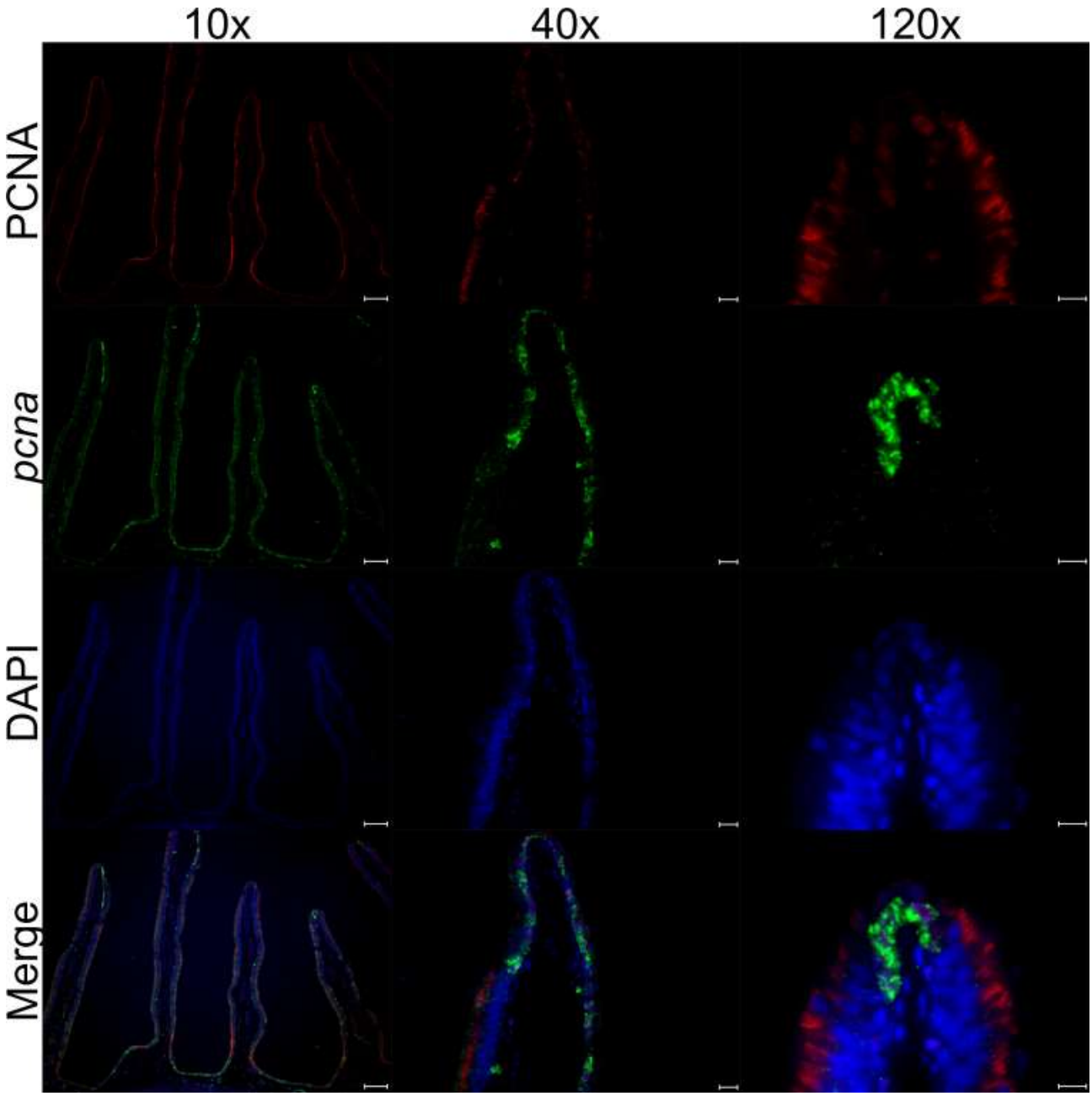

Figure 3.37: PCNA-antibody and PCNA *in situ* hybridization (TSA staining) signals are localized in different cells in the olfactory epithelia of *Lampetra fluviatilis*. Fluorescent double staining of *pcna* probe (green) and PCNA antibody (red) reveals that they localized in the same area, but not co-labelled in the olfactory epithelia of *Lampetra fluviatilis*. *Pcna* probe was visualized with TSA Alexa Fluor 488 and PCNA-antibody with an anti-mouse Alexa Fluor 594 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

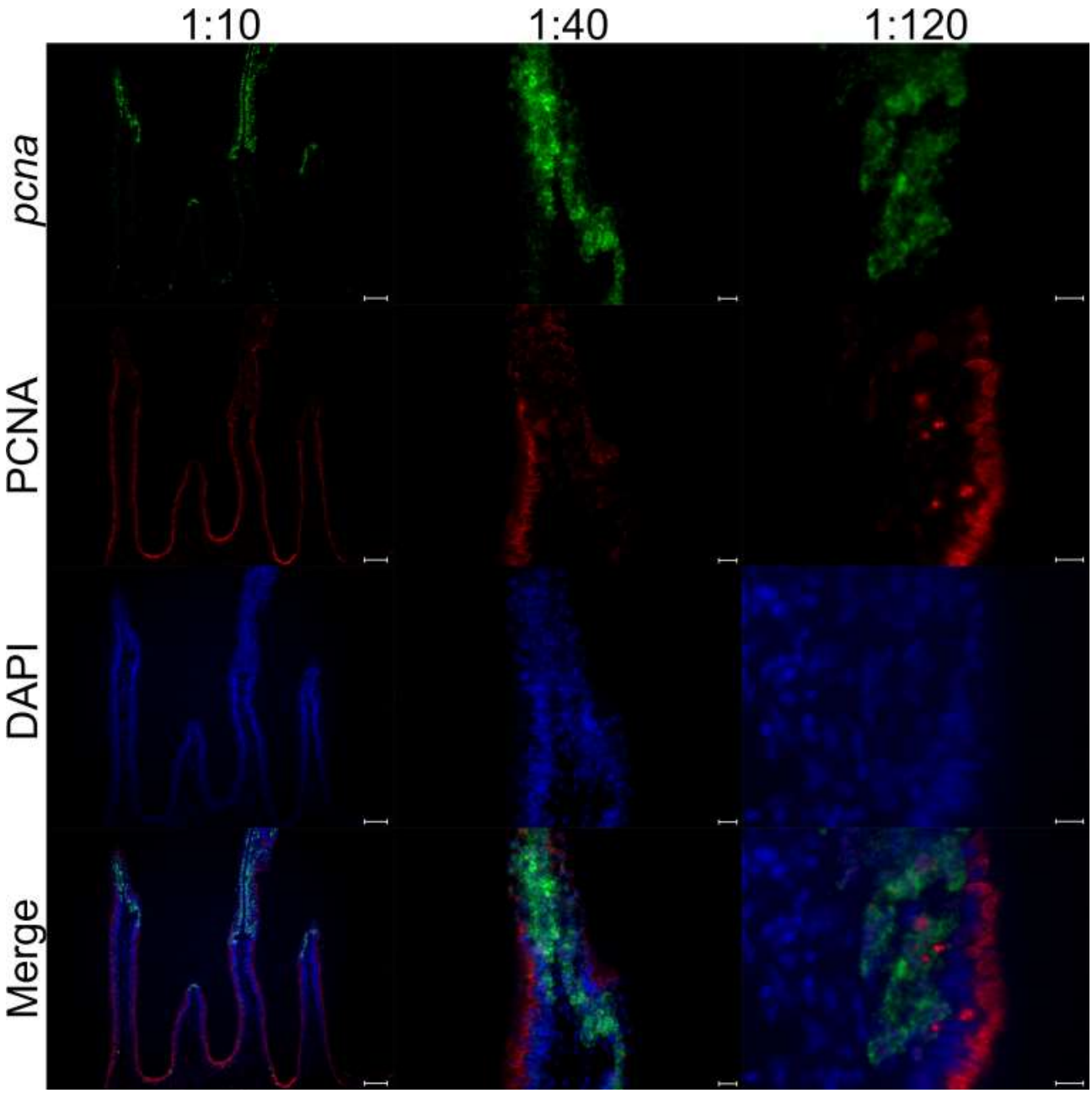

Figure 3.38: PCNA-antibody and PCNA *in situ* hybridization (TSA staining) signals are localized in different cells in the dorsal ventral cutted olfactory epithelia of *Lampetra fluviatilis.* Staining of PCNA probe (green) and antibody (red) in the same areas of the lamellae, but no co-labelling was observed. Fluorescent double staining of *pcna* (ISH) and PCNA (IHC) in the olfactory epithelia of *Lampetra fluviatilis*. *Pcna* probe was visualized with TSA Alexa Fluor 488 and PCNA-antibody with an anti-mouse Alexa Fluor 594 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

3.1.5.7.5 No expression of other markers like VgluT, ACIII and CasR probes in *Lampetra fluviatilis*

At first VgluT (vesicular glutamate transporter) was used as a marker for OSN, because it is known that mice express VgluT2 (Tatti et al., 2014) in their axonal terminals. But no *in*

situ staining was detected for the VgluT in the OE of *Lampetra fluviatilis*, visualized with NBT/BCIP(Fig. 3.39 A). The probes for ACIII (adenylate cyclase 3), which is activated after stimulation in nearly each olfactory OSN in the OE of vertebrates (Dal Col et al., 2007), also showed staining in the OE of *Lampetra fluviatilis* (Fig.3.39 B).

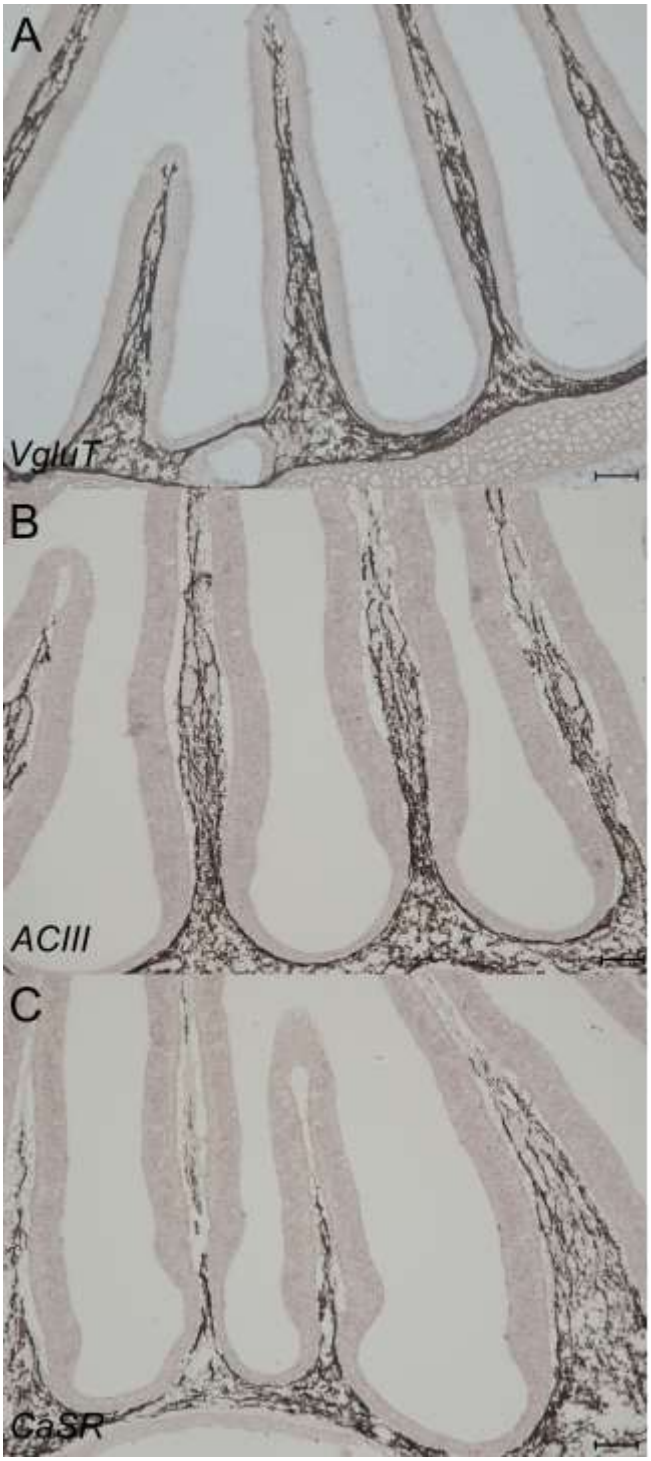

Figure 3.39: VgluT, ACIII and CaSR are not expressed in the olfactory epithelia of *Lampetra fluviatilis*. A)-C) No stained cells were observed for VgluT, ACIII or CaSR in the lamellae of *Lampetra fluviatilis*. Figure:In situ hybridisation of Vglut (A), ACIII (B) and CaSR (C) with an NBT/BCIP staining in the olfactory epithelia of *Lampetra fluviatilis*. Scale bar A-C) 50µm

The last possible marker for a closer investigation of A2c, was CaSR, the calcium sensing receptor. It is known that this receptor is localized in the cilia of the OSNs (Shih-Pin Lee et al., 2018). But here also ,after *in situ* hybridisations, different CaSR probes showed no staining in the OE of *Lampetra fluviatilis*. For each gene, two different primer pairs were designed (App. 6.1-6.3), based on searches in NCBI with homologous sequences of *Danio rerio*.

3.1.6. Presence of Adenosine-responsive cells in *Lampetra fluviatilis OE*

To investigate the staining of ATP-stimulated cells in the OE of *Lampetra fluviatilis* and to investigate if the expression zone is the same as for the A2c probe, two different stimulation experiments were done. After the OE was dissected and sliced, an IHC with a c-fos antibody, an immediately early gene marker, followed. The OE of the lamprey stimulated with water, showed no positive c-fos signal. The amino acid stimulated lampreys, exhibit single cells stained by the c-fos antibody. These single cells were localized in the olfactory sensory area of the OE, with an elongated cell body, starting close to the perephiral capsule and medial part of the lamellae. In the ATP-stimulated lampreys, an area near the tip of the lamellae was stained. The staining was inside the olfactory sensory area and in some cases long elongated cell bodies could be identified (Fig. 3.40).

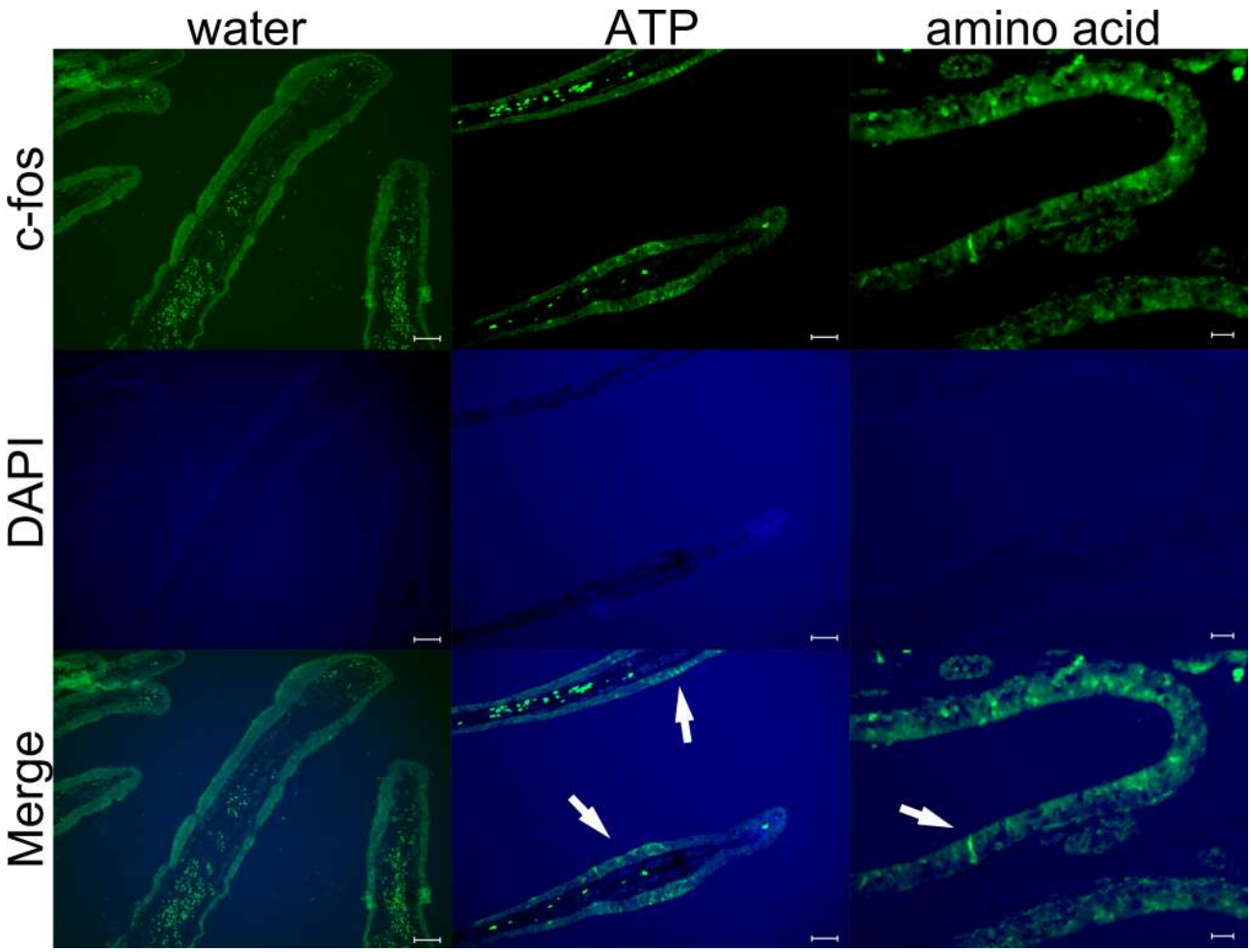

Figure 3.40: C-fos activity in the olfactory epithelia of *Lampetra fluviatilis* after stimulating with different odours. The lampreys gets either stimulated with 50µm ATP, 50µm aminio acids mix and water. ATP stimulated lampreys exhibit A2c-like signals (white arrows in middle merge pannel), whereas amino acid stimulated Lamprey exhibit single stained cells (arrows right merge panel). Lamprey stimulated with water exhibit no signal. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei.

Because ribosome phosphorylation often correlates with neural activity, PS6, a structural component of the ribosome that is phosphorylated downstream of PI3-K/mTOR, MAPK, and PKA signaling, was used as another immediately early gene marker (A. Knight et al. 2013). An IHC with the SP6-antibody was done with the rest of the series of slices from the same stimulated lamprey. The water control again showed no specific staining in contrast to the amino acid-stimulated lamprey, in which single cells inside the sensory area were stained. These cells consist of a roundish cell body with an elongated tip and were localized mainly in the medial to outer zone of the lamella. The ATP-stimulated lamprey showed a very weak staining in the apical cell layer of the lamellae, in which some roundish cells could be identified, which lay close to each other. But this staining pattern was not observed in every lamellae (Figure 3.41).

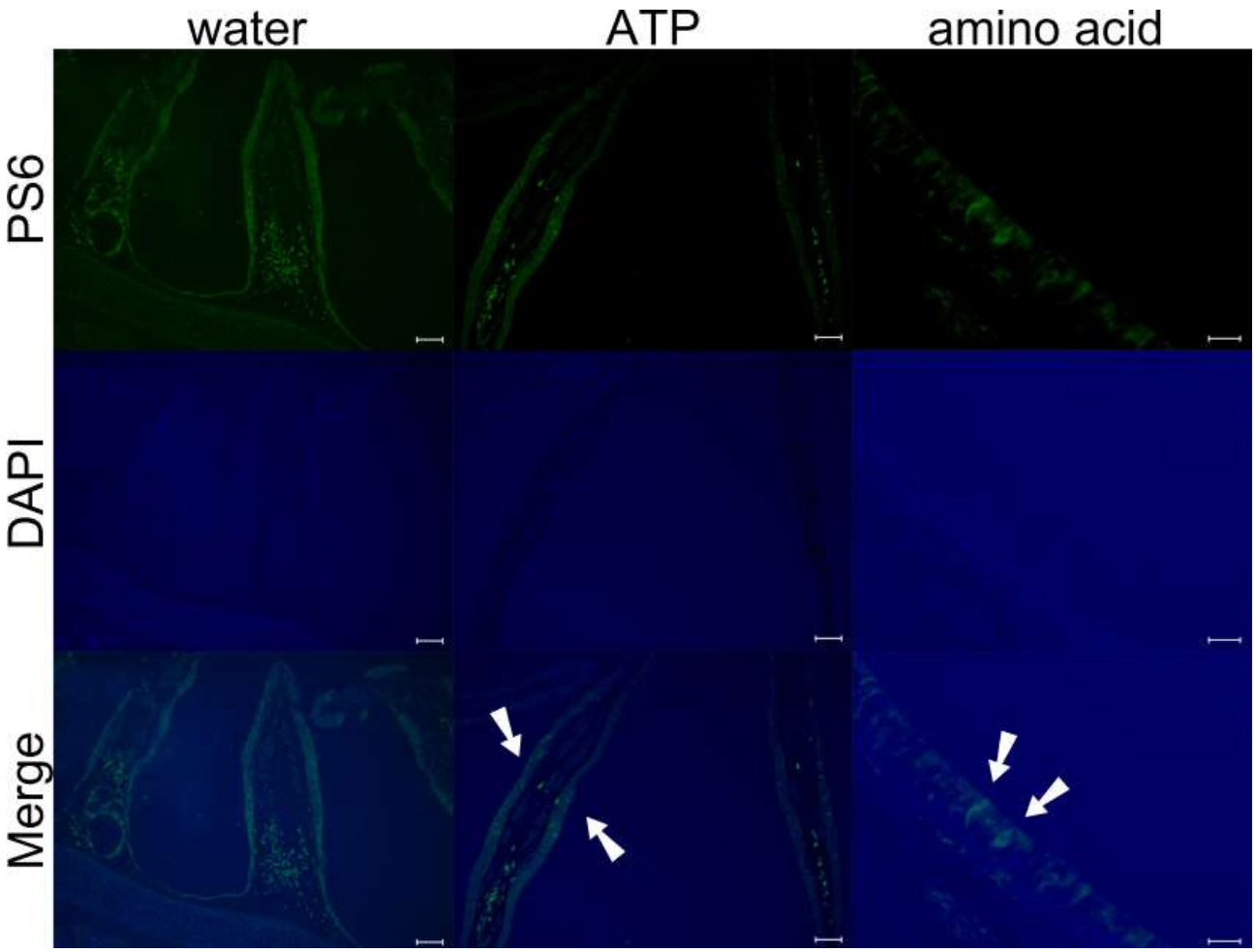

Figure 3.41: PS6 activity in the olfactory epithelia of *Lampetra fluviatilis* after stimulating with different odours. The lampreys gets either stimulated with 50µm ATP, 50µm aminio acids mix and water. ATP stimulated lampreys exhibit A2c-like signals (white arrows in middle merge pannel), whereas amino acid stimulated Lamprey exhibit single stained cells (arrows right merge panel). Lamprey stimulated with water exhibit no signal.Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei.

3.2 Expression of vomeronasal receptor genes in the jawless fish - *Lampetra fluviatilis*

Because of the unexpected expression pattern of the A2c receptor observed in *Lampetra fluviatilis*, this work was also focused on V1Rs, another group of olfactory receptors in lamprey. In former publications (Grus and Zhang, 2009; Libants et al., 2009) the existence of four V1Rs in the genome of *Petromyzon marinus* was predicted through phylogenetic studies, but their expression pattern was never investigated. *Via* blasting the V1R-related Ora amino acid sequence of zebrafish in NCBI, six possible *v1r* gene sequences were identified in the genome of *Lethenteron camtschaticum*, a closer relative of *Lampetra fluviatilis.* For each V1R different primer sets were tested with the cDNA of *Lampetra fluviatilis*

and used in either chromogenic or fluorescent *in situ* hybridisation.

3.2.1 No expression of V1R1 and V1R4 in *Lampetra fluviatilis* OE

For the V1Rs 1 and 4, three different primer sets were constructed for each gene, where only the primer set V1R C showed no product after the PCR (Fig 3.42; Tab.2.1). All other PCR products were used as a template for making *in situ* hybridisation probes. All probes were positively sequenced and several *in situ* hybridisations following the standardised protocol (2.6.3) were performed, but no staining for V1R1 or V1R4 could be observed in the main olfactory epithelia or accessory olfactory epithelia (Fig. 3.43).

