The teleost taar family of olfactory receptors:

From rapidly evolving receptor genes to ligand-induced behavior

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To my parents

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VI. Abstract

Trace amine-associated receptors (TAARs) have recently been shown to function as olfactory receptors in mammals. In this current study, the taar gene family has been delineated in jawless, cartilaginous, and bony fish (zero, 2, and >100 genes, respectively). I conclude that the *taar* genes are evolutionary much younger than the related OR and ORA/V1R olfactory receptor families, which are present already in lamprey, a jawless vertebrate. The 2 cartilaginous fish genes appear to be ancestral for 2 *taar* classes, each with mammalian and bony fish (teleost) representatives. Unexpectedly, a whole new clade, class III, of *taar* genes originated even later, within the teleost lineage. *Taar* genes from all 3 classes are expressed in subsets of zebrafish olfactory receptor neurons, supporting their function as olfactory receptors. The highly conserved TAAR1 (shark, mammalian, and teleost orthologs) is not expressed in the olfactory epithelium and may constitute the sole remnant of a primordial, non olfactory function of this family. Class III comprises three-fourths of all teleost *taar* genes and is characterized by the complete loss of the aminergic ligand-binding motif, stringently conserved in all 25 genes of the other 2 classes. Two independent intron gains in class III *taar* genes represent extraordinary evolutionary dynamics, considering the virtual absence of intron gains during vertebrate evolution. The dN/dS analysis suggests both minimal global negative selection and an unparalleled degree of local positive selection as another hallmark of class III genes. The accelerated evolution of class III teleost *taar* genes conceivably might mark the birth of another olfactory receptor gene family.

Ligands have only been identified for a handful of olfactory receptors of mammals and insects, while only a single teleost olfactory receptor have been deorphanized, a member of the OlfC family, OlfCa. Zebrafish TAAR olfactory receptors of classI are good candidates for having amines as possible ligands, due to the presence of the aminergic ligand binding motifs. This study identifies diamines as specific ligands for a taar receptor, DrTAAR13c. These diamines activate a sparse subset of olfactory sensory neurons, as indicated by c-Fos expression in olfactory epithelium. Diamines, putrescine and cadaverine, are foul-smelling aliphatic polycations that occur naturally as a result of bacterial decarboxylation of amino acids lysine and arginine, respectively. The concentration of diamines in their environment is correlated to the degree of putrefication.

In the behavioral assay, zebrafish exposed to even low concentration of diamines show dramatic, quantifiable aversion, while it shows attraction towards food stimulus and no response for water. The ligand spectrum of TAAR13c closely parallels the behavioral effectiveness of these diamines. This data is consistent with the existence of a defined neuronal microcircuit that elicits a characteristic behavior upon activation of a single olfactory receptor, a novum in the vertebrate sense of smell.

Zusammenfassung

Seit kürzerer Zeit hat sich herausgestellt, dass *trace amine-associated receptors* (TAARs) bei Säugetieren als Geruchsrezeptoren dienen. Die Familie der TAAR-Gene wird in der vorliegenden Studie für kieferlose Fische, Knorpel- und Knochenfische beschrieben (respektive Null, zwei und über 100 Gene). Es wird gefolgert, dass die TAAR-Gene evolutionär wesentlich jünger sind, als die verwandten Familien der OR und ORA/V1R Geruchsrezeptoren, welche bereits beim Neunauge, einem kieferlosen Wirbeltier vorkommen. Die zwei Taar-Genklassen mit Vertretern bei Säugetieren und Knochenfischen (Teleostei) scheinen jeweils von einem der beiden TAAR-Gene der Knorpelfische abzustammen. Mit der Klasse III der TAAR-Gene entsteht unerwarteterweise noch eine völlig neue Klade in der Linie der Teleosten. TAAR-Gene aller drei Klassen werden in Untergruppen olfaktorischer Rezeptorzellen des Zebrabärblings exprimiert, was ihre Funktion als olfaktorische Rezeptoren bekräftigt. Das stark konservierte TAAR1-Gen (Orthologe bei Haien, Säugetieren und Knochenfischen) wird nicht im olfaktorischem Epithelium exprimiert und kann daher einen letzten Vertreter dieser Familie darstellen, bei dem die ursprüngliche nicht-olfaktorische Funktion erhalten blieb. Die Klasse III enthält dreiviertel aller TAAR-Gene der Teleostei und ist durch den völligen Verlust der aminergen Ligandenbindungsstelle gekennzeichnet, welche bei allen 25 Genen in den anderen beiden Klassen durchgehend erhalten blieb. Zwei unabhängige Intron-Einschübe bei TAAR-Genen der Klasse III stellen eine aussergewöhnliche evolutionäre Dynamik dar, wenn die fast völlige Abwesenheit von Intron-Einschüben während der Evolution der Wirbeltiere in Betracht gezogen wird. Eine dN/dS Analyse legt eine minimale generelle negative Selektion als auch einen beispiellosen Grad lokaler positiver Selektion als weitere Merkmale der Klasse III Gene nahe. Die beschleunigte Evolution der Klasse III TAAR-Gene bei den Teleostei kann als Kennzeichen für die Geburt einer weiteren Famile olfaktorischer Rezeptorgene betrachtet werden.

Liganden wurden bisher nur für einige wenige olfaktorische Rezeptoren bei Säugetieren und Insekten gefunden, während dies bei den Knochenfischen nur für einen einzigen olfaktorischen Rezeptor der OlfC Familie gelang (OlfCa). Die olfaktorischen TAAR-Rezeptoren des Zebrabärblings aus Klasse I und II sind aufgrund des konservierten aminergen Ligandenbindungsmotifs gute Kandidaten dafür, Amine als Liganden haben.

