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Optical characterization of ligand-induced staining of olfactory receptor neurons in *Xenopus laevis*

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Table of contents

1 INTRODUCTION

1.1 Olfaction

Olfaction is defined as the ability to detect chemical compounds in the air (airborne-terrestrial animals) or water (waterborne-aquatic animals) at a distance, through their medium (Lambrechts and Hossaert-McKey 2006). It is also referred to as an organism's competency to sense and identify a chemical substance at a particular concentration from its source. An odorant is an organic or inorganic substance capable of evoking an olfactory response (Powers-Schilling 1995).

The sense of smell is one of the 5 primary senses in vertebrates. In higher vertebrates, the olfactory system is coupled in function with the endocrine system and the rest of the sensory organs e.g. the gustatory organ. Its specificity differs in various animals. Similar to the observation made in humans, perception of odour as stimuli is initiated by the olfactory system, specifically in the olfactory bulb (OB) (Kelliher and Wersinger 2009). The olfactory system consists mainly of the main olfactory organ of which the OB is a part and the vomeronasal (accessory) organ (VNO).

Although humans within an age range are said to have a good sense of smell, it is speculated that some animals have an extraordinary ability to detect an odour as it is a subjective phenomenon that cannot easily be studied, especially in lower animals. At present, it is believed to be the less comprehended than all other senses. This reveals some notable merits most animals have over humans when present in their territory.

Some animals possess a very good sense of smell in order to be able to identify their prey from afar, sense danger and detect poisonous food. In man, an odour, depending on its nature and intensity can bring about diverse behavioural changes. It can provoke undesirable, spontaneous responses such as vomiting, headache, etc. but in some cases, could also elicit a sense of euphoria and calmness in that environment.

It is worth mentioning that considering a basic aspect of life e.g. the continuity of life, which involves adaptation, reproduction and natural selection, perception of odour is one of the deciding factors. In mammals, olfaction can alter most of the behavioural

and physiological changes provoked by social interaction (Spehr *et al.* 2006). The main olfactory organ detects high volatile chemical signals from a distance (Brennan and Kendrick 2006). It has been reported in many species that chemical signals do not just serve as sexual attractants, but also induce sexual arousal and pre-copulatory postures in a variety of lower and higher animals (Gelez *et al.* 2004). In this context, the critical role of the VNO has also been shown by experimental excision (Spehr *et al.* 2006). It has been observed in a study that olfactory genes are differently expressed in two sexes and some sexual behaviour-related genes in mice are predominantly expressed in females or in males (Shiao *et al.* 2012). Nonetheless, it has also been stated that the VNO is involved in sexual interactions within a species of animals (Dulac and Torello 2003) but is not known to play a major role in perception of odorants.

Olfactory dysfunction in humans can arise from a variety of causes and profoundly influence a patient's quality of life. The sense of smell determines the preferred flavour of foods and beverages and also serves as an early warning signal for the detection of environmental hazards, such as decayed food, leaking toxic gas or air-borne pollutants. Hence, nutritional preference can be adversely affected by a dysfunctional sense. In human medicine, olfactory dysfunction can aid as an early diagnostic tool for Alzheimer's disease and could be one of many non-motoric symptoms present in Parkinson's (Salawu *et al.* 2010). This dysfunction is known to contribute to a diminished quality of life in these patients, comparable to that in animals.

1.2 Structural and functional organization of the olfactory system in higher vertebrates

Being an organ that enables animals to detect danger and respond within a short notice, it is located along an animal's natural direction of movement and facilitates direct contact with molecules in its environment. The main olfactory system and the vomeronasal system interact with one another functionally. The olfactory system enables a wide range of animals, be it terrestrial or aquatic, to detect and distinguish a variety of odours (Dulac and Torello 2003). In a larval frog, the olfactory organ could be seen abstractly with bare eyes, without any magnification. It is observed as a dark structure located medially to both eyes and tentacles. It is covered by a thin layer of moist skin (membrane), beneath which is a mucous layer. The vomeronasal organ (VNO) is a chemosensory organ, consisting of vomeronasal sensory neurons, which express chemoreceptors capable of detecting pheromones. It is widely referred to as the "pheromone primary coding site" (Igbokwe and Onwuaso 2009). VNO has the ability to regulate gonadotropin secretion via hypothalamic influenced neural pathway, thereby monitoring the activity of gonads (Alekseyenko *et al.* 2006). In amphibians, the VNO consists of receptor neurons, which mostly bare microvilli, whereas the olfactory neurons of the main olfactory organ either bare cilia or microvilli (Manzini and Korsching 2011). The medial amygdala, which is directly connected to the ventromedial hypothalamus and the preoptic has a site of projection (accessory olfactory bulb) for the axons of the receptor neurons of an amphibian VNO (Igbokwe and Onwuaso 2009). The VNO neurons convey olfactory signals to parts of the brain's limbic system-amygdala and several hypothalamic regions incorporated in primary motivated behaviour (Keverne 2002). According to (Mostafa *et al.* 2011), pheromones are described are naturally occurring chemical compounds secreted by animals to evoke behavioural and hormonal changes in the opposite gender.

The main olfactory organ is divided into the peripheral and the central part. The peripheral component as illustrated in figure 1 of the olfactory system starts from the mucous layer of the olfactory epithelia (OE) and ends at the OB. The olfactory mucosa, in the common mudpuppy for example, is located in the superficial region and contains about 100 million olfactory receptor neurons (ORNs) (Dionne 1992) of

the nasal cavity. It consists of pseudo-stratified cells and is made up of three main cell types:

1. Olfactory receptor neuron (ORN): characteristic for olfactory neurons is their bipolar structure; bearing axons at one end and a dendrite at the opposite. The dendrites end in knobs from which thin cilia protrude to the surface of their epithelium making contact via the mucus that cover the membrane with the stimulus containing medium. The axons lead to the direction of the olfactory bulb (OB) (Manzini and Korsching 2011). In vertebrates, the ORNs in the periphery are the primary sensing cells. There are 2 known different types of ORNs, ones endowed with cilia or with microvilli (Chakrabarti and Ghosh 2013). Furthermore, ORNs express 2 classes of receptors in the amphibian olfactory epithelium namely; I and II, which is similar to those in humans (Mezler *et al.* 1999).

2. Sustentacular cells: also known as the supporting cells provide the olfactory receptor neurons with physical and metabolic support (Chakrabarti and Ghosh 2013). They are either ciliated or secretory types of the supporting cells (Hansen *et al.* 1998). Compared to other cell types found in the olfactory epithelium, they are the most numerous (Getchell 1986) and project up to the basal membrane of the olfactory epithelia (OE) (Schild and Restrepo 1998). They provide support to the dendrite projections and allow for cell bodies of the olfactory neurons to be shielded from the nasal cavity.

3. Basal cells: are by function stem cells. They safeguard the loss of smell that may occur due to mucosal disease or mechanical damage by differentiating into either into sustentacular cells or ORNs (Døving and Trotier 1998). This takes about a period of 56 days in frogs following damaging of an ON (Graziadei and DeHan 1973). Basal cells can serve throughout out the life span of an organism by regenerating damaged olfactory epithelial cells in mature or immature animals (Mackay-Sim and Kittel 1991). They lie on top of the basement membrane, which in turn, is supported by the connective tissues of the lamina propria.

In addition to these three main cell types, lies the highly cellular lamina propria, which contains large secretory glands called the bowmann's glands, which lie within the olfactory epithelium. They produce mucus, which keeps the membrane moist so as to assist odorant molecules in dissolving and stimulating the olfactory receptors. In

vertebrates, the ORNs are bipolar neurons with an apical dendrite and an axon at the opposite end of the soma. The axons are small in diameter, unmyelinated and have the function to convey information centrally. The cilia of the ORNs bear different membrane proteins and are sites for sensory transduction (Gold 1999). Odorant molecules bind chemically to protein receptors in the membranes of the olfactory cilia. The type of receptor in each olfactory cell determines the types of stimulant (Powers-Schilling 1995). Binding to a receptor creates a receptor potential in the olfactory cell that generates impulse in the ON fibers. Receptor sensitivity may explain the variation in detection thresholds exhibited by different compounds (Powers-Schilling 1995). Contrary to the accessory system, the main olfactory system is linked to several cortical areas: to the cortical amygdala, piriform cortex, which is connected to the orbitofrontal and insular cortical (Jansen *et al.* 1998).

The central olfactory organ consists of the olfactory bulbs (OB) and the higher brain centre. The OBs are paired cortical layer that receive information from the olfactory epithelium. Signals from the OE are conveyed to the main, whilst the VNO transfers its signals to the accessory OB. Axons protruding from the ORNs terminate in multiple globular structures within the OB called the glomeruli (Guyton and Hall 2006). In order for the axons of the receptor cell to form the ON, they must pass through the cribriform plate, a thin sheet of bone. There are many more receptor cells than glomeruli: so about 10 million receptor cells converge on thousands of glomeruli. The spatio-temporal patterns of the glomerular activity represent the olfactory information (Wachowiak and Shipley 2006), and are made of several layers, which assist in the propagation of signal to the brain.

A glomerular layer consists of synapses existing between the axons of the olfactory neurons (ORNs), dendrites of mitral cells, tufted cells and periglomerular cells. The periglomerular and granule cells function by inhibiting the mitral cells and tufted cells.

The mitral cells layer consists of mitral cells, which are directly connected to higher brain centres. In other words, axonal projections arising from the mitral cells run laterally to higher cerebral structures, especially to the olfactory cortex and associated fields after having been excited in the OB (Nezlin and Schild 2000). A collection of mitral cells integrates signal input from synapses formed with converging axons of the receptor cells and relays them to the higher brain centres. The external plexiform layer contains the passing dendrites of mitral cells, granule cells and a few

tufted cells, which are similar in size to mitral cells (Schmidt *et al.* 2005). Some of the granule cell dendrites in the plexiform layer contribute to lateral inhibition through specialized dendro-dendritic synapses known as reciprocal synapses (Schmidt *et al.* 2005). Granule cells are inhibitory interneurons with their main target being the mitral cells (Guyton and Hall 2006). On their activation, they inhibit the mitral cells. This subsequently leads to a finer discriminatory effect of different odours.

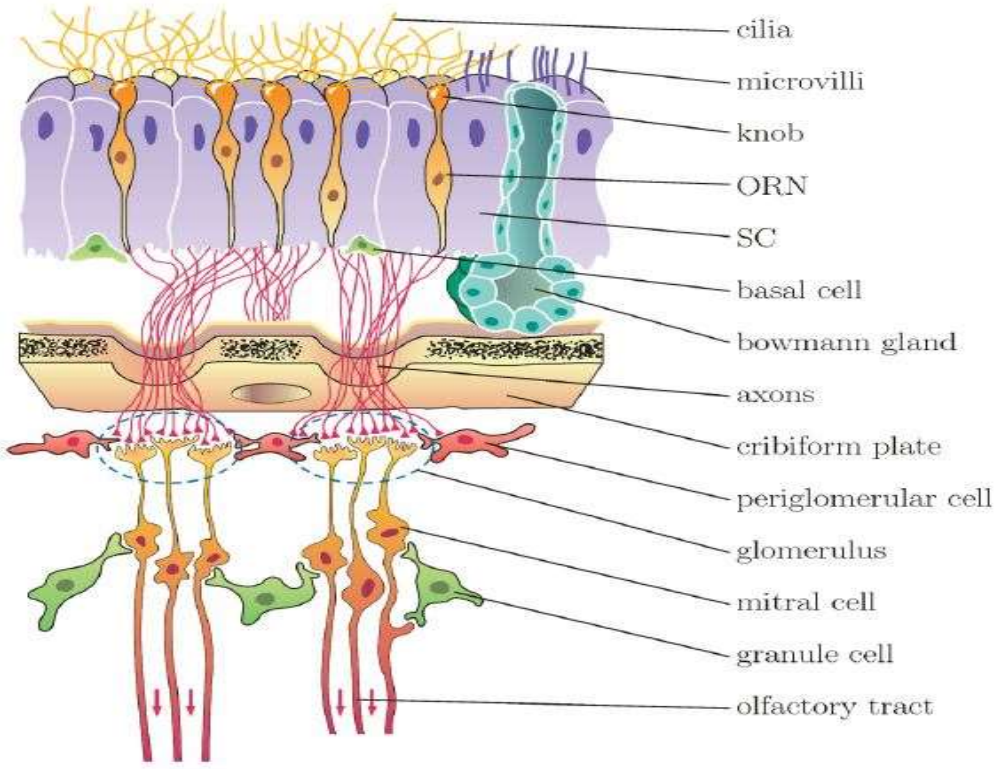


Figure 1: Histological organization of the main olfactory system. This figure represents the morphology of the olfactory system. Abbreviations: ORN: Olfactory receptor neuron; SC: sustentacular cell. Figure modified after Lang and Lang (2007); from “Basiswissen Physiologie”, page 429; 2nd edition; Springer Berlin Verlag.

1.3 Signal transduction mechanisms in ORNs

The olfactory system is rather a complex system. At present, there are many researches on anatomical, physiological and molecular levels on the sense of smell. Due to its complexity, it is yet to be completely understood. Nevertheless, its signal transduction pathway is widely known and forms a basis for understanding the olfactory system.

The chain of events that recognizes and converts the message is termed a “signal transduction” (Guyton and Hall 2006). Signal transduction starts primarily in the olfactory cavity, a thin, moist, mucus membrane that lines the olfactory cavity. The mucus membrane of the olfactory system is designed to strike a balance between efficient diffusion of odorant molecules and support of the immune system. Figure 2 below is an illustration of the signal transduction pathway. The first point of reaction begins at the olfactory receptors (ORs) of the cilia present on the dendrites of an ORN. Odorant molecules diffuse from water into the moist mucus membrane of amphibians, react with the cilia, and stimulate the receptors expressed on them (Guyton and Hall 2006). This in turn activates a G-protein coupled with transmembrane olfactory receptors. On activation, generation of a receptor potential on the cilial levels occurs. The receptor potential is then transmitted to the cell body of the ORN and is converted to an action potential frequency if the voltage reaches the threshold (Schild *et al.* 1994). The axons of the ORN combine to form olfactory nerves (ON), which in amphibians pass through the cribriform plate transmitting action potentials to the OB for processing (Wilson and Mainen 2006).

1.3.1 G-protein-coupled signal transduction

G–protein-coupled receptor is transmembrane receptor, which is expressed in the extracellular space and is also coupled intracellulary with GTP-binding proteins. On activation of the receptors by a ligand, a wide range of extracellular and intracellular processes including signal transduction cascades is elicited (Guyton and Hall 2006). One major G-protein involved in the olfactory signal transduction is known, G_{olf}. Each receptor protein is a long molecule that runs seven times through the membrane, folding inward and outward (Guyton and Hall 2006). G_{olf} is known to increase the levels of intracellular cyclic adenosine monophosphate (cAMP) by means of activating an enzyme called adenylyl cyclase. A single hormone molecule can lead to

formation of many cAMP molecules. Adenylyl cyclase is an enzyme that synthesizes cyclic AMP from adenosine triphosphate (ATP). Cyclic AMP functions as a "second messenger" to relay extracellular signals to intracellular effectors, particularly protein kinase A. The concentrations of intracellular cyclic AMP are largely dependent on adenylyl cyclase. Members of this family are responsible for transmitting information initiated by diverse signals such as hormones, neurotransmitters, odorants etc. (Berg *et al.* 2006). In an inactive state, guanyl nucleotide is bound to the G-protein, termed GDP (Berg *et al.* 2006). They are heterotrimeric in nature i.e., made of three different subunits; alpha, beta and gamma subunits, associated with the inner surface of the plasma membrane and transmembrane receptors (olfactory receptors) of hormones. The alpha subunit (G-α) belongs to the group of P-loop NTPase family. On excitation, this protein dissociates itself from the rest of the G-protein, binds the nucleotide and consequently activates adenylyl cyclase, which is attached to the interior part of the membrane near the receptor cell body (Guyton and Hall 2006). This process is known to terminate on its own without external influences, owing to the hydrolysis of GDP to GTP by the alpha-subunit. Signal amplification is a vital feature of signal cascades.