A

V1R1 amino acid sequence *of Lethenteron camtschaticum*

MANLAIVSVLIILILLGTIGNTCVLTMFLHIAVKNGELLPSDRSVINLSVAHLLASVFKNILYVFD
FFKMHLLNNLGCRFLLYSINLFRTLSISFTLLLSLFQFNKLQNSTYPGINFISLKFEKSTCLVSVL
LWIISSAIFTPLLFFDDILSSNNSSGLLQFSSCSLSPSGGYHLYFGIGTIILVDGFGAFAIVTMNV
LILKILWKHHEMVAARGWVHNANHMATLNAVKIVACLLITYIICWSINIIIRILLMRYGASLSAMS
MADIASVFSSLYYSFSPFIIIFGRTSIQKKIHNTLIRCPFIRKYTSEVSEVSINGHDQRNQNLTKA
TEIQLE

V1R4 amino acid sequence *of Lethenteron camtschaticum*

MHQAYHFAIYISMTVLGTFGNIFVFIFPLHESWKSKHVATADLLHVNLATGNLFNIAFRNIPALIY
TGTGHCPLGDSGCQVVMLGFLVTTAVTVWNTLVLSIFRFFKLSCSLRCQRILRKVETRRKPALCIL
LVWFACGALFAPTAVFTRRTGPLRANSSDLRNFYGCFSHITNKAYLYTLLSLSEIIPFLLMLGVSA
ATIAKLCRHKHRTEPQLRVNNWVGMPLRRAAHIHAAYLIVLLGLVFACCWGVYFVSTLIGIYMLDD
VSDSLVFTVRLASMVYPTISPYIIGLGNPKLMNRVKGCCTP

B

V1R1 nucleotide sequence *of Lethenteron camtschaticum*

ATGGCGAATCTCGCAATAGTTAGCGTGCTAATTATTTTAATATTACTCGGCACCATTGGAAACACG
TGCGTTTTGACCATGTTTCTTCACATCGCAGTGAAGAATGGGGAACTGCTCCCATCCGACAGGAGC
GTCATCAACTTGTCTGTCGCTCACTTGCTCGCCTCTGTATTTAAAAACATTTTATACGTTTTCGAC
TTCTTCAAGATGCATCTTTTGAACAACCTCGGATGCCGGTTTCTATTGTATAGCATAAACCTCTTC
CGAACTCTAAGCATCAGCTTTACTTTGTTACTTAGCCTCTTTCAATTTAATAAGTTGCAAAACTCA
ACGTATCCAGGTATCAATTTTATTAGCCTCAAGTTTGAAAAAAGTACATGTCTCGTCAGCGTGTTG
TTATGGATAATCAGCTCTGCAATTTTCACTCCGTTACTTTTTTTCGATGATATATTAAGCTCGAAT

AATAGCTCCGGATTGCTGCAGTTTTCTTCCTGTTCTCTTAGTCCATCAGGAGGTTACCACTTATAC
TTCGGCATAGGAACAATCATATTGGTGGACGGCTTTGGCGCATTTGCTATCGTGACCATGAACGTG
CTTATTTTGAAGATACTTTGGAAACACCATGAAATGGTTGCAGCCAGAGGGTGGGTTCACAACGCC
AACCACATGGCCACCCTTAACGCTGTAAAAATTGTCGCATGCTTGCTCATCACTTACATAATTTGC
TGGAGTATCAATATTATTATACGAATATTGCTCATGCGTTACGGTGCGTCTCTATCAGCCATGTCG
ATGGCAGACATTGCATCAGTATTTTCTTCACTGTACTACTCATTCAGTCCGTTCATAATTATCTTC
GGTCGTACCTCAATACAGAAGAAGATTCACAACACACTTATTCGATGTCCTTTTATCAGAAAGTAT
ACCTCTGAAGTAAGTGAAGTAAGTATAAATGGGCACGATCAAAGAAATCAAAACCTCACCAAAGCA

ACTGAAATTCAGCTTGAG

V1R4 nucleotide sequence *of Lethenteron camtschaticum*

ATGCACCAGGCATATCACTTCGCCATATACATCTCCATGACCGTATTGGGCACGTTCGGCAATATA
TTCGTCTTCATCTTTCCTCTGCACGAGTCGTGGAAGAGCAAGCACGTCGCCACCGCCGACCTGCTA
CACGTGAACCTGGCCACGGGAAACCTCTTCAACATCGCCTTCCGAAACATCCCGGCGCTGATCTAC
ACCGGCACGGGACATTGCCCCCTCGGAGACTCCGGCTGCCAGGTCGTCATGCTCGGCTTCCTCGTC
ACCACCGCGGTCACCGTGTGGAACACGCTCGTGCTGAGCATCTTCCGCTTCTTCAAGCTCAGCTGC
TCCCTTCGCTGCCAGCGCATCTTGCGCAAGGTGGAGACGCGGAGAAAGCCCGCGCTGTGCATCCTC
CTCGTGTGGTTCGCGTGCGGTGCCCTGTTCGCGCCCACCGCCGTGTTCACCCGGCGGACGGGCCCC
CTGCGCGCCAACTCGAGCGACCTGCGCAACTTCTACGGCTGCTTCTCGCACATCACCAACAAGGCG
TACCTGTACACGCTGCTCTCGCTCTCCGAGATCATCCCCTTCCTGCTCATGCTCGGCGTGAGCGCG
GCCACCATCGCCAAGCTGTGCCGCCACAAGCACCGCACGGAGCCGCAGCTGCGCGTCAACAACTGG
GTGGGGATGCCCCTCCGACGGGCGGCGCACATCCACGCCGCCTACCTCATCGTCCTGCTCGGCCTC
GTGTTCGCCTGCTGCTGGGGAGTCTACTTCGTGAGCACGCTCATCGGCATCTACATGCTGGACGAC
GTGTCGGACTCGCTCGTGTTCACGGTGAGACTCGCATCGATGGTGTACCCGACCATCAGCCCGTAC
ATCATCGGCCTGGGCAACCCCAAGTTGATGAACAGAGTGAAGGGCTGCTGCACGCCGTGA

Figure 3.42: V1R1 and V1R4 amino acid and nucleotide sequence of *Lethenteron camtschaticum.* A) Predicted amino acid sequences of *Lethenteron camtschaticum* V1R1 and V1R4 based on search with *Danio rerio* V1R1 and V1R4 amino acid sequence B) Corresponding nucleotide sequence of *Lethenteron camtschaticum* V1R1 and V1R4 with primers: V1R1 A (green); V1R1 B (light pink); V1R1 C (orange) V1R4 A (yellow); V1R4 B (blue); V1R4 C (pink)

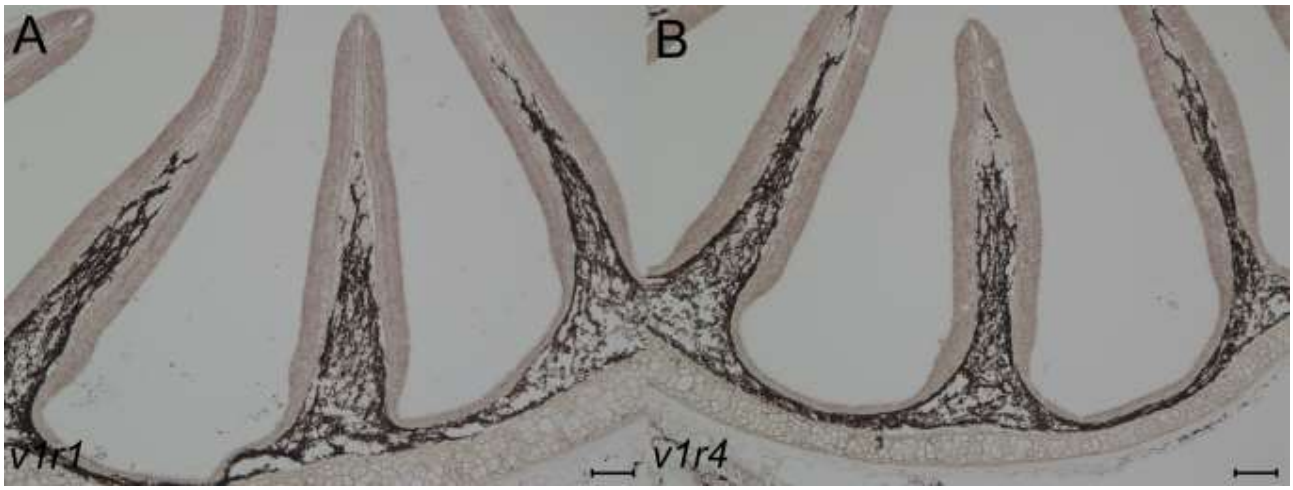

Figure 3.43: V1R1 and V1R4 are not expressed in the olfactory epithelia of *Lampetra fluviatilis*. A+B) *No expression of V1R1 or V1R4 in the lamellae of Lampetra fluviatilis. In situ* hybridisation of V1R1 and V1R4 with an NBT/BCIP staining in the olfactory epithelia of *Lampetra fluviatilis*. Scale bar A+B) 50µm

3.2.2 Expression of V1R2 in *Lampetra fluviatilis* OE

The next V1R receptor which was identified in the genome of *Lethenteron camtschaticum* was named V1R2. For this receptor also, three primer pairs were constructed and all of them were successfully used in a PCR reaction. With the sequenced templates an in *vitro* transcription was carried out, resulting in three probes. The probes were used with the standardized ISH protocol (2.6.3), followed by a chromogenic staining with NBT/BCIP, where nearly all cells in the olfactory and non-olfactory area were stained. The tips of each lamella and the apical layer in the sensory area showed no staining and formed a contrast to the cells underneath. At the beginning of the A2c expression zone and in the non-olfactory sensory area, all cells from basal to apical were stained. Also, the cells in the outer shell showed a dark staining. The V1R2-positive cells have a small egg-shaped to roundish cell body and from basal to apical four to six cells in a row were stained (Fig. 3.45). To investigate if the complete apical cell layer is stained, a fluorescent ISH together with a nucleus staining (DAPI) was performed.

A

V1R2 amino acid sequence *of Lethenteron camtschaticum*

MLSPALSPSHTHTHALSRSLSLCTASMLLTVLFVVLTVLGVCGNSAVLVAFSQLRRPSPADVVVIG
MSATHLLSSVFKNTPYVLASLDSLALDDGACKALLYSVNVLRSCSLAFTVILSALQLARVEPRGRP
RRLSAALSRLLSGRRGGGAARLVGATCALCALASAPLLALDVRSRGDGRAGGFLHISGCVVTPSDG
AAAFLEASNVLDVAALATLLAANALVLGFVLRHRRLVARNRGWVGGAGNAAALNAAKVIACLMVVA
FVCWGVNVCMRFTVVQRLSAEWTSTQADVRAFFTSVYYSLSPFIIIFGRSSIRGKVARAVRRCPCV
GPQRTRVLRLAVVDGGRGNRVWPGVAAR

B

V1R2 nucleotide sequence *of Lethenteron camtschaticum*

ATGCTCTCTCCCGCTCTCTCTCCCTCACACACACACACGCATGCTCTCTCCCGCTCTCTCTCGCTG
TGCACGGCCAGCATGCTGCTGACCGTGCTCTTCGTGGTGCTCACCGTCCTCGGCGTGTGCGGCAAC
TCGGCCGTGCTCGTCGCCTTCTCGCAACTGCGCCGCCCGTCTCCCGCCGACGTGGTGGTCATCGGC
ATGTCAGCGACGCACCTGCTCTCGTCCGTCTTCAAGAACACGCCGTACGTGCTGGCGTCGCTCGAC
TCGCTGGCGCTGGACGACGGCGCCTGCAAGGCGCTCCTCTACTCCGTCAACGTCCTGCGCTCCTGC
AGCCTCGCCTTCACGGTGATCCTCAGCGCGCTGCAGCTCGCGCGAGTCGAGCCCCGCGGCCGCCCG

CGCCGCCTCTCCGCCGCGCTCTCGCGCCTCCTGTCGGGCCGGCGCGGCGGCGGCGCCGCCCGCCTC
GTGGGGGCCACGTGCGCCCTCTGCGCGTTGGCGTCGGCGCCGCTGCTGGCGCTCGACGTGCGCTCG
CGAGGCGACGGGCGGGCCGGCGGCTTCCTGCACATCTCGGGCTGCGTCGTGACGCCCTCCGACGGC
GCCGCGGCCTTCCTGGAGGCCTCCAACGTCCTCGACGTGGCGGCGCTCGCCACCCTCCTCGCCGCC
AACGCGCTGGTGCTCGGCTTCGTGCTGCGGCACCGGCGGCTGGTGGCGCGGAACCGGGGCTGGGTC
GGGGGCGCCGGCAACGCGGCCGCCCTCAACGCGGCCAAGGTGATCGCGTGCCTCATGGTCGTGGCG
TTCGTCTGCTGGGGCGTCAACGTGTGCATGCGCTTCACCGTCGTGCAGAGGTTGAGCGCCGAGTGG
ACGAGCACGCAGGCCGACGTGCGCGCCTTCTTCACCTCCGTCTACTACTCCCTCAGCCCCTTCATC
ATCATCTTCGGGCGCTCGTCCATCCGCGGGAAAGTCGCCCGCGCCGTCCGCAGGTGCCCGTGCGTC
GGCCCGCAGCGCACGCGGGTCCTGCGGCTGGCGGTGGTGGACGGGGGGAGGGGCAACAGGGTGTGG
CCGGGGGTGGCGGCGCGCTGA

Figure 3.44: V1R2 amino acid and nucleotide sequence of *Lethenteron camtschaticum.* A) Amino acid sequences of V1R2 from *Lethenteron camtschaticum* based on search with *Danio rerio* amino acid sequence blasted at www.ncbi.nlm.nih.gov B) Nucleotide sequence of V1R2 from *Lethenteron camtschaticum* ; Primers: V1R2 A (green); V1R2 B (orange); V1R2 C (blue)

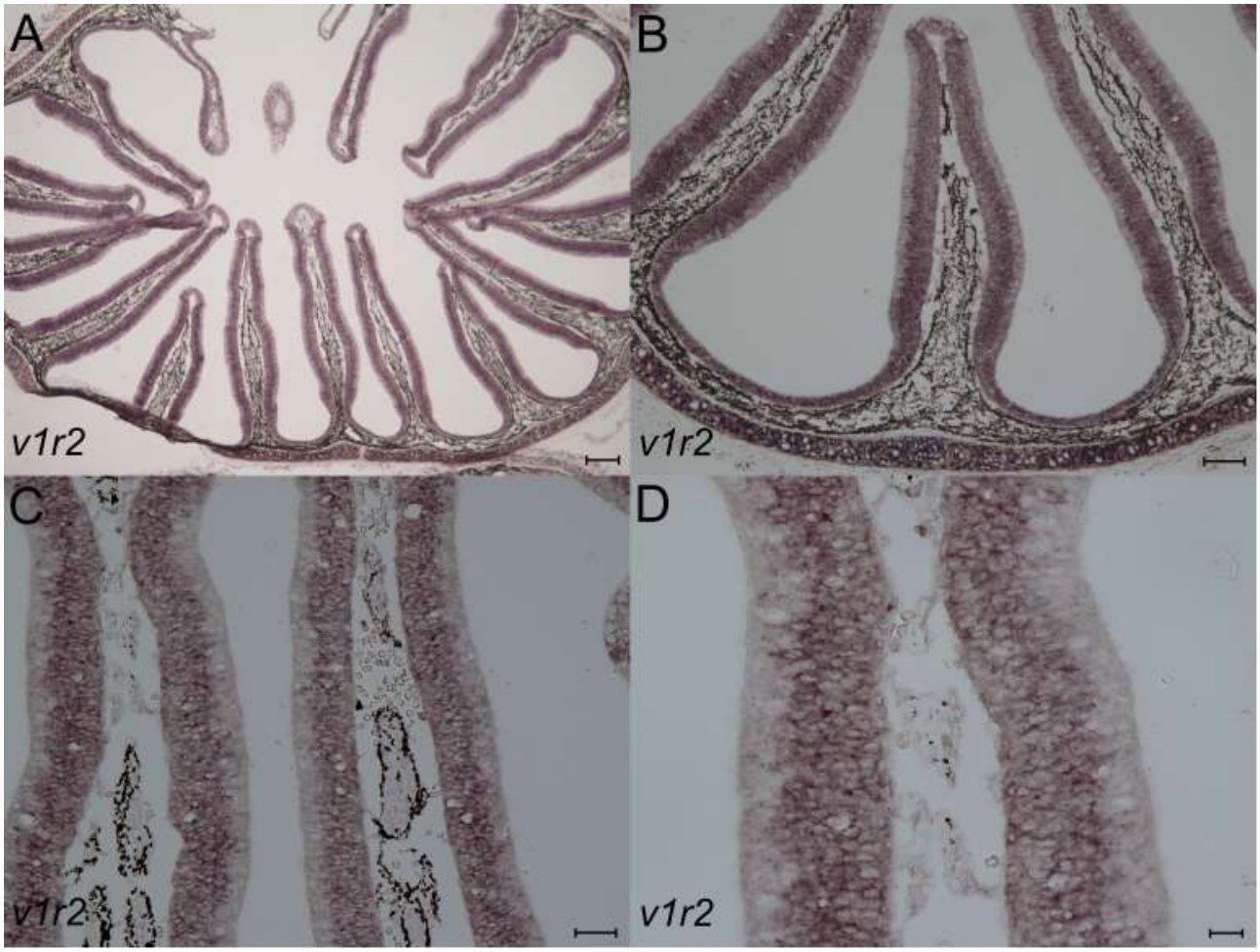

Figure 3.45: V1R2 (NBT BCIP staining) is expressed in the olfactory epithelia of *Lampetra fluviatilis.* A)-E) NBT/BCIP stainings of V1R2 in the olfactory and nonolfactory area, excluding the appical part of both areas. Scale bar A) 200µm B) 100µm C) 50µm D) 10µm

As observed in the NBT/BCIP staining (Fig. 3.45),the expression of the V1R2 probe is not only in the olfactory and non-olfactory areas of the lamellae, it is also in the cells of the outer shell. But mostly the apical cells in the lamellae are labelled for V1R2, whereas the bas-

al cells showed a weak or no staining. Also, after the fluorescent ISH, the positively-stained cells showed a roundish to egg-shaped structure (Fig. 3.46). After these results, the probes were sequenced and the resulting sequence was checked with tblastN for cross reactivity with other genes. (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The cross-reactivity result in the genome of *Petromyzone marinus* (kPetMar.pri) reveals that the nucleotide sequence of the used probe hits only an “olfactory receptor class A-like protein".

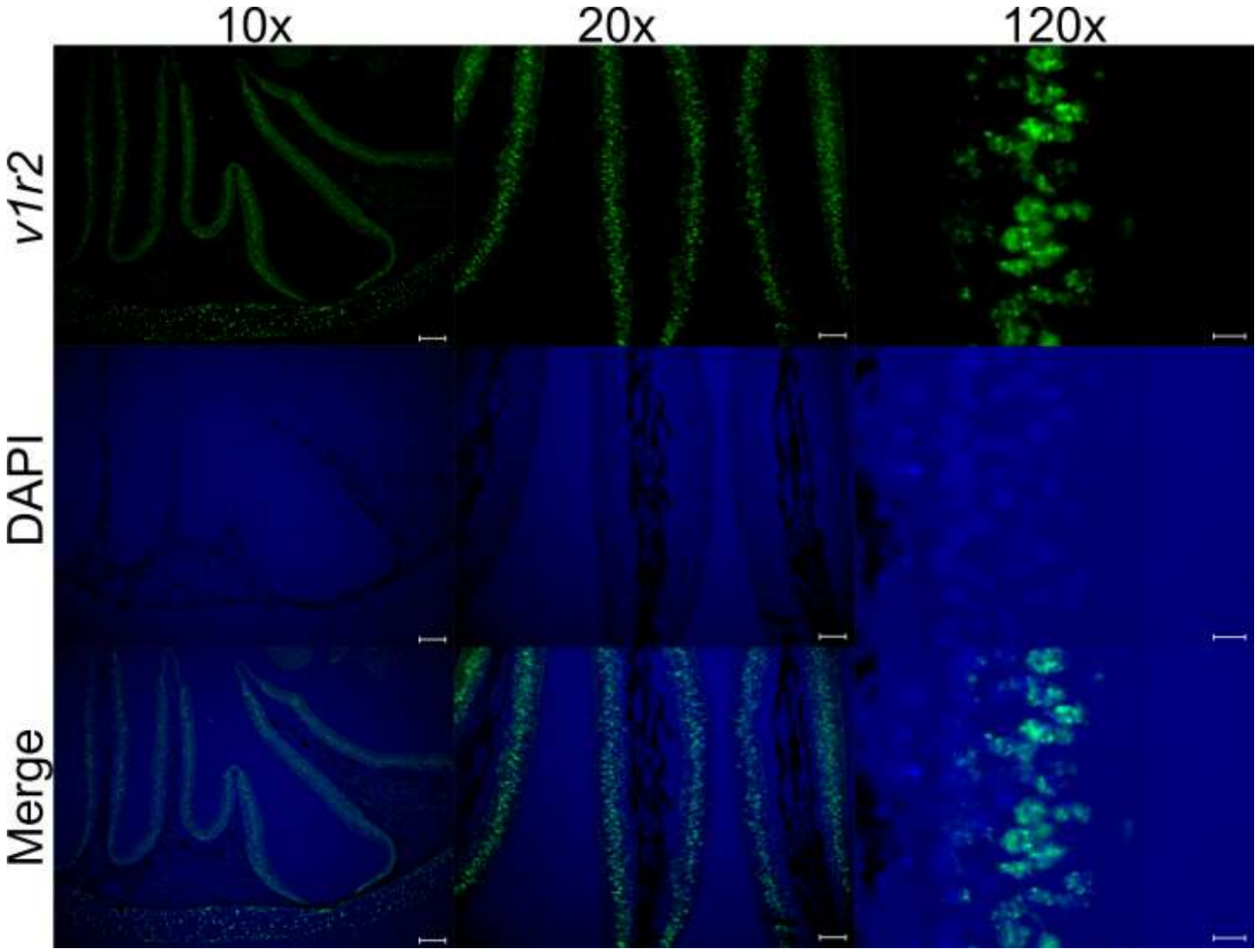

Figure 3.46: V1R2 expression (TSA staining) in the olfactory epithelia of *Lampetra fluviatilis.* V1R2 was visualized with TSA Alexa Fluor 488. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

3.2.3 Expression of V1R3 in *Lampetra fluviatilis* OE

The fourth of the six identified V1Rs was V1R3. It is the only V1R where only one primer pair was generated, because the resulting probe (Fig 3.47 B), after the *in vitro* transcription from the template, showed a clear staining after the ISHs. Five to ten single cells were stained per lamella in the olfactory a*rea of Lampetra fluviatilis* with the V1R3 probe after an

NBT/BCIP staining, but no cells could be found in the non-olfactory area or the tips of the lamellae. The stained cells had a roundish to egg-shaped cell body with a cap-like structure and nearly all cells are situated, in terms of height, in the medial part of the lamellae. And at the cap-formed tip, apical of the cells fin structures were visible, which look like dendrites (Fig. 3.48). For closer investigation of the expression pattern of V1R3, a fluorescent *in situ* hybridisation followed. The amount of stained cells, was the same as counted after the

A

V1R3 amino acid sequence *of Lethenteron camtschaticum*

MSSSSNLLLLLFVLCYTVSSSATSMSPPTRRHSPPPTLPPRPPLCPPAPPTLTSSSFSPSSRAGNG
EGPDDTAASTMNTEAMLLVIIHSVITAFGVVVNALILVVIANTACAYECLPPSDAILVNLAFVNLL
ISVVRNGPLLFWDAGVRMSLSDIGCKLCMGVWVVTRSVSVWTTLTLSMFHYLMVSRRHTSVKGKTS
ALRKAALTLLCVWAANVLFSLPAVVFSGNSAGNFTERLMLVSGTVRPLLGCVLRFASAREGLAYTT
ASMIINEIVPIVLMVTVNIKTLLILHRHRRQLHVASLMPSRVSTEVKAAKVIMVVVTIFVVCWGIH
VFAINYYNYRQD

B

V1R3 nucleotide sequence *of Lethenteron camtschaticum*

ATGTCATCATCCTCCAACCTCCTCCTCCTCCTCTTTGTACTCTGTTACACCGTCTCTTCCTCGGCC
ACCTCTATGTCTCCTCCGACTCGTCGTCATTCTCCTCCTCCTACTCTCCCTCCTCGTCCTCCTCTC
TGTCCTCCTGCTCCTCCTACTCTGACCTCCTCCTCCTTCTCTCCATCCTCCCGCGCAGGCAACGGT
GAGGGACCAGATGACACGGCGGCATCCACGATGAACACCGAGGCAATGCTCCTCGTCATCATCCAC
AGCGTGATCACGGCATTCGGCGTGGTGGTGAACGCGCTGATCCTGGTGGTGATCGCCAACACGGCG
TGCGCCTACGAGTGCCTGCCGCCCTCCGACGCAATCCTCGTCAACCTGGCGTTCGTCAACCTCCTC
ATCAGCGTGGTGCGCAACGGGCCGCTCCTCTTCTGGGACGCGGGCGTGCGCATGTCGCTGAGCGAC
ATCGGCTGCAAGCTGTGCATGGGCGTCTGGGTGGTGACGCGCTCCGTGAGTGTGTGGACCACGCTG
ACTCTGAGCATGTTCCACTACCTGATGGTGAGCAGGCGTCACACGAGCGTCAAGGGCAAGACGTCC
GCCCTGCGCAAAGCGGCGCTCACGCTGCTCTGCGTGTGGGCTGCCAACGTGCTCTTCAGCCTGCCC
GCTGTGGTCTTCTCCGGCAACAGCGCGGGCAACTTCACCGAGCGGCTCATGCTCGTGAGCGGGACC
GTTCGCCCGCTGCTCGGCTGCGTGCTCCGCTTCGCCAGCGCACGCGAGGGCCTCGCCTACACCACC
GCGTCGATGATCATCAACGAGATCGTGCCCATCGTGCTCATGGTGACGGTGAACATCAAGACGCTG
CTCATCCTCCACCGGCACCGGCGGCAACTGCACGTGGCCAGCCTCATGCCGTCGCGCGTCTCCACC
GAGGTGAAGGCGGCCAAGGTGATCATGGTGGTCGTGACCATATTCGTGGTCTGTTGGGGCATCCAC
GTGTTTGCGATCAACTACTACAACTACCGGCAAGAC

Figure 3.47: V1R3 amino acid and nucleotide sequence of *Lethenteron camtschaticum.* A) Predicted amino acid sequences of *Lethenteron camtschaticum* V1R3 based on search with *Danio rerio* amino acid sequence B) Corresponding nucleotide sequence of *Lethenteron camtschaticum* V1R3 with primers: V1R3 A (yellow)

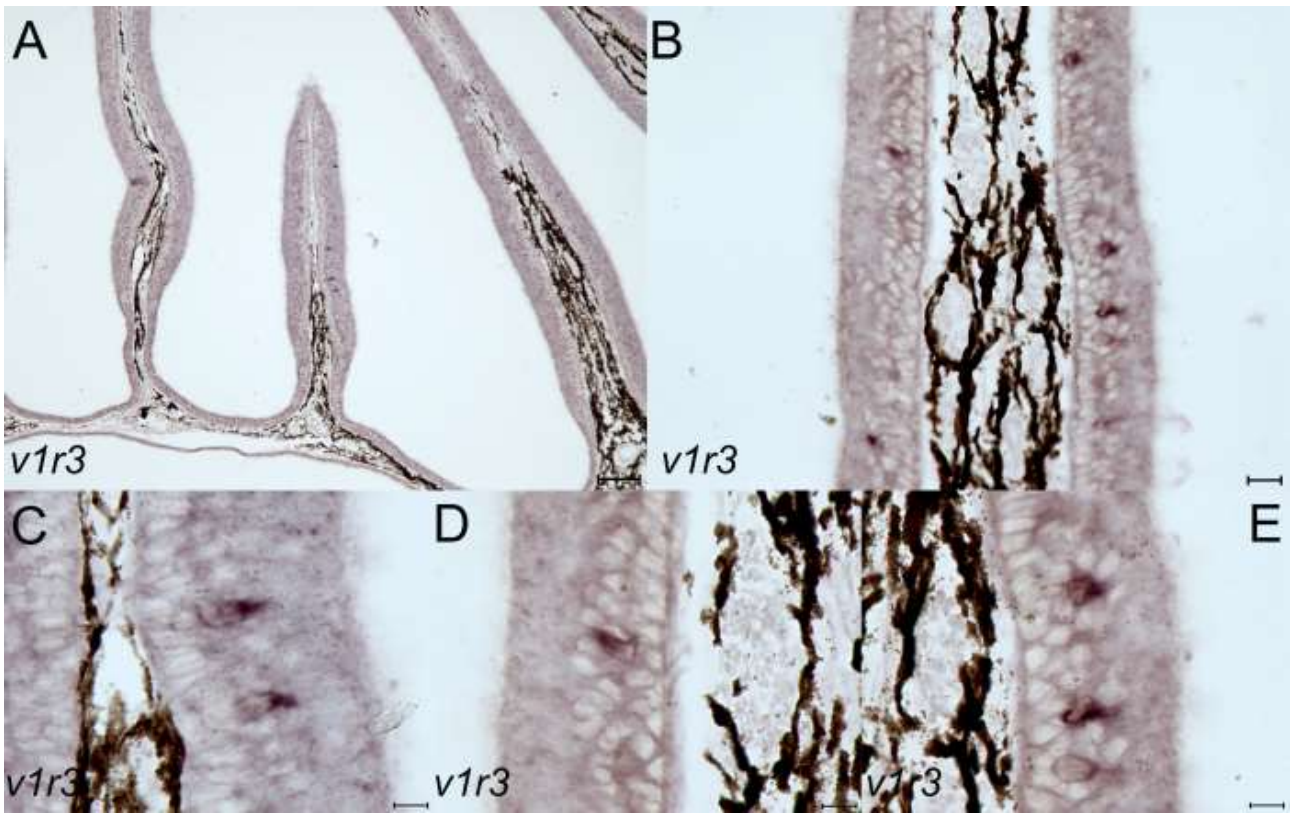

Figure 3.48: V1R3 (NBT/BCIP staining) is expressed in the olfactory epithelia of *Lampetra fluviatilis.* A)-E) NBT/BCIP stainings of V1R3 in the olfactory sensory area of *Lampetra fluviatilis*. Scale bar A) 200µm B) 100µm C) 50µm D) 10µm

NBT/BCIP staining, but the observed cell shape was mostly elongated. Also, at the apical side of the cell a fine structure was visible, which looked like the starting of an dendrite. Because of the DAPI staining, it could be shown that V1R3 is localized in the medial parts of the olfactory area, which exclude the tips and the non-olfactory area (Fig. 3.49).

For a closer characterisation of the single stained V1R3, different cell markers were used as described above (3.1.4).

At first a double staining of V1R3 ISH and actin IHC was done, with a clear staining of V1R3-positive cells, as described above. The actin-antibody staining was mostly randomly located at the most basal cells around their nucleus and sometimes bigger actin positive structures in the medial part of the lamellae were observed. Some weaker stainings at the apical layer, above the last nuclear layer could be identified, but no double labelling between V1R3 and actin was detected (Fig.6.6).