Die vorliegende Studie identifiziert Diamine als spezifische Liganden eines TAAR-Rezeptors (DrTAAR13c). Diese Diamine aktivieren eine geringe Anzahl olfaktorischer Rezeptorneuronen, die durch c-Fos Expression im olfaktorischen Epithelium identifiziert werden. Die Diamine Putreszin und Kadaverin sind faulig riechende, aliphatische Polykationen, die bei der Dekarboxylierung von Lysin und Arginin durch Bakterien auf natürliche Weise entstehen. Die Konzentration dieser Diamine in der Umgebung korreliert mit dem Grad der Verwesung. Im Verhaltensversuch zeigten Zebrabärblinge, die nur geringen Konzentrationen von Diaminen ausgesetzt worden waren, bereits ein deutliches aversives Verhalten, wohingegen ein Nahrungsstimulus anziehend wirkte, und Wasserzugabe keine Reaktion hervorrief. Interessanterweise ist die Ligandenspezifität des TAAR13c Rezeptors sehr ähnlich zur Wirksamkeit derselben Liganden in den Verhaltensversuchen. Diese Ergebnisse könnten auf die Existenz eines definierten neuronalen Mikroschaltkreises hinweisen, welcher durch Aktivierung eines einzigen Typs olfaktorischer Rezeptoren ein bestimmtes Verhalten auslöst, was für den Geruchssinn der Wirbeltiere ein Novum darstellt.

CHAPTER 1
INTRODUCTION

VII. INTRODUCTION

Animals in their natural milieu are surrounded by odors. These odors are rich source of information, and are perceived by sophisticated olfactory systems, that have evolved over time. The sense of smell helps species to localize prey, evade predators, explore food and recognize viable mates. In humans, memoirs, thoughts, emotions, and associations are more readily reached through the sense of smell than through any other channel. This suggests that olfactory processing is imperative and may differ fundamentally from processing in other sensory modalities. The molecular age in olfaction initiated in 1991 with the significant discovery of a large, multigene family of olfactory receptors in rat by Linda Buck and Richard Axel (Buck and Axel, 1991). The first cloned olfactory receptors consisted of a diverse repertoire of G-protein coupled receptors (GPCRs) with seven-transmembrane topology, and they were sparsely expressed in the olfactory epithelium. This Nobel Prize worthy pioneering discovery, together with availability of modern techniques and numerous completely sequenced genomes opened the way to characterize the gene families of olfactory receptors through exhaustive computational data mining in different species genome as well as by *in vitro* biology.

1. Olfactory system

The generalized initial point of olfactory system is the nose that contains the olfactory epithelium (O.E). The O.E contains olfactory sensory neurons (OSNs) that express olfactory receptor molecules (ORs) on their apical surfaces. The number of OR genes varies according to the species e.g. 388 in human, 155 in zebrafish and 1063 in mice (Nei et al., 2008). The olfactory system perceives myriad of odorants and translates the primary input into diverse odor perception. The primary event in olfactory perception is the recognition of odorants by odorant receptors (ORs), this may occur by diffusion or by the binding of the odorant to odorant binding proteins (OBPs) first, that lead to docking at the respective odorant receptor. One odorants receptor (OR) can bind to odorant of same or different chemical structures. Odorant receptors (ORs) that bind to the same types of odorants unite in the olfactory bulb and form glomeruli. The odorant information is then passed through the olfactory bulb (OB) to the olfactory cortex, in due course reaching the higher cortical areas involved in odour determination, as well as limbic areas supposedly mediating the emotional and physiological effects of odours (Kapur and Haberly, 1998)

.Odorants are perceived and encoded by different combinations of olfactory receptors (Malnic et al., 1999). In the nose, neurons expressing the same OR are scattered throughout olfactory epithelium (Vassar et al., 1993), however, in the olfactory bulb their axons converge at a specific glomeruli, where they form synapses with mitral and tufted relay neurons of olfactory bulb (Mombaerts et al., 1996; Ressler et al., 1994). This results in a rather stereotyped spatial map in which inputs from different ORs are targeted to different glomeruli. An odorant's receptor code is represented in the olfactory epithelium by a dispersed ensemble of neurons and in the bulb by a specific combination of glomeruli (Mori et al., 1999).

1.1. Mammalian olfactory system

Contrary to the fish, many terrestrial vertebrates, including rodents, have up to five main discrete and segregated olfactory systems, including a main olfactory system, which detects volatile odorants and a vomeronasal (accessory olfactory) system, which detects pheromones (Buck, 2000; Mombaerts, 2004). Recently, it has become obvious that there is functional overlap between the main olfactory epithelium and the vomeronasal organ. Certain pheromones activate neurons in the main olfactory system, and this activity has been found necessary for pheromone dependent behaviors (Mandiyan et al., 2005; Restrepo et al., 2004; Spehr et al., 2006b). Likewise, some general odorants categorized as non-pheromones activate the accessory olfactory system and modulate behavior in the absence of a functional main olfactory system (Sam et al., 2001; Trinh and Storm, 2003). In mammals, the olfactory information is processed through anatomically separated neural pathways. Volatile odorants are perceived by a large repertoire of olfactory receptors (ORs) expressed on the cilia and dendritic knob of the ciliated olfactory sensory neurons (OSNs) in the olfactory epithelium (OE), that project their axons to the main olfactory bulb (OB). Two additional receptor families (V1R, V2R) appear to detect pheromones and are expressed by microvillous sensory neurons in the vomeronasal organ that induce hormonal and behavioral responses through the accessory olfactory bulb (AOB). The axons from the accessory olfactory bulb project towards the amygdala and hypothalamus that are involved in aggression and mating behavior (Hasen and Gammie, 2009).