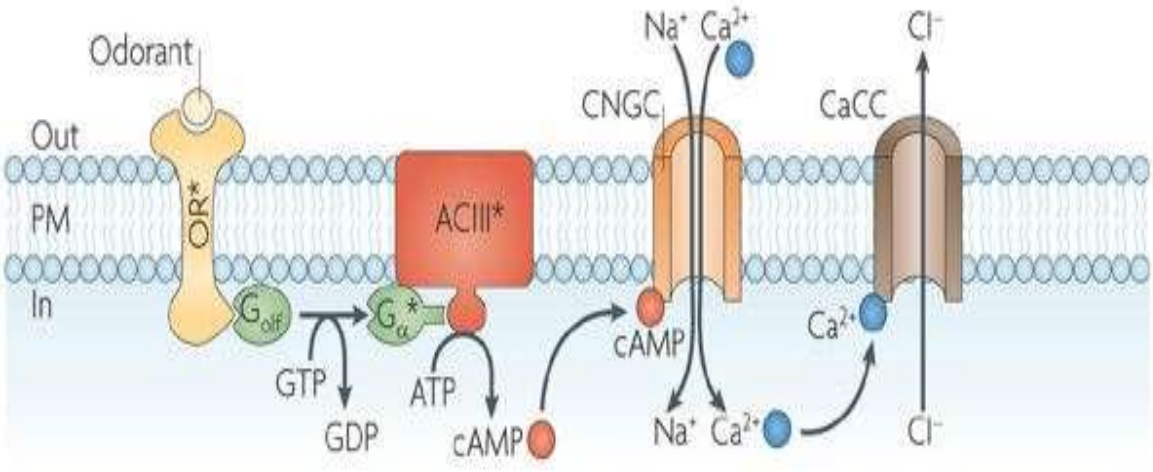


Figure 2: Signal transduction in ORNs. Odorant-induced signal transduction pathway. OR; olfactory receptor, ACIII; adenylyl cyclase III, GTP, guanosine-5'-triphosphate; GDP, guanosine diphosphate; ATP, adenosine-5'-triphosphate; CNGC, cyclic nucleotide-gated channel; CaCC, Ca^{2+}-activated Cl^- channel; cAMP, cAMP monophosphate; AMP, adenosine monophosphate. Modified after Kaupp 2010.

1.3.2 cAMP-coupled signal cascade

Studies have shown that in terrestrial vertebrates, a majority of ORNs possess a cAMP-mediated pathway while a few subgroups of ORNs exhibit alternative transduction cascades such as the IP3 (Ma 2007). cAMP is an intracellular second messenger. Its signal transduction involves a G-protein, which only binds to the transmembrane receptor on activation by a ligand molecule. Once a G-protein is activated on the interior of the cell, adenylyl cyclase (AC III) is activated by a dissociated alpha-subunit of a G-protein: this causes ATP to be catalysed to cAMP (Schild and Restrepo 1998). This process is an amplification of the signal transduction cascade, which means that the level of cAMP is exponentially increased on activation of AC III enzyme. Hence, the generation of a second messenger, cAMP by AC III can generally be said to provide a second level of amplification: each activated AC III is capable of converting many molecules of ATP to cAMP (Berg *et al.* 2006). In this context, the elevated levels of cAMP enables its binding to CNG channels thereby, making them permeable to calcium or sodium ion. Calcium and sodium ion influx increase the intracellular levels of calcium ions, depolarizes the cell membrane. Subsequently, calcium ions bind to calcium-activated chloride channels, which causes chloride ion efflux that results to the depolarization of the ORNs (Schild and Restrepo 1998).

1.3.3 IP3-mediated signal pathway

It is postulated that asides cAMP and IP3 that there may be other pathways for signal transduction which are yet to be exclusively researched on, and as a result not presently well understood. IP3 is one of the various second messengers that play an important part in the delivery of message inside a cell. Similar to cAMP, the IP3 can amplify a signal significantly by the generation of a second messenger (Berg *et al.* 2006). An increase in the intracellular calcium ion levels is known to be synonymous with cAMP and IP3 (Despopoulos and Silbernagl 2003). This means that when an α-subunit of a G-protein is activated, it binds to and activates the beta isoform of the phospholipase C (Berg *et al.* 2006). This is an enzyme that catalyses the cleavage of PIP2 to form DAG and IP3 (Despopoulos and Silbernagl 2003). The latter binds to intracellular IP3 gated calcium channels and triggers an increase in intracellular calcium ion levels, resulting in depolarization of the cell: a process similar to the cAMP-mediated pathway.

1.3.4 CNG-gated channels

Cation channels are widely known to be relevant components of the olfactory and visual systems (Trudeau and Zagotta 2003). They are cyclic nucleotide dependent and therefore, open and allow influx of cations into a cell. CNG- gated channels are classified under the voltage dependent ion channels although their activity is minutely voltage dependent. Similar to the K^+ ion channels, the CNG gated channels form heterotetrameric complexes (Kaupp and Seifert 2002) made up of four types of subunits. Through the binding of cyclic nucleotides they become activated and contribute together with cyclic AMP to cell depolarization. In the case of ORNs, the G protein, G_{olf} stimulates AC III to produce cAMP, which promotes opening of cAMP-gated channels and depolarization. The ability of CNG channels to be gated by cyclic nucleotides is, therefore crucial for organisms in monitoring their odorant environments (Reisert and Bradley 2005). Here also, sensory stimulation of vertebrate olfactory ORNs by odorants activates 7-transmembrane receptors, and triggers via a G protein, changes in the intracellular concentration of second messenger cyclic nucleotides (Reisert and Bradley 2005)

1.3.5 Forskolin signal pathway

Forskolin is a naturally occurring substance commonly found in Coleus forskohlii, a plant (Wagh *et al*. 2012). It is speculated to be a metabolism accelerator in the human body and is also widely used in biophysical studies (Garber *et al.* 1990). Its application in laboratory experiments have proven successful in the elevation of intracellular cAMP levels in cells, hence favourably chosen for researches related to intracellular cAMP levels and ion channels alteration. Acting as a ligand, it binds and activates a variety of AC IIIs (Barovsky *et al*. 1984). Upon activation of the enzyme AC III a chain of intracellular reaction similar to that in cAMP gated channels pathway is triggered off resulting to an increment in the levels of cAMP (see Figure 3).

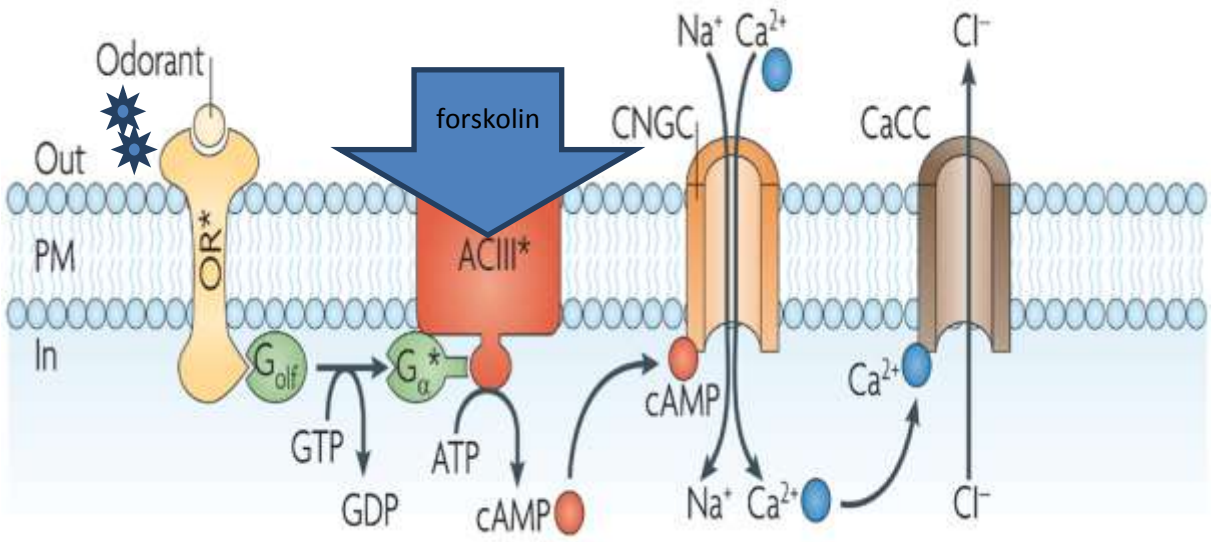


Figure 3: Forskolin-induced signal transduction pathway. This sketch represents the forskolin signal pathway. Abbreviations: OR; olfactory receptor, ACIII; adenylyl cyclase III, GTP, guanosine-5'-triphosphate; GDP, guanosine diphosphate; ATP, adenosine-5'-triphosphate; CNG, cyclic nucleotide-gated channel; CaCC, Ca2+-activated Cl- channel; cAMP, cAMP monophosphate; AMP, adenosine monophosphate. Modified after Kaupp 2010.

1.4 Molecular mechanism of odorant recognition

The accuracy of odour discrimination depends partly on the specificity with which odorants interact with appropriate sites (receptors) of olfactory neurons. ORs are not as specific as believed in other receptors of the same organism. An ORN per say, can respond to different odorants (Gliem 2010). It was also observed in a study that receptors expressed on the cilia which act as binding sites for odorants may through competition for binding sites allow for binding of different amino acids to a receptor (Rhein and Cagan 1983). However, the odorant molecules need to possess specific characteristics, owing to the structural barriers they may have to encounter and penetrate before gaining access to their target receptors. These structures maybe lipophilic or hydrophilic in nature and therefore, several criteria must be met for terrestrial vertebrates:

1) the substance must be volatile enough to permeate the air near the sensory area,

2) must be at least slightly water- and lipid-soluble to pass through the mucous layer and to the olfactory cilia and finally,

3) a minimum number of odorants must be in contact with the receptors for a minimum length of time. After the transportation of the odorant molecules through the mucous layer, a signal recognition and transmission occurs through the olfactory neurons (Schild and Restrepo 1998). Olfactory signalling is known to be terminated by phosphorylation (Boekhoff and Breer 1992) on of receptors via a negative feedback reaction, catalysed by specific kinases as discussed next in section 1.4.1 of adaptation mechanism in ORNs.

1.4.1 Ligand-induced endocytosis

Endocytosis is a process where vesicles are formed from the cell membrane and internalized. It functions as a transport mechanism for transporting molecules from the extracellular to intracellular space (Grant and Sato 2006). During endocytosis selected materials, which may alter cell function and morphology are ingested into the cell. These molecules depending on their sizes are transported differently into the cell. Endocytosis was first described in humans (Alberts *et al.* 2002). It was prompted by studies carried out on patients predisposed to atherosclerosis. As it was observed in such patients that the plasma cholesterol levels were escalated, implying that

cellular cholesterol uptake by means of endocytosis was at its limit (Alberts *et al.* 2002). It can further simply be described as the uptake of molecules by a cell. Eukaryotic cells are made of up cell membranes, which act as a barrier between the extracellular and intracellular environments (Robatzek *et al.* 2006). These cell membranes in vertebrates express receptors, which aid in detection and conveyance of molecules into cells for further signal processing (Le Roy and Wrana 2005).

There are different mechanisms of endocytosis namely; the clathrin-mediated and the non-clathrin-mediated endocytosic pathways (Robatzek *et al*. 2006). Endocytosis can further be differentiated by size of molecules ingested e.g. phagocytosis, during which large molecules like cell debris and microorganisms by cell vesicles are ingested and pinocytosis, which involves up-take of smaller molecules, mostly fluids (Guyton and Hall 2006). The classical clathrin-mediated endocytosis is the most widely understood of all endocytotic mechanisms. Here, clathrin polygons, adaptor proteins and membrane cargo receptors interact with each other during invagination of molecules from the extracellular space (Le Roy and Wrana 2005). Adaptor proteins attached to the membrane receptors at the extracellular end act as a bridge between the ligand-bound receptor and the clathrin polygons attached to them at the intracellular end by clathrin recruitment (McPherson *et al.* 2000). The receptors are termed ‘cargo receptors’ as they assist in transportation of molecules from the extracellular space into the cell. During endocytosis clathrin as shown in figure 4, with the aid of the adaptor proteins forms a lattice around the cell membrane and induces invagination of the ligand, receptor and part of the cell membrane (McPherson *et al*. 2000). Following invagination of the clathrin-coated vesicles, clathrin molecules detach themselves from the pit, which fuse with intracellular endosome causing release of the ligands and the receptors are recycled back to the cell membrane (Roepstorfff *et al*. 2008). A good example of a non-clathrin mediated endocytosis is observed in caveolae. They are cave-like flask-shaped pits found in cell membranes of certain tissues namely; adipocytes, smooth muscles and endothelial cells (Razani *et al.* 2002). They consist of cholesterol binding proteins called caveolin, assist in uptake of extracellular lipid molecules in these tissues, and are the most commonly known non-clathrin mediated-endocytotic pathway (Razani et al. 2002). Both the clathrin and non-clathrin endocytosis are said to be receptor-mediated.

Ligand-induced endocytosis is a down regulatory mechanism widely studied in humans (Sorkin and Von Zastrow 2002). It is described in a cancer study as a mechanism, which terminates further activation of EGF receptor (Roepstorfff *et al.* 2008). Binding of a stimulus to a receptor acts as an initial step in endocytosis, which may be followed by triggering of intracellular signal cascades or by invagination of ligand-receptor complex (Berg *et al* 2006). After internalization of the receptor-ligand complex to endosomes as in the clathrin-mediated endocytosis, the receptor may either be recycled back to the cell surface or transported to the lysosomes for degrading (Grant and Sato 2006). In addition, LIS has been observed by (Døving *et al*. 2009), in taste cells of juvenile brown trout taste buds. Another study on ovarian cells of Chinese rat, showed migration of a ligand-receptor complex into the intracellular space, where ligand-induced endocytosis was suggested as the mechanism involved as fluorescence on application of fluorescein-labelled anti-Flag monoclonal antibody and confocal microscopy accumulated intracellularly after Angiotensin II bound to AT1R-F (Merjan *et al*. 2001). Furthermore, notch, a transmembrane protein known for its role in cell-cell emybryonal development of multicellular organisms has been noted to be regulated by receptor endocytosis (Le Borgne *et al.* 2005) showed that N proteins are activated during endocytotic signalling upon ligand-receptor endocytosis. In plants it was first described by a group of researchers, who observed the process in FLS2 of an *Arabidopsis.* A study, which described ligand-induced internalization of receptors to be dependent on receptor activation, cytoskeleton and proteasome functions (Robatzek et al. 2006).

Similar to other sensory receptors, sensory adaptation also occurs in olfactory receptors of vertebrates in an exposure-dependent manner (Guyton and Hall 2006). The obliteration of ORN response to stimuli is a repercussion of adaptation (Reisert and Matthews 1999), which could involve ligand-induced endocytosis. Sensory adaptation is a phenomenon, whereby olfactory neurons alter their sensitivity to a stimulus after a prolonged exposure (Reisert and Matthews 1999), which may have been constant or intermittent. In olfactory cells, adaptation is a mechanism known to be specific for different receptors (Guyton and Hall 2006) and serves as a protection of a cell and its components by diminishing its response to sustained stimuli (Schmidt *et al.* 2005).

According to a study by (Zufall and Leinders-Zufall 2000), response of olfactory receptors to stimuli tends to diminish remarkably within few seconds mainly, when receptors are continuously stimulated owing to an effective down regulation of intracellular signal transduction, which is essential for rapid reaction of organisms to fluctuating stimuli.