Because of the clear double labelling of HuC and G_{olf} (Fi3.32) and to investigate if V1R3 is localized in HuC-positive cells, a double labelling of V1R3 ISH and HuC IHC was performed. The result was a yellow staining, which means that all positive labelled cells of

V1R3 are co-labelled with the HuC-antibody. Acetylated tubulin, which is a stabilizing part of the cilia in ciliated OSNs, was also investigated in a double labelling experiment with V1R3. Therefore a V1R3 ISH was performed, followed by an IHC with an acetylated tubulin antibody. The localization of acetylated tubulin was above the apical layer of DAPI-stained nucleus and only missing at the tips of the lamellae. The stained dendrites of the cell bodies of V1R3 did not full reach the acetylated tubulin-positive stained layer (Fig. 3.51) in the lamellae. In some cases it looked as if the V1R3 cells were connected to the acetylated tubulin layer, because fine dendrite-like structures, which were stained by acetylated tubulin antibody were close to the tip of the V1R3-positive cells, but a clear double staining was never observed (Fig 6.7).

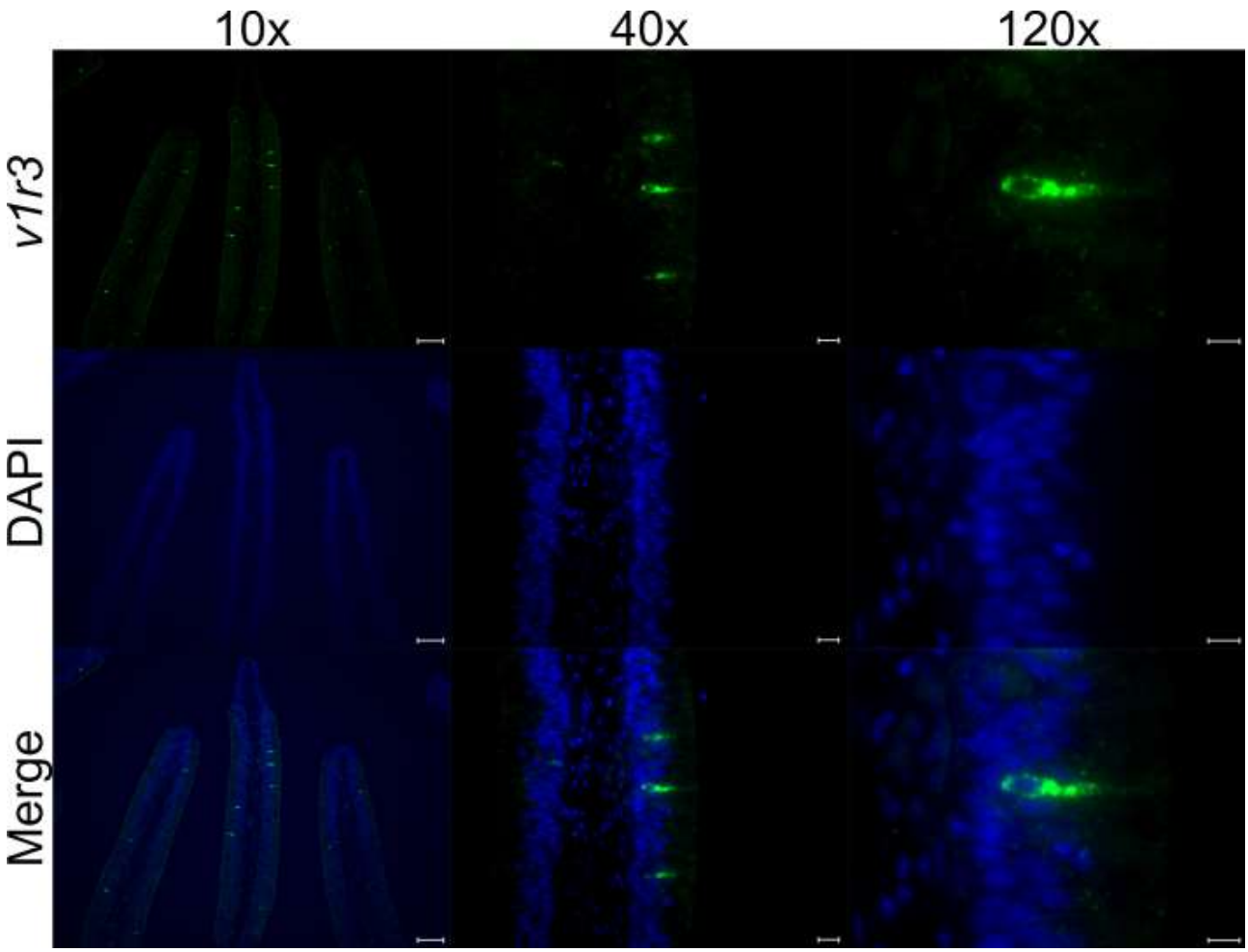

Figure 3.49: V1R3 (TSA staining) expression in the olfactory epithelia of *Lampetra fluviatilis*.V1R3 was visualized with TSA Alexa Fluor 488. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

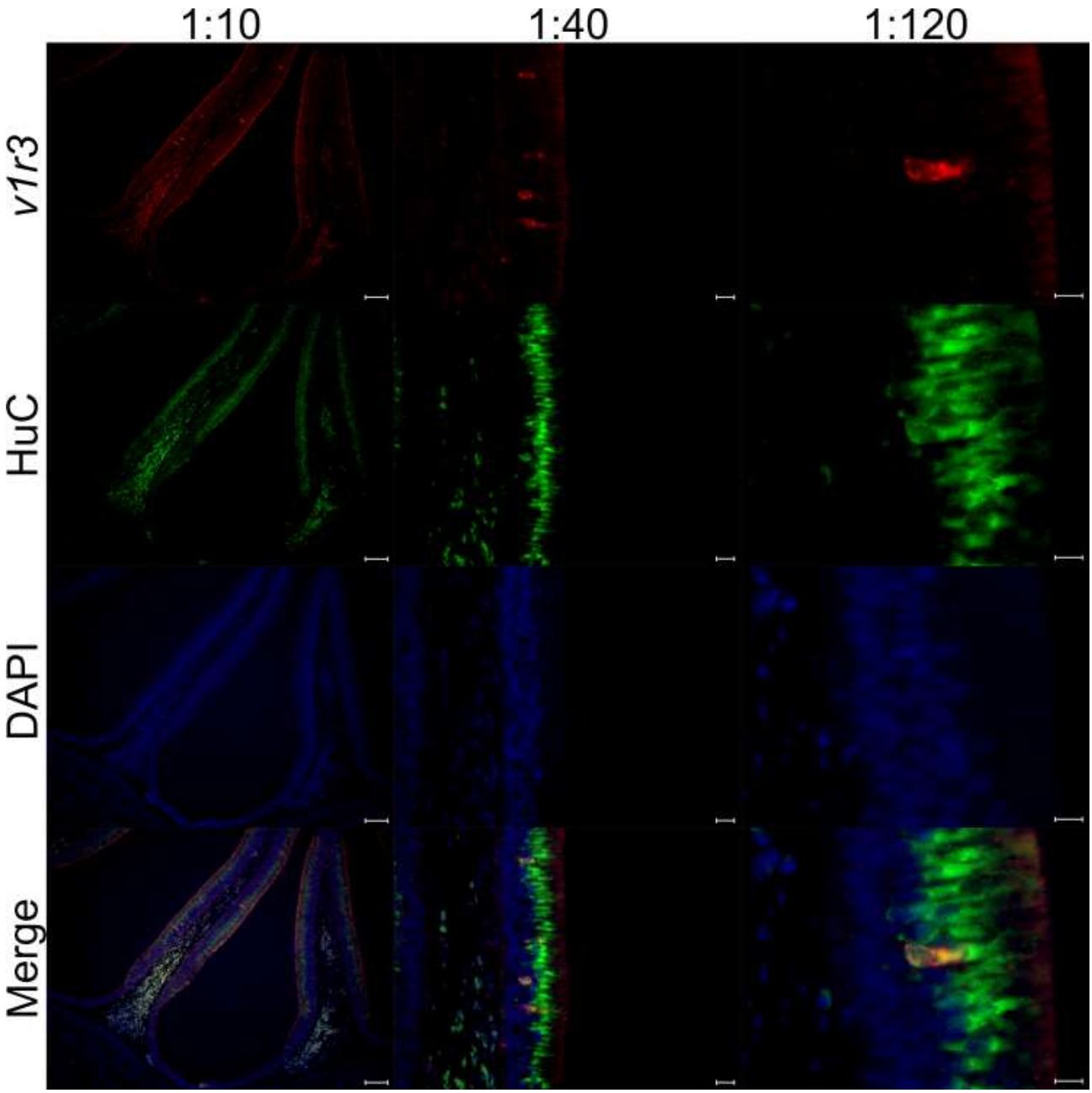

Figure 3.50: V1R3 is co-expressed with the neuronal marker HuC in the olfactory epithelia of *Lampetra fluviatilis* Fluorescent double staining of V1R3 (ISH) and HuC (IHC) in the olfactory epithelia of *Lampetra fluviatilis*. V1R3 was visualized with TSA Alexa Fluor 633 and HuC with an anti-mouse Alexa Fluor 488 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

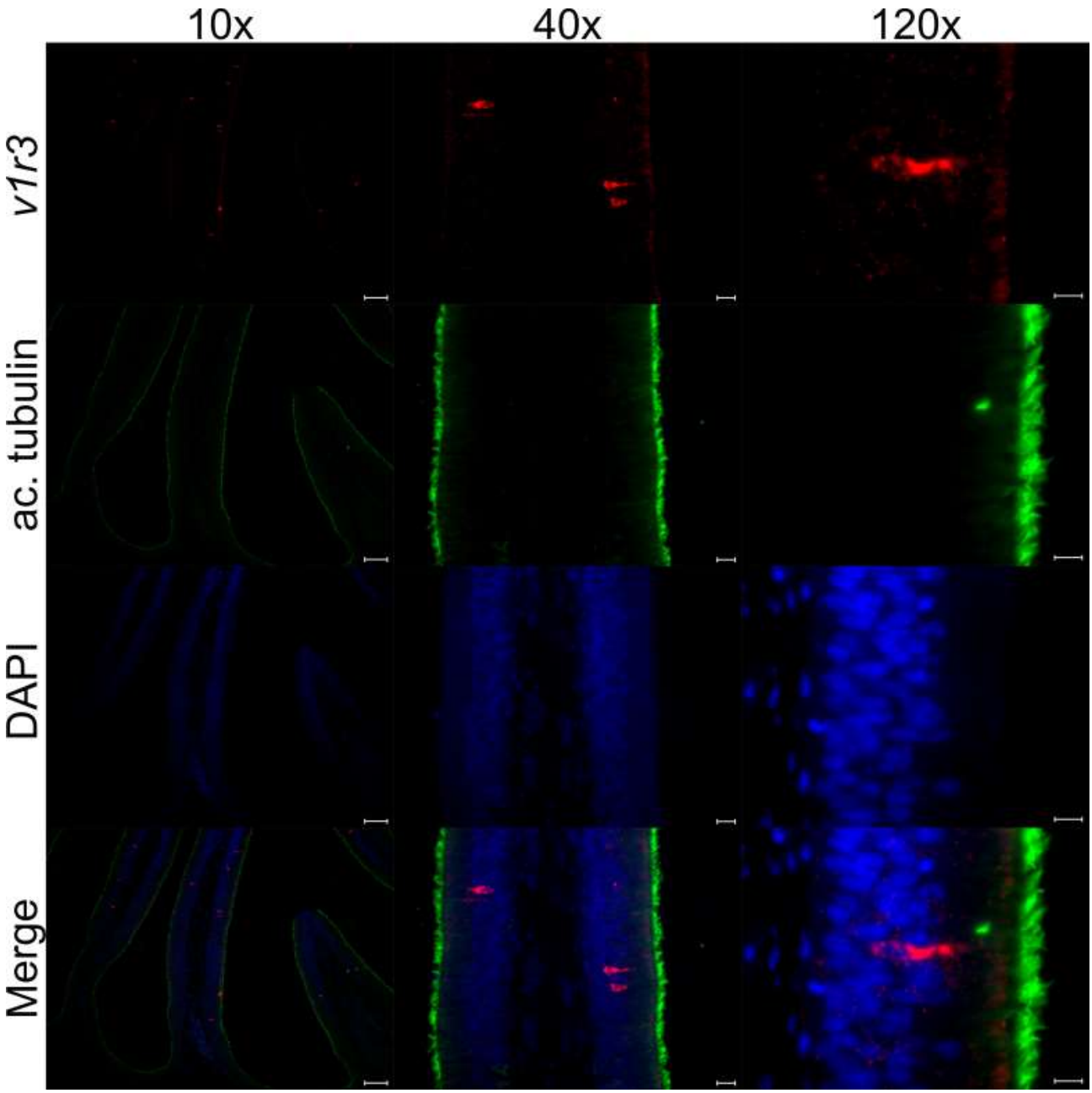

Figure 3.51: Apical localization of acetylated tubulin immunostaining does not overlap with cytoplasmic V1R3 *in situ* hybridization signal. Fluorescent double staining of V1R3 (ISH) and acetylated tubulin (IHC) in the olfactory epithelia of *Lampetra fluviatilis.* V1R3 was visualized with TSA Alexa Fluor 633 and acytelated tubulin with an anti-mouse Alexa Fluor 488 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

3.2.4 Expression of V1R5 in *Lampetra fluviatilis* OE

The next V1Rto be found in the genome of *Lethenteron camtschaticum*, was named V1R5. The first primer set V1R5 A showed no product after the PCR with the cDNA of *Lampetra fluviatilis*, whereas with the primer set V1R5 B and C PCR, products of the right size were

received. After the verification of the nucleotide sequence *via* sequencing, probes for both working primer sets were made (Fig 3.52). But only product for the primer set C was successfully used in the ISH staining. After performing the NBT/BCIP ISH,with the probe of the V1R5 primer set C, approximately three to five cells in the olfactory area of a lamella were stained. The V1R5-positive cells, in terms of height, are situated in the medial part of the lamella. They had a roundish to egg-shaped cell body with a long dendrite, reaching the apical border of the lamella. In the tips and the non-olfactory area no V1R5-positive labelled cells were observed (Fig. 3.53). Because of limiting OEs of *Lampetra fluviatilis* in the laboratory, only a single fluorescent *in situ* was done, with the same the results observed in the NBT/BCIP staining (Fig. 3.54).

A

V1R5 amino acid sequence *of Lethenteron camtschaticum*

MLPPQFRNFLFYGLLALGGLLGNTTVMAVTLQSLSKLPTADLLFVLLATCNLVNNLLRLGTSLSEA
TGTCLLTRAGCPLYYFTVRFTEVLAIWITLLVSVFRLAKVRSPFGVNAILGRMMEQRRCAVAIGSV
SAVLGALLHPLLQFTIPFDGNGSVPWCQCIIEPSSYYIRYSMSTVLLIFLEVLPTVLMVVTNVRTI
CFLRATKVQWQQGGWATSRPFCQEVRAVKLLSALVCVFLFSWGSHATSIVLVTSNPATVNPQLVQA
VRILSTMYRFPEPVHHRLGQPEVSRSSFWLHPKLPQVSRSEAHGGCRPVRPNSPLMRSEKEYNDGN

B

V1R5 nucleotide sequence *of Lethenteron camtschaticum*

ATGCTGCCACCGCAGTTCAGGAACTTCCTTTTCTACGGGCTGTTGGCCCTGGGCGGTTTGTTGGGG
AACACAACCGTCATGGCAGTGACTCTGCAGAGCCTCTCCAAGCTACCCACGGCCGACCTGCTCTTC
GTGCTGCTCGCCACGTGCAACCTCGTCAACAACCTCCTGCGCCTCGGCACCTCCCTGTCGGAGGCG
ACGGGAACGTGCCTGCTGACGCGCGCCGGCTGCCCGCTCTACTACTTCACCGTCCGCTTCACCGAG
GTGCTGGCCATCTGGATCACGCTGCTCGTCAGCGTGTTCCGCCTCGCCAAGGTGCGCAGCCCCTTC
GGCGTCAACGCCATCCTGGGCCGCATGATGGAGCAGCGCCGCTGCGCCGTGGCTATCGGTTCCGTG
TCCGCCGTGCTTGGCGCGCTGCTGCACCCGCTGCTCCAGTTCACGATACCTTTTGACGGCAACGGC
AGCGTGCCCTGGTGCCAGTGCATCATAGAACCCTCCAGCTACTACATCAGATACTCCATGTCCACC
GTCCTGCTGATCTTTCTGGAGGTTTTGCCCACGGTCTTGATGGTGGTCACCAACGTGAGGACCATC
TGCTTCCTGCGTGCCACCAAAGTGCAGTGGCAGCAGGGCGGCTGGGCCACGAGCCGGCCGTTCTGT
CAGGAGGTGCGAGCCGTGAAGCTGCTCTCCGCCCTAGTCTGCGTCTTCTTGTTCAGCTGGGGCAGC
CACGCCACCAGCATCGTGTTAGTCACGAGCAACCCGGCCACAGTGAACCCCCAGTTGGTGCAAGCC
GTCCGCATTCTCTCCACCATGTATCGCTTCCCTGAGCCCGTACATCATCGGCTTGGGCAACCAGAA
GTTTCGAGATCGAGTTTTTGGCTGCACCCAAAACTGCCTCAAGTGTCGCGCTCCGAAGCGCACGGT
GGCTGCCGTCCAGTACGACCTAACTCACCTCTGATGCGCAGCGAAAAGGAATACAATGATGGTAAC
TAA

Figure 3.52: V1R5 amino acid and nucleotide sequence of *Lethenteron camtschaticum.* A) Predicted amino acid sequences of *Lethenteron camtschaticum* V1R5 based on a search with *Danio rerio* amino acid sequence B) Corresponding nucleotide sequence of *Lethenteron camtschaticum* V1R5 with primers: V1R5 A (yellow); V1R5 B (green); V1R5 C (pink)

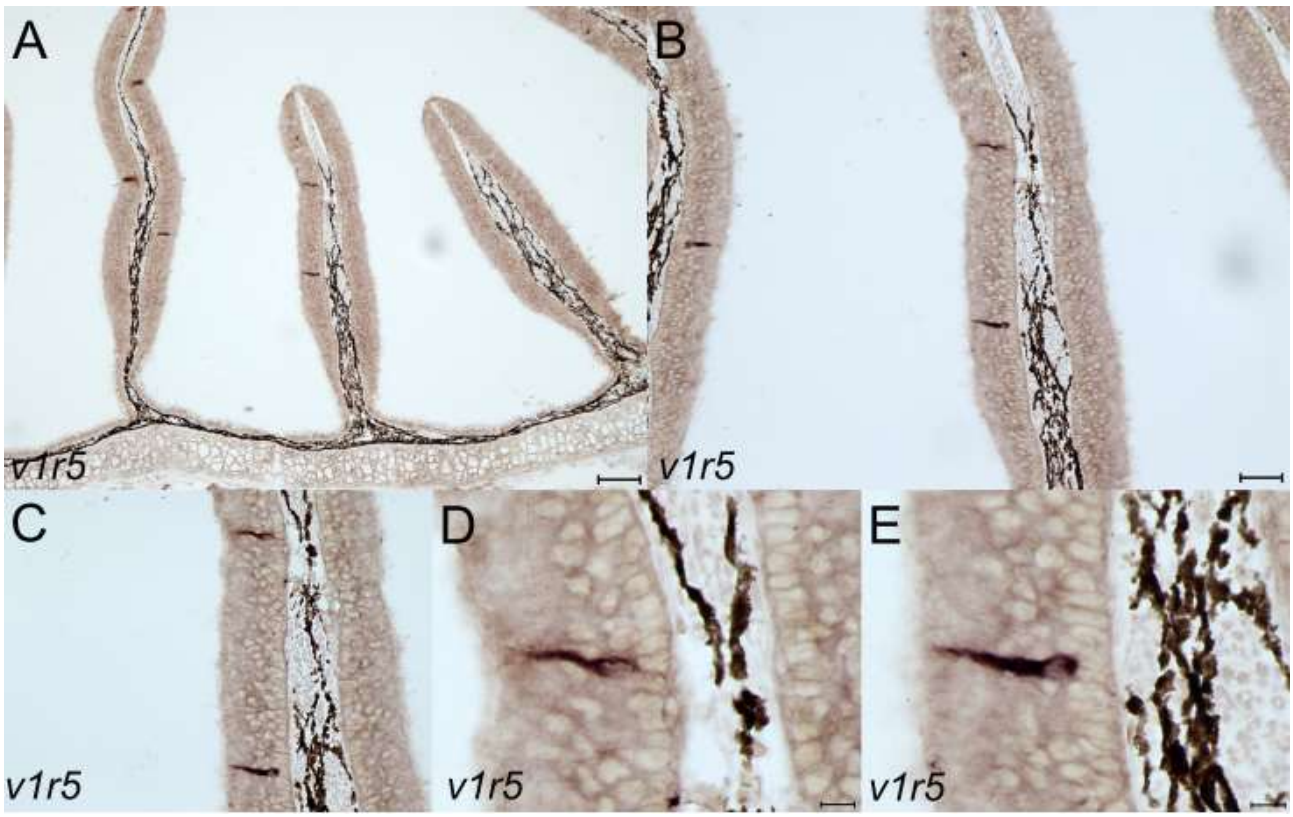

Figure 3.53: V1R5 (NBT/BCIP staining) is expressed in the olfactory epithelia of *Lampetra fluviatilis.* A)-E) V1R 5 cells are sparsely expressed in the lamellae of *Lampetra fluviatillis*. NBT/BCIP stainings of V1R5 in the olfactory sensory areo of *Lampetra fluviatilis*. Scale bar A) 100µm B) 50µm C) 30µm D+E) 10µm

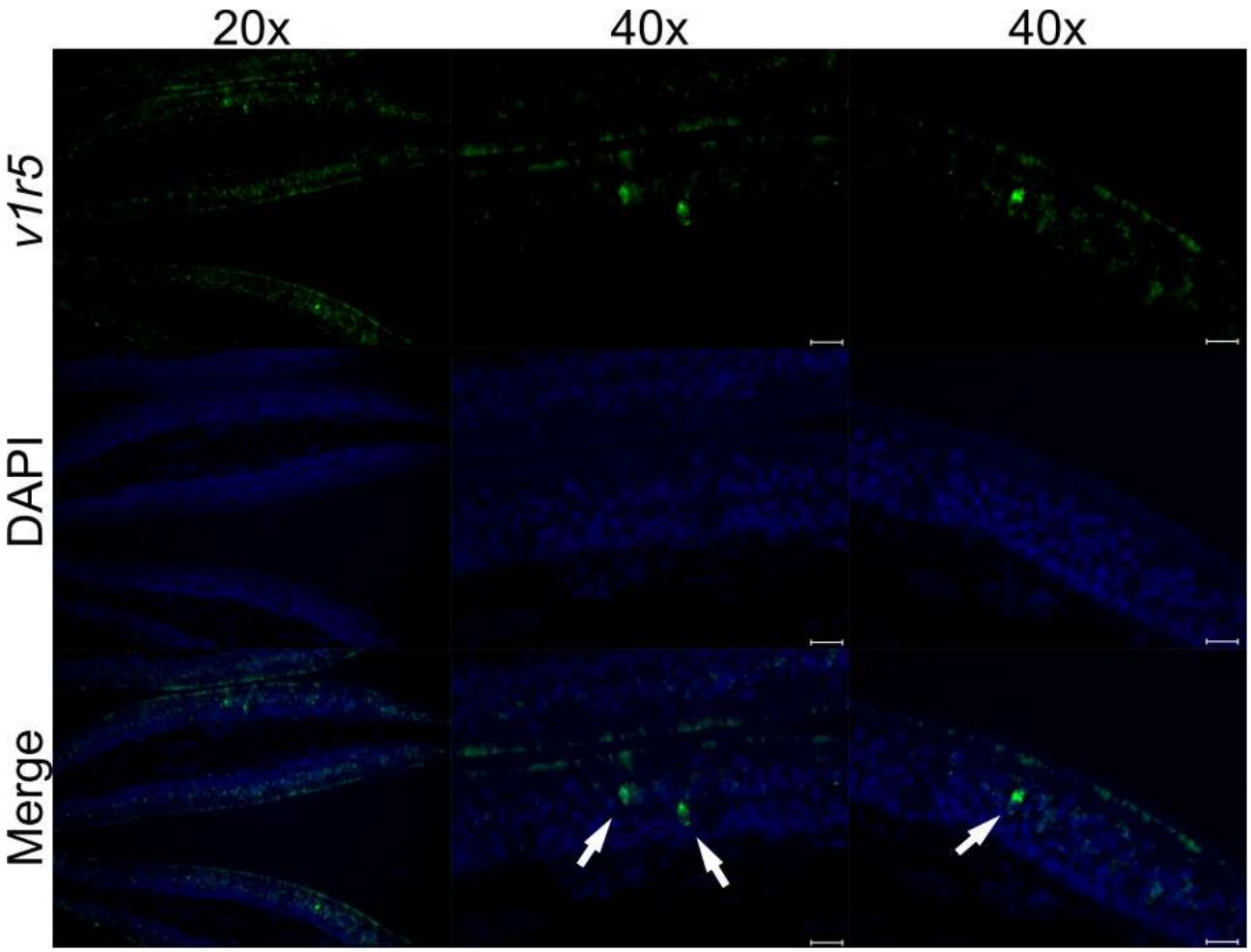

Figure 3.54: V1R5 expression (TSA staining) in the olfactory epithelia of *Lampetra fluviatilis.* V1R5-positive cells are expressed in the lamella of *Lampetra fluviatilis.* V1R5 was visualized with TSA Alexa Fluor 488 (white arrows). Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 20x) 50µm 40x) 20µm

3.2.5 Expression of V1R6 in *Lampetra fluviatilis* OE

The last of the V1Rs to be identified in *Lethenteron camtschaticum* was V1R6. For this gene two different primer sets, V1R6 A and B, were made (Fig. 3.55).