Organ	Receptors	Ligands
MOE	ORs, TAARs, GC-D	general odors, MHC class I peptides volatile amines, CO_2 (bicarbonate)
VNO	V1Rs,V2Rs, FPRs	volatile pheromones, MHC class I peptides, formyl peptides
GG	TAARs, V2r83	alarm pheromones
SO	ORs	general odors

Table.1. Mammalian olfactory organs and their respective receptors with possible ligands

A third mammalian organ, the septal organ of Masera (S-O), also contains sensory neurons ((Kaluza et al., 2004; Tian and Ma, 2004) that express odor receptors (Table.1). The S.O was recently shown to perceive multiple volatile odorants that are also detected by the main olfactory epithelium (Grosmaitre et al., 2007; Ma et al., 2003).Interestingly, a subset of OSNs from both the SO and the main olfactory epithelium may respond to mechanical pressure and thus may report changes in air pressure induced by sniffing (Grosmaitre et al., 2007). Recently, another mammalian organ named the Grueneberg ganglion (GG) was found to subserve olfaction (Fleischer et al., 2006; Fleischer et al., 2007). The Grueneberg ganglion (GG) located in the vestibule of the anterior nasal cavity is considered as an olfactory organ based on the presence of the olfactory marker protein (OMP), expression of V2R and TAARs olfactory receptors and olfactory neurons axonal projection to the olfactory bulb (Fleischer et al., 2007). These neurons are activated by volatile alarm pheromones and are required for the freezing behavior in mice, indicating a role in pheromonal signaling (Brechbuhl et al., 2008).

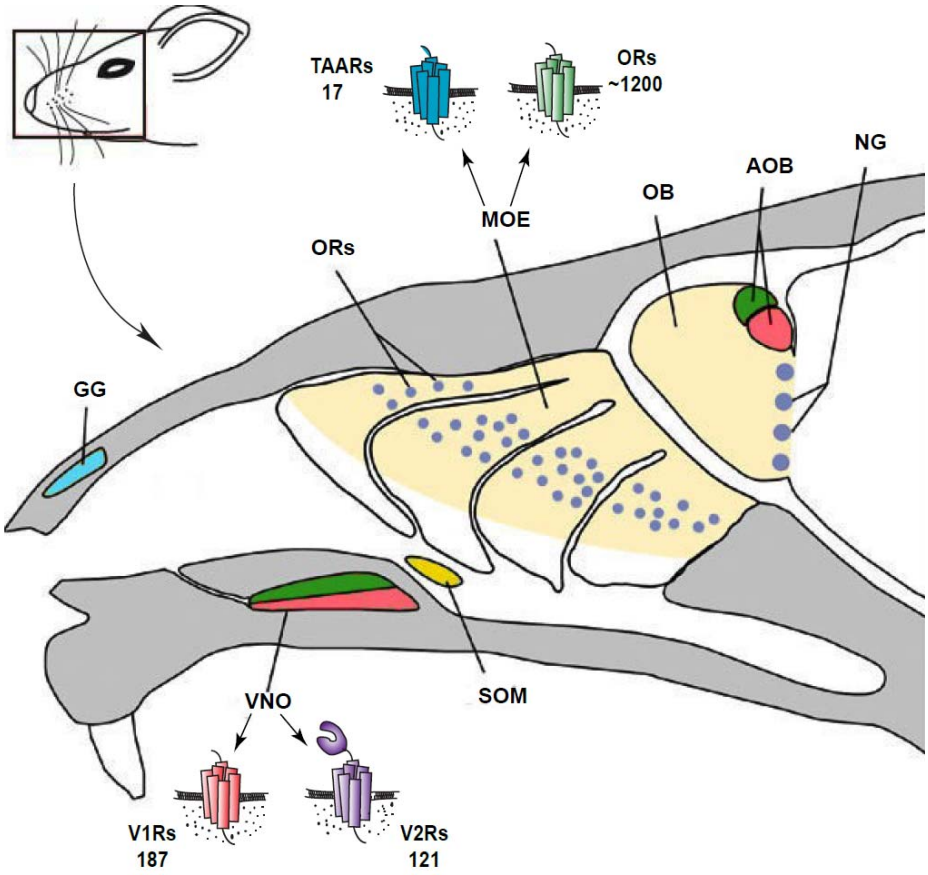

Fig. 1. Schematic representation of mouse olfactory systems. Main olfactory epithelium (MOE), olfactory bulb(OB), accessory olfactory Bulb(AOB),Grueneberg ganglion (GG), Vomeronasal organ (VNO), septal organ of Masera, guanylyl cyclase D (GCD), necklace glomeruli (NG), trace amine associated receptors (TAARs), olfactory receptors (ORs),vomeronasal receptors type1(V1Rs), vomeronasal receptors type2(V2Rs).

1.2. Zebrafish olfactory system

Zebrafish is equipped with only one olfactory system, the main olfactory system that contains a single olfactory epithelium as first site of odor perception. The olfactory epithelium has two distinguished areas: central sensory area and peripheral non-sensory area. The sensory area contains 3 types of olfactory sensory neurons (OSNs) called ciliated, microvillous and crypt OSNs that project their axons to the OB (Hansen and Zielinski, 2005). Ciliated, crypt and microvillous OSNs can be labeled with OMP, S100 and TRPC2 neural markers respectively (Germana et al., 2004; Sato et al., 2005). Ciliated

OSNs express odorant receptors (ORs) and trace amine associated receptors (TAARs), crypt OSNs may express a vomeronasal receptor type1 (V1Rs, also called ORAs in zebrafish) (Hansen and Zielinski, 2005; Saraiva and Korsching, 2007) and Microvillous OSNs express vomeronasal receptors type2 (V2Rs, also called OlfCs in zebrafish (Alioto and Ngai, 2006). Mitral and tufted cells of the OB synapse with incoming axons from OE and transfer the signals to the olfactory cortex. These three types of OSNs show several different properties with respect to their morphology, relative position in the OE, and molecular expression(Yoshihara, 2009).