To comprehend the mechanism of olfactory adaption, previous studies were carried out focusing on G-protein γ-subunit (Yamada *et al*. 2009). GPCRs are a point of focus in adaption processes in ORN. They are as already described transmembrane receptors coupled to a G-protein, and the first site where adaptation occurs. During adaptation GPCRs are capable of desensitization, which means that the number of responsive receptors to a stimulus is down regulated (Xiao *et al.* 1998). Furthermore, a series of events are also notable. These include uncoupling of G-protein from the transmembrane receptor and phosphorylation (Ferguson and Caron 1998). An event of extended odorant exposure also resulted in intracellular accumulation of arrestin2 in vesicles (Mashukova *et al.* 2006), which can be mediated through G-protein receptor kinase (Ferguson and Caron 1998). Although the precise mechanisms of action at the cellular level remain incompletely understood, it is known (Murmu *et al.* 2011) that various signalling pathways (e.g. in insects) such as calcium/calmodulin complex, CNG channels, cAMP, and the inositol 1,4,5-triphosphate receptor (InsP3R) may be involved in adaptation processes of olfactory neurons.

Most calcium-mediated signal transduction cascades act through the protein calmodulin, a calcium binding protein that is present in every eukaryotic cell. Calmodulin possesses four binding sites for calcium (Berg et al. 2006). Activation of the protein mostly occurs only when all four sites are occupied by calcium (Berg et al. 2006). The resultant calcium/calmodulin complex binds to CNG channels, leading to disruption of the olfactory process by diminishing cell sensitivity (Trudeau and Zagotta 2003). To terminate the olfactory process, hydrolysis of cAMP triggers activation of Ca2+ calmodulin dependent phosphodiesterase. Calcium/calmodulin kinase II-dependent attenuation of adenylyl cyclase is another mechanism, which participates in the adaptation of an ORN. In order not to deviate from the centre of the study, which is ligand-induced endocytosis in ORNs as a possible mechanism of adaptation, it would be profitable to mention that the olfactory receptors could also be endocytosed as an adaptation mechanism variation. For this to be achieved, β-

arrestins which belong to a small family of proteins involved in signal transduction cascades and play a vital role not only in the rhodopsin system (Mashukova *et al.* 2006) but also in the internalization of ORNs in larval tadpoles (Bruening 2009) are involved. On binding to phosphorylated transmembrane receptors, it may uncouple receptors from heterotrimeric G proteins and relays them to clathrin-coated pits for endocytosis (Berg *et al*. 2006). These numerous mechanisms involved in olfactory transduction cascade may suggest that olfactory receptors are not particularly designed for constant activation. Adaptation of ORNs to odorants can be abolished by the inhibition of clathrin-mediated endocytosis, showing the physiological relevance of odorant receptor desensitization mechanism (Mashukova *et al*. 2006). With this findings and considering the similarities in both mechanisms, it is discussed whether both mechanisms could be said to be exactly the same.

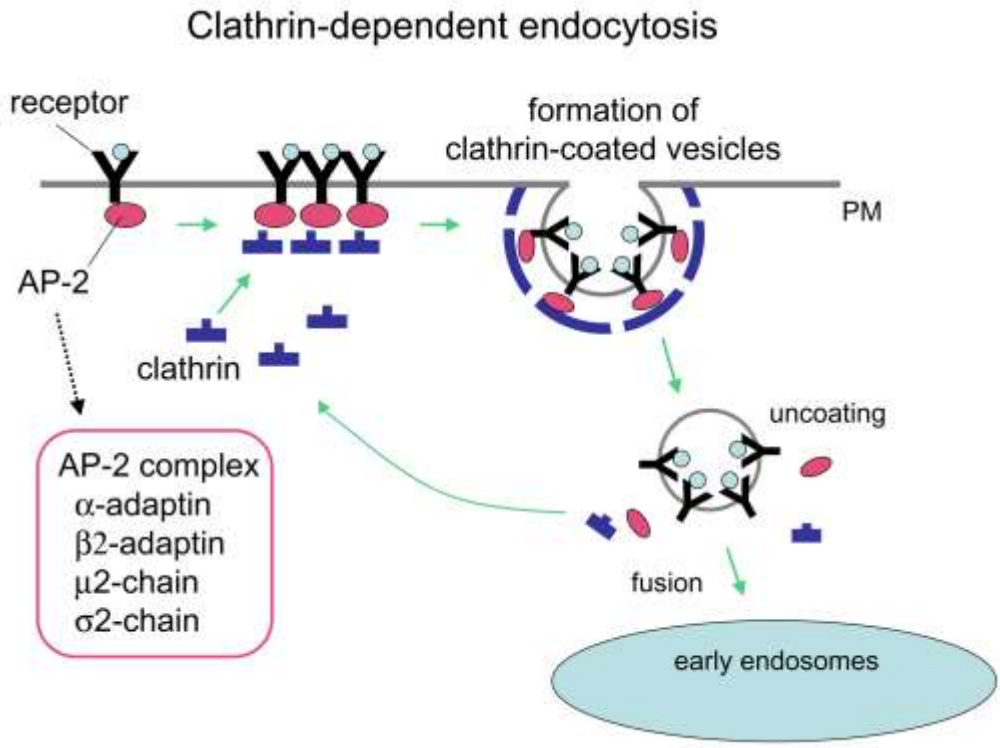


Figure 4. Clathrin-dependent endocytosis. This Diagram illustrates the mechanism of clathrin-mediated endocytosis. Adaptor protein (AP2). Modified after Grant and Sato 2006.

1.5 Goal of the thesis

As mentioned in the introduction section 1.1, olfaction is a vital procedure in both lower and higher animals. In ORNs, odorants play a significant role in the response of organisms to stimuli perceived by them. In this study we aimed primarily at developing a relatively new staining method, based on previous experiments conducted in fish (Døving *et al*., 2009) that could be implemented in finding out by optical characterization of ligand-induced staining (LIS) is possible and efficient in the ORNs of X.l and also whether LIS is an indirect method by which ligand-induced endocytosis can be visualized. To do so, supplementary experiments were developed utilizing LIS as a basis to discover whether or not LIS occurs through CNG channels and also if LIS is temperature dependent or not. The results of this thesis may assist in understanding better the initial event in olfactory adaptation in ORNs of larval *X.l.*

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 The experimental model-*Xenopus laevis*

The African *Xenopus* (see Figure 4) also known as the African clawed frog was first described in the beginning of the nineteenth century, the *X.l* was first described by a french naturalist (Gurdon and Hopwood 2000). It belongs to the family of Pipidae and to the anuran order. Its body is flattened. It has a smooth skin, a small head, and small up turned, lidless eyes. The animal is also blunt snouted, ventrally whitish, sometimes spotted, and has a dorsal olive brown colour with dark markings as shown in Figure 4. It has no tongue and therefore, has to push down food into its mouth and down its throats with its hands. Its forefeet are unwebbed, but with hind feet fully webbed with sharp black claws on their inner toes. Their larva called a tadpole is streamlined in shape, translucent, brownish in colour, with tentacles at the mouth corners and has a slender tail ending in a filament.

It originates from different countries in Africa like Angola; Cameroon; Central African Republic, Congo, Mozambique, Nigeria, South Africa etc. It is poikilothermic, inhabits of a variety of aquatic habitats, which may have been human modified including, ponds, drainage ditches, flood channels, golf course ponds and sewage plant ponds. The highest densities of this species are found in permanent well-vegetated waters with soft substrates that do not freeze. It is known to survive in the above-mentioned environments, which are conducive for them outside their place of origin. An african frog can deep into mud, leaving a hollow which persists in the dry season enabling them survive without nutrition. It migrates over land on rainy nights and also when their moist source dries up. The optimal habitat temperature for survival and reproduction ranges between 20-25°C. Respiration occurs with the aid of the lungs and they swim on surface of water to breathe.

The *X.l* can feed on a variety of foods; recent metamorphs, aquatic invertebrates, fish, amphibians, stripes of liver, including its own larvae, whereas animals in larval stages feed on algae. Reproduction takes place in fresh water. The female species mature earlier than the males and lay up to thousands of eggs, which they deposit in small groups. To initiate breeding, frogs were retained in an aquarium at about 20°C,

separated according to sex and fed with pond sticks. This was followed by a repeated injection of human chorionic gonadotropin (Sigma, Deisenhofen, Germany) into the dorsal lymph sac after which the different sexes were paired in dark aquaria. After about 24 hours, the embryos were gathered in a separate aquarium at a temperature of 20°C. They were then fed with algae to facilitate growth.

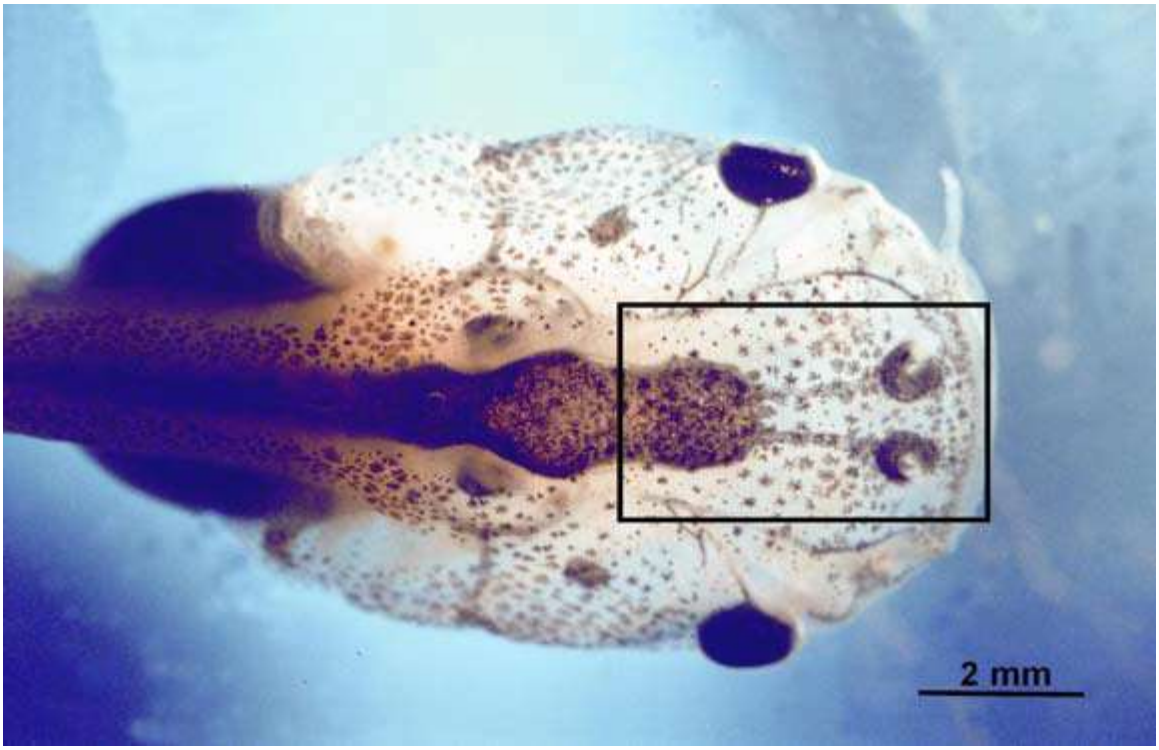


Figure 5: Diagram of the experimental model animal, *X.l*. Tissue block used for experiment indicated with black rectangle. Modified after Gliem, 2007

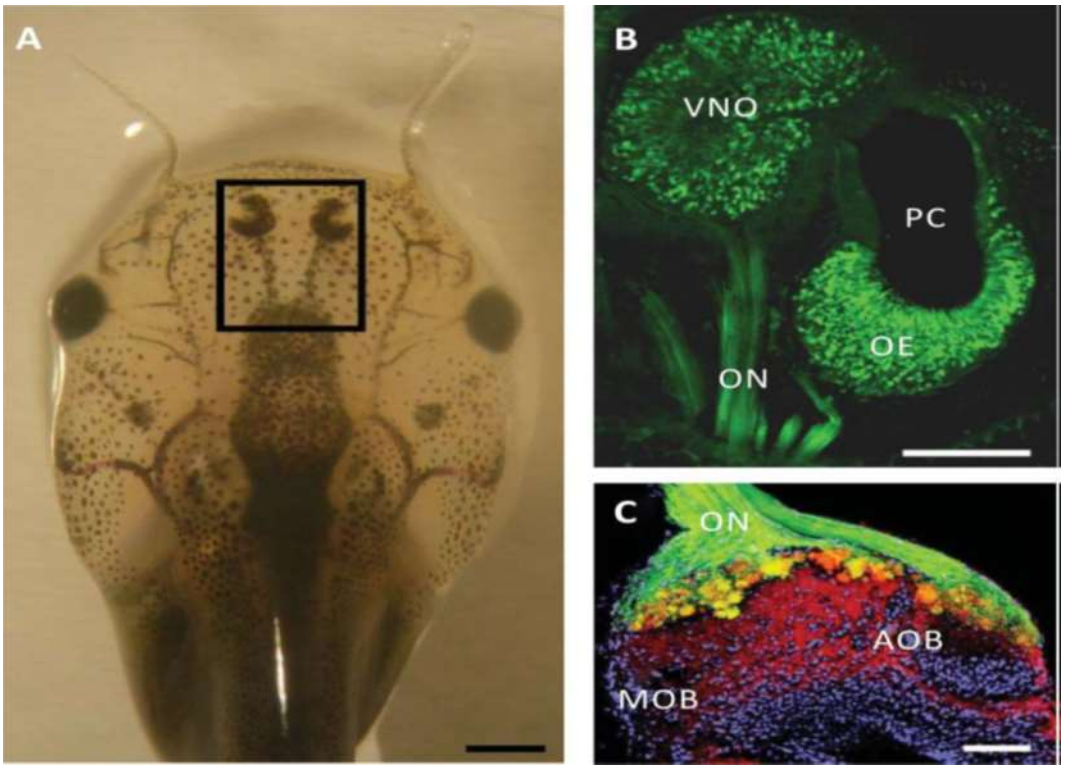


Figure 6: Organization of the olfactory system of *X.l.* (A) Larval *X.l* (stages 51-52). Olfactory system indicated medially to both eyes with black rectangle. (B) Horizontal overview over olfactory epithelium and vomeronasal organ (VNO, vomeronasal organ, OE, olfactory epithelium, ON, olfactory nerve, PC, principal cavity) (scale bar 200µm). (C) Horizontal overview of OB, olfactory bulb (ON, MOB, main OB, accessory OB); (scale bar 100µm). Figure modified after Menini, 2010.

2.1.2 Fluorophore

A laser microscope facilitates viewing of living and non-living samples with laser light. Laser light is channelled through an objective lens to the sample, which has already been stained with a fluorescent dye. Fluorescent dyes have a property of absorbing light with a shorter wavelength and higher energy and emitting from its source, the sample, light with a longer wavelength and less energy, which should or should not be detected, as the case may be. There are many fluorophores utilized in tissue staining for laser scanning microscopy. For this study, 20µM Alexa 488 dextran and Texas red dextran dyes were used. They are both excited with the wavelengths 488nm and 561nm respectively. The dyes applied were purchased from Invitrogen, Oregon, USA and stored in a refrigerated dark bottle to hinder decomposition. All Alexa dyes and their conjugates are fluorescent and photo-stable. They are insensitive to pH between 4 and 10 (Panchuk–Voloshina *et al.* 1999). They could be conjugated with proteins or dextran sugars. Fluorescein conjugates, although still maintaining appreciable fluorescence emission, are quite photolabile (Benchaib *et al.* 1996) and their fluorescence intensity is pH-sensitive, with maximal emission in the basic range (Nakamura *and* Strittmatter 1996). Photo-stability and fluorescence emission intensity and specificity are to be considered when choosing dye for microscopy.