A

V1R6 amino acid sequence *of Lethenteron camtschaticum*

MNHTERAIVSLRILLWLASLLGNSLLLLSLLLLSGGLQTRRAKTAPSSAAGPSRSQRGRTFHLFLA
NLATANLVGTCLADMPDLVSIAMVEMTRPRDLEGDILTVVCPALKFASAVAETGAICFTLLICVYR
YQKLCGWPAFSGALRSAPLDQQRLARLCCLLAWATTVAFSLPSIFMGSEWEAPIILMPYSINSTSS
TFTNLTSSSFTNLSSSSSSSTNSSSLSMHRDTCPSDLFTCPKARRCAVSHMAYKYIYLLGTNVCPL
VVVFVASLRIVLTLMRSRRAAVKGPSSAEPSSGSVKTRGGSGPVLMALMLFEVSWCAHLALQFLAD
SDRLIYWSDADFFITGVYTSTSPYIFGIGSGLLPPKSPARATG

B

V1R6 nucleotide sequence *of Lethenteron camtschaticum*

ATGAACCACACCGAGAGAGCCATTGTGTCTCTGCGCATCCTGCTGTGGCTGGCCAGCCTGCTGGGC
AACTCGCTCCTCCTGCTCTCGCTTCTGCTGCTCAGCGGCGGGCTTCAGACGCGCAGGGCCAAGACC
GCGCCGAGCAGCGCCGCGGGGCCCAGCCGGAGCCAGCGTGGCCGCACCTTCCACCTGTTCCTCGCC
AACCTGGCGACGGCCAACCTGGTGGGCACGTGCCTCGCCGACATGCCGGACCTGGTGAGCATCGCC
ATGGTGGAGATGACGAGGCCCAGAGACCTCGAGGGAGACATCCTCACCGTCGTTTGCCCCGCGCTC
AAGTTCGCGTCGGCGGTGGCCGAGACGGGCGCCATCTGCTTCACGCTGCTCATTTGCGTCTATCGC
TACCAGAAGTTGTGCGGCTGGCCGGCGTTCAGCGGCGCCCTGCGGTCTGCACCTCTCGACCAGCAG
CGCTTGGCTCGCCTCTGCTGTCTCCTCGCCTGGGCCACCACCGTGGCCTTCTCACTCCCGTCCATC
TTCATGGGCAGCGAGTGGGAAGCGCCCATCATTCTGATGCCGTACTCTATCAACTCAACATCATCA
ACATTCACCAACCTAACATCGTCATCATTCACTAACCTATCATCATCATCATCATCATCCACCAAC
TCGTCGTCGTTGTCAATGCACCGAGACACGTGTCCGTCGGACCTCTTCACGTGCCCCAAGGCCCGC
CGCTGCGCTGTCTCCCACATGGCCTACAAGTACATCTACCTCCTGGGCACCAACGTCTGTCCCCTC
GTCGTGGTCTTCGTCGCGTCGCTGCGCATCGTCCTCACGCTGATGCGCTCTCGGAGGGCCGCGGTG
AAGGGGCCGTCCTCTGCCGAACCCTCCTCCGGAGGATCTGTCAAGACCCGCGGAGGTTCGGGCCCC
GTGCTGATGGCCCTGATGCTGTTCGAGGTGTCCTGGTGCGCCCACCTGGCGCTGCAGTTCCTGGCC
GACTCGGACCGCCTCATCTACTGGTCTGACGCTGACTTCTTCATCACGGGAGTCTACACCTCCACC
AGCCCCTACATCTTCGGCATCGGCAGTGGCCTTCTGCCGCCCAAATCTCCCGCCAGAGCCACCGGC

Figure 3.55: V1R6 amino acid and nucleotide sequence of *Lethenteron camtschaticum.* A) Predicted amino acid sequences of *Lethenteron camtschaticum* V1R6 based on search with *Danio rerio* amino acid sequence B) Corresponding nucleotide sequence of *Lethenteron camtschaticum* V1R6 with primers: V1R6 A (yellow); V1R6 B (green);

After getting positive and sequenced PCR products with both primer sets, the resulting probes were used for the ISHs. The ISH with an NBT/BCIP staining of V1R6, reveals three to twelve stained cells per lamella. The V1R6-positive cells are localized, in terms of height, mostly in the apical layer of the olfactory area and their cell body is egg-shaped with a dendrite reaching the most apical layer of the lamella (Fig. 3.56). The same results for V1R6 were observed after a fluorescent ISH (Fig. 3.57). To investigate if V1R6 is also co-labelled with HuC, a double staining of V1R6 ISH and HuC IHC was performed. The double staining revealed a co-labelling of all V1R6 cells with the HuC-antibody. To investigate if these V1Rs are localized in microvillious or ciliated neurons, a combination of V1R6 ISH and IHC with, either beta-actin antibody for microvillous OSNs or acetylated tubulin for ciliated OSNs, was done. The double staining of V1R6 ISH and beta-actin IHC revealed no clear results, because the beta-actin antibody stained different structures in the olfactory and non-olfactory area, which are mostly situated at the basal part of the lamellae, spanning several cells. The apical stained structures are smaller and not located at the same positions as the V1R6-positive cells (Fig. 6.8). The double staining of V1R6 probe together with the acetylated tubulin antibody, showed that cilia in the most apical

layer of the olfactory and non-olfactory area were stained, but no clear double staining of either was observed in the experiments (Fig. 3.59).

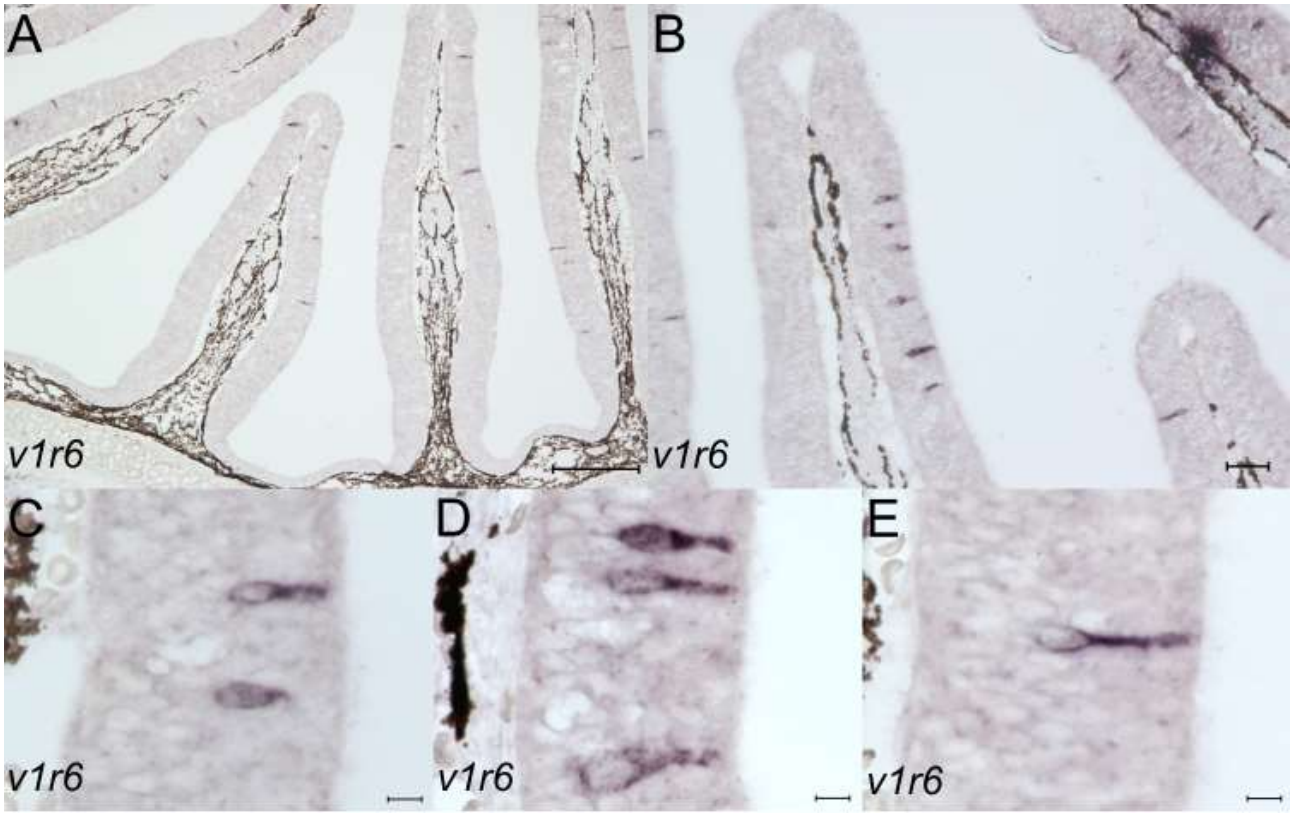

Figure 3.56:V1R6 (NBT/BCIP staining) is expressed in the olfactory epithelia of *Lampetra fluviatilis*. A)-E) Single V1R6 receptors expressed in the OE. NBT/BCIP stainings of V1R6 in the olfactory sensory area of *Lampetra fluviatilis*. Scale bar A) 200µm B) 100µm C) 50µm D) 10µm

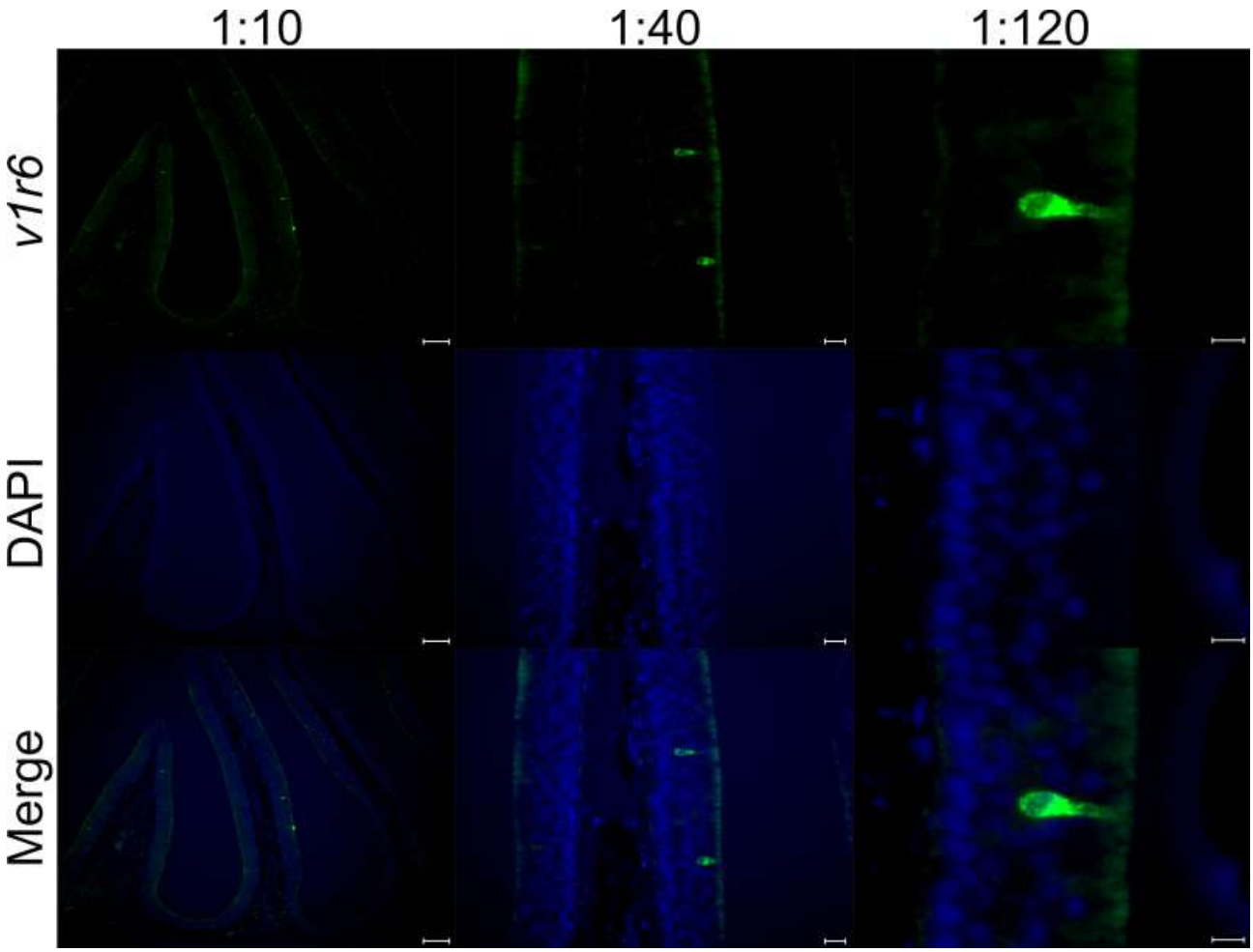

Figure 3.57: V1R6 (TSA staining) expression in the olfactory epithelia of *Lampetra fluviatilis.* V1R6 positive cells are localized in the apical layer of the lamella. V1R6 was visualized with TSA Alexa Fluor 488. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

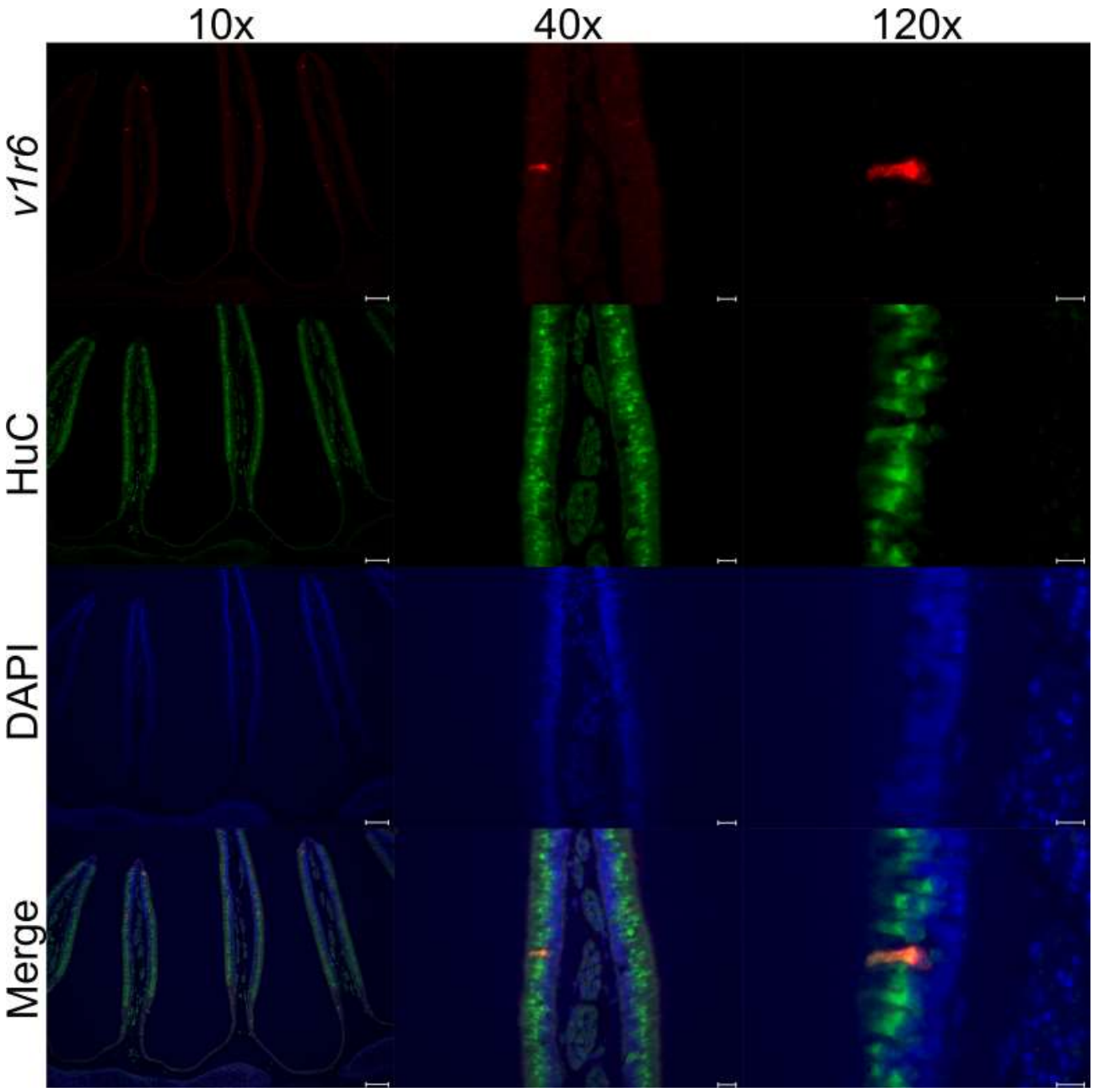

Figure 3.58: V1R6 is co-expressed with the neuronal marker HuC in the olfactory epithelia of *Lampetra fluviatilis*. V1R6 (red) is expressed in HC positive cells (green). Fluorescent double staining of V1R6 (ISH) and HuC (IHC) in the olfactory epithelia of *Lampetra fluviatilis*. V1R6 was visualized with TSA Alexa Fluor 633 and HuC with an anti-mouse Alexa Fluor 488 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

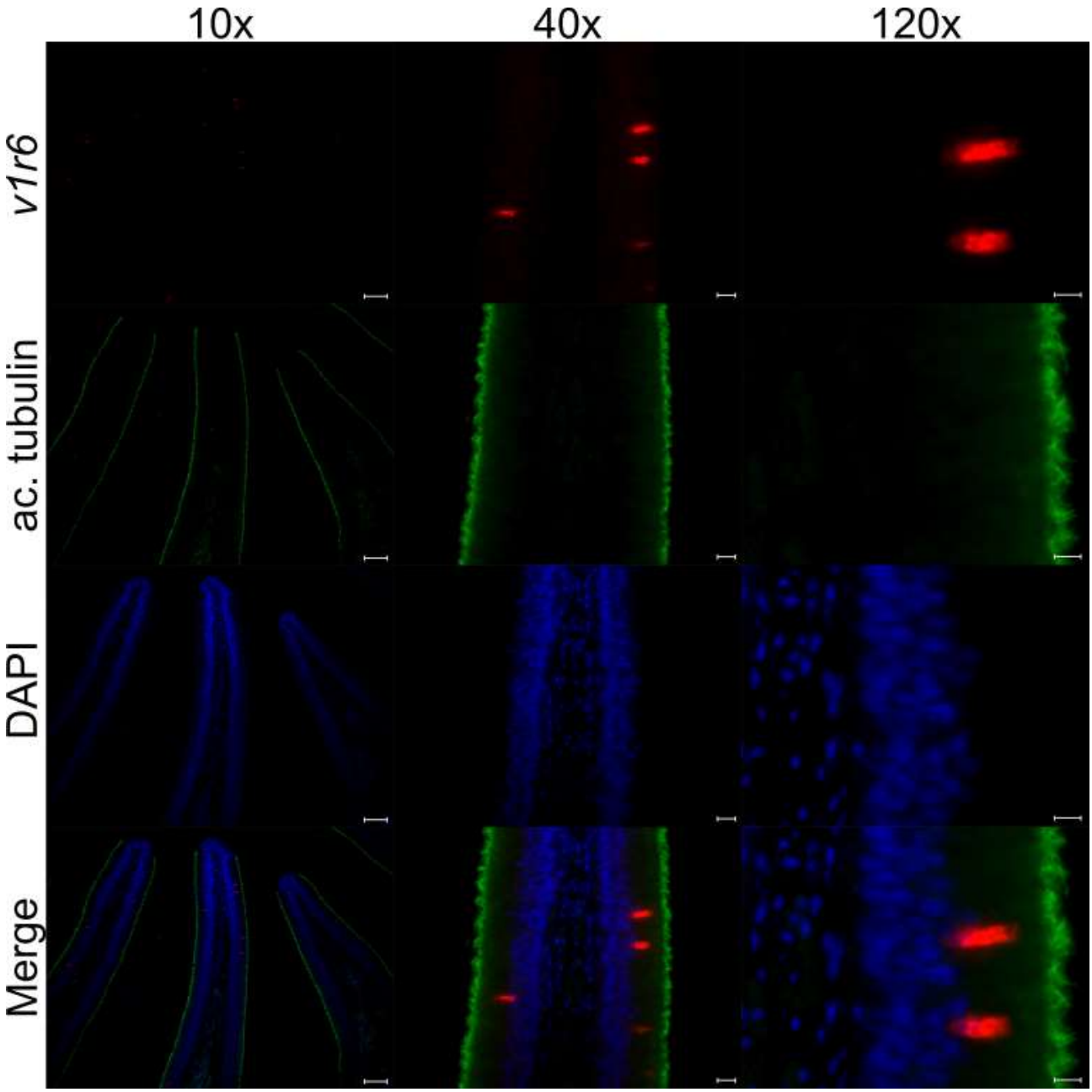

Figure 3.59: Apical localization of acetylated tubulin immunostaining does not overlap with cytoplasmic V1R3 *in situ* hybridization signal. V1R3 (red) and acetylated tubulin are expressed in different layers, no co-labelling was detected. Fluorescent double staining of V1R6 (ISH) and acetylated tubulin (IHC) in the olfactory epithelia of *Lampetra fluviatilis.* V1R6 was visualized with TSA Alexa Fluor 633 and acetylated tubulin with an anti-mouse Alexa Fluor 488 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

3.2.6 Different expression zones distinguishable in analysis of laminar height for different V1Rs in *Lampetra fluviatilis*

It is known that the height is characteristically different between all four known populations of olfactory sensory neurons in zebrafish, ciliated, microvillous, crypt and kappe neurons (Ahuja et al. 2014). To investigate if there are clear differences between the single V1Rs and/or between the V1Rs and ORs, an ISH with a further published OR (Berghard and Dryer, 2019), LOR3 was performed. The LOR3-positive OSNs are localized without exception in the olfactory area and have an egg-shaped structure where sometimes a dendrite at the apical site shows up (Fig. 6.9). The distribution of all four measured OSNs (V1R3 (n=322), V1R5 (n=196), V1R6 (n=247) and LOR3 (n=564)), are significantly different (tested with KS-test7, data not shown). V1R3, together with LOR3 receptor cells, are more apically localized than V1R5 and V1R6, resulting into two different expressing zones (Fig. 3.60).

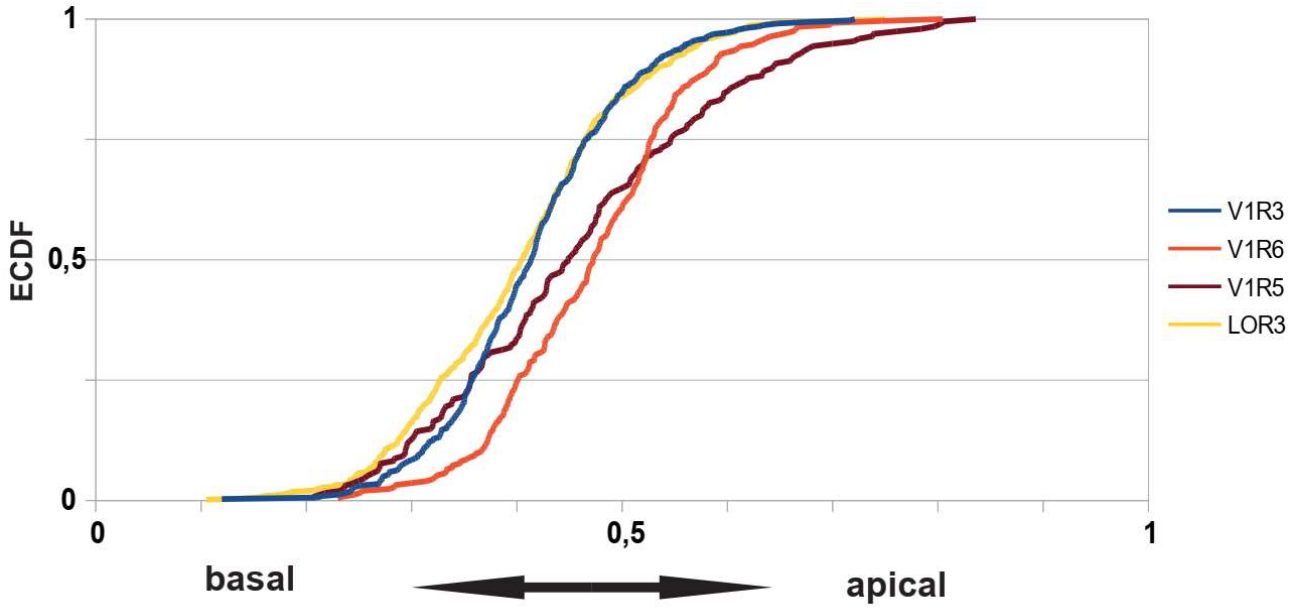

Figure 3.60: Quantitative assessment of laminar height distributions of three V1R and one OR of *Lampetra fluviatilis*. Laminar height of V1R-positive cells was quantified for three V1R receptors (V1R3 (n=322),V1R5 (n=196) and V1R6 (n=247)) and one OR (LOR3 n=564) of *Lampetra fluviatilis*. Height within the lamina was normalized to maximal laminar thickness. The resulting distributions of relative laminar height (from 0, most basal to 1, most apical, i.e. bordering to the lumen).

3.2.7 Analysis of the radial coordinates of three V1Rs in *Lampetra fluviatilis* reveals two different expression zones

The radial distribution of the three V1Rs and LOR3, revealed two different radial expression zones. Where LOR3, V1R3 and V1R5 are localized close to each other and not significantly different from each other (KS-test), V1R6 is significantly different to all others and localized more to the centre of the OE (Fig. 3.61).The KS-test reveals that only the radial distribution of V1R6 is significant different from all other tested receptors (Tab.6.2).

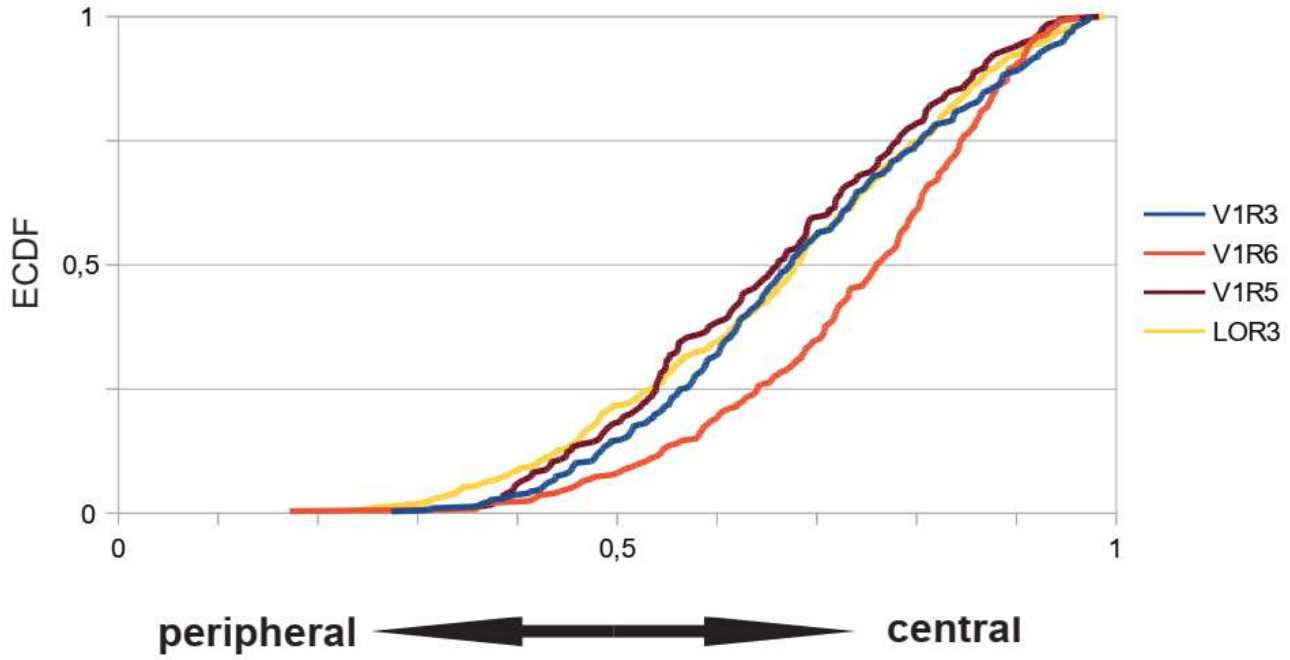

Figure 3.61: Quantitative assessment of lamellar radius distributions of three V1Rs and one OR shows two different expression zones in *Lampetra fluviatilis*. Laminar radius of four V1R receptors V1R3 (n=297), V1R5 (n=195), V1R6 (n=247) and LOR3 (n=563) were quantified for *Lampetra fluviatilis*. Radial position within the section was normalized to maximal radius, i.e. length of the lamella containing the respective labelled cell. (from 0, most peripheral to 1, most central).

3.2.8 Expression of V2R1 and V2R2 in the olfactory epithelia of *Lampetra fluviatilis*

Until today no V2R genes have been discovered or described in the genome of lampreys. During phylogenetic studies in our laboratory, two possible V2Rs were identified in the genome of *Lethenteron camtschaticum*, named V2R1 and V2R2. For each of these V1Rs

three to four different possible probes were chosen, based on the identified amino acid sequence (Fig 6.4 and 6.5) of the genes. But neither several chromogenic NBT/BCIP ISH (Fig.3.62) nor fluorescent ISH (data not shown) revealed any staining s in the olfactory epithelia of *Lampetra fluviatilis.* Only in the typical weak background staining chromogenic NBT/BCIP ISH was observed, which also developed in the negative control with water. In all experiments LOR3 was used as a positive control, and showed its typical expression pattern (Fig 6.9).