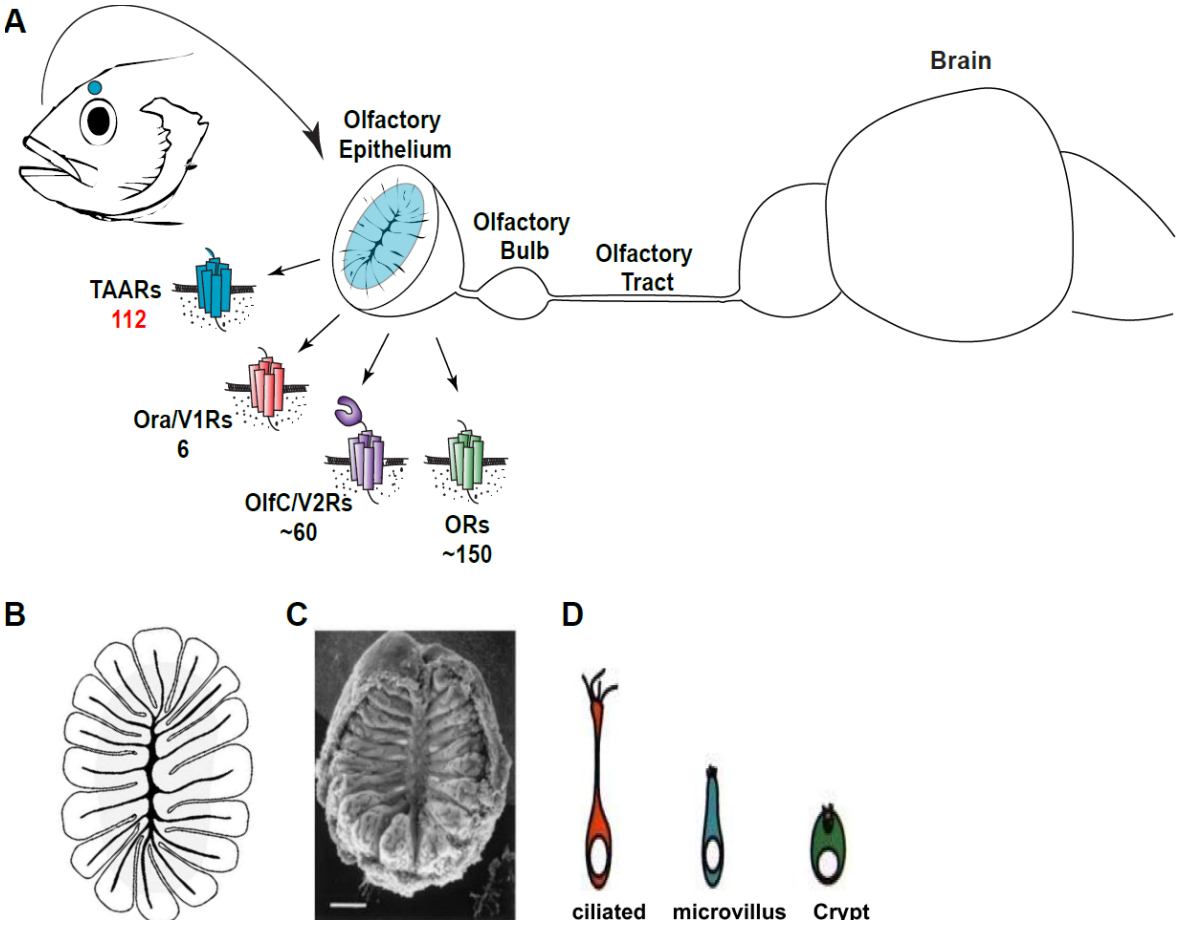

Fig. 2. General Organization of zebrafish fish olfactory system. (A) spatial organization of the olfactory system and four olfactory receptor families expressed in the olfactory epithelium. (*B*) Schematic representation of a horizontal cross-section through an olfactory rosette. (*C*) Scanning electron micrograph of an olfactory rosette of an adult zebrafish. (D) Types of OSNs expressed in olfactory epithelium. Numerous tiny hair-like cilia protrude from the dendrites of ciliated olfactory receptor cells and house the different olfactory G protein-coupled receptors. (SEM courtesy Prof. Sigrun I. Korsching).

1.3. Olfactory sensory neuron (OSNs)

The olfactory epithelium of fish contains three types of morphologically distinguished and functional olfactory sensory neurons (OSNs): Ciliated, Microvillous and Crypt. The three types of OSNs show different properties with respect to their morphology, relative position in the OE, and molecular expression. Zebrafish is equipped with only one olfactory organ that expresses all three types of olfactory sensory neurons (Korsching, 2009).The relationships among cell morphology, molecular signatures, and axonal terminations of different OSNs suggest that the two segregated neural pathways are responsible for coding and processing of different types of odor information in the zebrafish olfactory system (Miyasaka et al., 2005).