2.1.3 Odorants

As stimuli, various odorants with final concentrations 0.1mM (for amines), 1mM, 5mM were applied (see table 1). These include a mixture of 19 amino acids, 13 amines, an ox-bile acid solution and 5 alcohols (Sigma, Deisenhofen, Germany). Amino acids are principle components of proteins. They are found almost in all vital aspects of nature. They are major building blocks of not just our nutrition, but also what we are. The human skin, for example, which is the largest and one of the most important organs, is predominantly made of proteins. Furthermore, protein deficiency or collagen defects stirs up a big discussion in not just the elderly but also the young in various cases like marfan syndrome. This study does not only focus on protein as a whole but also on its building block: amino acids. Biochemical reactions, involving enzymes are very necessary processes in living organisms. Amino acids are useful as edible substances and also as olfactory stimulus like other odorants for appetite, sexual behaviours, emotion-related responses in some aquatic animals.

Structurally, an amino acid (AA) consists of a central carbon atom, called the alpha carbon, linked to an amino group, a carboxylic acid group, a hydrogen atom and a distinctive “R” group which is often referred to as the side chain (Berg *et al.* 2006). Only L amino acids are constituents of proteins and also, only L amino acids were used for this experiment. The letter L refers to them having an anti-clockwise rotation, and being with D isomers, are mirror images of each other (Berg *et al*. 2006). For this study 19 different amino acids (see list table 1) were mixed and dissolved in double-distilled water before application. 10 mM of a mixture of different 19 amino acids, were dissolved in 30ml of distilled water and then filled up to 40 ml in a falcon tube. These were distributed in 25 different 1.6 ml eppendorf tubes, refrigerated and thawed before each use.

Alcohol (AL) for example, is in present times synonymous with pleasure, cosmetics etc. It is widely used in laboratories as a cleansing agent or a basic component of most solutions. It is, however, used as a stimulus for the OE of a *Xenopus*. In chemistry, it is a compound with an –OH functional group. It can either be lipophilic or hydrophilic depending of the structure of their alkyl chain. This suggests whether or not it can easily pass through a lipophilic structure.

An amine (AM) is by definition any member of a family of nitrogen-containing organic compounds that is derived, either in principle or in practice, from ammonia (NH3). Though known medically to be carcinogenic, they are also widely used in most biochemical and physiological experiments. Naturally occurring amines include the alkaloids, which are present in certain plants; the catecholamine neurotransmitters (i.e., dopamine, epinephrine, and norepinephrine) and a local chemical mediator, histamine that occurs in most animal tissues.

Compound	Type	Quantity (g)
	L-Methionine	0.0603
	L-Leucine	0.0535
	L-Arginine	0.0851
	L-Tryptophan	0.0833
	L-Isoleucine	0.0530
	L-valine	0.0473
	L-histidine	0.0846
Amino Acids	L-asparagine	0.0533
	L-aspartat	0.0537
	L-glutamin	0.0587
	L-cystine	0.0492
	L-alanine	0.0360
	L-glycine	0.0303
	L-glutamate	0.0594
	L-phenylalanine	0.0674
	L-proline	0.0465
	L-serine	0.0465
	L-threonine	0.0481
	Type	**Quantity (g)**
	2-phenylthylamine	0.0630
	Tyramine	0.0699
	Isobutylamine	9.962
	Cyclohexylamine	8.687
	Hexylamine	7.493
	3-methylbutylamine	8.529
Amines	N,N-dimethylamine	9.136
	2-methylbuylamine	8.042
	1-formylpiperidine	8.914

	2-methylpiperidine	8.290
	N-ethylpiperidine	6.547
	1-ethylpiperidine	7.206
	Piperidine	10.022
	Type	**Quantity(M)**
Alcohols	α-terpineol	5.75
	β-ionone	4.71
	2-phenylethylacohol	8.26
	3-Phenylpropylacohol	7.20
	Citral	5.54
	Type	**Quantity(g)**
Bile Acids	Ox-bile	0.176

Table 1. List of odorants applied. Final concentration of mixture of above listed odorant solutions 5 mM, 10 mM, 10 µM and 100 µM.

2.2 Ligand-induced endocytosis

2.2.1 Tissue preparation of the *X.l*

An outlined general procedure for preparing and preserving the olfactory mucosa of the experimental specimen, *X.l.* Odorants were applied before or after staining. To begin, tadpoles in suitable stages were anaesthetized by immersion, for about 2 min in a mixture of crushed ice and double distilled water. This enabled a more conducive avenue for excising a tissue slice containing the olfactory mucosa and ONs. Immobilized tadpole was transferred into a silicon- filled petri dish for sacrifice by dissection of the midbrain from spinal cord (midbrain), after which a tissue block containing the olfactory mucosae and nerves was transferred into a ringer solution at room temperature (20°C). Prior to slicing, Microtome slicer (VT 1200S; Leica Microsystems GmbH, Wetzlar, Germany) was calibrated, tissue slice glued onto a stage and sliced at a thickness of about 200 µm. The major components of the sliced tissue were the olfactory mucosa and both ONs leading to the forebrain. To enable microscopy of the sliced tissue, the tissue slice was transferred into a petri dish with a ringer solution at room temperature and fixed with a platin framed net. Microscopy followed without any further delay to prevent apoptosis of olfactory cells.

All experiments were carried out with tadpoles of *X.l.* purchased from (Kaehler, Hamburg Germany; Nasco USA), fed with pond stick food, incubated in aquaria at about 20°C and bred. All tadpoles used were in stages 52-54 of development. All procedures for animal handling and tissue dissection were carried out in conformity to the outline of policy of the Goettingen University Committee for Ethics in Animal Experimentation. Slicing proceeded immediately after preparation of tissues.

2.2.2 LIS of olfactory mucosa

Tadpoles were let to swim in distilled water for about 2 min after which they were transferred into a falcon tube containing a dye and 15 ml of either a mixture of 19 amino acids, bile acids, amines or alcohols at a concentration of 5mM or 10mM. They were incubated at different times ranging from 30s to 15 min, transferred into distilled water for about 1.5 min and then anaesthetized in preparation for slicing.

All chemicals were purchased from sigma unless stated otherwise. 20 µM of Texas red dextran dye was mixed with 15ml double distilled water in a rinsed falcon tube.

Three beakers, each numbered 1-3 were rinsed with double distilled water and kept ready. Beaker 1 and 3, inscribed respectively, were filled with double distilled water. Tadpole of *X.l* between stadium 52 and 54 was put in beaker 1, removed after 1-2 min, then transferred to beaker 2 with the solution of Texas red dextran (Invitrogen, USA) and an odorant and left to swim at incubation times 30 secs, 1 min, 2 min, 5 min, 10 min and 15 min. Thereafter, tadpole was transferred in beaker 3 with a spoon rinsed with distilled water. Solution with dye and odorant was filtered particle- free with a syringe consisting of a membrane filter and then transferred into another previously rinsed falcon tube. Filter and syringe were consequently discarded for new ones after each third use. After 2 min, tadpole was immersed in ice water to anaesthetize and slicing was proceeded. This procedure was carried out for AA mixture, AM, BA and AL mixture in their respective concentrations. 3 animals (6 epithelia) were used for each odorant at a particular concentration and incubation time.

For the negative control experiments, no odorants were applied. This serves as a basis for comparing standard experimental conditions as mentioned above with negative control experiment condition.

For 20 µM AlexaFluor-488 dye (Invitrogen, USA), the same procedures as mentioned above were followed.

The animals were allowed to swim in tap water for less than a minute and then transferred into a falcon-tube containing a solution of dye and an odorant and left at different incubation times from 30 sec, 1 min, 2 min, 5 min and 10 min and 15 min respectively but in this case, the 5-minute incubation time was mostly considered for this experiment. They were anesthetized by immersion in a mixture of crushed ice and tap water in a beaker for about 2 min so as to prevent them from suffocating then were transferred on to a dissection dish filled with silicon substance to avoid stickiness. There they were pinned down on a petri dish filled with silicon (laterally at both ends distal from the eyes) to prevent possible reflex movements, which may still arise from the spinal cord. Dissociation of the brain from the spinal cord took place at the level of the medulla oblongata with an anatomical dissection blade. A block of tissue consisting of the OE, and the ON was excised (see figure 6) carefully, preventing squashing of the soft tissue and the glued with cyanacrylate glue (Carl-Roth GmbH, Karlsruhe, Deutschland) on to the stage of a vibroslicer (VT 1200S,

Leica, Bensheim, Deutschland). Caution was taken while applying the glue as it may lead to gluing or skin burn. Lastly, the stage containing the tissue block was transferred to a bath containing ringer solution and then set to cut horizontally at a speed of 0.04mm per second into 170-200 µm thick slices. At the course of slicing, the attached microscope was used for slicing observation.

Negative control of experiment: for the negative control experiments, similar process was repeated as stated in the tissue preparation in chapter 2.2.1 except that no odorants were added to the solution containing fluorescent dyes. The incubation times were 30 sec, 1, 2, 5, 10 and 15 minutes.

2.2.3 Tissue fixation

The stability of the sliced tissue is important during microscopy to optimize tissue imaging and avoid artefact. To achieve this, a hand-made grid with a nylon net obtained from a pair of ladies pantyhose was used. Fine nylon threads were separated with the aid of a magnifying glass and rolled horizontally across the long sides of a thin, platinum frame, which was cut to fit into the depression of the petri dish. The net was glued with cyanacrylate glue at both ends where the net threads were run to prevent them from slipping off. Both surfaces of the platinum frame were lined with threads close to each other. On one hand, the threads should not be too close to another to enable imaging of tissue, whereas on the other, they should not be too far from another to avoid movement and therefore, damage of the mucosa. To increase the distance between two threads, a blade was used to scratch off the threads at one surface. This provides enough room for the olfactory epithelium (OE) of the mucosa to fit in for proper microscopy.

2.3 Temperature and ligand-induced endocytosis

2.3.1 Temperature and olfactory adaptation

As stated in section 2.3 of 'methods' that experiments on LIS carried out during this study were done under certain conditions including room temperature (27°C). Also considering a publication (Døving *et al*. 2009) which revealed that low temperatures (about +2°C) and disruption of microtubules with an anti- neoplastic drug, nocodazole caused a substantial reduction in the efficacy of LIS of taste cells in a juvenile brown trout (Døving *et al*. 2009). Further experiments including the application of lower temperatures (+2°C) other than outlining methods for determining whether LIS occurs in our laboratory animal were conducted in order support the hypothesis that temperature may have an effect on LIS method in ORN of a *X.l*. A temperature experiment was conducted to observe if a change in temperature negatively or positively affects LIS.

2.3.2 General procedure

The temperature experiments carried out aimed at observing the outcome of reduced temperature on LIS. Initially, a mixture 20 µM Alexa 488 dextran dextran dye and 10 mM of AA-solution were dissolved in ddH_2O and distributed equally in 4 wells of the 96-well plate, which was placed in an icebox covered with a lid. Eppendorf-tube and petri dish were rinsed with ddH_2O, filled with ddH_2O and then placed into the covered icebox. Containers containing odorant, dye solution, and ddH2O were allowed to cool to about 3-4°C and after 20 min, tadpoles were anesthetized. Tissue blocks were cut out from immobilized tadpoles and transferred to the eppendorf-tube containing cold (1-2°C) ddH_2O for about 10 minutes, after they were again transferred into four wells containing dye and odorant mixture and incubated for 5 min. This was proceeded by slice preparation, fixation and imaging. This procedure was repeated using tap water in place of ddH2O.

For negative control experiments e attempts were made under lower temperatures at room temperature of about 3-4°C using 1.5 mM AA, instead of at room temperature (27°C). This served as a comparison for the outcome of both conditions.

2.4 Test with forskolin

2.4.1 Staining and tissue preparation.

A test with the application of forskolin was carried out to observe indirectly, whether staining (dye uptake) would occur through opening of CNG channels

Eppendorf tubes were rinsed with double distilled water. 5 µl of 10 mM forskolin stock solution were pipetted into an eppendorf tube. 50 µl of 200 µM Alexa488 stock solution and 945 µl of double distilled water were added. The mixture was vortexed and distributed as 200 µl in 5 different wells of a 96-well plate, which was previously well rinsed with double distilled water.

As mentioned above, 5 tadpoles (stages 52-54) were anaesthetized in ice water for about 2 min and killed by transection of the spinal cord from the brain. A tissue block consisting of the OE and ON was cut out. All tissue blocks were first transferred into a petri-dish with double distilled water at room temperature (20°C) and tap water after 3 min, each tissue block was immersed in a separate well containing 200 ml of a mixture of 50 µM forskolin stock solution, 20 µM Alexa 488 dextran stock solution and 945 ml of double distilled water for 5 min at RT. Tissues were then transferred to a BPYR solution after which each was glued to a vibrating blade microtome slicer stage and consequently cut into a thin slice of about 170-200 µm. The utilized wells of the 96 well plated were marked to prevent further use and rinsed. Sliced tissues were fixed with a platin framed net in BPYR solution for immediate imaging under microscope with optimal Alexa 488 dextran laser settings.

2.4.2 Concept of microscopy

Laser scanning microscopes are ideal for biological and physiological imaging due to their outstanding spatial and temporal resolution. It enables viewing of biological tissues, either fixed or alive. It is known to produce in focus images and either eliminate or diminish out of focus light compared to other forms of microscopy. Basically, as demonstrated in figure 6, it is major components are the detector, laser source, pinhole, dichromatic scanning mirror, and collimator. The pinhole aperture is located in a conjugate plane with the stage where the sample is placed. Its principle is based on the laser light acting as excitation light for the fluorescence in the sample. When the molecules in the objective get excited, they emit a fluorescent light which passes through the objective lens and then focused on then camera. The camera is coupled with a computer for modification and processing. To optimize the sharpness of the image, the out of focus light is hindered from getting to the camera by the use of a pinhole. This has a special function for selecting which light that passes through it. Two laser lights used for this experiment were of wavelengths 488 nm or 561nm. The di-chronic mirror enables light coming from the sample to be transmitted, the laser light to be reflected in x and y axes and conveyed to a detector. For signal detection a scan head mounted on the microscope is used. The entire system is coupled to a computer program for control and processing (Zeiss, Jena, Germany).The microscope used was an LSM 710 (Carl Zeiss micro-imaging, Jena Deutschland) with an adjustable objective stage which simultaneously prevents instability of the fixed sample whilst scanning. It is coupled to a computer with which the laser microscope was navigated and a laser box containing different sources of laser light. To block or diminish out of focus light, filter was placed in use.

To obtain optimal images, pinhole was set between 60-90 units. A laser intensity of not more than 2% for Alexa dye laser with wavelength 488 and with Texas red dextran dye 561 were applied. These settings could automatically be saved and called up using the smart setting option for either dyes.