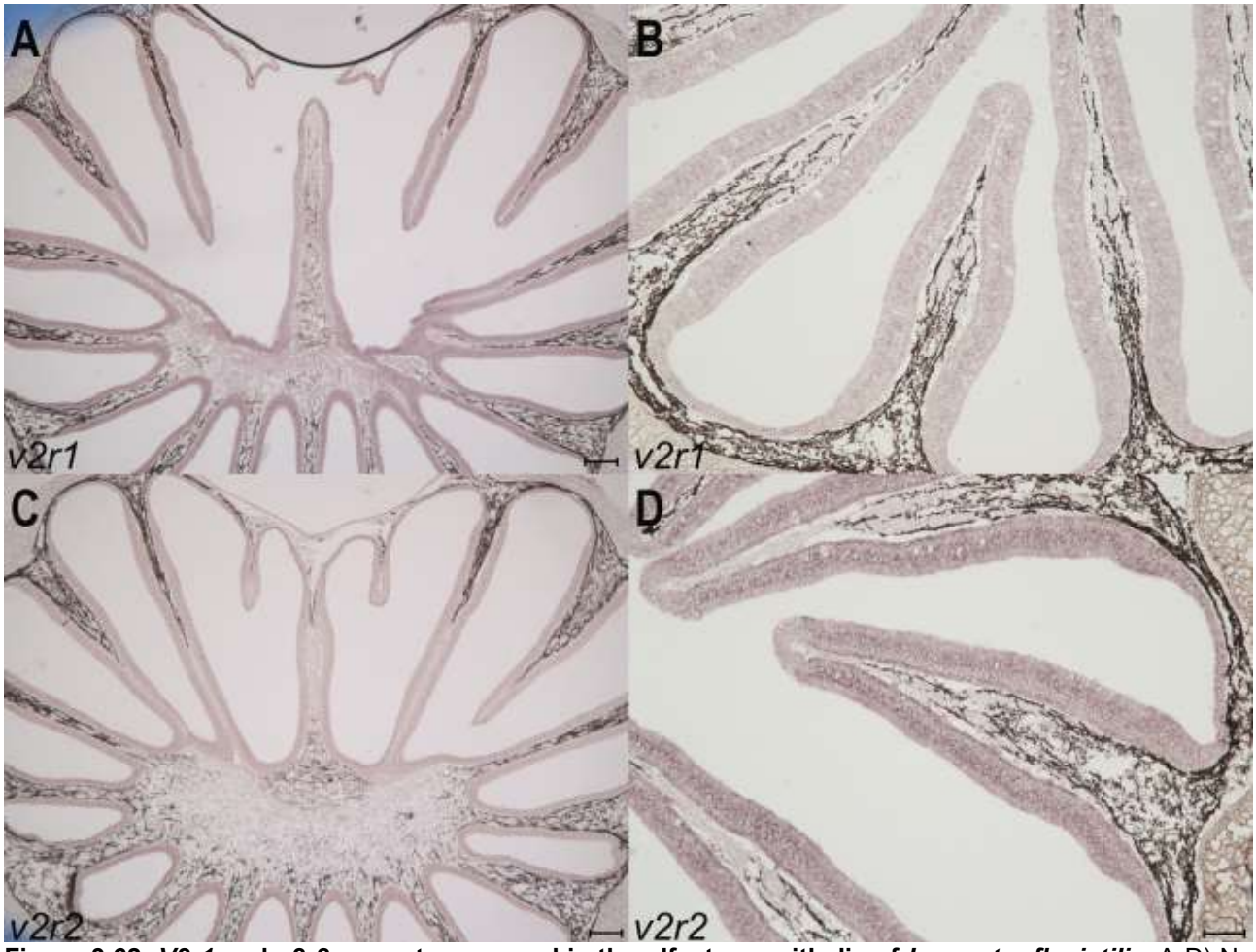

Figure 3.62: *V2r1* and *v2r2* are not expressed in the olfactory epithelia of *Lampetra fluviatilis*. A-D) No signal was detected for V2R1 or V2R2. *In situ* hybridisation of *v2r1* and *v2r2* with an NBT/BCIP staining in the olfactory epithelia of *Lampetra fluviatilis*. Scale bar A+C) 200 B+D) 100

3.3 Nested expression domains for Ora receptors in zebrafish olfactory epithelium

One of the questions which arise from the localization of olfactory receptors in the nose is, if there is a spatial expression pattern within the different gene families in one species. In

previous studies in zebrafish the spatial distribution of single odorant receptors of one gene family were shown to localize in a specific expression pattern, but never before a complete gene family been examined (Weth et al., 1996; Ahuja et al., 2018). The *ora* gene family with their six members, discovered by our laboratory in 1996 (Saraiva and Korsching,1997), offered the opportunity to investigate this question for a complete gene family for the first time.

3.3.1 Comprehensive analysis of spatial expression patterns for a complete olfactory receptor family

To investigate the complete expression of the *ora* gene family, with NBT/BCIP and TSA two different staining methods were used and compared with each other. This became necessary because two receptors, Ora5 and Ora6, showed a low expression in the OE and the visualization could not be achieved with the phosphatase-related NBT/BCIP staining, which was established in the laboratory. It was therefore necessary to establish a further staining method that improved the signal. The choice fell on the TSA method developed in earlier years, which amplifies the signal by depositing fluorophore-conjugated tyramide at the antigen binding site. This enables the detection of very rare targets in a cell.

At the beginning for Ora1-4 the NBT/BCIP method was used by Shahrzad Bozorg Nia, a former PHD Student of the lab, with three complete epithelia for each gene. The localization of Ora1-4-positive cells on complete series of horizontal cryostat sections from adult zebrafish olfactory epithelia, reveals a typical odorant expression pattern in zebrafish (Fig. 3.63). For the four diffrent *ora* genes, stained with NBT/BCIP, the average number of labelled cells per section ranges between 1.5 and 3.2, which is within the range of frequencies reported for other olfactory receptor genes in zebrafish (Ahuja et al., 2018) .

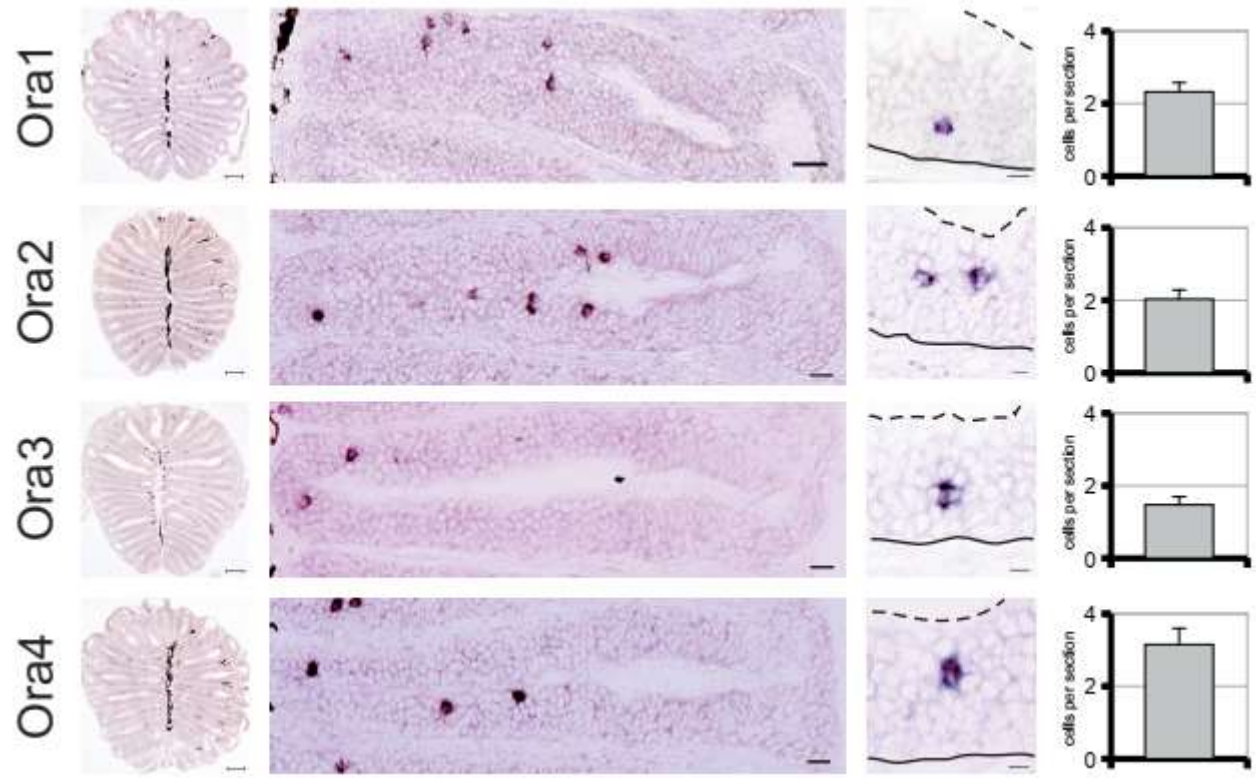

Figure 3.63: Spatial expression pattern of Ora1-4 in the olfactory epithelia of *Danio rerio*. NBT/BCIP stainings of Ora1-4-positive cells in the complete epithelia (left), in a single lamella (middle) and at higher magnification (right). Right graphs: Ora1, Ora2 and Ora3 approx. 2 cells and Ora4 approx. 3 cells per section in a complete epithelia. Scale bar (left) 100µm (middle) 50µm and (right) 10µm.

For visualizing Ora5- and Ora6, as reported before, the TSA method was used for nine (Ora5) and eleven (Ora6) OE. Also, for Ora1-4 seven to eight epithelia were stained to compare the number of cells with the NBT/BCIP method. The advantage of this method was considerably less background staining, resulting in an easier identification of the stained olfactory neurons. The number of cells stained with the TSA method for Ora1-4 in the OE, was two to three times greater compared to the cells stained with NBT/BCIP (Fig 3.64). Stained cells for Ora5 and Ora6 showed, compared to the other four Oras, less expression in the OE, with less than one cell per section (Fig.3.64).

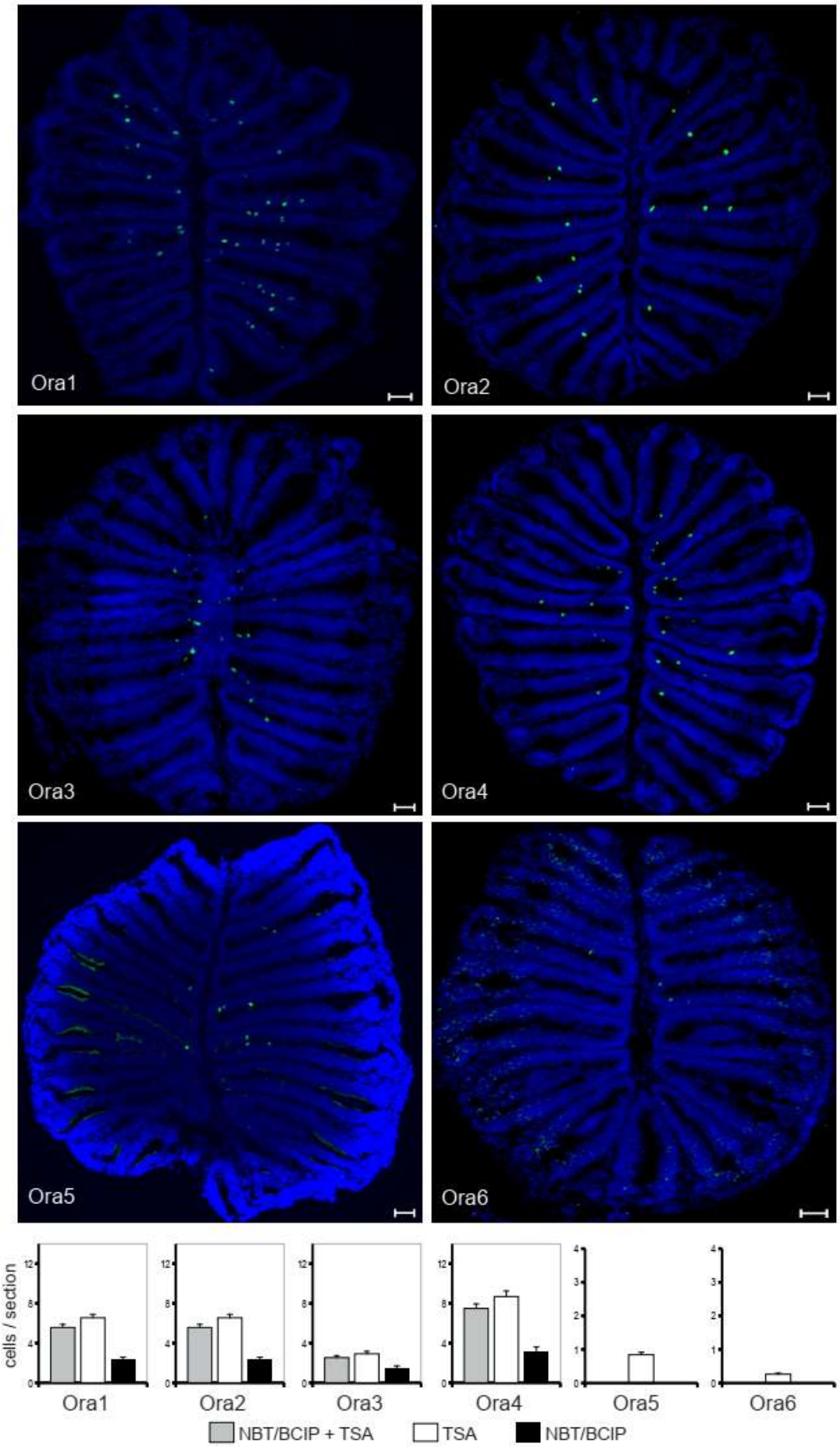
Ora1
Ora2
Ora3
Ora4
Ora5
Ora6
cells / section
0
4
8
12
0
1
2
3
4
Ora1
Ora2
Ora3
Ora4
Ora5
Ora6
NBT/BCIP + TSA
TSA
NBT/BCIP

Figure 3.64: Spatial expression pattern of Ora1-6 in the olfactory epithelia of D*anio rerio* with the TSA method and comparison of different staining methods, reveals no major difference. Top panels: TSA stainings of Ora1-6-positive cells (green) in the complete epithelia. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) inblue to visualize the localisation of the TSA signal relative to the nuclei. **Under pan-e**l: comparison of cells per section for all *ora* genes, between the two different staining methods NBT/BCIP and TSA shows only for Ora4 a minor difference.

3.3.2 Two major expression domains for *the ora* gene family in zebrafish are distinguishable in quantitative analysis of radial distribution, each populated by more than one *ora* gene

Previous studies in zebrafish showed significant differences in localization in relation to the radius in members of the OR family. Different expression zones could be shown for individual ORs (Weth et al., 1996). To investigate if *ora* genes also reveal different expression zones, related to radius, ten to eleven olfactory epithelia were evaluated per *ora* gene, with approx. 1000 to 2500 measured cells for Ora1 to 4, 330 for Ora5 and 130 for Ora6, and plotted in an ECDF diagram (Fig. 3.65 upper panel). Two different *in situ* staining methods,TSA and NBT/BCIP, were used, which showed no difference based on the data. For each gene, the individual epithelia were summarized in a common curve and made visible using a single ECDF diagram. This shows that Ora3-positive cells are found closest to the middle raphe, followed by Ora4, 5 and 6, forming together a first expressing zone. Ora1 and Ora2 are localized further from the middle raphe and are part of the second expressing zone for the radial distribution (Fig. 3.65 lower panel). The use of a KS test showed significant differences between the single Oras, except Ora6 with Ora4 and Ora5 (Tab. 6.4).

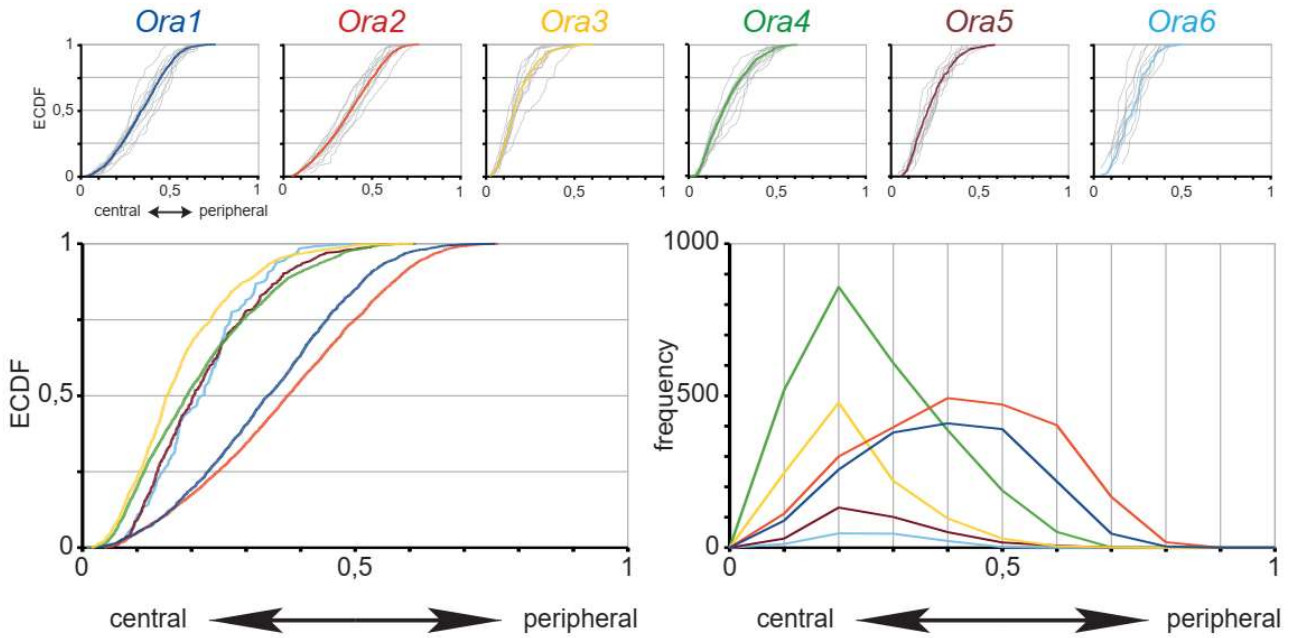

Figure 3.65: Two major expression domains are distinguishable in quantitative analysis of radial distribution, each populated by more than one *ora* gene in *Danio rerio*. Upper panels: The resulting distribution of relative radius of all six *ora* genes shown as ECDF. Coloured line mean graph, Light grey curves in the ECDF plot for each of the tested *ora* genes represent the distribution for individual olfactory organs. **Left under panel:** Overlay of the six distributions shown individually in upper smaller panels. **Right under panel:** Radial distribution histogram of all six *ora* genes. Radial position within the section was normalized to maximal radius, i.e. length of the lamella containing the respective labelled cell. (from 0, most central to 1, most peripheral).

3.3.3 Three major expression zones in zebrafish are distinguishable in quantitative analysis of height distribution in the *ora* gene family

To date, the expressing OSN has only been identified for the Ora4 receptor. Which are localized in the crypt neurons. The height position in the lamellae can perhaps give further information on the search for the OSNs in which the remaining Oras are presented. The same cells that were used to determine the radius, were used to determine the height of the lamellae. Also no difference between the two *in situ* stainings were observed. Ora1-positive cells are located more basally, compared to all others, forming a more basal expressing zone, whereas Ora5 is localized most apically of all Ora receptors and forming a single zone. Ora2, Ora3, Ora4 and Ora6 are located between these two zones and form their own expressing zone (Fig. 3.66). The KS test showed a significant difference between all Ora receptors (Tab.6.5).

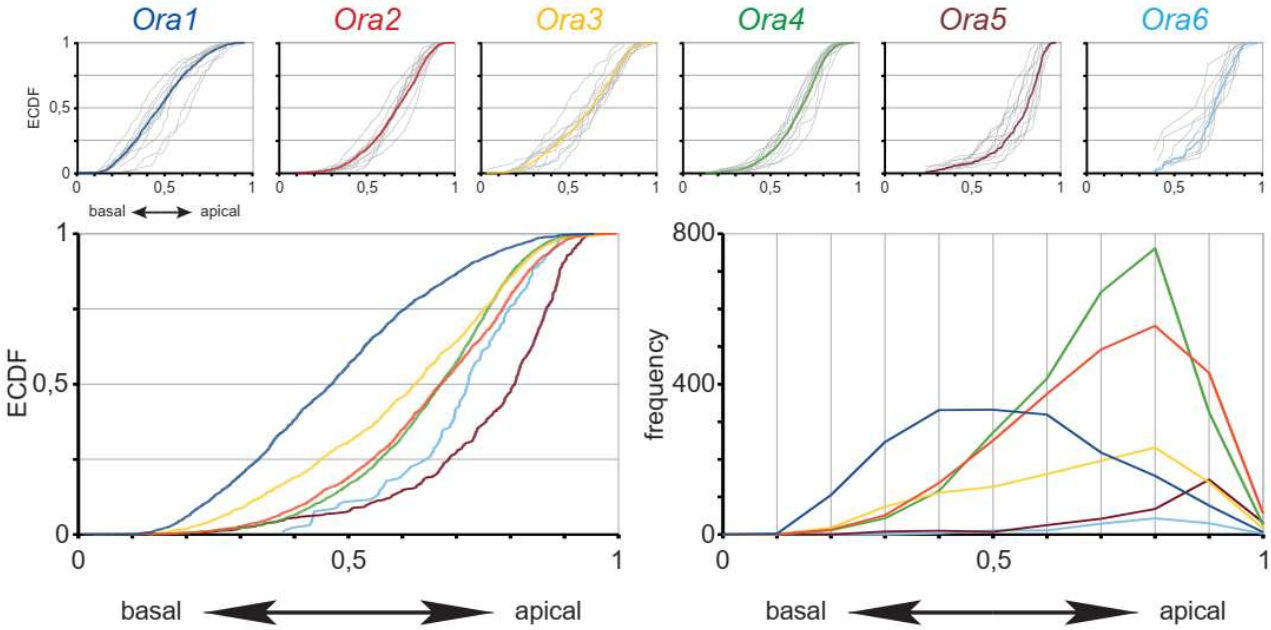

Figure 3.66: Three major expression zones are distinguishable in quantitative analysis of height distribution for six *ora* genes.
Upper panels: The resulting distribution of relative height of all six *ora* genes shown as ECDF. Coloured line mean graph, Light grey curves in the ECDF plot for each of the tested *ora* genes represent the distribution for individual olfactory organs. **Left under panel:** Overlay of the six distributions shown individually in upper smaller panels. **Right under panel:** Height distribution histogram of all six *ora* genes. Height position within the section was normalized to maximal height, i.e. length of the lamella containing the respective labelled cell. (from 0, most central to 1, most peripheral).

3.3.4 No significant differences in height within the olfactory organ of zebrafish between different *ora* genes

Another question that was raised was whether there are differences, within a gene family, in the localization in the height in the olfactory organ. Most labelled receptor cells are above the centre of the olfactory organ. It looks as if Ora3- and Ora4-positive cells are localized more apically in the olfactory organ than the rest of the Ora receptors (Fig.3.67 left lower panel). The data suggests that both are also significantly different from the other Ora receptors, but if the frequency of cells compared to the others is considered, a distinction is hardly possible (Fig. 3.67 right lower panel) The KS-Test reveals that the height within the OE is only significant different for Ora2/Ora4 and Ora4/Ora5 (Tab.6.6).

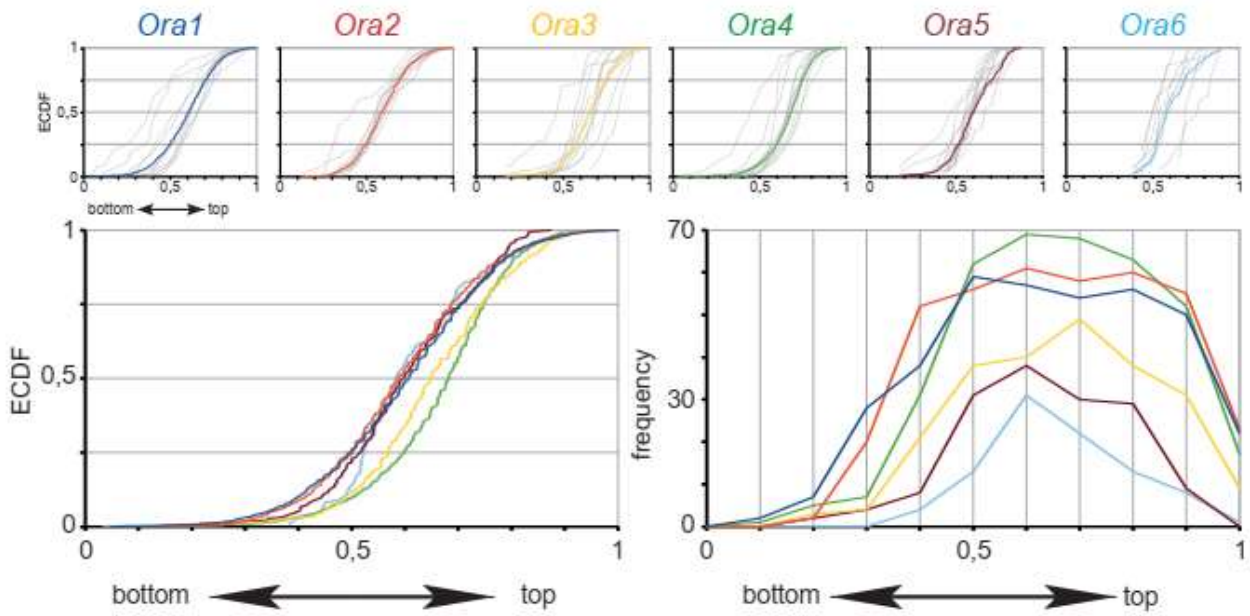

Figure 3.67: No significant differences in height within the olfactory organ between different *ora* genes. **Upper panels:** The resulting distribution of relative z axis (height within the organ) of all six *ora* genes shown as ECDF. Coloured line mean graph, Light grey curves in the ECDF plot for each of the tested *ora* genes represent the distribution for individual olfactory organs. **Left under panel:** Overlay of the six distributions shown individually in upper smaller panels. **Right under panel:** Height distribution histogram of all six *ora* genes. Height within the olfactory organ was quantified as section number in a series of horizontal sections, and normalized to the total number of sections containing sensory epithelium, using the same set of cells, for which laminar height was determined. Relative height within the organ ranges from 0 (top section, near to the opening of the bowl-shaped olfactory organ) to 1 (bottom-most section).

3.3.5. Three-dimensional analysis of spatial expression patterns shows significantly different expression zones for all six *ora* genes

In order to get a better overview about the distribution of the Ora receptors in *Danio rerio*, the radius and height in the lamellae and radius and height in the entire epithelium were compared. For this purpose, the first and third quartiles of each measurement of radius, laminar height and height in the organ were three-dimensionally schematically compared for all Ora receptors in diagrams (Fig 3.68). This graphic representation shows very clearly that the individual *ora* genes occupy their own mutually different expression zones inside the lamella. For example, OSNs that are positive for Ora5 receptors are found very apically and centrally within the lamella, while OSNs positive for Ora2 receptors are more basal and outer (Fig 3.68 left panel). When looking at the height within the OE, it can be clearly seen that there are no differences between the individual *ora* genes based on the

data between the first and third quartile (Fig 3.68 right panel).