1.3.1. Ciliated olfactory sensory neurons

Ciliated sensory neurons with their somata rooted in the deep layer of the olfactory epithelium, have long dendrites (Fig. 2d) and express ORs and possibly TAARs in the zebrafish olfactory epithelium, the main sensory organ in teleosts (Hansen et al., 2004; Speca et al., 1999). Volatile odorants are perceived by a large repertoire of odorant receptors (ORs) sparsely expressed in the OE and the information is transmitted to the main olfactory bulb (OB).The signal transduction of ciliated OSN uses cyclic nucleotide-gated channel A2 subunit, and olfactory marker protein (Friedrich and Korsching, 1998; Hansen et al., 2003; Sato et al., 2005). Ciliated OSNs project their axons mostly to the dorsal and medial regions of the OB, whereas the microvillous OSNs project their axons to the lateral region of the olfactory bulb (Sato et al., 2005). The LOT is involved in the perception of amino acids (von Rekowski and Zippel, 1993) that induce feeding behavior (Hamdani et al., 2001), whereas the mMOT is involved in the perception of alarm reaction (Hamdani et al., 2000). The axons of ciliated OSN, which bind the same odors synapse with mitral cells, to form glomeruli in the medial and ventral regions of olfactory bulb. Transgenic fish labeled with molecular cell markers, OMP for ciliated OSN have been generated in recent years (Sato et al., 2005).

1.3.2. Microvillous olfactory sensory neurons

Microvillous OSNs are located in the apical layer of olfactory epithelium of teleosts and express OlfCs (mammalian V2R-type receptors homologue) and transient receptor potential channel C2 (TRPC2) (Hansen et al., 2004; Morita and Finger, 1998). Microvillous OSNs have short dendrites that possess microvilli for stimulus detection (Fig. 2d). In mammals Microvillous OSNs express vomeronasal receptors2 (V2R) in the vomeronasal organ. Pheromones (olfactory cues capable of inducing stereotypical social and sexual behaviors among conspecifics) are perceived mostly by V2R receptors expressed by microvillous OSN that project their axons to the accessory OB. The lateral region of the OB is innervated by the microvillous OSNs (Hamdani et al., 2002; Hansen et al., 2003). In zebrafish, Microvillous neurons are also involved in perception of amino acids and nucleotides (Friedrich and Korsching, 1998; Hansen et al., 2003) and probably project through the LOT that elicits feeding behavior (Sato et al., 2005).

1.3.3. Crypt sensory neurons

Crypt cells (CCs), a third type of OSN located in the OE of actinopterigians (ray-finned fishes) and some other vertebrates (Hansen and Finger, 2000), were described in teleosts in 1998 (Hansen and Finger, 2000; Morita and Finger, 1998). Crypt cells are absent in both sarcopterigians (lobe-finned fishes), tetrapods and in American alligator (*A. mississippiensis)* (Hansen, 2007; Hansen and Finger, 2000). Crypt cells have a typical morphology, clearly distinguished from that of common olfactory receptor neurons (ORNs). Crypt cells are ovoid cells and with a crypt-like apical invagination where cilia protrude, as their exceptional characteristic (Fig. 2d). Crypt cells are located in the upper third of the OE and scattered along the olfactory lamellae (Catania et al., 2003; Ferrando et al., 2006; Hansen et al., 2003). Their presence and distribution in fishes seem to vary from specimen to specimen and from season to season, suggesting a certain variability and feedback control of the expression of the CN population (Hamdani el and Doving, 2006; Hansen and Finger, 2000). Although the precise function of crypt ORNs in olfactory pathways is still tentative, it has been shown in crucian carp (*Carassius carassius*), that their axons project through the lateral bundle of the medial olfactory tract (lMOT), which mediates reproductive behavior (Weltzien et al., 2003), to a central region in the ventral

olfactory bulb (Hamdani el and Doving, 2006), whose neurons are triggered by pheromones (Lastein et al., 2006).

1.4. Olfactory receptor gene family repertoire

The discovery of olfactory receptors (Buck and Axel, 1991) opened a new age for molecular study of GPCRs. So for, five olfactory receptor gene families, all of them G protein-coupled receptors, have been identified and characterized in mammals(Liberles et al., 2009; Riviere et al., 2009), while for teleost have four olfactory receptor gene families have been described up to now (Korsching, 2009). They include the odorant receptors (OR), vomeronasal receptor (V1R/ORA and V2R/OlfC), formyl peptide receptor (FPRs, found only in mammals) and trace amine-associated receptors (TAARs). The number of identified olfactory receptors expanded rapidly by data-mining due to the availability of complete genome of several model organisms, not only in rodents but also in other mammals, amphibians, fish and birds. Olfactory GPCRs families involved in perception of pheromones were identified (Belluscio et al., 1999; Dulac and Axel, 1995) . Recently a new class of GPCRs named trace amine-associated receptors (TAARs) was recognized in rodents (Liberles and Buck, 2006), zebrafish and other species (Berghard and Dryer, 1998; Gloriam et al., 2005). Olfactory receptor gene families vary between species considering that each species have their own characteristic set of chemical signals that are important for survival and reproduction. The remarkable species-specific and ambiance related discriminatory capacity of the chemosensory system is directly linked to the diversity of the olfactory receptor gene families (Dryer, 2000). ORs, FPRs and TAARs belong to the classA (rhodopsin-like) GPCRs, with short extracellular N- terminal ligand binding domain and short cytosolic C-terminal domain. V1Rs are also considered closed to classA. Although ORs and V1Rs do not share considerable sequence homology, both are Class-(rhodopsin-like) GPCRs. Widespread features among ORs and V1Rs include an intronless coding region, exclusively monogenic (Rodriguez et al., 1999) and monoallelic (Roppolo et al., 2007) expression, a scattered and mainly clustered chromosomal organization (Del Punta et al., 2002), and a sparsely distributed tissue expression pattern consistent with the ‘one neuron – one (or a few) receptor(s)’ hypothesis (Feinstein et al., 2004).V2Rs belong to classC, which is structurally close to

the metabotropic glutamate receptor, with an additional large N-terminal extracellular domain (Feinstein and Mombaerts, 2004).