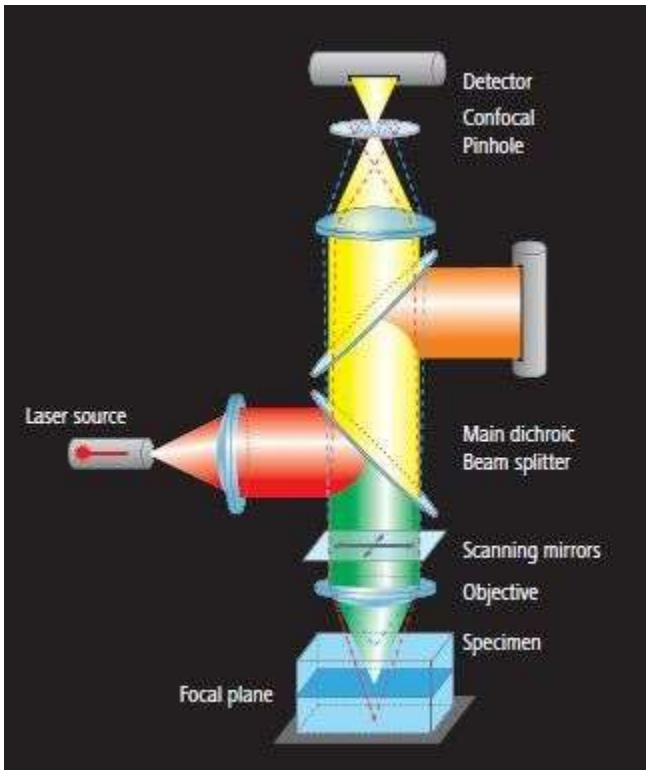


Figure 7 : Illustrative sketch of the concept of microscopy. Modified after Carl Zeiss from www.zeiss.de

3 RESULTS

3.1 Ligand-induced staining in ORNs of larval *X.l* positive control experiment

To comprehend the mechanism of LIS in *X.l* several experimental procedures were followed. This enabled the studying of the effect of each odorant in the ORN of *Xenopus laevis*. As stated in chapter 2.1.3 of 'Materials and Methods', amino acids, bile acids, alcohols, amines, specific fluorescent dextran dyes namely; Texas red and Alexa dextran dyes were applied and a laser microscope utilized for imaging of the prepared tissues. This study among other aspects, aimed at finding out whether staining by LIS does occur in selected experiment models by applying 5 mM or 1 mM AA, AM, AL and BA as odorants. The figures below illustrate the findings of the experiment after tissue blocks had been dissected, sliced and fixed for microscopy. Figure 8 represents an image of the olfactory epithelium, with focus on a single olfactory neuron, indicating with the aid of arrows the soma and dendrite of an ORN. Further images represent a set of images of olfactory mucosa stained by incubation for 15 minutes either with 20 µM Texas red or Alexa 488 dextran dyes and odorants. Images of olfactory mucosa treated with 1 mM, 5 mM or in the case of amines 0.1 mM of odorants, and in each case, treatment with 20 µM Texas red or Alexa 488 dextran dye for 10-15 minutes showed adequate mucusal staining. On each is set of images, the effect of staining at specific conditions with bile acids is compared to amino acids, amines and alcohols respectively. This is the case with amines. High concentrations of amines above 0.1 mM unlike the rest of the odorants resulted to cell toxicity. The following figures (8-15) represent ligand-induced staining in olfactory mucosa of *X.l*. They are a direct comparison of the outcome of staining with 0.1 mM, 1 mM and 5 mM bile acids to other odorants namely, amino acids, amines and alcohols. Figure 9 illustrates a tissue block of the experimental animal containing the principal cavity and olfactory mucosa and olfactory cells stained with 5mM bile acids and 5 mM amino acids and dyed with 20 µM Texas red dextran. Figures 10 and 11 further represent stained olfactory mucosa at a condition of 1 mM bile acids compared to 1 mM alcohols, and 20 µM Texas red dextran for 15 minutes. For direct comparison of staining effect of bile acids to amines, each odorant was applied at a concentration of 0.1 mM, as shown in figure 12. On application of Alexa 488 dextran dye, stained ORNs were aslo observable in the olfactory mucosa as illustrated in

figures 13 and 14, where 1mM of bile, amino acids and alcohols, as well as 20 µM of Alexa-488 dextran dye were applied for 15 minutes. Same conditons were repeated as in Texas red dextran dye test on staining of olfactory cells with amines. The staining effect on treatment with 0.1mM bile acids was compared to that of 0.1mM amines after a 15-minute incubation period. Each olfactory mucosa tissues in the above mentioned images represented contains layers of ORNs distributed in the periphery lateral, intermediate and medial of the epithelium about 70µm thick.

The images from the negative control experiments reveal as shown in epithelia of figure 16 that no cells stained after 15 minutes of incubation. Figures 16 (negative control images) differ from the results of figures 10, 11 and 12, a positive control experiment in that the stained structure in figure 15 exhibits a different staining pattern on treatment with 20 µM Texas red dextran and 20 µM alexa-488 dextran dye.

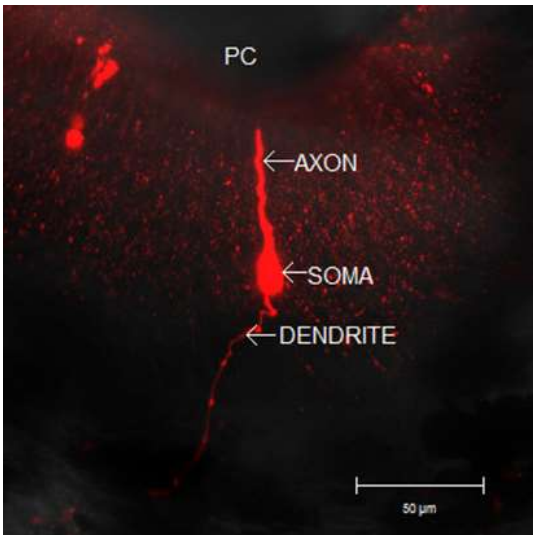


Figure 8. Zoomed image of sliced OE. Arrows indicate parts of a single stained ORN. Stimulus: 10 mM BA, dye: 20 µM Texas red dextran, incubation time: 10 min

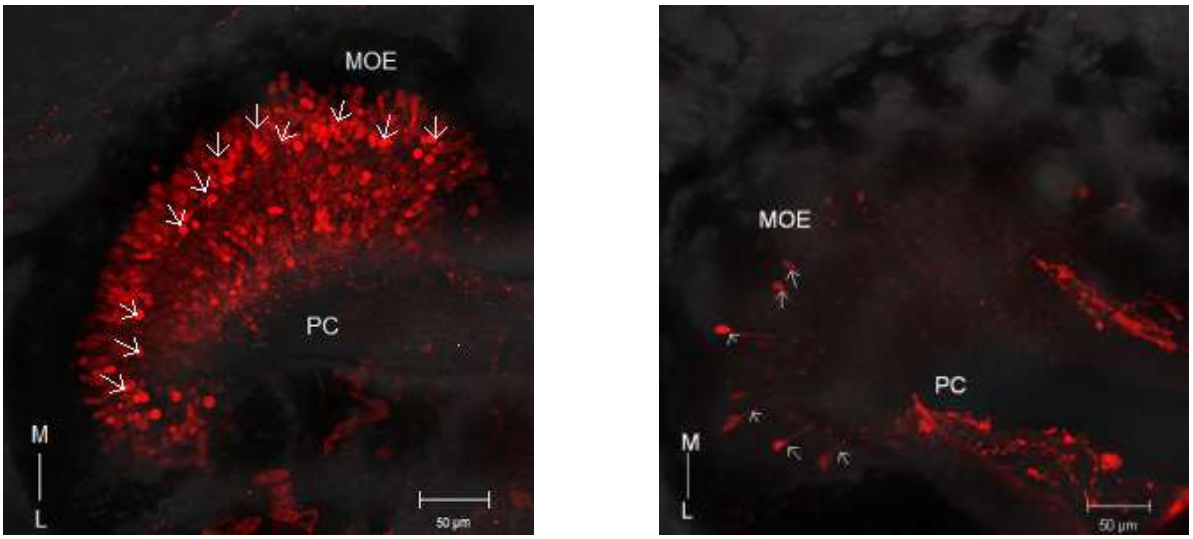


Figure 9. Images of sliced olfactory epithelia (merged z-stacks). Arrows indicate red stained ORNs. Stimulus: 5 mM BA (to the left), 5 mM AA (to the right). 20 µM Texas red dextran dye, incubation time: 10 min

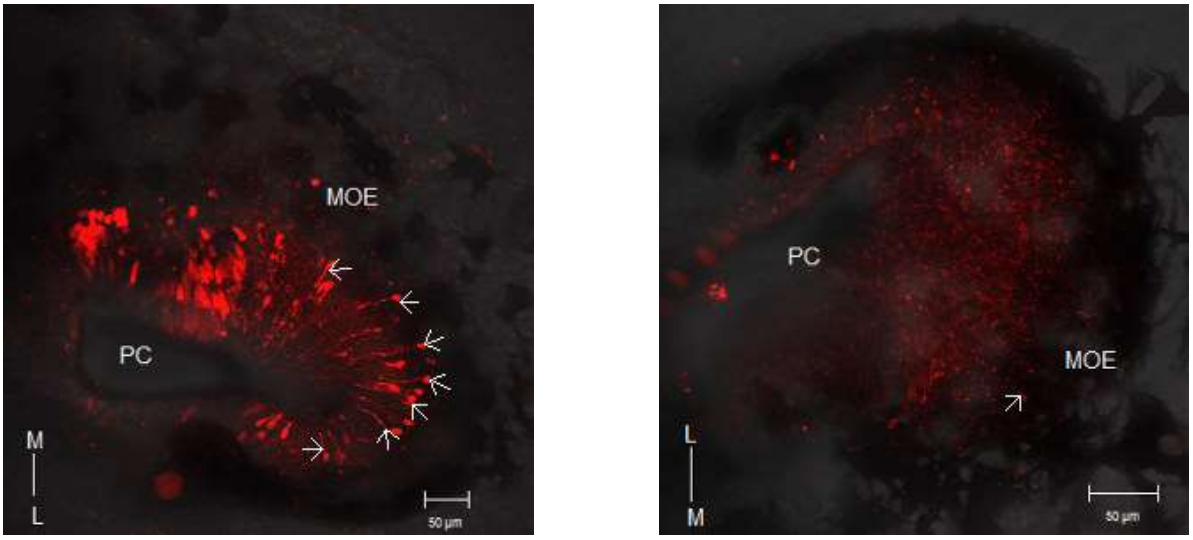


Figure 10. Images of sliced olfactory epithelia (merged z-stacks). Arrows indicate red stained ORNs. Stimulus: 1 mM BA (to the left), 1 mM AA (to the right). 20 µM Texas red dextran dye, incubation time: 15min

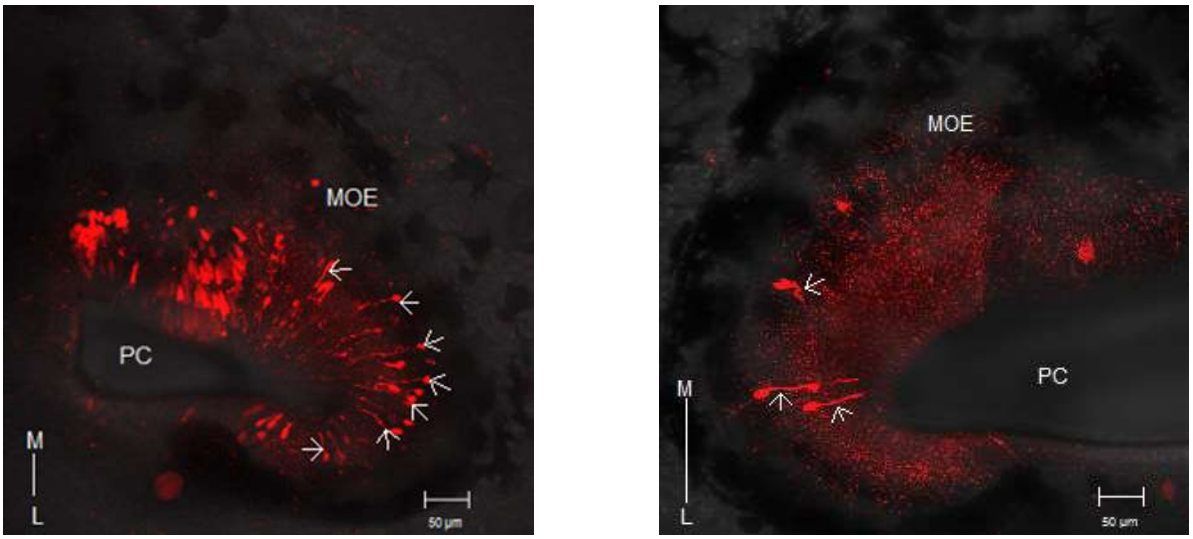


Figure 11. Images of sliced olfactory epithelia (merged z-stacks). Arrows indicate red stained ORNs. Stimulus: 1 mM BA to the left, 1 mM AL (to the right). 20 µM Texas red dextran dye, incubation time: 15 min

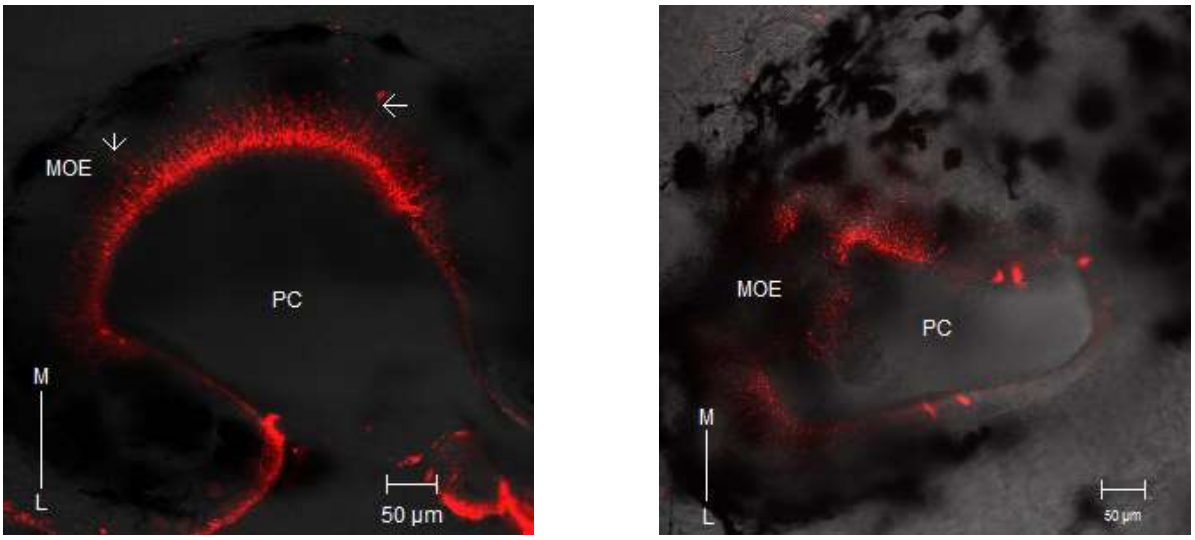


Figure 12. Images of sliced olfactory epithelia (merged z-stacks). Arrows indicate red stained ORNs. Stimulus: 0.1 mM BA to the left, 0.1 mm AM (to the right). 20 µM Texas red dextran dye, incubation time: 15 min

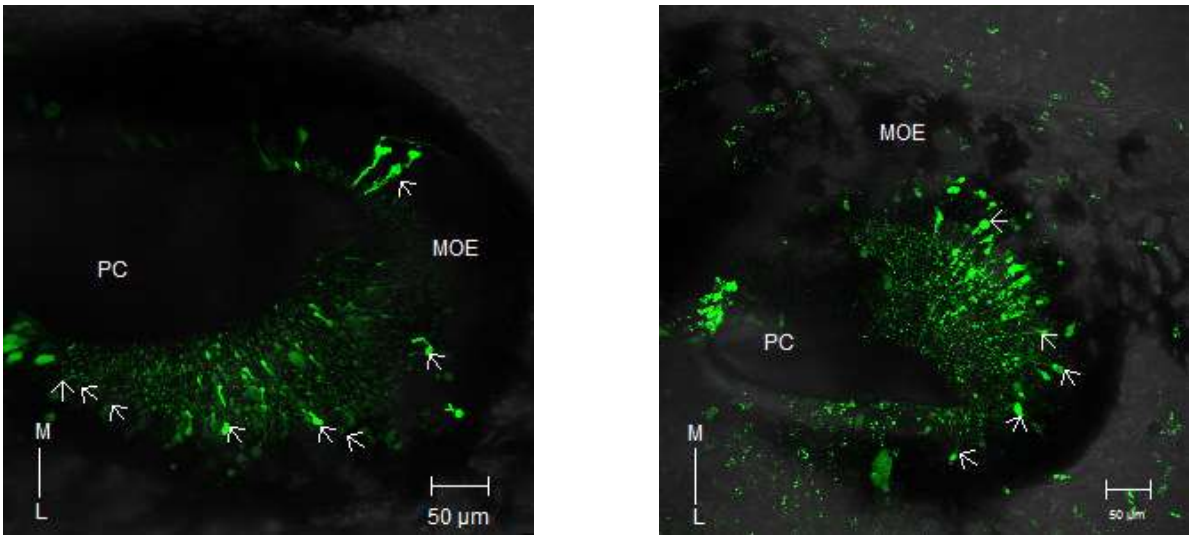


Figure 13. Images of sliced olfactory epithelia (merged z-stacks). Arrows indicate red stained ORNs. Stimulus: 1 mM BA to the left, 1 mM AA (to the right). 20 µM Alexa 488 dextran dye, incubation time: 15 min