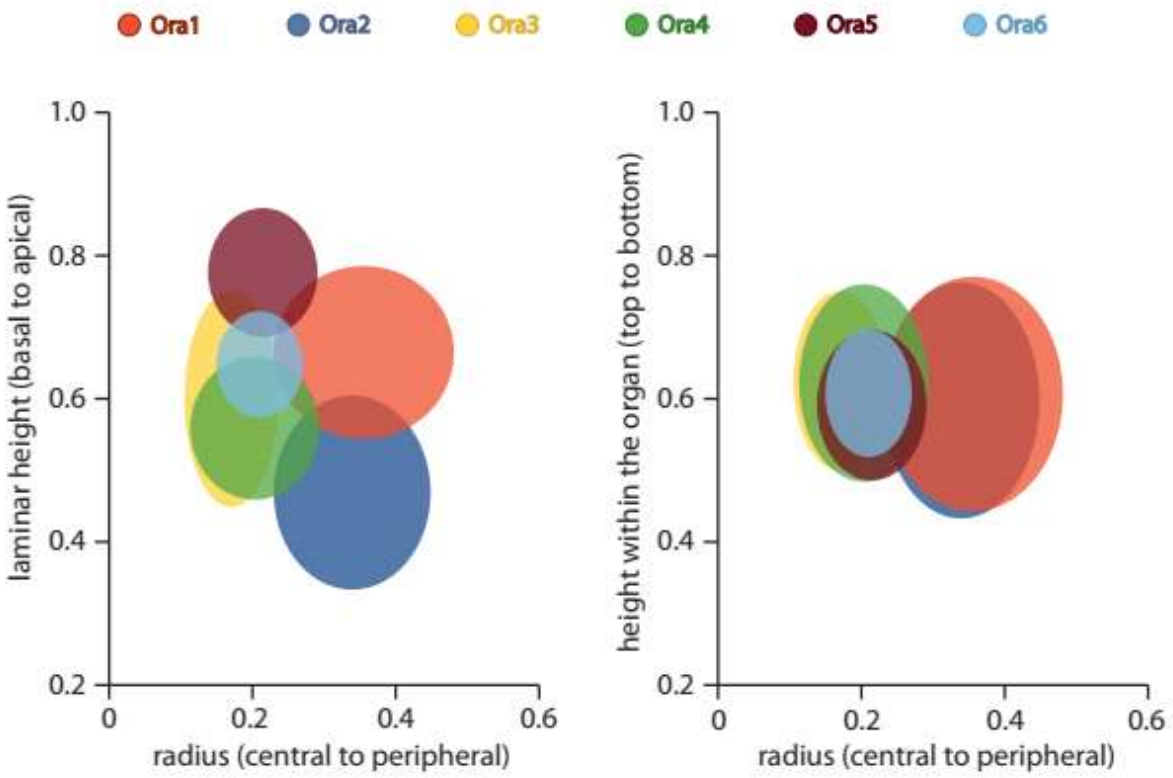

Figure 3.68: Three-dimensional analysis of spatial expression patterns shows significantly different expression zones for all six *ora* genes. Comparison of spatial distribution parameters between *ora* genes. Schematic representation of spatial distributions for different *ora* genes, by ellipses ranging from the 1st to the 3rd quartile value for x and y parameter. Colour code for *ora* genes as before. Left panel Radius (x axis) is depicted vs. laminar height (y axis). Right panel: Radius (x axis) is depicted vs. height within the organ (y axis).

4. DISCUSSION

Olfactory receptors of different families are mainly localized in the olfactory epithelia of all vertebrates. They can detect a large variety of chemical compounds, which can guide the animal in its natural enivronment. This study investigates different receptor families in aquatic animals, especially their expression, spatial distribution and tries to determine their function more closely.

The overwhelming majority of olfactory receptor genes in a multitude of different vertebrate species shows a highly characteristic spatial pattern of expression: neurons expressing the same receptor are distributed sparsely within the sensory surface, with adjacent neurons expressing different receptor genes. It is assumed that this coding strategy protects the entire representation of the environment by the multitude of existing olfactory receptors against limited mechanical and chemical damage of the OE, because partial damage would weaken all representations, but not eliminate particular receptor populations. Here we found that the A2c receptor in lamprey deviates from this pattern, although the A2c receptor in other fish and frog conforms to the typical pattern. It was shown that an important general characteristic of olfactory receptors is a widely not random distribution (Bayramli et al., 2017), which also correspond with our results found here.

4.1 The spatial pattern of expression for A2c in lamprey does not suggest an olfactory function, in contrast to that observed in higher aquatic vertebrates

The recently discovered A2c receptor, the first known olfactory receptor for nucleosides, was shown to be expressed in zebrafish OSNs. Phylogenetic studies also showed that this receptor is only present in amphibians and fish. It was not found in reptiles, mammals and birds, which suggests that it was lost in the aquatic - terrestrial transition. In zebrafish A2c-expressing cells showed a similar spatial distribution to that described for other olfactory receptors for zebrafish, such as ORs or OlfCs (Wakisaka et al., 2017; Weth et al. 1996;

Ahuja et al., 2018).

Here we wanted to examine to what extent the function of the A2c as an olfactory receptor might be conserved across the tree of life. To that end we show here for the first time, the A2c-expressing neurons in closely-related fish (common carp) and not so closely-related fish (eel and lamprey) to zebrafish, as well as in an amphibian (*Xenopus*). For carp this is the first time an ISH has been published for olfactory epithelia. The OE of these two teleosts look very similar in structure, presumably due to the evolutionary proximity of carp and zebrafish, which both belong to the Cyprinidae family. This similarity also applies to the spatial distributions of the A2c-positive neurons, although these are localized a little further away from the middle raphe in carp compared to zebrafish. This difference might reflect a species difference, but could also reflect the different stages of development employed - while adult zebrafish were used for the experiments, the carp were still juvenile fish.

The European eel (*Anguilla anguilla),* also a member of the teleosts, but not so closely related to zebrafish as carp, showed the same spatial distribution of A2c-positive OSNs at the juvenile freshwater stage. Compared to both carp and zebrafish, the A2c-positive OSNs were found much closer to the middle raphe in the single lamellae. Both the size of the different olfactory receptor gene families and their physiological relevance can be expected to differ considerably between species (Korsching, 2020), in particular not closely related ones such as eel and cyprinids. Since the spatial position of cells expressing an individual olfactory receptor gene results from an interplay with all the other olfactory receptors sharing the same sensory surface it follows that differences in the location of particular expression zones is to be expected. Thus the observed differences between the preferred positions of A2c-expressing cells in eel, zebrafish and carp (from more central to less central) is not surprising.

It is known that sexually immature eels, which live in fresh water until maturation, have a very acute sense of smell, compared to their mature stage in salt water. In former experiments, artificially matured eels of advanced maturity, showed a marked degeneration of the sensory epithelium with an apparent loss of the columnar organization, and also a reduction in the number of mucous cells (Pankhurst & Lythgoe, 1983). It would be interesting to examine whether mature eels in the ocean still possess a similar number of A2c-positive OSNs, or whether there was a loss of such neurons.

The African clawed frog (*Xenopus laevis*), a member of the tetrapods with an aquatic lifestyle even in the adult constitutes a link between fish and land-living animals. It

expresses A2c-positive OSNs in the main olfactory epithelium. The spatial distribution of A2c-positive OSNs is similar to formerly published expression patterns for other olfactory receptors, but the frequency was less compared to some other olfactory receptors, with one to a maximum of three cells per section (Syed et al., 2015; Syed et al. 2017).

It would be interesting to see where A2c is expressed in the adult olfactory epithelia. Three *a2c* genes were identified in the genome of *Xenopus laevis*, *a2c1, a2c.2a and a2c.3b*. Only the probes for A2c1 showed an expression pattern in the metamorphic stage. For A2c2a and A2c.2b we found for both genes the intact Coding sequence (CDS) (Fig 3.7), but signals were detected in the olfactory epithelia was detected. A possible explanation is that genes are pseudogenes, and their promoter region could be damaged. This could be explained by the allotetraploid hypothesis, because 48 million years ago the subgenomes of Xenopus laevis and Xenopus tropicalis diverged from each other and in the time from around 34 -18 million years resulting in a whole genome duplication in Xenopus laevis. Transposable elements were active, which leads to diploids in Xenopus laevis during this period, resulting in increasing numbers of pseudogenes. (Session et al., 2016)

Taken together, in all bony vertebrate species analysed, A2c was found in sparsely distributed cells, which is a hallmark of olfactory receptor gene expression, consistent with a function of A2C as olfactory receptor in all these species.

Next, we examined A2C expression in the olfactory sytem of lamprey, which are much earlier-diverged vertebrates and possess a single unpaired nose, located in the middle of the head, whose overall morphology is different from that of teleosts, although the same organization of the sensory surface in lamellar folds is present. Nevertheless the expression pattern of other olfactory receptors in lamprey was similar to that observed in zebrafish or frog (Freitag et al., 1999; Berghard and Dryer, 1998). In contrast, the expression pattern of A2c was completely different. We could show that the A2c-labelled cells form a single zone adjacent to the sensory epithelia, either at the tip or mostly at the bottom of the individual lamellae, i.e. in contrast to the expression pattern of A2c in the teleost and tetrapod species analysed, lamprey A2c-expressing cells are contiguous and not dispersed. We also checked a possible expression in the AOO, which has been shown to contain OSNs (Green et al.2017)), but found no expression of A2c there. Since for other olfactory receptors such as ORs an expression in nonsensory tissues was reported, often in the heart, testis or kidney (Pronin et al., 2014), we also investigated the expression of

A2c in eyes and heart. No expression was found in these tissues.Thus A2c appears to be specifically expressed in the nose, but not in the sensory surface of the nose or the eye.

The surprising spatial distribution of A2c-expressing cells in lamprey does not support a function as olfactory receptor in this species. This led us to investigate the nature of A2c-expressing cells more closely.

4.2 A2c-expressing cells in lamprey are negative for several neuronal markers

Various established molecular markers were used to examine the unusual cells expressing A2c. The selected markers for the olfactory sensory area were HuC and G_{olf}, which were shown in former studies to label the whole sensory area in the olfactory epithelia in different vertebrates, as for example zebrafish and frog (Date-Ito et al, 2008; Oka and Korsching 2011). Here we could show, that A2c is co-expressed with HuC in zebrafish and carp confirming the neuronal nature of A2c-expressing cells in these species. In lamprey A2c was not co-expressed with either HuC or G_{olf}, but formed its own region adjacent to the olfactory sensory area defined by these markers, which leads to the assumption that A2c is not localized in olfactory sensory neurons. We could also show, that G_{olf} and Huc are perfectly co-labelled in the complete sensory area. In zebrafish it was shown that A2c is co-expressed with G_{olf} (Wakisaka et al., 2017). A hypothesis consistent with these expression patterns would be that the A2c-positive cells in lamprey could be evolutionary progenitor cells of olfactory neurons, whereas in eels and carp they represent mature OSNs.

Here we also tested TRPC2, an accepted marker for microvillous neurons of bony vertebrates, which was recently discovered in the genome of sea lamprey (*Petromyzon marinus*) (Grus and Zhang 2009). Unexpectedly, the TRPC2 labelling was not restricted only to the olfactory sensory area as Huc or Golf, but also labels other parts of the OE and no co-labelling with A2c was observed.

We could also show, that acetylated tubulin, a common marker of ciliated cells, is localized in the same region as A2c, but not directly co-expressed with A2c. This finding is reminescent of the earlier descriptions of the non-sensory area in the zebrafish, in which ciliated

cells were also identified with the help of electron microscopy (Hansen and Zeiske, 1998). But even in the ciliated cells of the so-called non-sensory area in the zebrafish, acid-sensing ion channels (ASIC2) were identified, which leads to the assumption that the non-olfactory area can also take on sensory tasks (E. Viña et al., 2015). At this point it would be interesting to find out whether the acid-sensing ions channel 2 found in the zebrafish also occur in the lamprey and are perhaps localized in the same zone in which A2c was found. Interestingly, however, earlier studies in larvae of *Petromyzon marinus* showed that the antibody against acetylated tubulin only marks the olfactory sensory area (Frontinni et al. 2003). This could indicate that ciliated cells migrate into the non-olfactory area later in the course of development into an adult animal and take on tasks there that are important at a later stage.

Since the attempted *in situ* hybridization with further neuronal markers and receptors, vGlut CasR and ACIII, which could reveal the functionality and identity of the A2c-positive cells, has failed, it would be interesting to look for further possible markers to solve the mystery of the unusual A2c localization. One of these markers could be Calcium-binding proteins (CBPs) such as parvalbumin, calretinin, calbindin1 and S100, which have roles in intracellular signalling and/or buffering of calcium during neuronal activity and shown to be localized in different OSNs in zebrafish (Kress et al., 2015). This could maybe solve the question if these A2c-positive cells are OSNs, as in the other investigated species.

Based on general morphology, the region in which A2c is localized is identical to two of three proliferative regions, identified in earlier studies (Thornhill, 1970). To confirm this assessment we used a PCNA-antibody, established as a marker for proliferating cells (Bayramli et al., 2017; Ino and Chiba, 2000). The PCNA-antibody labels exactly the region in the lamprey OE where A2c is localized. Our results contradict those of an earlier study, where a different PCNA-antibody labelled in larval *Petromyzon marinus,* a cell layer near the basal lamina, but in adult animals a weak diffuse unspecific staining was observed (Hua et al., 2000).

Our PCNA-antibody was verified by us in zebrafish, where it was localized after double labelling with A2c in the expected stem cell-forming zone (Oehlmann et al., 2004; Bayramli et al., 2017) and no double labelling with A2c occurred. In the lamprey, however, both were localized in exactly the same non-olfactory sensory region, but there was no clear double labelling at the cellular level. It is still unclear whether they are expressed in the same cells. In stimulation experiments with ATP in lamprey, which activates the A2c re-

ceptor, similar regions, as in the A2c ISH, could be shown by immediately early gene markers. The control with amino acids and water also showed the expected results, but due to the lack of test animals, only one animal was stimulated for each of these experiments, which is definitely too few for a significance statement. In both cases this could suggest that A2c may play a role in the development of olfactory sensory neurons. A2c could even directly affect the differentiation pathway of the OSNs, because it is known that purines (ATP and ADP) are in fact mitotic signals for olfactory stem cells in frog and fish (Hassenklöver et al., 2009; Demirler et al., 2020). Our preliminary experiments suggest the same ligand for A2c in lamprey. However should be noted that the comparison of PCNA ISH and IHC has brought unexpected observations which present problems for the above hypothesis.

Interestingly, the attempt at double labelling between PCNA antibodies and labelled PCNA-RNA showed that protein and RNA appear to be present in different cells, because firstly there was no direct double labelling and the zone in which the protein is localized is much larger. The PCNA RNA could only be detected in the tips of the lamellae, while the protein was mainly located in the tips and directly adjacent to the lower part of the olfactory sensory zone. These findings could indicate that the PCNA-antibody in the lamprey does not actually mark PCNA and thus an unknown protein, which happens to be localized in exactly the same zones as A2c. But it could also be that, with regard to the different distribution of PCNA RNA and protein in the tips, the cells, which are stem cells, are renewed relatively quickly within the lamella, and thus a shift in the expression pattern occurs. In zebrafish it has been shown that immature neurons migrate from sensory-adjacent regions into the sensory surface (Oehlmann et al., 2004, Bayramli et al., 2017), so it is conceivable that the proliferative regions in the lamprey nose might serve the same purpose. However, the absence of PCNA-RNA in the basal PCNA antibody-positive areas doesn't support this theory. In the course of this thesis no final solution could be found to explain this enigma. Maybe A2c was recruted later by an olfactory receptor, as it happens for the Fprs in mice (Dietchi et al., 2017)

4.3 Half of all identified V1Rs and none of the V2Rs are expressed in the olfactory epithelium of Lampetra fluviatilis

Another group of olfactory receptors in which we were interested were the vomeronasal receptors. In former publications (Grus and Zhang, 2009; Libants et al., 2009) the existence of four V1Rs in *Petromyzon marinus* was predicted, through phylogenetic studies, but their expression had never been analyzed. Here we identified in the genome of *Lethenteron camtschaticum* six potential V1Rs and two V2Rs.

For all genes several probes based on the cDNA of *Lampetra fluviatilis* were made, but in four cases, V1R1, V1R4, V2R1 and V2R2 no stained OSN was detected either in the OE or AOO. It appears unlikely that this was a technical problem, since several primer pairs were used for each gene. On the one hand, this could be due to the fact that we work with adult animals and these receptors may be only expressed at younger life stages. Because observations showed that the density of OSNs in juvenile lamprey stages is much higher than in adults, in contrast to the situation in the mouse OE, where it is exactly the other way around (VanDenbossche et al. 1995). It could mean that these receptors, which are not found expressed in the adult animal, are only localized in younger life cycles and are no longer needed in the adult animal. Another possibility is, that the RNA of these genes has only a regulatory function in the adult Lamprey (ref viell lncRNA).

Three of the V1Rs, V1R3, V1R5 and V1R6 in *Lampetra fluviatilis*, showed a staining and spatial distribution as known from OR receptors in lamprey or other vertebrates (Laframboise et al., 2007; Sato et al., 2005). Furthermore the same three V1Rs, V1R3, V1R5 and V1R6, showed a clear double labelling with the OSN marker HuC, confirming their expression in olfactory neurons. In the case of V1R3, the double labelling with acetylated tubulin shows that these are probably ciliated OSNs. This would also coincide with previous findings, that OSNs in lamprey are ciliated (Thornhill,1967; VanDenbossche et al., 1995). Interestingly, V1Rs and V2Rs are expressed in evolutionarily later-derived vertebrates such as rodents in microvillated cells (Menco et al., 2001). Unfortunately, neither the staining with actin nor with TRPC2 in *Lampetra fluviatilis*, which are both possible markers for microvillated cells, showed usable results here. When examining the spatial distribution of

the individual V1Rs, we were also able to show that there were differences between the individual V1Rs in terms of radius and height in the lamella in the lamprey. Unfortunately, due to the lack of experimental animals, it was not possible to investigate the third dimension, height within the entire organ. These results coincide with earlier findings in the zebrafish, in which a spatial distribution could be shown for some members of the OR family (Weth et al.,1996) with regard to the radius of the lamella. A spatial distribution in terms of radius and height in the lamella could also be demonstrated for the OlfC family (Ahuja et al., 2017).

4.4 V1R-related *ora* genes in zebrafish show clearly segregated if broadly overlapping expression zones

As already mentioned, earlier studies in our laboratory had shown different spatial distribution within the Olfc and OR family showed different distribution patterns for single members of this families (Ahuja et al., 2017; Weth et al., 1996).

Here we wanted to investigate the spatial distribution of a complete family of olfactory receptors. The choice fell on the V1R-related ORAs in the zebrafish, which, with their present six members, represent an ideal object of investigation. Initial work was done by a former PHD student, Shahrzad Bozorg Nia (Bozorg Nia, 2015). Here the bulk of the experiments was performed and analysed. While writing this thesis, it turned out that Ora3 can be divided into ORa3a and Ora3b. Whether these two show a different spatial pattern is currently being investigated by a PHD student, Shazib Hussain.

At least 11 complete olfactory epithelia/gene were examined with the much more sensitive *in situ* method TSA. For this project, based on the number of stained cells, it was shown that fluorescence-based staining is definitely to be preferred, compared to chromogenic methods, because not only were the expression patterns for *ora5* and *ora6* shown and evaluated for the first time, but also because the complete gene family of Oras together could be examined. We were able to show that o*ra* 5 and *ora* 6 have less cell numbers, compared to *ora* 1-4. These findings are consistent with the lower FPKM (Fragments Per Kilobase of transcript model per Million fragments) values obtained in RNA sequencing experiments published in former studies (Saraiva et al, 2015). When looking at the radius and height of the individual *ora* genes, it turned out that apparently every member of the

ora family is localized in its own zone. In this case the relative height and radius are probably regulated by different processes, because no co-variation of preferred radius or height was observed between different *ora* genes. We were also able to show that there is nearly no difference in height within the organ, similar to what was observed recently for the members of the Olfc family (Ahuja et al., 2017).

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6. SUPPLEMENTARY INFORMATION

A

Vglut amino acid sequence *of Lampetra fluviatilis*

MEAFKERVFVPGKEKLKEAAGKSLRLLHRRMSKQEGEIELTEDGRPVQVKPPKEPLCECRCFGMPK
RYVIAVMSGLGFCISFGIRCNLGVAVVDMVNNSSYHSGDKLIMQKAEFNWDSGTVGMIHGSFFWGY
IVTQIPGGFISNKFAANRVFGLAIVLTSTLNMFIPTAARVHYGLVIFVRILQGLVEGVTYPACHGI
WAKWAPPLERSRLATTAFCGSYAGAVVAMPLAGILVQYAGWSSVFYVYGSFGLFWYIFWLCIAYES
PAKHPTISDEEKTYIEESIGESYGILGSTQKFKTPWKKFFTSMPVYAIIVANFCRSWTFYLLLISQ
PAYFEEVFGFEISKVGIISALPHLVMTIVVPIGGQLADFFRSKKIMSTTTVRKMMNCGGFGMEATL
LLVVGFSHGKGIAITFLVLAVGFSGFAISGFNVNHLDIAPRYASILMGISNGVGTLSGMVCPLIVG
AMTKGKTREEWQNVFLIAALVHYGGVIFYGLFASGEKQPWADPEETSDEKCGFINEDELGGDDFAE
GEAETALGDGGVAGAGGGGLAGASGGKSYGATAQSKGSWPNGYQSKDAPGQGGDRENYVYADPGER
DSS

B

Vglut nucleotide sequence *of Lampetra fluviatilis*

ATGGAAGCGTTCAAGGAGCGCGTGTTCGTTCCCGGCAAGGAGAAGCTCAAGGAAGCGGCGGGGAAA
TCGCTTCGTCTCCTCCACAGGCGCATGTCCAAGCAAGAGGGTGAGATCGAACTGACCGAGGATGGG
CGGCCCGTGCAAGTGAAGCCTCCCAAGGAGCCCCTCTGCGAGTGCCGCTGTTTCGGAATGCCCAAG
CGTTACGTCATCGCCGTGATGAGCGGCCTGGGCTTCTGCATCTCCTTTGGGATCCGTTGCAACTTG
GGCGTGGCCGTGGTGGACATGGTCAACAACAGCTCGTACCACTCGGGAGATAAGCTCATCATGCAG
AAAGCGGAATTCAACTGGGACTCTGGGACTGTGGGGATGATCCACGGATCGTTCTTCTGGGGCTAC
ATCGTCACACAAATCCCAGGAGGTTTCATCTCCAACAAATTCGCTGCCAATCGAGTATTTGGCCTT
GCCATCGTCCTAACGTCCACACTGAACATGTTCATCCCGACAGCGGCGCGGGTGCACTATGGCCTC
GTTATCTTCGTGCGTATCTTGCAGGGACTCGTTGAGGGGGTCACGTACCCGGCTTGCCACGGAATC
TGGGCCAAGTGGGCCCCGCCGTTAGAACGCAGTCGACTGGCCACCACGGCCTTCTGTGGTTCCTAT
GCAGGAGCCGTGGTGGCAATGCCCTTGGCCGGGATATTGGTGCAGTATGCAGGATGGTCGTCAGTG
TTTTATGTGTACGGATCGTTCGGCCTTTTCTGGTACATATTCTGGTTGTGCATTGCCTACGAGAGC
CCGGCCAAACATCCCACCATCTCGGACGAAGAGAAGACCTACATCGAGGAGAGCATCGGCGAATCA
TACGGGATTTTGGGATCGACGCAGAAATTCAAGACTCCCTGGAAGAAATTCTTCACCTCCATGCCG
GTCTACGCCATCATCGTGGCCAACTTCTGCCGGAGCTGGACCTTCTACCTGCTTCTCATCTCGCAG
CCCGCCTACTTCGAGGAGGTGTTTGGCTTTGAGATCAGCAAGGTGGGCATCATCTCGGCTCTTCCT
CATCTGGTGATGACGATCGTGGTGCCCATCGGAGGACAACTGGCCGACTTCTTCCGCAGCAAGAAG
ATCATGTCCACCACCACCGTGCGGAAGATGATGAATTGTGGAGGCTTCGGCATGGAAGCCACTCTG
CTGCTCGTGGTCGGCTTCTCGCACGGCAAGGGCATCGCCATCACGTTCCTCGTCCTCGCCGTCGGC
TTCAGCGGCTTCGCCATCTCAGGCTTCAACGTCAACCACTTGGACATCGCCCCTCGCTATGCAAGT
ATTCTCATGGGCATCTCCAACGGCGTGGGGACCCTGTCGGGAATGGTGTGCCCTCTCATCGTGGGC
GCCATGACAAAGGGCAAGACTCGCGAGGAGTGGCAGAACGTCTTCCTCATCGCGGCGCTGGTGCAC
TACGGCGGCGTCATCTTCTACGGCTTGTTCGCCTCCGGCGAGAAGCAGCCGTGGGCCGACCCCGAG
GAGACGAGCGACGAGAAATGCGGCTTCATCAACGAGGACGAGCTCGGCGGGGACGACTTCGCGGAG

GGCGAGGCCGAGACGGCGCTGGGCGACGGTGGCGTCGCCGGTGCCGGCGGCGGCGGCCTTGCGGGG
GCCTCGGGCGGGAAGTCGTACGGCGCGACGGCGCAAAGCAAAGGGAGCTGGCCCAACGGCTATCAG
AGCAAGGATGCGCCGGGCCAGGGGGGAGACAGGGAAAACTACGTGTACGCCGATCCCGGCGAGAGG
GACTCCTCCTGA

Figure 6.1: Vglut amino acid and nucleotide sequence of *Lampetra fluviatilis*. A) Predicted amino acid sequences of *Lampetra fluviatilis* Vglut based on published seuences (Twickel et al. 2018) Corresponding-nucleotide sequence of *Lampetra fluviatilis* Vglut with primers: Vglut (pink)

A

ACIII amino acid sequence of *Lethenteron camtschaticum*

MPRARAFSEPEYSAEYSADYSVSLPSDPGTGVEQNREVTVRSTGCCLCLPRFMRLTFRPESLENLY
QTYFRRQRHDTLLVLVAFAALFDCYVIVMCAVIYTDDKLASVAVAAVGLAANALLLLLCWFRALPD
RILHKFIPYTLWILIGAQIFCYLGLSFSRFHEASDTVDWQAFFTFAFFLTLPLRLTPIVLMSAVSC
GIHTLVLGVTIAQQQQQQQQVEDAELIVQVGAHPRSHLSLAVCLSLSLCLHLSLYLSLSICACLST
PCRPRRIFFCSTVLHHAGCSMGAVYMRVVVVASACVRSILFADIVGFTQLSSACSAHELVKLLNEL
FARFDKLAATYSQLRIKILGDCYYCICGVPEFREDHAACCIYMGLAMVNAISRVHISQVTFECLKG
EFEVEPGDGGARNEYLREKGIVTYLVVPPSPPPRNGINGVKLSLTSCDETSPKLINTTEWSGALQA
NLTPERDELDSRVVNPSFPNPRRRLRLRDLAVRVIDAQQNEQELNRLLNEALIERETARALKGKYT
NRVSMRFVDPALETRFSVEKEKQSGAAISCSCVVLLFSFIVELCLDP