Human can perceive a vast number of volatile chemicals yet human are considered to have a poor sense of smell compared to the other animals like rodents, dogs and snake. Humans have about 350 functional odorant receptors (Niimura and Nei, 2003) much less than the ~1000-1200 in the mouse and rat genomes, respectively (Young et al., 2003; Zhang et al., 2004b). In fish the numbers are several fold smaller, ranging from 86 to 155 putatively functional OR genes in fugu and zebrafish, respectively (Nei et al., 2008). There are more ORs than all other known GPCRs combined that make ORs one of the largest gene families known so far (Dryer, 2000). In rats, OR comprise about 6% of their total functional genes, emphasizing the importance of olfaction to the species. The olfactory repertoire of teleost fish is smaller in size (OR, ORA), comparable (olfC), or even larger (TAAR) than the corresponding mammalian gene repertoires (Dryer, 2000; Nei et al., 2008). Despite smaller repertoire size, teleost OR and ORA families show higher divergence than their mammalian counterpart (Korsching, 2009). Olfactory receptors families are evolutionary dynamic that is evident with positive selection in teleost ORs. However, it is still not evident whether the putatively selected amino acid changes are correlated with a novel gain of function. The *ora* genes are subject to strong negative selection, and in fact are being conserved among all teleost species investigated. A small subset of “olfactory” genes may have other non-olfactory functions, in addition to or instead of a primary olfactory role. The highly conserved TAAR1 (shark, mammalian, and teleost orthologs) is not expressed in the olfactory epithelium of zebrafish and mouse and may represent the sole remnant of a primordial, non-olfactory function of this family (Liberles and Buck, 2006). Human OR, hOR17-4, is expressed in the nose as well as in the testis, responding to the chemical bourgeonal, thus allowing sperm to undergo chemotaxis to find the egg cell (Spehr et al., 2006a).

Evolution history of olfactory gene families in several species revealed that gene gain and loss is fundamental and had major significance in defining the current total number of genes in these families (Young and Trask, 2002). High species specificity and rapid evolution are characteristics of olfactory receptor gene families. Local gene duplication is the most probable cause of gene birth. The duplicate genes can follow many evolutionary

trajectories. If the new gene is functionally redundant, one of the copies may be removed from the functional repertoire by inactivating mutation. In contrast, if the new copy acquires mutations that allow it to recognize a novel, useful odorant molecule, then it is likely that natural selection will favor the retention of the new, modified sequence. Species-specific expansion and loss of genes and even whole subfamilies is a persistent phenomenon in the mammalian receptor families (Grus et al., 2005; Lane et al., 2004; Zhang et al., 2004a). The rate of nucleotide substitution (dN/dS) induces diverse selective pressure. Nucleotide substitutions in genes, coding for proteins, can be either synonymous (no change in the amino acid or non-synonymous (changes in the amino acid), and this ratio of the rate of non-synonymous substitutions (dN) to the rate of synonymous substitutions (dS), can be used as an indicator of selective pressure acting on a protein-coding gene (Bielawski et al., 2000; Yang and Bielawski, 2000). Higher rates of non-synonymous to synonymous substitutions are a signature of positive selection. Usually, most non-synonymous changes are expected to be eliminated by purifying selection, but under certain conditions Darwinian selection may lead to their preservation. Conversely, if changes in the sequence eliminate useful ligand-recognition patterns, they would be subject to ‘‘negative or purifying selection’’, i.e. the numbers of synonymous substitutions would be more frequent than the non-synonymous ones, as is observed for genes in general. The incidence of positive selection in the genome is generally associated with transcription factors and some receptor families, including olfactory receptors (Bustamante et al., 2005), although the frequency of positive selection is conflict-ridden (Studer et al., 2008). Ratio of synonymous and non-synonymous substitutions may provide information about the degree of selective pressure. Numerous studies have found support for amino acid signatures of positive selection on the olfactory receptors in mammal and fish species (Hughes and Hughes, 1993). However, it remains unclear whether the putatively selected amino acid changes are linked with a novel gain of function.

1.4.1. Odorant receptor family (OR)

Olfactory receptors are members of a large family of seven-transmembrane (TM)-domain G-protein coupled receptors (GPCRs), comprising about 6% of their total functional genes in rat, emphasizing the importance of olfaction to the species. ORs are small (~1 kb),

intronless and are expressed in the ciliated neurons, in a monogenic pattern i.e. a particular olfactory sensory neuron expresses only one OR (Buck and Axel, 1991; Mombaerts, 2004; Sato et al., 2007). The TM regions are connected by three extracellular and intracellular loops, with an extracellular amino-terminus and an intracellular carboxy-terminus. Olfactory receptors possess highly conserved motifs, hyper variable protein regions are also found in the third, fourth and fifth TM region (Trabanino et al., 2004). MAYDRYVAIC is the highly conserved amino acid motifs within and across species located at TM3 end (Liu et al., 2003). OR genes occur in clusters in vertebrate genomes (Niimura and Nei, 2003). Despite this fact, the evolutionary dynamic nature of this family is characterized by rapid expansion, gene duplication, extensive gene loss via pseudogenization, and diversifying selection (Alioto and Ngai, 2005; Young and Trask, 2002). Since the cloning of the first rodent OR genes in 1991, ORs have been isolated from C. elegans, drosophila, lamprey, teleosts, amphibian, avian and humans (Nei et al., 2008). Vertebrate ORs contain introns and sequence identity between vertebrates and invertebrates are very low (Dahanukar et al., 2005). ORs of *C.elegans* share only ~10% sequence identity with vertebrate OR genes. This leads to the question whether non-vertebrate and vertebrate OR genes derive from a common ancestor (Gaillard et al., 2004). Vertebrates can detect and discriminate higher number of different volatile chemicals than the number of ORs encoded in the genome. This perception is achieved through a mechanism known as the 'combinatorial receptor code' i.e. one odour molecule can be recognized by several ORs, and one olfactory receptor can recognize several odour molecules (Malnic et al., 1999).