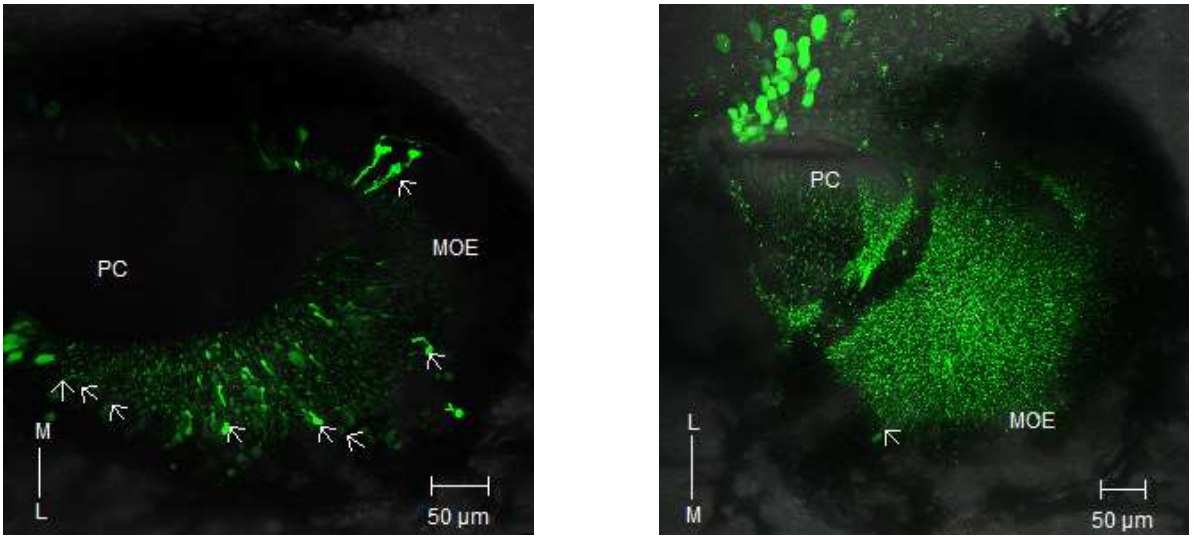


Figure 14. Images of sliced olfactory epithelia (merged z-stacks). Arrows indicate red stained ORNs. Stimulus: 1 mM BA to the left, 1 mM AL (to the right). 20 µM Alexa 488 dextran dye, incubation time: 15 min

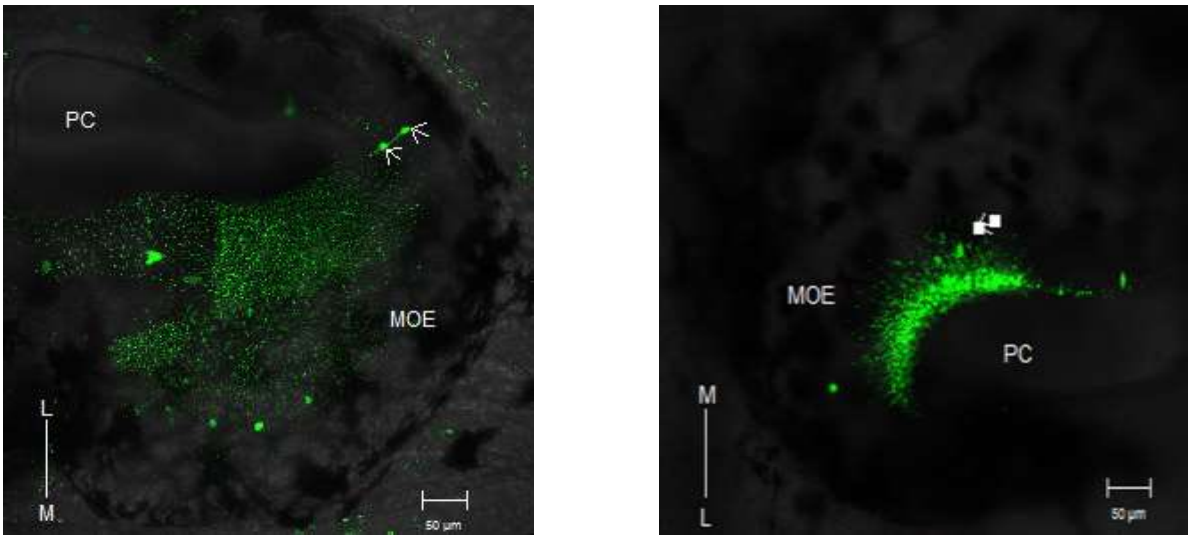


Figure 15. Images of sliced olfactory epithelia (merged z-stacks). Arrows indicate red stained ORNs. Stimulus: 0.1 mM BA to the left, 0.1 mM AM (to the right). 20 µM Alexa 488 dextran dye, incubation time: 15 min

3.2 Negative control experiment (LIS)

A negative control experiment was carried out. Here (see figure 16), no odorant was applied to the fluorescent dye solution with the aim of observing whether or not dye uptake would occur.

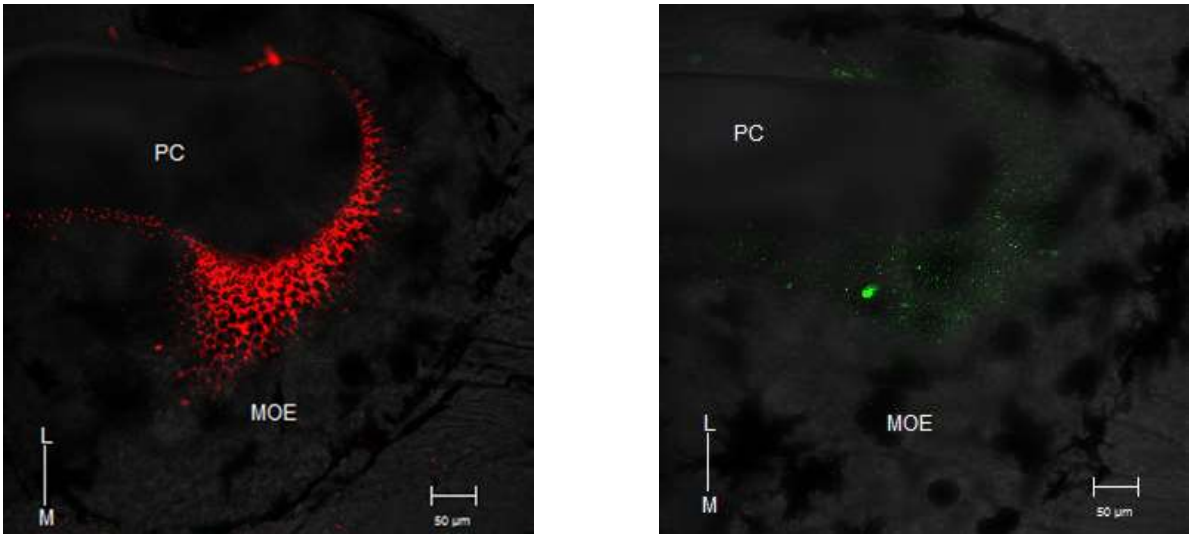


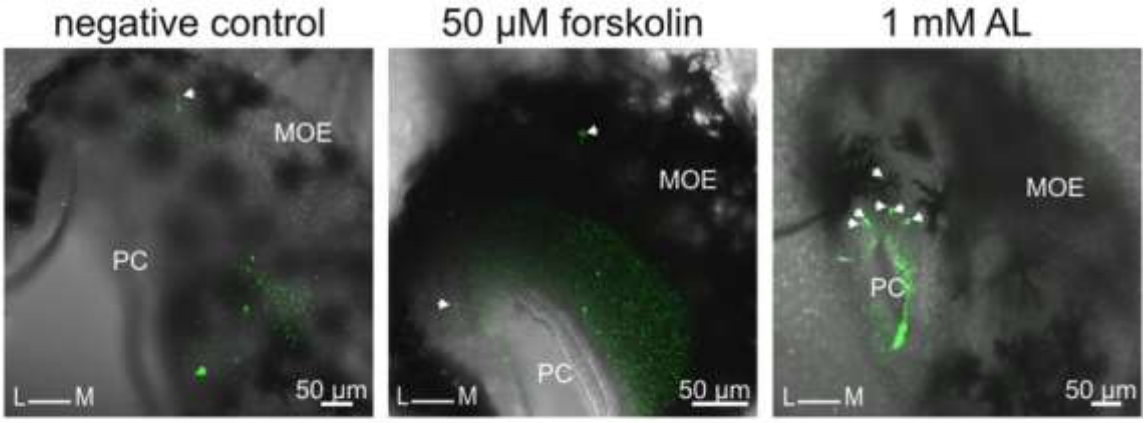
Figure 16. Negative control of the LIS experiment with an exclusion of an odorant as stimulus (merged z-stacks). Dye: 20 µM Texas red dextran dye to the left and Alexa 488 dextran (to the right), incubation time: 15 min. No stained cells shown.

3.3 Test with forskolin

There are various mechanisms of interest speculated to be involved in the observed staining of ORNs namely; via diffusion through open cAMP/CNG cation channels, and through adaptation of olfactory receptor neurons, mainly via receptor-mediated endocytosis. In order to confirm my hypothesis that staining in ORNs does occur through the latter and not via diffusion through cAMP activated cation-channels, a suitable method using forskolin as a deciding substrate was established to study the possible mechanism pathways. Forskolin increases the intracellular cAMP levels by triggering a chain of similar reactions as already explained in section 1.3 (see section 1.3). It achieves this by directly activating ACIII (Insel and Ostrom 2003) which in turn initiates the opening of cAMP-dependent cation channels (CNG-channels) by increasing intracellular cAMP-levels.

For this experiment, 50 µM forskolin and 20 µM Alexa 488 dextran stock were pipetted 96 well plate was rinsed ddH_2O and 5 wells filled with 200 µl of odorant and dye solution.Tissue blocks of 5 randomly selected tadpoles were prepared and each was placed in a well containing solution. After 5 minutes tissue blocks were transferred into a perti dish containing Bpyr. This was proceeded by slicing and imaging. From this experiment one could state as shown in Figure17A, that no cells were stained. In other words this suggests that little or no odorants and dye molecules were taking up by the ORNs compared to the ORNs original in the LIS experiment. Additionally, the above lined out forskolin test was carried out using the following conditions; ddH_2O and forskolin, tap water and forskolin, ddH_2O only and tap water only, with the last two conditions representing the negative control experiments. From the following diagram below (Fig. 19), one could say that less or no cells were stained in the negative control experiments. Images obtained from the tests with forskolin with or withoutout the application of odorants are represented in figures 17-19. Figures A indicated few cells stained with 1 mM of AL or no cells stained as in the negative and forskolin tests.

17



18

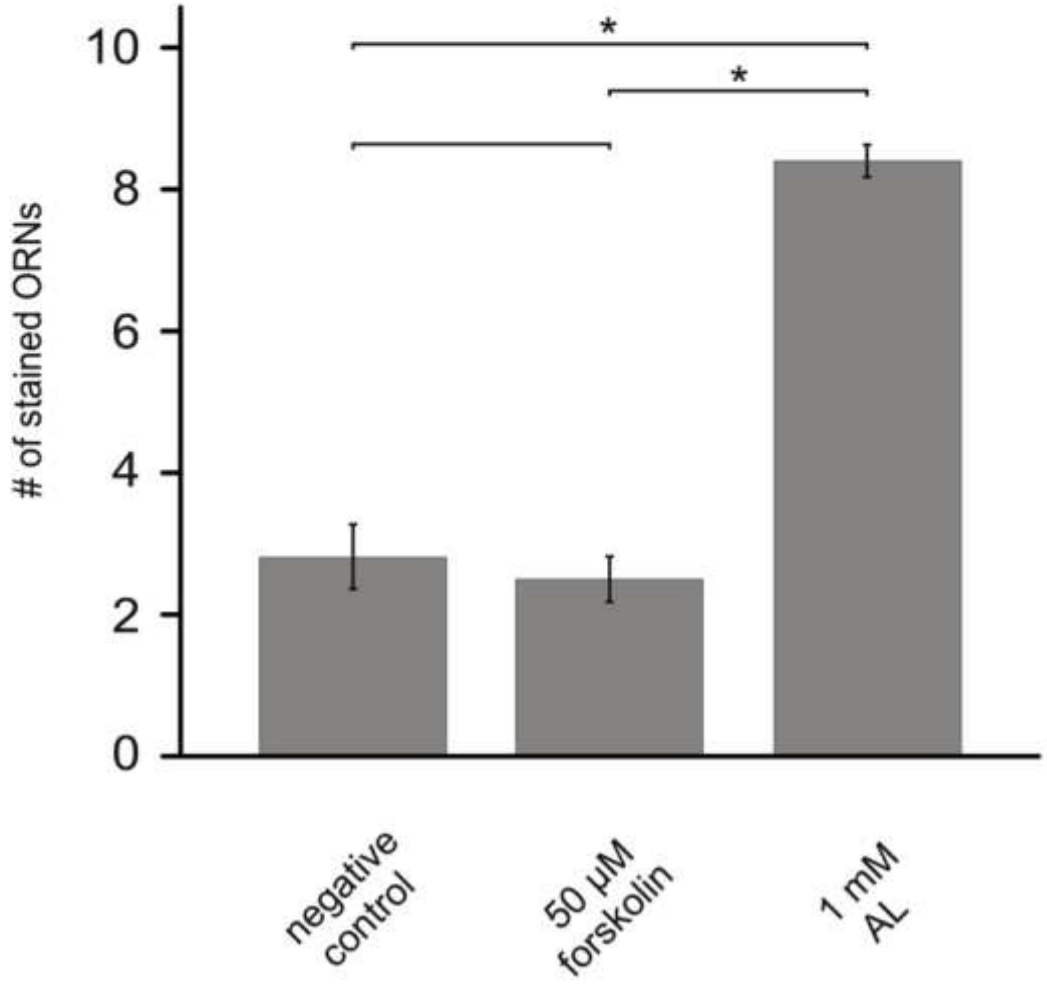


Figure 17 and 18

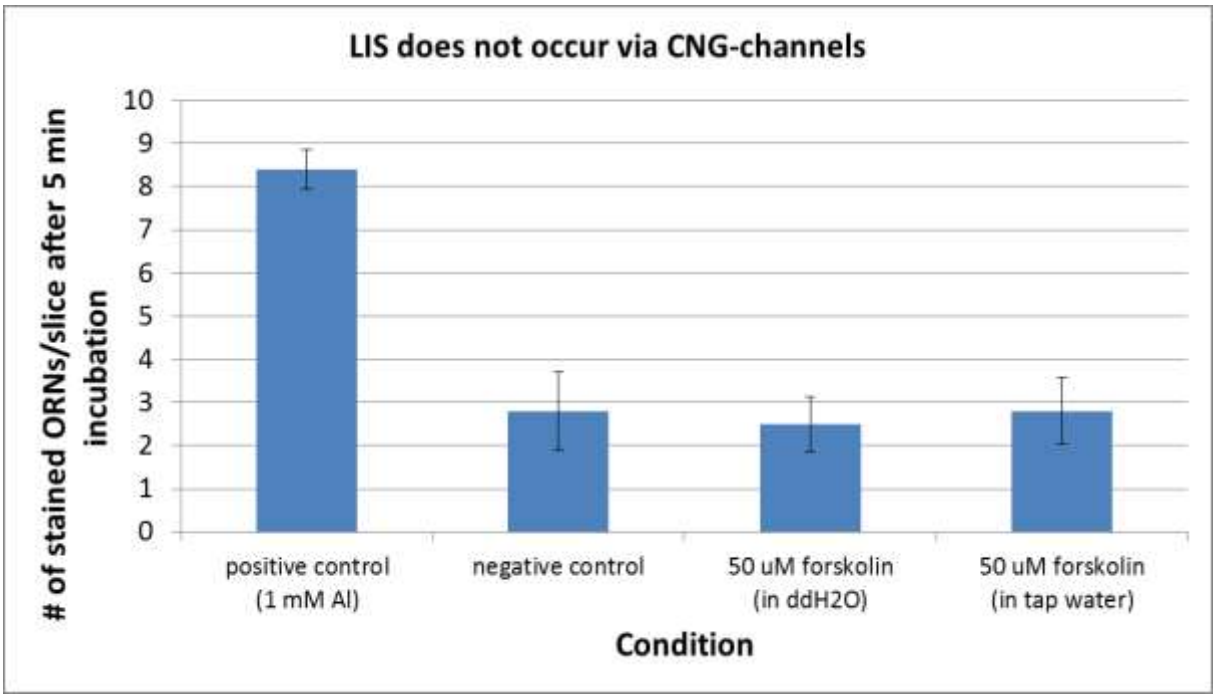

LIS does not occur via CNG-channels
# of stained ORNs/slice after 5 min incubation
10
9
8
7
6
5
4
3
2
1
0
positive control (1 mM Al)
negative control
50 uM forskolin (in ddH2O)
50 uM forskolin (in tap water)
Condition