B

ACIII nucleotide sequence of *Lethenteron camtschaticum*

ATGCCTCGCGCCAGGGCCTTCTCGGAGCCCGAGTACTCGGCCGAGTACTCGGCGGACTACTCTGTG
AGCCTCCCGTCGGACCCGGGCACGGGCGTGGAGCAGAACCGCGAGGTGACCGTGCGCAGCACGGGC
TGCTGCCTGTGCCTGCCGCGCTTCATGCGCCTCACCTTCAGGCCCGAGTCGCTGGAGAACCTCTAC
CAGACGTACTTCCGGCGCCAGAGGCACGACACGCTGCTCGTGCTCGTGGCCTTCGCGGCCCTCTTC
GACTGCTACGTCATCGTCATGTGCGCCGTCATCTACACCGACGACAAGCTGGCCTCCGTGGCGGTG
GCGGCCGTGGGCCTGGCCGCCAACGCGCTGCTGCTGCTGCTCTGCTGGTTCCGGGCGCTGCCCGAC
CGCATCCTGCACAAGTTCATCCCCTACACGCTGTGGATCCTCATCGGGGCGCAGATCTTCTGCTAC
CTGGGCCTGAGCTTCTCGCGCTTCCACGAGGCGAGCGACACGGTGGACTGGCAGGCCTTCTTCACC
TTCGCCTTCTTCCTCACGCTGCCGCTCCGCCTCACGCCCATCGTGCTCATGAGCGCCGTGTCGTGC
GGCATCCACACGCTCGTGCTGGGCGTCACCATCGCCCAGCAACAGCAGCAACAGCAGCAGGTCGAG
GACGCTGAGCTCATCGTGCAGGTGGGTGCGCACCCCAGATCGCACCTCTCTCTCGCTGTCTGTCTC
TCTCTCTCTCTATGTCTTCATCTGTCTCTCTATTTATCTCTCTCTATATGTGCCTGCCTCTCGACG
CCCTGTCGGCCACGGCGGATTTTTTTTTGTTCGACCGTACTTCACCATGCCGGTTGCTCCATGGGC
GCAGTGTATATGCGAGTTGTTGTTGTTGCTTCCGCGTGCGTGCGCAGCATCCTGTTTGCGGACATC
GTGGGCTTCACGCAGCTGTCGTCGGCGTGCAGCGCGCACGAGCTCGTCAAGCTGCTCAACGAGCTC
TTCGCGAGGTTCGACAAGCTGGCCGCGACGTACTCACAGCTGCGCATCAAGATCCTGGGCGACTGC
TACTACTGCATCTGCGGGGTCCCCGAGTTCCGCGAGGACCACGCGGCCTGCTGCATCTACATGGGG
CTCGCCATGGTCAACGCCATCTCGCGCGTGCACATCTCCCAGGTGACGTTCGAGTGCCTGAAGGGC
GAGTTCGAGGTGGAGCCGGGCGACGGCGGAGCGAGGAACGAGTACCTCCGCGAGAAGGGCATCGTC
ACCTACCTCGTGGTGCCGCCCAGCCCGCCGCCCCGCAACGGCATCAACGGCGTCAAGCTGTCGCTG
ACGTCGTGCGACGAGACTTCCCCCAAGCTCATCAACACCACAGAGTGGAGCGGCGCGCTGCAGGCC
AACCTCACGCCCGAGAGGGACGAACTCGACTCGCGGGTGGTGAACCCCTCCTTCCCCAACCCGCGG
CGGAGGCTGCGCCTGCGCGACCTGGCGGTGAGGGTGATCGACGCGCAGCAGAACGAGCAGGAGCTC

AACCGGCTCCTCAACGAGGCGCTGATCGAGCGCGAGACGGCGCGAGCGCTGAAGGGCAAGTACACA
AACCGCGTGTCGATGCGCTTCGTGGACCCCGCGCTCGAGACGCGCTTCTCTGTCGAGAAGGAGAAG
CAAAGCGGCGCCGCCATCAGCTGCTCGTGCGTCGTGCTGCTCTTCTCTTTCATCGTCGAGCTCTGC
CTTGACCCA

Figure 6.2: ACIII amino acid and nucleotide sequence of *Lethenteron camtschaticum.* A) Predicted amino acid sequences of *Lethenteron camtschaticum* ACIII based on search with *Danio rerio* amino acid sequence B) Corresponding nucleotide sequence of *Lethenteron camtschaticum* ACIII with primers: ACIII A (yellow); ACIII B (blue); ACIII C (orange)

A

CasR amino acid sequence of *Lethenteron camtschaticum*

AWKDGDLNLGGLFPIHFGVLSRADSLTVRPEAVECVRFNFRGLRWMLAMAFAVEEINADNLLLPGL
RLGYKMADTCNTVSKALEATLGFVARNKLGALGMGGVCNCTPAPRANTVSVVGATSSGVSTAVANL
LSLFRIPQVSYASSSRVLSNKQQYRSFMRTILSDEQQATALAELVREFGWTWVGLIASDDDYGRPG
IERFREENEEEVICVEFSETVSLYDPPERIHELADMIANATARVIVVFASGTDLDPLMREVARRNL
TGRTWIGSEAWVSSSLIARPEIFHVVGSTLGFVQRPGHIPGFREYLQKVHPQRHPNNVFVRQLWEA
TFNCTLKAEAASSDNCTQSVQSHEQGINECVKGAANAPRPFCTGRENISAVKTPFLDYTDLRITYN
VYLAVYAIAHALHDMLFGCREGAGLFEGGKCTNIQQLEPWQLLRYLQVVRFRNNKGEEVQFDEKGD
PRGGYSLVNWQQSPKDGSVQLVEVGSYDGRKPSGQRLLCNGSKQISKGYSREIPTSVCTQPCAPGT
RKGILEGEQVCCFECISCAEGEYNNESDANSCLGCPEDSWPSANHTMCVPKREEYLSWREPFGVCL
AFVGCLGAVLTLSVAAVFVKHRGTAIVKATNVELSYLLFFSLFCCFLSSLFFIGKPSTVACKLRQP
TFGISFVLCVSCVLVKTHRVLQVFESQIPGRGGVRKLLGLDHQLLLVFLFTLVQVVICAVWLYTAP
PTFEEDDTFKNDVIYLSCDEGSQLALGFVIGYTCLLAGVCFLFAFRARKLPENFNEAKFITFSMLV
FFIVWLSFIPAYVSSQGKFVAAVEVIAILVSSFGLLSCIFFHKCYIILLKPERNTPEEVRCSTAAH
SFKTAARVNLHTASSIGSGGSAGSM

B

CasR nucleotide sequence of *Petromyzon marinus*

GCGTGGAAGGACGGCGACCTGAACCTGGGTGGGCTCTTCCCCATCCACTTCGGCGTGCTGAGCCGC
GCCGACAGCCTCACGGTGCGACCCGAGGCCGTGGAGTGCGTCCGGTTCAATTTCCGCGGCCTGCGT
GGATGCTCGCCATGGCCTTCGCCGTCGAAGAGATCAACGCTGACAACTTGCTGCTGCCGGGCCTCA
GGCTGGGCTACAAGATGGCCGACACGTGCAACACGGTGTCGAAGGCGCTGGAGGCGACGCTGGGCT
TCGTGGCGCGCAACAAGCTGGGAGCCCTGGGCATGGGCGGCGTCTGCAACTGCACGCCGGCGCCGC
GCGCCAACACCGTGTCCGTGGTGGGTGCCACCAGCTCCGGGGTCTCCACCGCCGTGGCCAACCTCC
TGAGCCTCTTCCGCATCCCGCAGGTGAGCTACGCGTCGTCGAGCCGCGTGCTCAGCAACAAGCAGC
AGTACCGCTCGTTCATGCGCACCATCCTGAGCGACGAGCAGCAAGCAACGGCGCTCGCCGAGTTGG
TGCGCGAGTTCGGCTGGACCTGGGTGGGCCTCATCGCGTCGGACGACGACTACGGGCGGCCGGGCA
TCGAGCGCTTCCGCGAGGAGAACGAGGAGGAGGTGATCTGCGTTGAATTCTCTGAGACAGTGTCGC
TCTACGACCCGCCAGAGCGGATCCACGAGCTGGCCGACATGATCGCGAACGCGACGGCACGGGTCA
TCGTCGTGTTCGCCAGCGGCACCGACCTGGACCCGCTGATGCGCGAGGTGGCACGGCGCAACCTAA
CGGGGCGCACTTGGATCGGCAGCGAGGCCTGGGTGAGCTCCTCGCTCATCGCCCGCCCCGAGATCT
TCCACGTGGTGGGCAGCACGCTGGGCTTCGTGCAGAGGCCAGGGCACATCCCGGGCTTCCGCGAGT
ACCTGCAGAAGGTGCACCCGCAGCGCCACCCGAACAACGTGTTCGTGCGGCAGCTGTGGGAGGCCA
CCTTCAACTGCACGCTGAAGGCCGAGGCGGCGTCGAGCGACAACTGCACCCAGTCGGTTCAGAGCC
ATGAGCAGGGGATTAATGAATGTGTGAAAGGCGCAGCCAACGCCCCGCGGCCCTTCTGCACCGGCC

GTGAGAACATCAGCGCGGTGAAGACGCCGTTCCTGGACTACACGGACCTGCGCATCACCTACAACG
TCTACCTGGCCGTGTACGCCATCGCGCACGCGCTGCACGACATGCTCTTCGGCTGCCGCGAGGGCG
CTGGCCTCTTCGAGGGCGGCAAGTGCACCAACATCCAGCAGCTGGAGCCATGGCAGCTCCTGCGCT
ACCTGCAAGTGGTGCGATTCCGAAACAACAAGGGCGAGGAGGTGCAGTTTGACGAGAAGGGCGACC
CCAGGGGTGGGTACAGCCTCGTCAACTGGCAGCAGTCGCCCAAGGACGGCTCCGTGCAGCTGGTGG
AGGTGGGCAGCTATGATGGGCGCAAGCCCTCGGGCCAGCGCCTCCTTTGTAATGGAAGTAAACAAA
TCAGCAAGGGATACAGTAGAGAGATACCCACGTCCGTCTGCACCCAGCCGTGCGCTCCGGGGACGC
GCAAGGGCATCCTGGAAGGGGAGCAGGTGTGCTGCTTCGAGTGCATTTCCTGTGCAGAAGGAGAGT
ACAACAACGAATCCGATGCCAACAGCTGCCTCGGGTGCCCCGAAGATTCCTGGCCGTCGGCGAACC
ACACGATGTGCGTCCCCAAGCGGGAGGAGTACCTGTCGTGGCGAGAGCCCTTCGGCGTGTGCCTGG
CGTTCGTCGGCTGCCTCGGCGCCGTGCTCACACTCAGCGTGGCCGCCGTCTTCGTCAAGCACCGCG
GCACGGCCATCGTGAAGGCCACCAACGTCGAGCTCTCCTACCTGCTCTTCTTCTCGCTCTTCTGCT
GCTTCCTGAGCTCGCTCTTCTTCATCGGCAAGCCGAGCACGGTCGCCTGCAAGCTGCGCCAGCCCA
CGTTCGGCATCAGCTTCGTCCTGTGCGTCTCGTGCGTCCTCGTCAAGACCCACCGCGTCCTCCAGG
TGTTCGAGTCGCAGATCCCCGGCCGCGGCGGCGTGCGCAAACTGCTGGGCCTCGACCACCAGCTGC
TGCTCGTCTTCCTCTTCACGCTCGTGCAGGTCGTCATCTGCGCCGTGTGGCTCTACACGGCGCCGC
CCACCTTCGAGGAGGACGACACGTTCAAGAACGACGTCATCTACCTGTCCTGCGACGAGGGCTCGC
AACTCGCGCTGGGCTTCGTCATCGGCTACACGTGCCTGCTCGCCGGCGTCTGCTTCCTGTTCGCGT
TCCGCGCGCGCAAGCTGCCCGAGAACTTCAACGAGGCCAAGTTCATCACGTTCAGCATGCTGGTGT
TCTTCATCGTTTGGCTCTCGTTCATCCCGGCCTACGTGAGCTCGCAGGGCAAGTTCGTGGCCGCCG
TCGAGGTGATCGCCATCCTGGTGTCCAGCTTCGGGCTCCTGAGCTGCATCTTCTTCCACAAGTGCT
ACATCATCCTGCTCAAGCCCGAGCGCAACACGCCCGAGGAGGTGCGCTGCAGCACGGCCGCCCACT
CCTTCAAGACGGCGGCGCGCGTCAACCTCCACACGGCGTCGAGCATCGGCAGCGGCGGCAGCGCGG
GCAGCATG

Figure 6.3: CasR amino acid and nucleotide sequence of *Petromyzon marinus.* A) Predicted amino acid sequences of *Petromyzon marinus* ACIII based on search with *Danio rerio* CasR amino acid sequence B) Coressponding nucleotide sequence of *Petromyzon marinus* CasR with Primers: CasR A (green)

A

V2R1 amino acid sequence of *Lethenteron camtschaticum*

MEPLCLLAIGLILLHITPHIETRLFIESGGDVILGGLFPLHNSVINLPLEFNAVPTSADCVMLNER
ALTRLYTMLFTIEEINNRSDLLPNMKLGYRVIDSCTDVMKAVEASYEFSLFGSTQKSPLVVIGDAY
SFLTIPAAYTLGLKHIPMISYSASAPSLSDKTRFPTFMRTIPSDTFQSKALAELVGHFGWTWIGIL
GSDDDYGRQGLGKFQSDAHEYGVCFDFQIWVPKKIAINDINSIVNVIHNSTVKVIIAFVIDAELEP
IIKEMARRQIVGKTFIASESWISSPIIHKPQYADVLEGTIGFDMISADMNKLSDFLRNSKPTTDNP
FMTELWQETFHCTPHPPMENASGLADAARLPACSGDESLGSVTSSFFNPSEMTVPYAVYLAVYAVA
HALHELAACTTSTGVFAGGHCANLSDIQPWQVMRYLKKVDFKDNGYNIRFDANGDPVAYYILKQWQ
RKDDGNMKMVEVGSYTNLNNNTNSTNNGHLIINDELLFWIGKQLKAPDSLCVAACPEGSRKEYEAG
QPSCCYRCVQCSDGEFSKTKDANNCVRCPPDSWSTGTHTDCFVKPVQYLKWDSVEGLILHIAAIVG
LFLTFDVLFIFCKYRETPIVKASNFKISLMLLVCLFCNFLCIYVFVGIPKPWMCIARQPFFGVSFS
SCLSCILVKTISMIIAFKPNQTRNDTFHRQMTRAEIPIVAVVIAIEVALCVVWYFLASPRVFRNEN
IKADTIFLQCDEGSPFNFAFIIAYLYVLALICLMLSFMVRKLPNNFNEGKFVMFSALTFFIVWISF
IPAYILSNEHRVVVEIISIILSGYGILIFLFFHKCYIILLQPQKNTREHVDRQLRNYIEREEEDNV
G

B

V1R1 nucleotide sequence of *Lethenteron camtschaticum*

ATGGAACCATTATGTTTACTAGCTATAGGCTTAATCTTACTGCATATAACTCCTCATATAGAAACCA
GGCTGTTTATAGAGTCTGGTGGAGACGTTATATTAGGTGGATTGTTTCCACTGCACAACAGCGTAAT
TAATCTACCACTTGAATTCAACGCCGTTCCTACATCTGCGGATTGCGTAATGTTAAACGAGAGGGCT
CTGACAAGACTCTACACGATGCTGTTCACAATCGAAGAGATCAATAATAGATCTGATCTTCTGCCCA
ATATGAAGCTTGGGTATAGAGTCATAGACTCGTGCACTGATGTTATGAAGGCTGTGGAGGCCTCCTA
CGAATTCTCACTGTTTGGGTCGACACAGAAATCTCCGCTGGTAGTCATAGGAGATGCCTATTCTTTT
TTGACGATTCCTGCAGCATACACATTGGGTTTAAAGCACATTCCAATGATAAGTTACTCTGCAAGTG
CACCAGCCTGAGTGACAAGACAAGGTTTCCAACGTTCATGAGGACGATCCCCAGCGACACATTCCAG
TCAAAGGCTCTTGCCGAGCTTGTCGGTCATTTCGGCTGGACGTGGATTGGCATTTTGGGCTCAGACG
ATGACTACGGCAGGCAGGGCCTCGGAAAGTTTCAAAGCGATGCCCACGAGTATGGCGTCTGCTTCGA
CTTTCAAATCTGGGTGCCTAAGAAAATCGCGATTAATGACATCAATAGCATCGTGAATGTGATTCAC
AATTCCACAGTGAAAGTAATTATTGCATTTGTAATTGACGCCGAACTTGAACCTATCATCAAAGAGA
TGGCCAGAAGACAAATTGTTGGGAAAACGTTCATCGCAAGTGAATCTTGGATTAGTTCTCCAATCAT
TCACAAACCGCAGTATGCCGACGTTCTTGAAGGCACCATAGGGTTTGACATGATTAGCGCCGACATG
AACAAGCTCAGCGATTTCCTTAGAAACAGCAAACCTACAACAGACAACCCGTTCATGACTGAGCTGT
GGCAGGAGACATTTCACTGTACACCGCATCCACCAATGGAGAACGCATCTGGACTTGCGGACGCCGC
GCGCCTTCCGGCTTGCAGTGGAGACGAGTCTCTGGGCAGCGTGACCTCGTCGTTCTTCAACCCGAGC
GAGATGACCGTGCCGTACGCGGTGTACCTGGCGGTGTACGCCGTGGCCCACGCACTGCATGAGCTCG
CCGCCTGCACAACGTCGACCGGTGTCTTTGCGGGAGGCCACTGCGCCAATCTGTCTGACATCCAGCC
ATGGCAGGTTATGAGGTACTTAAAGAAAGTTGACTTCAAAGATAACGGCTACAACATTAGATTTGAC
GCAAATGGAGATCCCGTTGCCTATTACATCTTGAAGCAATGGCAGCGGAAGGACGATGGGAACATGA
AGATGGTAGAAGTCGGGTCCTACACCAATCTAAACAACAACACGAACAGCACCAACAATGGACATCT
CATCATCAACGATGAGCTCCTCTTTTGGATAGGGAAACAGCTGAAGGCTCCGGATTCATTATGCGTG
GCTGCTTGTCCAGAAGGAAGCAGGAAGGAATACGAAGCGGGGCAGCCTTCCTGCTGCTACAGATGTG
TGCAATGCTCAGATGGCGAGTTCAGCAAGACTAAAGACGCCAATAACTGCGTGAGGTGCCCTCCGGA
CTCCTGGTCAACGGGAACGCACACCGACTGCTTTGTTAAACCCGTGCAGTACCTAAAGTGGGACTCT
GTCGAAGGGCTCATTCTTCACATTGCCGCGATCGTTGGCCTCTTTCTCACGTTCGATGTACTCTTCA
TTTTCTGCAAGTATAGAGAAACACCAATCGTAAAGGCGTCAAATTTCAAAATAAGCCTCATGCTATT
GGTTTGTTTGTTCTGCAATTTCCTCTGCATCTACGTGTTTGTCGGCATACCAAAGCCCTGGATGTGC
ATTGCCCGACAGCCCTTTTTTGGTGTCAGCTTCTCGTCCTGCTTGTCTTGCATCTTGGTCAAAACAA
TCTCAATGATAATTGCGTTCAAGCCCAATCAAACCAGGAATGACACATTCCACAGACAAATGACACG
CGCAGAAATCCCTATCGTAGCCGTGGTTATTGCCATAGAAGTTGCATTGTGCGTGGTCTGGTACTTC
CTAGCATCTCCGAGAGTATTTAGGAATGAGAACATCAAAGCCGACACAATCTTCCTGCAATGCGACG
AAGGCTCTCCATTCAACTTTGCGTTTATTATCGCGTATTTGTACGTTTTGGCATTGATTTGTTTAAT
GCTATCATTCATGGTACGCAAGCTTCCTAATAACTTCAACGAGGGCAAGTTCGTGATGTTCAGTGCC
TTAACGTTCTTTATTGTGTGGATTTCATTCATCCCCGCTTACATTCTGAGTAACGAGCACAGGGTCG
TTGTTGAGATCATCTCTATCATTCTCTCTGGCTACGGAATCCTTATTTTTCTGTTCTTCCACAAATG
CTACATCATTTTGCTTCAACCACAGAAAAATACAAGAGAGCATGTAGATAGACAGTTGAGGAATTAT
ATAGAAAGAGAGGAGGAAGACAATGTTGGATGA

Figure 6.4: V2R1 amino acid and nucleotide sequence of *Lethenteron camtschaticum.* A) Predicted amino acid sequences of *Lethenteron camtschaticum* V2R1 based on search with *Danio rerio* amino acid sequence B) Corresponding nucleotide sequence of *Lethenteron camtschaticum* V2R1 with primers: V2R1 A (green); V2R1 B (pink); V2R1 C (blue)

A

V2R2 amino acid sequence of *Lethenteron camtschaticum*

ISLYMRYLTWMYTMLYAIDEINERQDLLPDIRLGYDIYDSCTNVMKSLEAGVALMKATEDLTRPPL
VGVIGDGNSKQTVVLAQMLGLHNVPLISYAASAPALGNKAEFPTFMRTIPGDSSQSKALAELVGHF
RWTWIGTLGSDDEYGRQGLSHFENEVASMYKVCFSFRLWIPKNAQYDDITKIVDTIADSNAMSIVV
FAIDTDFEPVLKEVVRRNIVDRIWVASEGWITSPYLNKPEYAPTLEGTIGFDVAQGNVQDIMDYLR
NPMRVAENPFGDEYLKEAFGCTLPTAGPNHSDTSTDTNNATSAGNFPSAAGMGIPFAVYLAVYTVA
HALHDLLDCDTKRGADEKSSCANVSNIQPWQVIMLMKMHDLNFQQNNYSVKFFKNGDPLPHYVLKN
WQRQKDGTLVIKNVGTYEYSKEGTNASALHFTSEPMWKNSSSTVPASMCSVPCIKGQRKEFGPGWS
AQCCYKCVSCSDGSYSDKDDAINCTDCTPNEMSSENHTSCVPKPLEYLRWGSGEGITLVVLAMLGF
CFTLAVTVIFVRYHDTPIVKASNRTLYFTLLFSLGCMFLGTLTFFGEPAPWQCFVQQPCFGISFSL
CLSCTLVKAVEMVVAFKPSEVFTNKLKIIMKFEVVIVALLTSIEVVICVLWLAILQPQVTMQPSLK
SINVECQKSSLFLIPILSYIYLLGLVCVVLAFLVRKVPKNFNEGKLVLLGMLTFFIVWISFIPAYY
VTPGKYMVAVEVISIILSGYGIIGFLFFRKCYIILWKPQNNTRWRVNNDQLCQRERDN

B

V1R2 nucleotide sequence of *Lethenteron camtschaticum*

ATAAGCCTGTACATGCGGTACCTCACATGGATGTACACCATGCTGTACGCTATCGACGAGATCAAC
GAGAGGCAAGATCTTCTGCCCGACATCAGGCTCGGTTATGACATCTATGACTCCTGCACGAACGTC
ATGAAGTCCCTCGAGGCGGGCGTGGCATTGATGAAAGCAACGGAGGACCTCACGAGACCACCTCTC
GTAGGGGTCATCGGAGATGGAAACTCAAAGCAGACCGTTGTCTTGGCCCAGATGCTCGGCTTACAC
AACGTGCCACTGATAAGCTATGCTGCCTCGGCACCTGCCCTCGGCAATAAAGCGGAGTTCCCCACC
TTCATGCGCACCATCCCCGGTGACAGCTCGCAGTCAAAGGCACTGGCGGAGCTGGTCGGGCACTTT
CGCTGGACCTGGATCGGCACACTGGGCTCGGACGACGAGTATGGCCGCCAGGGCCTTTCCCACTTC
GAAAACGAAGTAGCATCGATGTACAAAGTGTGCTTTAGCTTCAGGCTGTGGATCCCAAAGAATGCT
CAGTACGACGACATCACGAAAATCGTCGACACCATTGCCGACTCGAATGCGATGAGCATCGTCGTA
TTCGCCATCGACACCGACTTTGAGCCGGTCCTCAAGGAAGTGGTCAGACGCAATATAGTGGACAGG
ATCTGGGTGGCCAGCGAGGGGTGGATCACATCTCCCTATTTAAATAAACCCGAGTACGCACCCACG
CTGGAAGGGACCATAGGATTTGACGTGGCCCAAGGCAACGTCCAGGACATCATGGACTACCTGCGG
AACCCCATGCGGGTAGCGGAAAATCCGTTCGGGGACGAGTATTTGAAAGAGGCTTTCGGCTGCACG
CTCCCCACCGCAGGGCCAAATCATTCTGACACCTCAACTGATACAAATAATGCCACCTCTGCAGGC
AATTTCCCTTCTGCTGCGGGGATGGGCATCCCGTTCGCCGTCTACTTAGCCGTCTACACGGTAGCA
CACGCACTCCACGACCTCCTCGACTGCGACACGAAGAGGGGAGCTGATGAGAAAAGCAGTTGCGCC
AATGTGTCAAACATCCAGCCGTGGCAGGTGATTATGTTAATGAAGATGCACGATTTAAACTTCCAA
CAAAATAATTACAGTGTAAAGTTCTTCAAGAATGGCGACCCGTTGCCACACTACGTCCTCAAGAAC
TGGCAGAGGCAGAAAGACGGGACCTTGGTTATCAAGAATGTGGGCACTTACGAGTACAGCAAGGAA
GGGACCAATGCAAGCGCTCTCCATTTCACCTCCGAGCCCATGTGGAAGAACTCCAGCTCAACGGTA
CCTGCATCCATGTGTTCAGTTCCTTGTATAAAAGGCCAGAGAAAGGAATTTGGACCTGGCTGGAGT
GCGCAATGTTGTTACAAGTGCGTCAGCTGCTCAGATGGAAGCTACAGCGATAAAGACGATGCCATC
AACTGCACAGACTGTACCCCCAACGAGATGTCCTCAGAAAACCACACCTCCTGCGTGCCCAAGCCG
TTGGAATACTTGCGTTGGGGATCGGGCGAGGGCATCACACTCGTGGTCCTGGCCATGCTGGGCTTC
TGCTTCACCCTGGCGGTGACTGTGATCTTCGTCAGATATCACGACACCCCCATCGTGAAGGCATCA
AACCGTACCTTGTACTTCACACTGCTCTTCTCGCTTGGCTGCATGTTCTTGGGCACGCTGACTTTC
TTCGGGGAGCCTGCGCCATGGCAGTGCTTTGTGCAACAGCCATGTTTTGGCATCAGCTTCTCCCTG
TGTCTCTCCTGTACCCTGGTGAAGGCGGTGGAGATGGTGGTCGCCTTTAAGCCCAGTGAAGTTTTC

ACTAATAAGCTGAAAATCATCATGAAGTTTGAGGTTGTCATCGTGGCTCTGCTCACCAGTATCGAG
GTGGTTATATGCGTGCTATGGCTGGCGATCTTGCAGCCACAGGTCACGATGCAGCCCAGTCTGAAA
TCCATCAACGTCGAATGTCAAAAGTCCTCCTTATTCCTGATCCCGATTCTCAGCTATATCTACCTG
CTGGGTCTCGTCTGCGTGGTCTTGGCGTTTCTGGTGCGAAAGGTTCCGAAGAATTTTAACGAGGGC
AAGTTAGTGCTGCTGGGAATGCTGACTTTCTTCATTGTGTGGATCTCATTCATCCCAGCCTACTAC
GTCACACCTGGAAAGTACATGGTGGCCGTGGAGGTCATATCCATCATACTGTCTGGATACGGGATT
ATTGGATTTTTGTTTTTCCGCAAATGTTACATCATTTTGTGGAAGCCACAAAATAACACTCGATGG
CGTGTAAATAATGATCAGCTGTGCCAAAGAGAGAGAGACAACTAA

Figure 6.5 : V2R2 amino acid and nucleotide sequence of *Lethenteron camtschaticum.* A) Predicted amino acid sequences of *Lethenteron camtschaticum* V2R2 based on search with *Danio rerio* amino acid sequence B) Corresponding nucleotide sequence of *Lethenteron camtschaticum* V2R2 with primers: V2R2 A (green); V2R2 B (orange); V2R2 C (blue); V2R2 D (pink)