The evolutionary origin of Zebrafish dates back to the most common ancestor of teleost and tetrapods as evident by the comparison of teleost fish, amphibian, and mammalian OR repertoires (Alioto and Ngai, 2005; Niimura and Nei, 2005). Some OR genes even go back to the common ancestor of jawed and jawless fish (Freitag et al., 1999). The zebrafish OR repertoire is several folds larger than that of two pufferfish species, which have less than 50 OR genes (Alioto and Ngai, 2005; Niimura and Nei, 2005). Interestingly, teleost OR genes do show signs of positive selection, although the evolutionary rate of teleost is slow compared to tetrapods (Alioto and Ngai, 2005). Many Teleost ORs are located in clusters in the genome although some genes are sparsely present (Alioto and Ngai, 2005). Within the gene clusters, subfamilies are largely contiguous and subfamily

members usually exhibit the same transcriptional orientation, suggesting tandem duplication as a mechanism of gene expansion.

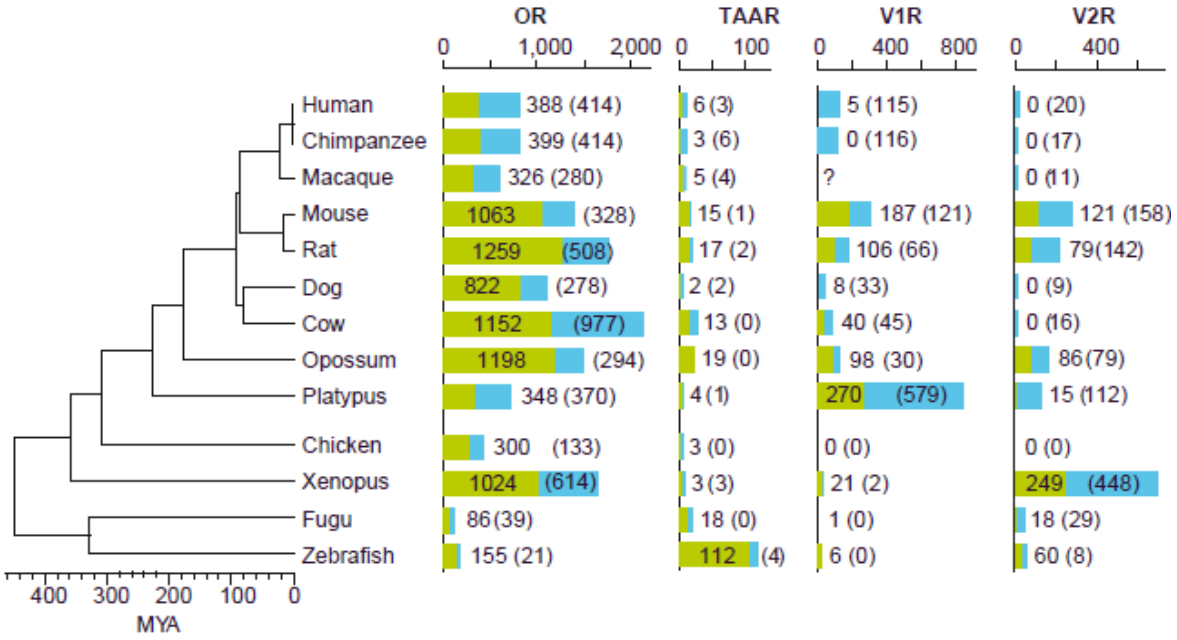

Fig. 3. Numbers of olfactory receptor genes in different species. The green and blue bars represent the numbers of functional (intact) genes and pseudogenes (disrupted genes), respectively. The numbers next to each bar represent the number of functional genes and the number of pseudogenes, which is shown in parentheses. A question mark indicates that data are unavailable. ORs, odorant receptors; TAARs, trace amine associated receptors; V1R, Vomeronasal receptors type1; V2R, Vomeronasal receptors type2.

1.4.2. Trace amine-associated receptor family (TAAR)

In addition to ORs, olfactory system also contains other chemosensory receptors to detect chemical stimuli. TAARs were identified in 2001 (Borowsky et al., 2001).Trace amine associated receptors (TAARs) are close relatives of G protein-coupled aminergic neurotransmitter receptors as dopamine and serotonine receptors and recognize derivatives of the classical monoamines such as ß-phenylethylamine, octopamine, tryptamine, and tyramine (Lindemann and Hoener, 2005). Initially, TAARs have been considered neurotransmitter receptors as well, based on the expression and effects of some family members in the central nervous system (Lindemann and Hoener, 2005). However, recently, Liberles and Buck (Liberles and Buck, 2006) reported for several