Figure 19

Figure 17-19: Ligand-induced staining does not occur via cAMP-gated channels. (17) Slices of the OE stained with Alexa 488 dextran-dextran (merged z-stacks). Arrows indicate labeled ORNs. The left image shows the result from a negative control experiment where the tissue was incubated for 5 min in ddH2O containing 10 µM Alexa 488 dextran-dextran whereas the tissues of the middle and the right images were incubated in the same solution but additionally containing either 50 µM forskolin or 1 mM AL-mixture respectively. (18) The histogram summarizes the results obtained in all slices tested for cellular staining under the three experimental conditions. While on average 2,8 ORNs (+0,91 SEM; n = 5 slices) were stained in negative control experiments, 2,5 (+0,64 SEM; n = 13 slices) and 8,4 (+0,46 SEM; n = 5 slices) receptor neurons got labeled when 50 µM forskolin or 1 mM AL-mixture was present. T-test analysis yielded no significant difference between the negative control group and forskolin-treated slices ($p=0,84$; $p>5\%$). However, a significant difference was found between each of these groups and the data from 1 mM AL-treated slices ($p=0,003$; $p<5\%$ for negative control - 1 mM AL // $p=0,000005$; $p<5\%$ for 50 µM forskolin - 1 mM AL). Abbreviations: AL, alcohol/ketone/aldehyde mixture; PC, principal cavity; MOE, main olfactory epithelium; L-M, lateral to medial.

3.4 Temperature experiment

To support the hypothesis that a change in temperature may alter the adaptation capability of ORNs, a temperature based experiment was conducted. As confirmed in previous study by (Døving et al. 2009), that extreme temperature sensitivity of endocytosis poses a significant problem for aquatic poikilotherms, in which body temperature is determined primarily by water temperature (Padrón *et al.* 2000) and also knowing that decreased temperature (+2°C) and disruption of microtubules with nocodazole profoundly reduced the number the taste cells stained, indicating endocytotic uptake of dye and transport towards the cell soma in vesicles (Døving *et al.* 2009). Due to this finding, I assumed that low temperature may have a negative effect on the adaptation of ORNs in *X.l* and conducted experiments where LIS-experiments were conducted at a temperature of 3-4°C. For negative control experiments, attempts were made at room temperature instead of at 3-4°C using 1.5 µM AA. The outcome of the temperature test as represented in figures 20-22 indicate no stained ORNs at temperatures between 2-5 degree celcius on application of 1 mM AA and 5 mM AA. Furthermore, the negative control test conducted at room temperature yielded more stained cells compared to the positve tests.

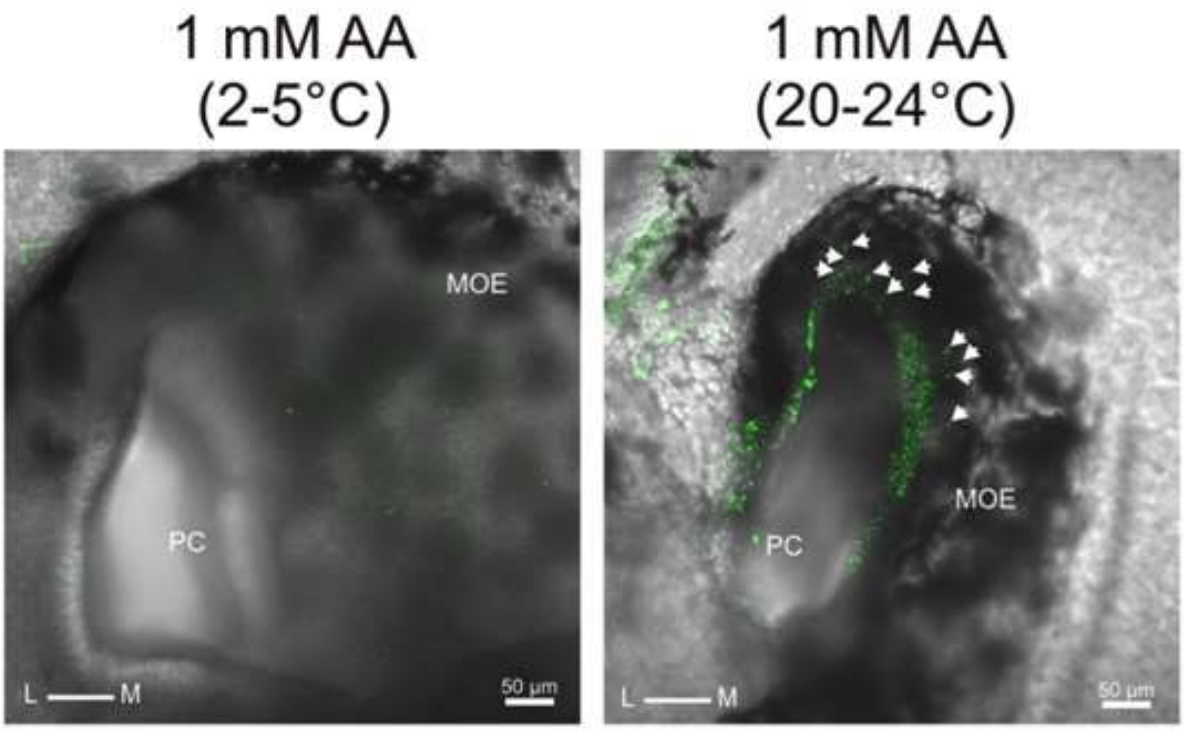


Figure 20

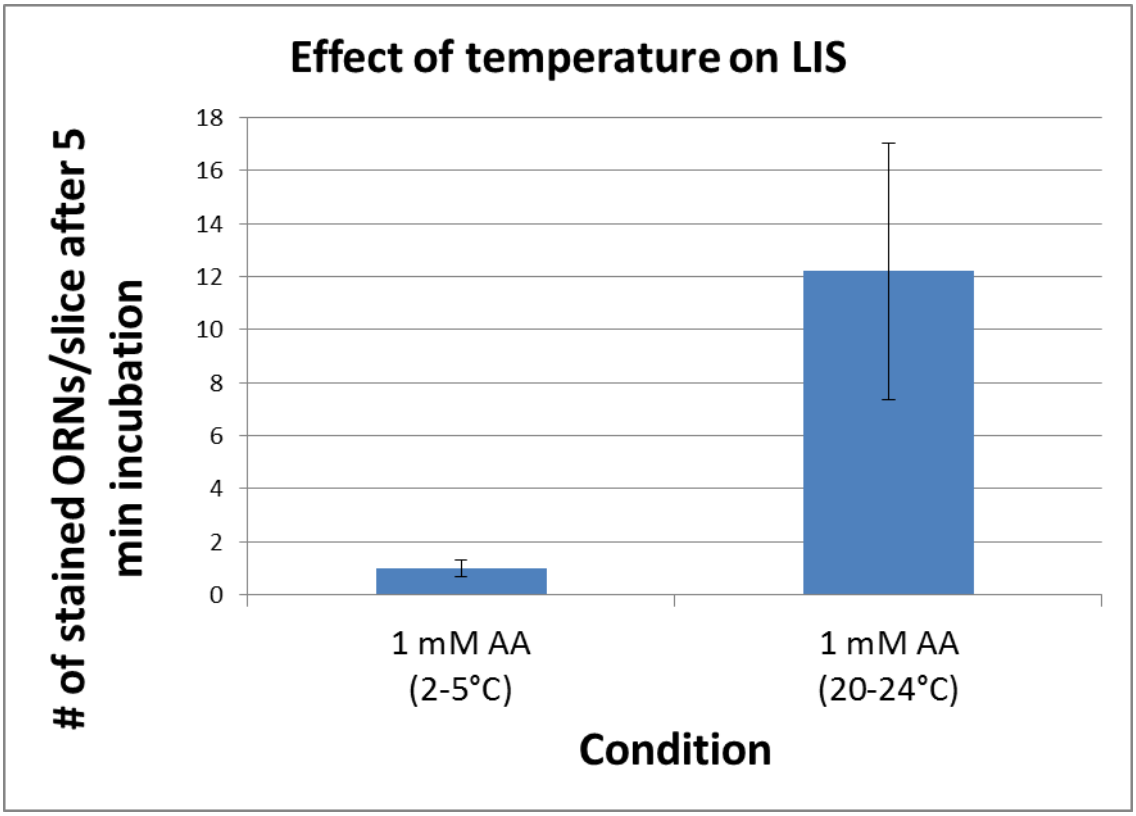


Figure 21

Figure 20-21 : Effect of low temperature on odor-induced staining of ORNs (Part I). (20) Slices of the OE stained with Alexa 488 dextran-dextran (merged z-stacks). Arrows indicate labeled ORNs. The left image shows the result from an experiment where the tissue was incubated for 5 min at 2-5°C in ddH2O containing 10 µM Alexa 488 dextran-dextran and 1 mM AA. The tissue of the right image was incubated in the same solution but at a temperature of 20-24°C (21) The histogram summarizes the results obtained in all slices tested for cellular staining under the two experimental conditions. While on average 1 ORN (+0,31 SEM; n = 8 slices) was stained at 2-5°C, 12,1 (+4,84 SEM; n = 5 slices) receptor neurons got labeled at a temperature of 20-24°C. T-test analysis yielded no significant difference between both groups (p=0,11; p>5%). Abbreviations: AA, amino acids; PC, principal cavity; MOE, main olfactory epithelium; L-M, lateral to medial.

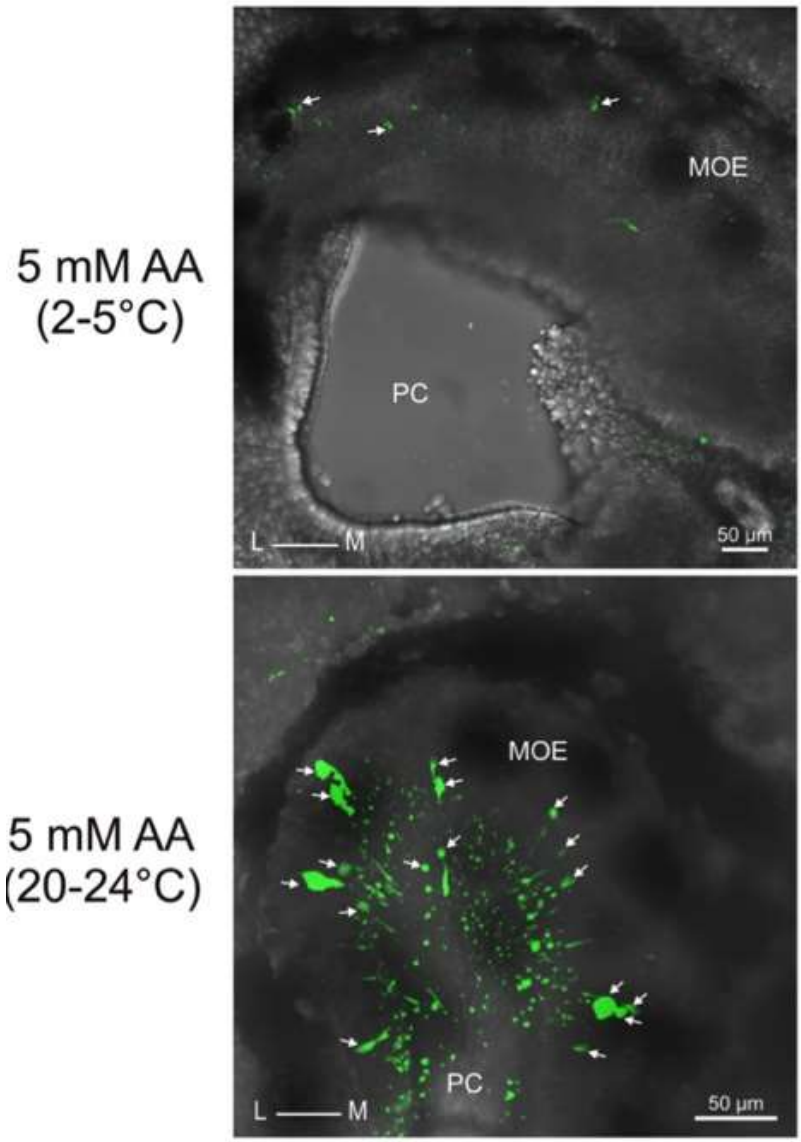


Figure 22

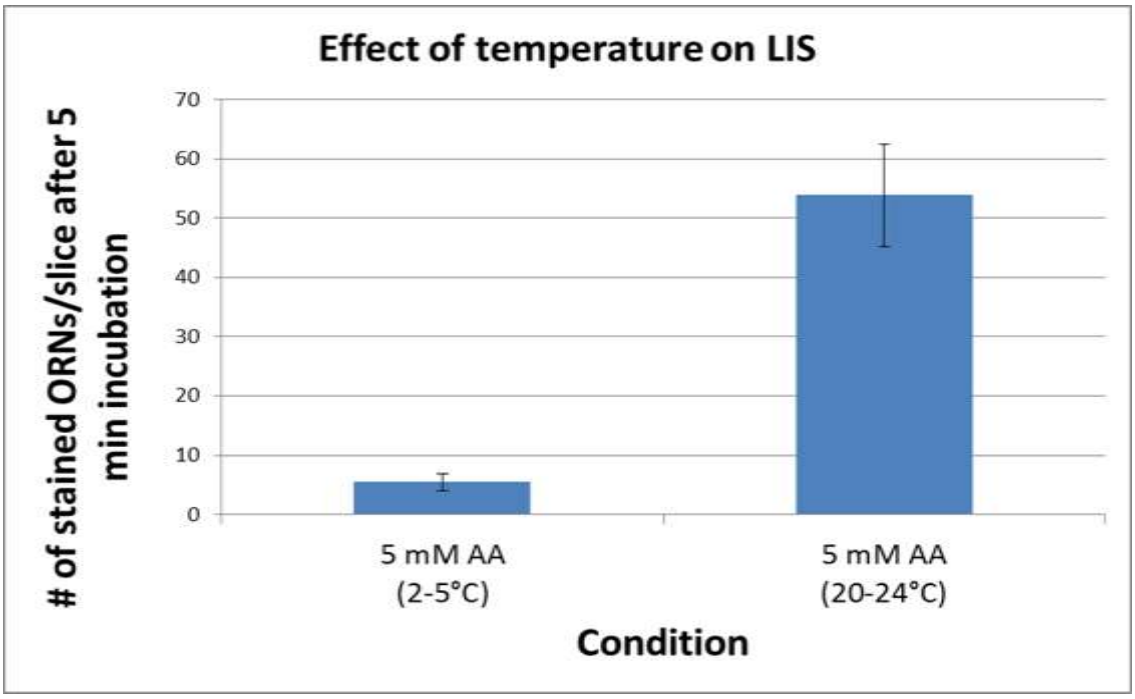


Figure 23: Effect of low temperature on odor-induced staining of ORNs (Part II). (A) Slices of the OE stained with Alexa 488 dextran-dextran (merged z-stacks). Arrows indicate labeled ORNs. The left image shows the result from an experiment where the tissue was incubated for 5 min at 2-5°C in ddH2O containing 10 µM Alexa 488 dextran-dextran and 5 mM AA. The tissue of the right image was incubated in the same solution but at a temperature of 20-24°C (B) The histogram summarizes the results obtained in all slices tested for cellular staining under the two experimental conditions. While on average 5,5 ORNs (+1,44 SEM; n = 4 slices) were stained at 2-5°C, 53,9 (+8,67 SEM; n = 8 slices) receptor neurons got labeled at a temperature of 20-24°C. T-test analysis yielded a significant difference between both groups (p=0,001; p<5%). Abbreviations: AA, amino acids; PC, principal cavity; MOE, main olfactory epithelium; L-M, lateral to medial.