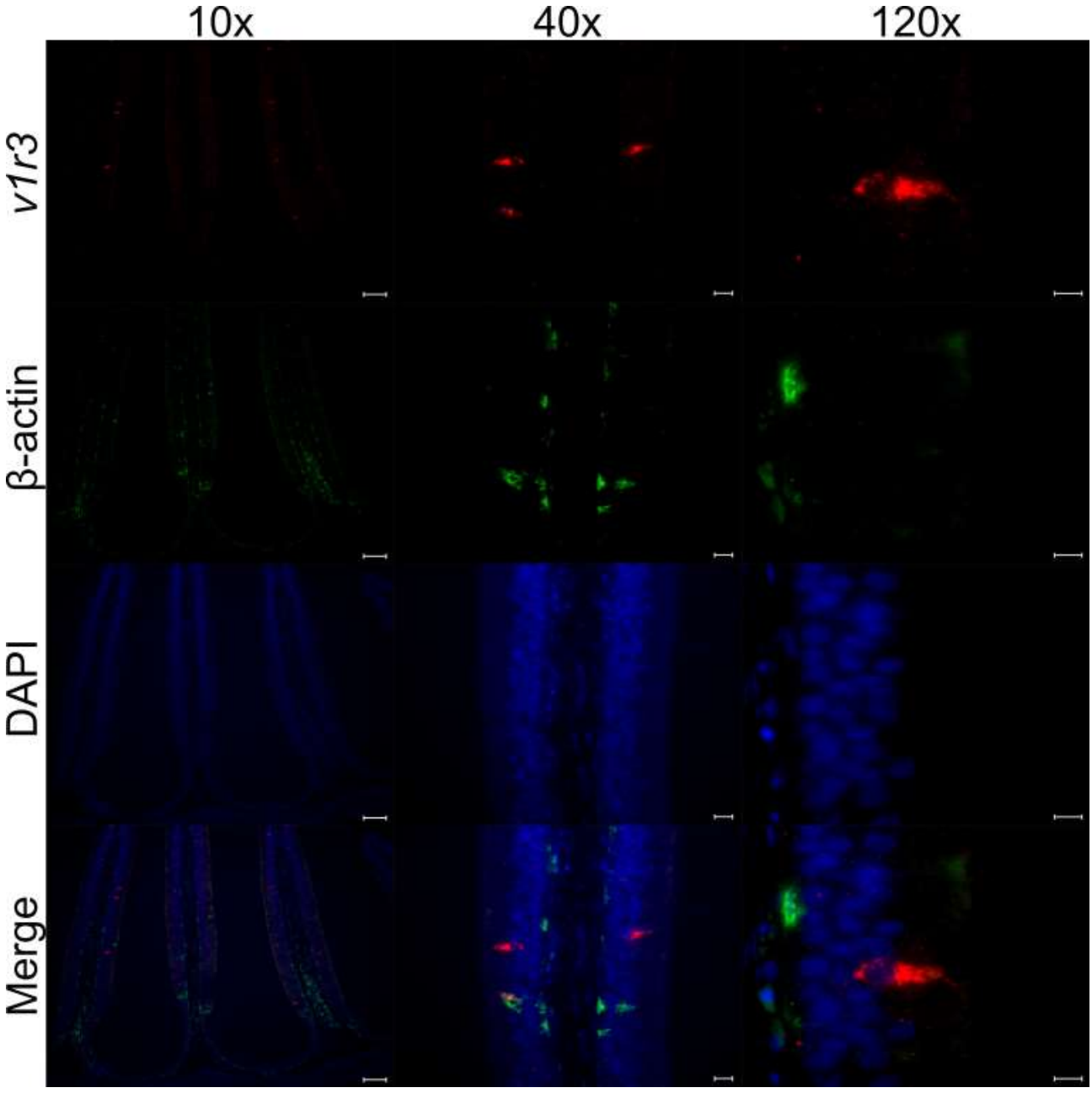

Figure 6.6: *V1r3* is not coexpressed with β-actin in the OE of *Lampetra fluviatilis*. V1R3 probe (red) and β-actin-antibody (green) are not coexpressed in the lamellae. Fluorescent double staining of *v1r3* (ISH) and β-actin (IHC) in the olfactory epithelia of *Lampetra fluviatilis*. *V1r3* was visualized with TSA Alexa Fluor 633 and β-actin with an anti-mouse Alexa Fluor 488 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

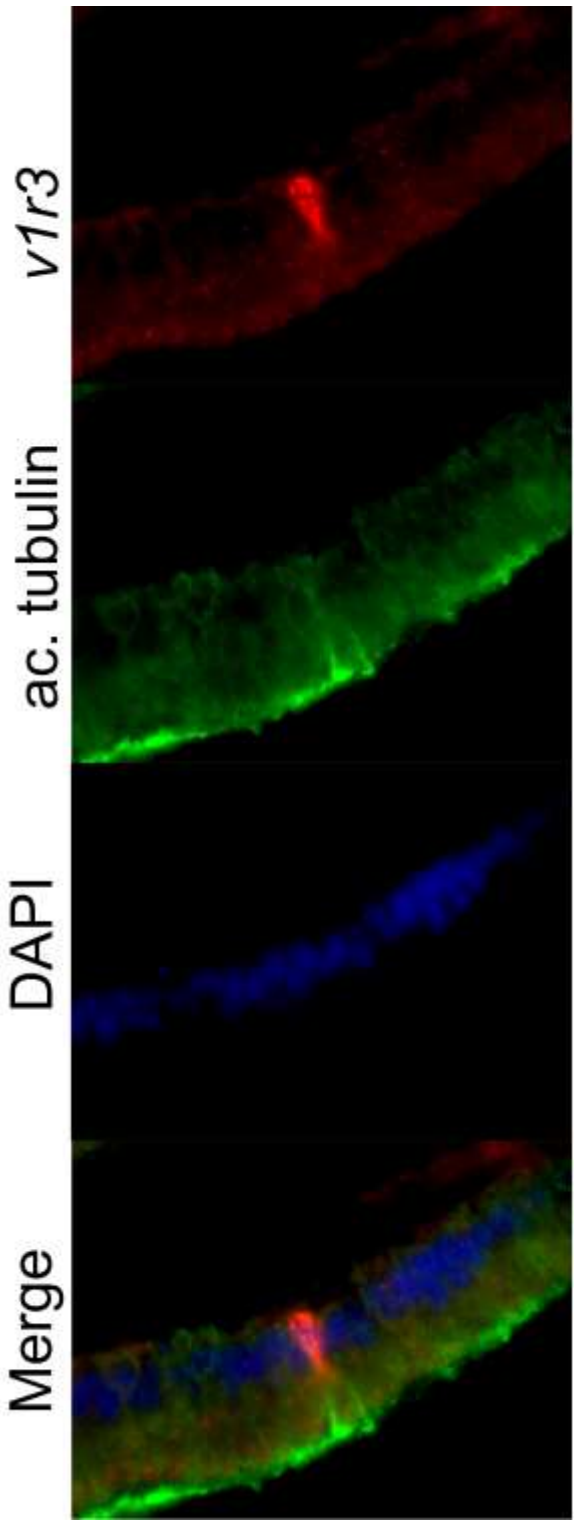

Figure 6.7: *V1r3* is not directly coexpressed acetylated tubulin in the OE of *Lampetra fluviatilis*. Possible connection between V1R3 receptor (red) and filaments stained with tubulin-antibody (green) in the lamellae. Fluorescent double staining of *v1r3* (ISH) and acetylated tubulin (IHC) in the olfactory epithelia of *Lampetra fluviatilis. V1r3* was visualized with TSA Alexa Fluor 633 and acytelated tubulin with an anti-mouse Alexa Fluor 488 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei.

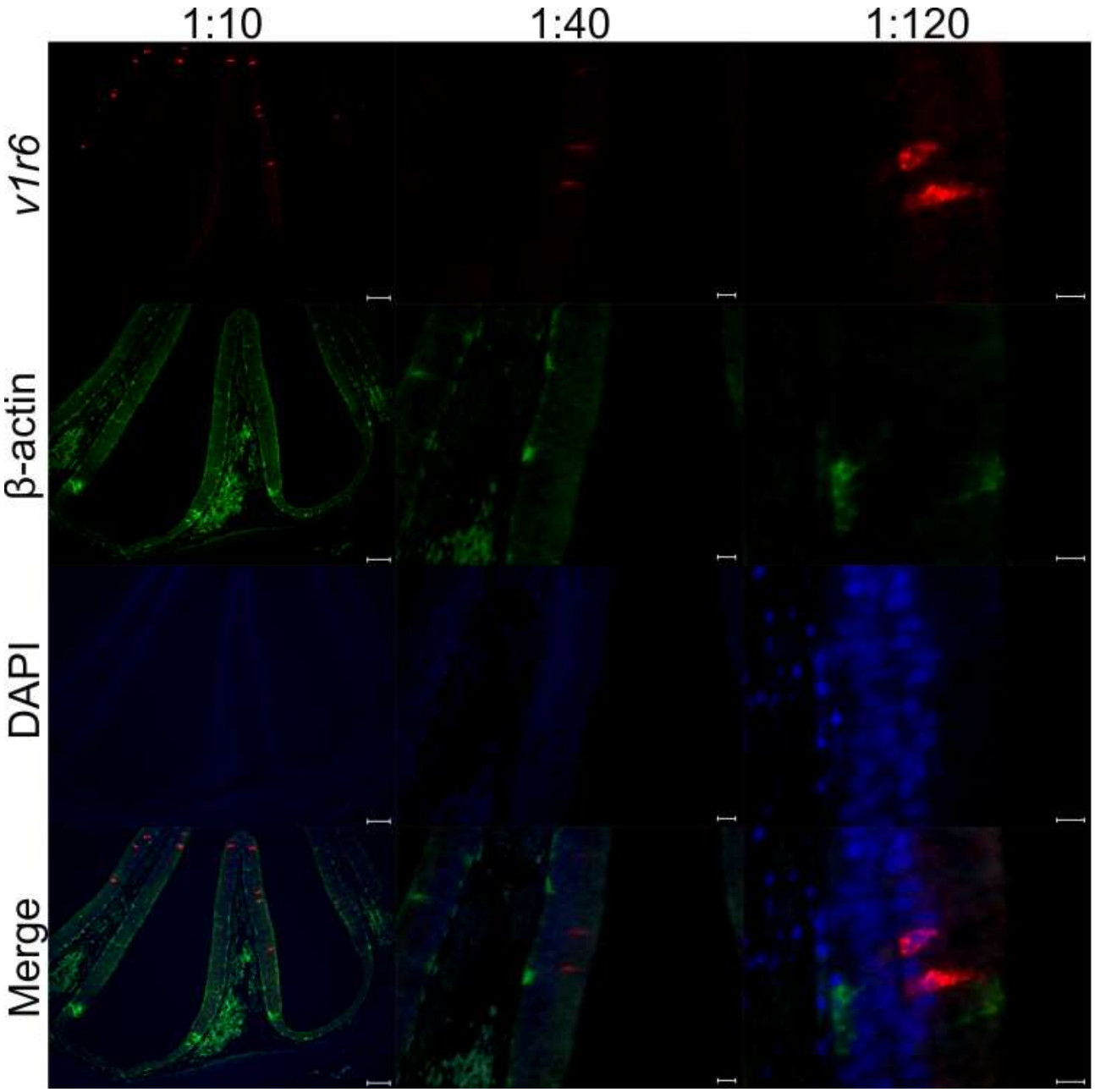

Figure 6.8: *V1r6* is not coexpressed with β-actin in the OE of *Lampetra fluviatilis*. V1R6 probe (red) and β-actin-antibody (green) are not coexpressed in the lamellae. Fluorescent double staining of *v1r6* (ISH) and β-actin (IHC) in the olfactory epithelia of *Lampetra fluviatilis*. *V1r6* was visualized with TSA Alexa Fluor 633 and β-actin with an anti-mouse Alexa Fluor 488 antibody. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) to visualize the localisation of the TSA signal relative to the nuclei. Scale bar for 10x) 100µm 40x) 20µm 120x) 10µm

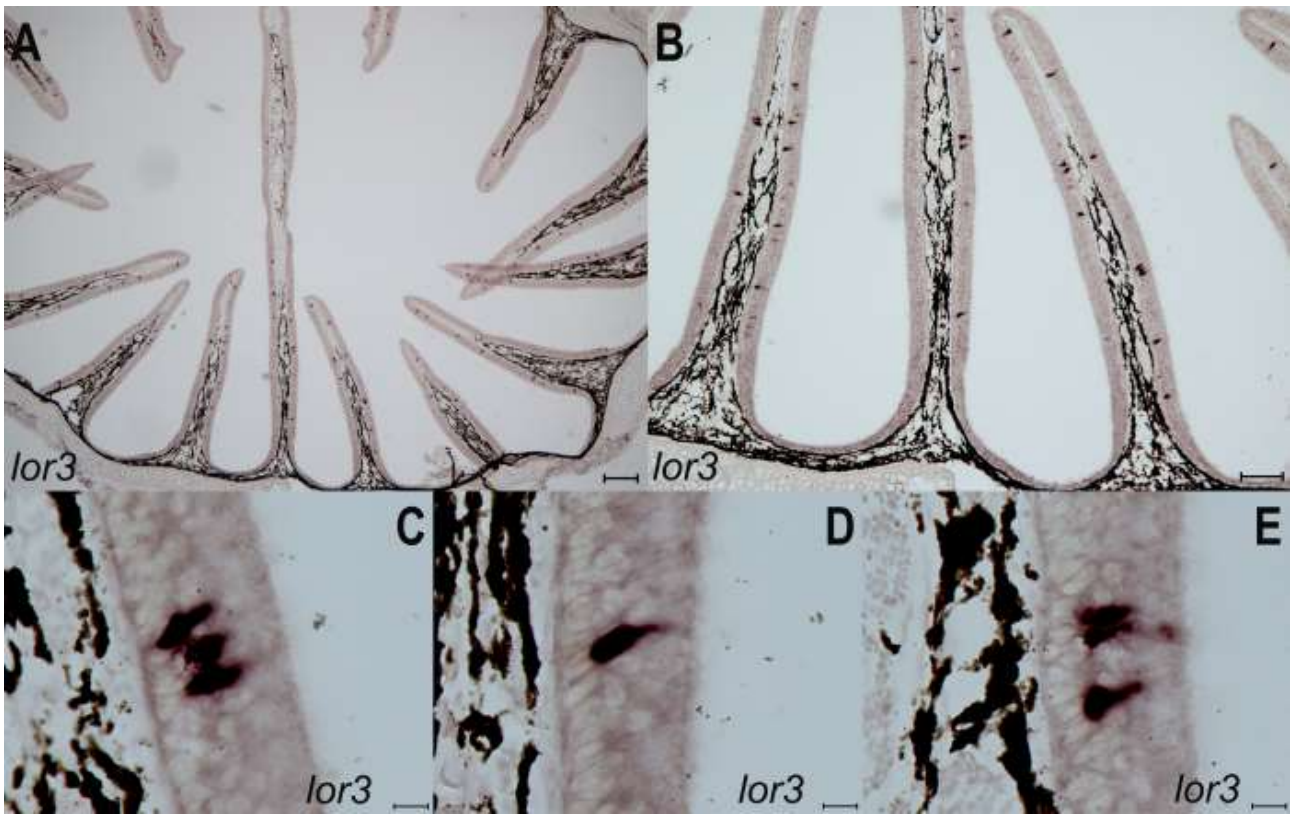

Figure 6.9: *Lor3* expression in the olfactory epithelia of *Lampetra fluviatilis*. A)-E) LOR3 is expressed in the olfactory sensory area in the lamellae. NBT/BCIP stainings of *lor3* in the olfactory area of *Lampetra fluviatilis*. Scale bar A) 200µm B) 100µm C) - E) 10µm

a2c	CARP	EEL	ZF
EEL	0,533 (2.2e-16)		
ZF	0,258 (2.083e-08)	0,397 (2.2e-16)	
LAMP	0,257 (2.489e-09)	0,769 (2.2e-16)	0,402 (2.2e-16)

Table 6.1: Kolmogorov–Smirnov test for radial measurement of A2c-positive cells in different fish species, reveals highly significant difference between all species. The distribution of A2c-positive cells for carp (n=244), eel (n=299), zebrafish (n=324) and lamprey (n=444) are significant different. All distributions numbers shown the the absolute max distance between the ECDFs of two samples and numbers in brackets shown the p-values (statistical significance). p<0.01= data significant

Gene	*v1r3*	*v1r5*	*v1r6*
v1r5	0,105 (0,1515)		
v1r6	0,224 (2,6e-06)	0,257 (1,0e-06)	
lor3	0,074 (0,2326)	0,058 (0,724)	0,22 (1,5 e-07)

Table 6.2: Kolmogorov–Smirnov test for radial measurement of three different *v1r* genes and one *or* gene in *Lampetra fluviatilis*. Three different V1Rs, *v1r3* (n=297); *v1r5* (n=195) and *v1r6* (n=247) and one OR *lor3* (n=563) where tested. The test shows that only the radial distribution of V1R6 is significant different from all other tested receptors. Numbers shown the absolute max distance between the ECDFs of two samples and numbers in brackets shown the p-values (statistical significance). p<0.01= data significant

	v1r3	*v1r5*	*v1r6*
v1r5	0,211 (4.035e-05)		
v1r6	0,3 (2.288e-11)	0,192 (0.0006391)	
lor3	0,11 (0.006917)	0,216 (2.203e-06)	0,307 (1.665e-14)

Table 6.3: Kolmogorov–Smirnov test for height inside the lamella measurement of three different *v1rs* genes and one *or* gene and in *Lampetra fluviatilis*. Three different V1Rs, *v1r3* (n=322); *v1r5* (n=196) and *v1r6* (n=247) and one OR *lor3* (n=564) where tested. The test shows that *v1r3* is significant different from *v1r5* and *v1r6* and *lor3* is significant different from V1R5 and V1R6. Numbers shown the absolute max distance between the ECDFs of two samples and numbers in brackets shown the p-values (statistical significance). p<0.01= data significant

	ora1	*ora2*	*ora3*	*ora4*	*ora5*
ora2	0,11 (4,9e-11)				
ora3	0,505 (2,2e-16)	0,55 (2,2e-16)			
ora4	0,371 (2,2e-16)	0,422 (2,2e-16)	0,151 (2,0e-15)		
ora5	0,377 (2,2e-16)	0,436 (2,2e-16)	0,201 (1,85e-09)	0,12 (0.000333)	
ora6	0,430 (2,2e-16)	0,49 (2,2e-16)	0,244 (2,28e-06)	0,146 (0,1076)	0,753 (0,6647)

Table 6.4: Kolmogorov–Smirnov test for radial measurement of six different *ora* genes in *Danio rerio*. The radial distance in the lamella was measured as described in 2.8 for *ora1* (n=1788) *ora2* (n=2359) *ora3* (n=1075) *ora4* (n=2612) *ora5* (n=338) and *ora6* (n=129). The radial distribution between all Oras is significant different, except *ora6* with *ora4* and *ora5*. Numbers shown the absolute max distance between the ECDFs of two samples and numbers in brackets shown the p-values (statistical significance). p<0.01= data significant

	ora1	*ora2*	*ora3*	*ora4*	*ora5*
ora2	0,409 (2,2e-16)				
ora3	0,296 (2,2e-16)	0,125 (1,64e-10)			
ora4	0,432 (2,2e-16)	0,081 (1,63e-07)	0,15 (2,8e-15)		
ora5	0,628 (2,2e-16)	0,323 (2,2e-16)	0,401 (2,2e-16)	0,400 (2,2e-16)	
ora6	0,558 (2,2e-16)	0,205 (6,9e-5)	0,308 (6,7e-10)	0,2 (0,0001073)	0,286 (4,5e-07)

Table 6.5: Kolmogorov–Smirnov test for height in the lamella of six *ora* genes in *Danio rerio.* The height distance in the lamella was measured as described in 2.8 for *ora1* (n=1792) *ora2* (n=2359) *ora3* (n=1074) *ora4* (n=2613) *ora5* (n=338) and *ora6* (n=129). The height of all six investigated Oras is significant different. Numbers shown the absolute max distance between the ECDFs of two samples and numbers in brackets shown the p-values (statistical significance). p<0.01= data significant

	ora1	*ora2*	*ora3*	*ora4*	*ora5*
ora2	0,047 (0,7865)				
ora3	0,111 (0,059)	0,097 (0,13)			
ora4	0,15 (0,012)	0,134 (0,004)	0,069 (0,544)		
ora5	0,150 (0,016)	0,15 (0,014)	0,123 (0,129)	0,167 (0,006)	
ora6	0,187 (0,0116)	0,173 (0,024)	0,109 (0,42)	0,162 (0,046)	0,09 (0,78)

Table 6.6: Kolmogorov–Smirnov test for height within the OE of six *ora* genes in *Danio rerio.* The height within the OE was measured as described in 2.8 for *ora1* (n=373) *ora2* (n=387) *ora3* (n=233) *ora4* (n=375) *ora5* (n=151) and *ora6* (n=92). The height within the OE is only significant different for *ora2/ora4*, and *ora4/ora5*. All other pairs show no significant differences between the height within the OE . Numbers shown the absolute max distance between the ECDFs of two samples and numbers in brackets shown the p-values (statistical significance). p<0.01= data significant

7. APPENDIX

7.1 Erklärung zur Dissertation

"Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit − einschließlich Tabellen, Karten und Abbildungen −, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie − abgesehen von unten angegebenen Teilpublikationen − noch nicht veröffentlicht worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.
Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Sigrun Korsching betreut worden."

Köln, 13.04.2021

7.2 Publications

AHUJA G., BOZORG NIA S., ZAPILKO V., SHIRIAGIN V, **KOWATSCHEW D**., OKA Y., KORSCHING SI. (2014) Kappe neurons, a novel population of olfactory sensory neurons. Sci Rep. 4:4037.

AHUJA G., REICHEL V., **KOWATSCHEW D**., SYED AS., KUMAR KOTAGIRI A., OKA Y., WETH F., KORSCHING SI. (2018) Overlapping but distinct topology for zebrafish V2R-like olfactory receptors reminescent of odorant receptor spatial expression zones. BMC Genomics. 19:383.

TWICKEL AV., **KOWATSCHEW D**., SALTÜRK M., SCHAUER M., ROBERTSON B., KORSCHING S., WALKOWIAK W., GRILLNER S., PEREZ-FERNANDEZ J. (2019) Individual Dopaminergic Neurons of Lamprey SNc/VTA Project to Both the Striatum and Optic Tectum, but Restrict Co-release of Glutamate to Striatum Only. Curr Biol. 29:1-9.

LIESSEM S., **KOWATSCHEW D.**, DIPPELC S., BLANKEA A., KORSCHING SI., GUSCHLBAUERA C., HOOPERD S.L., PREDELA R., BÜSCHGES A. (2021) Neuromodulation Can Be Simple: Myoinhibitory Peptide, Contained in Dedicated Regulatory Pathways, Is the Only Neurally-Mediated Peptide Modulator of Stick Insect Leg Muscle J. Neurosci.41(13):2911-2929.

DIERIS M., **KOWATSCHEW D.**, AND KORSCHING SI. (2021) Olfactory function in the trace amine-associated receptor family (TAARs) evolved twice independently. Scientific Reports

Publications in review:

KOWATSCHEW D. AND KORSCHING SI. An ancient adenosine receptor gains olfactory function in bony vertebrates.

Publications in preperation:

KOWATSCHEW D., BOZORG NIA S., HASSAN S., KORSCHING SI. Spatial coding logic for a complete family of olfactory receptor genes, the V1R-related ora genes shows clearly segregated if broadly overlapping expression zones.

7.3 DANKSAGUNG

An dieser Stelle möchte ich allen allen beteiligten Personen meinen großen Dank aussprechen, die mich bei der Anfertigung meiner Doktorarbeit unterstützt haben und die vielleicht hier nicht namentlich erwähnt werden!

Mein besonderer Dank gilt meiner Doktormutter Prof. Dr. Sigrun Korsching für die ausgezeichnete Betreuung und die enorme Unterstützung bei der Umsetzung der gesamten Arbeit. Durch Dich habe habe ich mich nach meinem persönlichen Empfinden enorm weiterentwickeln können, gerade weil Du mir in den gemeinsamen Projekten freie Hand gewährt hast und nur in den kritischen Phasen die Zügel angezogen hast. Außerdem war es immer eine Freude für mich auch außerhalb der Arbeit, mit Dir und Deiner Familie gemeinsame schöne Stunden zu verbringen.

Des Weiteren möchte ich mich bei Prof. Dr. Kay Hofmann und Prof. Dr. Peter Kloppenburg bedanken, welche sich unkompliziert die Zeit genommen haben mich zu prüfen und mich somit wahrscheinlich auf den letzten Metern zum Abschluss meiner Doktorarbeit begleiten.

Ein großes Dankeschön geht an Alan Metcalfe und Dr. Katharina „Katie“ Dittrich, welche sich die Zeit und Nerven genommen haben die Arbeit in ein verständliches Englisch zu bringen. @ Alan: Danke dafür, dass ich durch Dich die wunderbare Welt der Klassik zu schätzen gelernt habe, was zu totaler Entspannung führte, wenn auch die Experimente misslangen! @Katie: Danke für das ich Dich damals in Göttingen kennenlernen durfte, und ich hoffe auch weiterhin auf lustige Abende, an denen wir zusammen die Leber baumeln lassen können.
Mehmet Saltürk ich möchte Dir hiermit herzlich danken, dass Du nicht nur ein guter Freund geworden bist, sondern für mich und den restlichen Labormitgliedern immer ein fantastischer Labormanager warst und es weiterhin für die andern sein wirst. Ich hoffe Dein Traum vom Lebensabend in Antalya wird bald wahr und wir sehen uns irgendwann bald dort am Strand mit einem kühlen Bier in der Hand und lassen dann die alten Zeiten Revue passieren. Ich wünsche Dir auch ewig aufgepumpte Reifen an Deinem Fahrrad und freue mich auf neue 100 km Touren auf dem Drahtesel mit Dir.

Ein weiter großer Dank geht an meinen guten Freund Dr. Venkatesh „Hans“ Krishna, es war eine Ehre und Freude mit Dir zusammengearbeitet und auch außerhalb der Uni gefeiert zu haben und es wird mir eine Freude und Ehre sein Dich weiterhin als meinen Freund zu haben, egal wo Du auf der Welt gerade steckst. Vielleicht schaffen wir es irgendwann einmal Nordlichter in Nordnorwegen zu sehen, anstatt uns nur den Hintern mit überteuerter Kaltschale in der Hand abzufrieren.
Danke auch an Dr. Ivan Ivandic, ich habe die Zeit mit Dir und Deinem großartigen Humor extrem genossen.
Einen Lieben Dank auch an Günes Birdal, für das letzte schöne gemeinsame Jahr im Labor! Ich bin gespannt wer von uns als erstes alle Biere dieser Welt verköstigt hat ;)!
Ein weiterer Dank geht an Alexandra Scharn, die mich während ihrer Zeit als Bachelor und Master einen lange Zeit im Labor und außerhalb des Labors begleitet hat. Es war immer witzig mit Dir, obwohl ich mir nicht immer sicher war, ob Du trotz Deiner Kopfhörer alles verstanden hast ;)!
Ein großer Dank geht auch an Dr. Milan Dieris für die fantastische Zusammenarbeit in unseren gemeinsamen Projekten und die witzige Zeit im Göttinger Nachtleben zusammen mit Dr. Katharina Dittrich, sowie unsere gemeinsame Tour durch Indien.
Herzlichen Dank auch an Dr. Gaurav Ahuja, welcher mich in der ersten Zeit im Labor in die Geheimnisse des wissenschaftlichen Arbeitens eingewiesen hat.
Auch danken möchte ich Dr. Arndt von Twickel, für die hervorragende Zusammenarbeit während unseren Neunaugenprojekten!
An dieser Stelle auch einen großen Dank an Tanja Rayle, welche unser guter Geist des Labors ist und die mit ihrer Arbeit im Zusammenhang mit der Verwaltung des Labors uns immer den Rücken frei gehalten hat.
Lieben Dank auch an die Bachelorstudenten Liane, den beiden Marcos, Adil und Matthias, welche das Labor zu einer Wohlfühloase für mich gemacht haben.
Danke auch an ein paar meiner guten Freunde Andreas „Ölf Rakija“ Brändlein, Vladislav „The Machine“ Terziev, Torsten „Der Philosoph“ Neuber, Boris „Riders on the Storm“ Traub, sowie die gesamten Jungs meiner Fußballmannschaften, die mir immer wieder zeigten, dass es auch ein Leben außerhalb des Labors gab.
Ein weiter riesiger Dank geht an meine Familie die mich allzeit mit Liebe, Zeit und Geld unterstützt haben. Благодаря чичо за ракията! @ Marek und Peter: wir haben zusammen Nordkorea überlebt, dagegen war die Zeit im Labor ein Zuckerschlecken ;)! @ Carolin: Danke für Deine Liebe und Geduld!!!!!