4 DISCUSSION

Modern science has made it possible to better understand the molecular mechanism of olfaction at all levels of the olfactory system. Prior to advanced knowledge of olfaction, it is long known that sense of smell and animal behaviour are coupled by function (Keverne 2002). In mammals the olfactory cells located in the olfactory epithelium by means of receptors expressed on dendrites make the initial contact to stimulus and are known for their ability to recognize scores of odorants (Firestein 2000). Due to the fact that sense of smell largely influences behaviour, choice of food, reproduction and social interaction between animals, a deeper knowledge on its anatomy and function is of interest to researchers. Olfaction has been studied in several animals. Although the similarity between olfaction in humans and lower animals has not yet been completely understood, similar G-protein-coupled mechanisms, which occur in other systems other than the olfactory system in humans have also been observed in the olfactory system of lower animals (Weickert *et al*. 2000).

Adaptation to stimulus has not been described in human pain receptors in contrast to olfactory receptors. The failure of pain receptors to adapt has been attributed to its allowance to alert a person of underlying tissue damage (Guyton and Hall 2006). A research on the taste cells of rat, which revealed that taste receptor cells activated by hydrochloric acid induced a cAMP-coupled sour taste transduction, intracellular pH decrease and receptor adaptation by pH recovery mechanism (Lyall *et al.* 2001).

In addition, it is gathered from a study by (Døving *et al*. 2009) that a ligand-specific induction of endocytosis occurs in taste cells of juvenile brown trout taste buds and is dependent on the presence of adequate stimuli. Døving's study suggests that cell staining was via ligand-induced endocytosis. This finding prompted my interest in studying ligand-induced endocytosis in another type sensory cell of a different experimental animal namely *X.l*. Using newly generated methods, adequate staining of living animals was successful. These methods enabled proper and careful preparation of animals. Hence, I was able to view living, stained olfactory receptor cells, and evaluate the LIS mechanism outcome on application of an odorant and fluorophore dextran dye. Using forskolin, the role of CNG channels in staining, and the effects of low temperature on LIS in the experimental animal were observed.

Figure 8 depicts a single stained ORN possibly via LIS, while figure 9 represents adequately stained ORNs in *X.l*. On application of 5mM BA as stimulus and 20µM Texas red dextran for 15 minutes, one could also observe that not just ORNs were stained but also what could be presumed to be unspecific epithelial structures or mere accumulation of fluorescent dye. This results stir up the question of by which exact mechanism staining in ORNs occurs. As mentioned earlier (chapter 1.4.1 of 'Introduction') we suspect that ligand-induced endocytosis might be responsible for the stained ORNs as cell staining in Hana3A cells of mouse for instance, showed OR2AG1 by means of clathrin-mediated endocytosis upon activation with amyl butyrate (Mashukova *et al.* 2006). Furthermore, same study revealed that ß-arrestin, a molecule responsible for desensitization of receptors is mobilized upon stimulation of olfactory receptor (Mashukova *et al*. 2006). Clathrin-mediated endocytosis is one of the several endocytotic mechanisms in eukaryotic cells. It is described as the uptake molecules into a cell using clathrin-coated pits from the surface (McMahon and Boucrot 2011). Similar events occur in EGFR sites and transferrin upon activation. Receptor-mediated endocytosis of transferrin was observed to be inhibited in clathrin-deficient cells in HeLam cells, implying that metabolism of transferrin is receptor-mediated endocytosis dependent (Motley *et al.* 2003).

4.1 Ligand-induced staining in larval *X.l*

Odorant receptors are ciliary proteins located in the mucus layer of the OE. They assist primarily in odorant detection and in transduction of odorant information. The notion of the adaptation mechanisms in fish shown by Døving *et al*. 2009 induced the idea of studying the adaptation mechanism in *X.l.*

In the course of this study, I was able to reveal that LIS is a suitable method for staining ORNs. The results of these experiments have revealed as also observed by Døving *et al.* 2011 and Chen 2012 that the newly applied method (LIS) can successfully be applied to induce staining of ORNs in an aquatic specie. With emphasis of this study lying on whether ligand-induced staining is an applicable method for labelling odour specific subsets of ORNs in the experimental animal, the outcome of the experiment carried out with different odorants (AA, AL, AM and BA) and two different fluorophore dextran dyes (Alexa 488 dextran and Texas red dextran dyes) confirmed that cell staining does occur at different concentrations for each

odorant. Though stained cells as illustrated in the ‘Results’ were observed for each odorant and dextran dye at room temperature, BA as represented in figures 9-15 of ‘Results’, in comparison to AA, AM, AL revealed a remarkable staining efficacy than other odorants with the same concentration of dye in all experiments (a quantitative evaluation was not part of this thesis and was conducted in another projects). Images produced on staining after incubation time of 15 minutes on treatment with Texas red dextran dye and Alexa-488 dextran dye were compared to one another. The first red-stained 7 images presented at the ‘Results’ section were treated with Texas red dextran dye and respective odorants for 10-15 minutes. Staining with BA (to the left) was compared to staining with other odorants; AA, AM and AL (to the right). In figure 9, OE stained with 5mM BA was compared to that stained with 5mM AA. On reduction of the concentration of odorants to 1mM, maximum staining using BA was still observed as shown in figure 10. The images of figure 10, which compares the outcome of staining with 20µM Texas red dextran dye, 1mM BA to 1mM AA for 15 minutes revealed as in figure 9 that BA had a better staining efficacy than AA. The same staining difference can also be noticed in figures 11, where BA further showed a better staining effect than AL on application of 1mM of BA and AL, and 0.1mM BA and AM in figure 12. Similar methods were applied on the green-stained images as represented in figures 13-15 were the olfactory epithelia were staind for 15 minutes with 20µM Alexa-488 dextran dye and 1mM of each of the above-mentioned odorant. Figure 13-15 compares staining with BA (to the left) to staining with AA,AM and AL (to the right) one can observe only very few stained cells are seen on the OE incubated for 15 minutes with1 mM AA compared to that of 1mM BA. Same results can be seen in figure 14, were 1mM BA is compared to 1mM AA and in figure 15 to 1mM AL. In figure 15 0.1mM BA stained better than 0.1mM AM, a silimar finding to that of the above- mentioned test with Texas red dextran dye. For the LIS negative control tests, no odorants were applied and the OE were incubated in a Texas red dextran dye (to the left) and Alexa-488 dextran dye solution (to the right) for 15 minutes. The outcome as shown in figure 16 illustrates staining by accumulation of dye in the epithelium by cells other than the ORNs.

In addition, this experiment was however, only able to reveal that ligand-induced staining takes place in ORNs of *X.l* but could not concede the precise mechanism through which it occurs. To address this, another method was invented with the

application of forskolin to investigate whether cAMP/CNG gated channels or the olfactory receptors are responsible for cell staining.

The staining and slicing method in forskolin test was practically similar to that in LIS experiment, with the only difference being that tissue blocks were cut out before staining was done, for economical reasons. Forskolin is an adenylate activator, which leads to smooth muscle relaxation, revealed its ability to increase intracellular levels of cAMP and also markedly, inhibiting vasopressin-induced calcium ion concentration increase (Lincoln et al.1990). In rat olfactory CNG-channel forskolin-induced calcium ion influx through CNG channels was stated to be inhibited by phosphodiesterase (PDE) (Rich *et al.* 2001) and also induced cell repolarization in rats, which is suggested to be responsible for the relaxtaion of the smooth muscles in previoulsy phenylephrine-stimulated rat tail artery (Rembold and Chen 1998).

As mentioned earlier in section 1.3 of the introduction, forskolin activates ACIII which in turn, elevates the intracellular cAMP levels via the induction of an ion flux through cAMP-activated CNG-channels. Therefore, forskolin was specically chosen to observe indirectly, whether staining (dye uptake) would occur through opening of CNG channels. The results showed, as represented in figures and diagrams 18-20 that hardly any ORNs were specifically stained in comparison to the original LIS experiments implying that LIS does not occur via CNG channels. The fluorescence seen in the image of figure 18 is either artifact or accumulated fluorescent dye in epithelium. Figure 8 is a zoomed image of a stained olfactory epithelium. Here one can identify the axon and dendrite of most ORNs. A morphology not seen in the forskolin test images or with the accumulated fluorecence in the epithelium. Forskolin experiments also included a negative control where no forskolin was added to the solution applied. The results of this test were similar to that of the negative control of LIS experiments (no stained cells observed) as represented in figures 18-20. This outcome supports our hypothesis that staining in ORNs does not occur through CNG channels. Generally, one can state that figure 18 A. represents the results of a negative control LIS test, a test with forskolin and a positive control test with 1mM AL at an incubation of 5 minutes respectively. It is observable that the outcome (right image) of the negative control test, that no cells were stained due to the absence of stimulus (odorant), the test with 50µM forskolin also yielded a similar result to the negative LIS test as hypothesized, implying that staining of cells does not occur via

CNG channels whereas, the image representing the positive control test with 1mM AL yielded stained ORNs. These results confirms that ligand-induced staining occurs via ligand-induced endocytosis. Parallel to the microscope images, a histogram was constructed as shown in figure 18 B and figure 20. This histogram similar to the images of 18 A. compared the outcome of each condition in terms of staining effect. It indicates that only 2.8 and 2.5 ORNs were stained in the negative control test and forskolin test respectively, while 8.4 ORNs were stained on application of 1mM AL after 5 minute incubation. During forskolin test, the effect of tap and double distilled water were also tested and according to figure 18, no significant difference was observed.

In Døving's study, Iow temperature (+2°C) and disruption of microtubules with nocodazole caused a substantial reduction in the number of taste cells stained, indicating endocytic uptake of dye and transport towards the cell soma in vesicles (Døving *et al.* 2010). To analyze the inhibition of olfactory adaptation through ligand-induced endocytosis in *X.l*, a method involving decreased temperature was implemented. Since it is known that receptors are protein in nature (Venkatakrishnan *et al.* 2013), hence a significant change in temperature may impede staining in taste receptor cell (Døving et al. 2009). In my study, decreased temperatures ranging between 2-5°C as confirmed by figures 21-24 of 'Results' on application of 1mM AA and 5mM AA also revealed no stained cells in this experiment on larval *Xenopus laevis* ORNs. In figure 21, the images and histograms show few or no stained ORNs on application of 1Mm AA at temperatures between 2°-5°C, whereas at room temperature of about 25°C significantly more cells were stained. The histograms indicated that just about 5.5 ORNs were stained with 5 mM of AA at an incubation period of 5 minutes at temperatures ranging from 2°-5°C, while over 50 ORNs were stained with 5 mM AA at an incubation of 5 minute at room temperature

With a successful verification of the 3 different hypotheses, namely, that ligand-induced stainining takes place in *X.l*, that ligand staining, presumably receptor-mediated endocytosis and not via CNG channels and furthermore, that low temperature has a negative influence on ligand-induced endocytosis, provision has been made for further ideas related to these experiments. Recalling that ligand-

induced staining occurs via a receptor-mediated endocytosis, further trials with the application of the presented method together with immunohistochemistry could be done in the future to simultaneously gather information about cell-ligand sensitivity and protein expression. These experiments could aim, firstly, at staining simultaneoulsy, with various fluorophore dextran dyes specific subsets of ORNs that may respond to different odorants and also detect subsets of ORNs that may exist in specific regions of the olfactory mucosa. Secondly, preparing tissues including not just the OM, but also axons and the OB to reveal the possiblity of staining projections to the bulbus in a *X.l.* A study by Chen on LIS in larval *Xenopus laevis,* where calcium responses of statined *ORNs* were present provided evidences that both [Ca2+]in and [Ca2+]out are influencing factors of LIS (Chen 2012). In addition, Chen revealed that imbalances in intracellular calcium ion levels upon influx at elevated extracellular calcium ion levels may disrupt the adaptation mechanism in ORNs.

For further analysis of the inhibition of receptor-mediated endocytosis, methods using drugs may be introduced in future researches. This already proved efficacious with the exposure of the OE of crucian carp, *Carassius carassius*, and brown trout, *Salmo trutta*, to nocodazole (Døving *et al.* 2010), a drug that selectively affects the transport of an apical membrane protein (Eilers *et al.*1989) and therefore presumably inhibits receptor-mediated endocytosis. Experiments to further prove that the observed process is an endocytic one could be carried out using imidazo-pyrazine derivative BIM-46174; a drug which acts as a selective inhibitor of heterotrimeric G-protein complex (Prévost *et al.* 2006). Reagents known to inhibit enzyme translocation and desensitization mediated by odorants while disabling odorant-stimulated phosphorylation of GPCRs (Boekhoff *et al.*1994) may also be worth considering for further tests.

5 SUMMARY

This thesis aimed at investigating ligand-induced staining (LIS) as a new labelling technique to visualize ORN subpopulation in larval *X.l*. In the course of this study, it was at first validated that LIS is a suitable technique for this experimental model animal. Secondly, tests carried out with forskolin furthermore revealed that the observed staining does not occur via activated cAMP/CNG channels. To generate first evidence that an odorant-induced, receptor-mediated endocytosis could be a plausible explanation for the observed staining, a temperature experiment was conducted, knowing that a decrease in temperature affects receptor-mediated endocytosis in sensory cells of vertebrates. As a decrease in staining efficiency was observed in these experiments, in summary it can be hypothesized that, presumably based on an adaption mechanism, a receptor-mediated endocytosis is the reason for the observed ligand-induced staining.

6 LIST OF FIGURES

7 LIST OF ABBREVIATIONS

AA	amino acid
ACIII	adenylcyclase III
AL	alcohol
AM	amine
ATP	adenosine triphosphate
BA	bile Acid
$[Ca^{2+}]i$	intracellular Ca2+concentration
Camp	cyclic adenosine monophosphate
CNG	cyclic nucleotide-gated
G_{olf}	olfactory-specific guanosine triphosphate (GTP)-binding protein
GTP	guanosine triphosphate
IP3	IP_3-Rezeptor
MOE	main olfactory epithelium
OE	olfactory epithelium
ON	olfactory nerve
OR	olfactory receptor
ORN	olfactory receptor neuron
PC	principle cavity
PIP2	phosphatidylinositol 4,5-bisphosphate
VNO	vomeronasal organ
X.l.	xenopus laevis

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CURRICULUM VITAE

Der Lebenslauf wird aus Datenschutzgründen nicht mit veröffentlicht.