mammalian *taar* genes, some of whom they could deorphanize, the expression in olfactory sensory neurons. Thus, the *taar* genes joined a growing number of GPCR families that serve as olfactory receptors (Liberles and Buck, 2006). Surprisingly, the fish *taar* gene repertoire appeared to be much larger than the mammalian repertoire (Gloriam et al., 2005), whereas the opposite holds true for the other olfactory receptor families. After the cloning of the first TAAR receptors in mammals (Borowsky et al., 2001), TAAR genes have been found in genomes from lower vertebrate species (Gloriam et al., 2005). The first study evaluating teleost *taar genes* (Gloriam et al., 2005) made use of very incomplete databases, and thus many of its conclusions, including the size of the family, the phylogenetic reconstruction, the genomic location, the frequency of pseudogenes, the absence of introns, and the suggested nomenclature are now outdated. Still valid are its observations that the *taar* gene family exhibits rapid evolution and correspondingly remarkably species-specific repertoires. A follow-up study confirmed these observations using a more complete data set (Hashiguchi and Nishida 2007), double the number of *taar genes* found in stickleback (Hashiguchi and Nishida, 2007). The selective pressure acting on teleost *taar genes* takes the form of positive selection, of which incidences have been observed in the OR, V1R, and V2R families. Currently, *taar gene* repertoires have been established for fugu, stickleback, medaka, and zebrafish. Fugu has the smallest repertoire, less than 20 genes, followed by medaka with 25 genes, stickleback with 49 genes, and zebrafish with 109 genes (Hashiguchi and Nishida, 2007).

Taar genes occur in a single cluster in tetrapods, evidence of a genesis from local gene duplications, possibly via illegitimate crossover during meiotic recombination. In teleosts, *taar genes* form two large clusters (Hashiguchi and Nishida, 2007), presumably resulting from the whole genome duplication occurring early in the teleost lineage (Nakatani et al., 2007) . Additionally, several isolated genes and small groups are found; however, due to the still unfinished genome build in zebrafish, this may not be the final distribution. The most recent common ancestor of tetrapods and teleosts (of lobe-finned and ray-finned fishes) presumably already had a small cluster of *taar genes*. Whereas all mammalian and all zebrafish *taar genes* are monoexonic, an intron was found in many medaka, fugu, and stickleback genes (Hashiguchi and Nishida, 2007), consistent with an intron gain early in the evolution of neoteleosts, i.e., relatively late in vertebrate evolution. This is rather remarkable since several whole genome scanning studies found very little evidence for

any intron gains during all of vertebrate evolution (Coulombe-Huntington and Majewski, 2007) and may be related to the apparently low selective pressure in the *taar* gene family. TAAR genes were shown to co-express G*α*Olf, suggesting that they are expressed at least in ciliated neurons (Liberles and Buck, 2006). In this thesis I have analyzed both the scope and the evolutionary history of the TAAR gene family in fish. Natural ligands identified for mouse TAARs have been detected in mouse urine which is known to be a major source of social cues (Liberles and Buck, 2006). Therefore, it has been suggested that TAARs may be highly relevant for social communication and individual recognition.

1.4.3. Vomeronasal receptors family type1 (V1R)

Vomeronasal receptor family is expressed in the accessory olfactory organs named Vomeronasal organ. The vomeronasal organ is a tubular crescent shape paired structure located separately from the nasal cavity. The vomeronasal sensory neurons are formed in the olfactory placode along with other sensory olfaction neurons. Vomeronasal receptors in vomeronasal sensory epithelium are lining an elongated cavity (lumen) inside the bone capsule which encloses the organ. The only way of access for stimulus in VNO is a thin duct that opens onto the floor of the nasal cavity inside the nostril ((Dulac, 2000). The vomeronasal receptors are GPCRs and are often referred to as pheromone receptors since vomeronasal receptors have been tied to detecting pheromones. The axons of vomeronasal receptors transducer signals through accessory olfactory bulbs (AOB) to olfactory Amygdala. There have been two types of Vomeronasal receptors, each found in distinct regions: V1R, located on the apical compartment; V2R located on the basal compartment of the VNO (Buck, 2000; Dulac, 2000).

Mammalian V1Rs are homologues of teleost ORA family. Telesost ORA family belongs to classA GPCRs, hence named odorant receptors A (ORA). ORA in teleost are expressed in the main olfactory epithelium as teleost lack vomeronasal organ. ORA receptors have short N-terminal and high sequence diversity sequence diversity in transmembrane domains. V1R display a 1 kilobase, intronless genomic structure (Buck and Axel, 1991), while teleost homolog ora genes have introns in two of six genes (Saraiva and Korsching, 2007). Ora genes have been the most recent of the four teleost olfactory receptor families (ORs, TAAR, ORA, OlfC) .The first member of this family was uncovered in 2005 (Pfister

and Rodriguez, 2005). The teleost ORA receptor gene family is relatively small with only 6 members compared to over 100 genes in the corresponding rodent V1R gene family. Ora genes form a monophyletic clade, supporting their identification as a single family separate from the other chemosensory receptor families. Ora genes have been identified already in the lamprey (Saraiva and Korsching, 2007). Orthologues (closest homologs between species) are more closely related to each other than any paralog Ora genes (closest homologs within species), indicating that all six family members are evolutionarily much older than the speciation events in the teleost lineage. Noticeably, ora genes are highly conserved among all teleost species analyzed so far, such that individual orthologs for all six genes can be detected in all five teleost species analyzed so far (bar a single gene loss in the pufferfish genus) (Saraiva and Korsching, 2007). ora genes show no evidence for positive selection, in contrast to the other olfactory receptor families including the mammalian V1R family ((Saraiva and Korsching, 2007). Contrary to the other olfactory receptors families, ORA genes do not occur in cluster in teleost genome, four of the six ora genes are arranged in closely linked gene pairs across all fish species studied. 2-heptanone, a putative pheromone, was identified as a ligand for one member of the V1R family (V1Rb2) (Boschat et al., 2002), but no follow-up studies have been done with this ligand. V1R genes are linked to reproductive behavior (Del Punta et al., 2002). All six ora genes are expressed specifically in the olfactory organ of zebrafish, in sparse cells within the sensory surface (Saraiva and Korsching, 2007), consistent with the expectation for olfactory receptors and similar to the expression of the tetrapod subclade V1R.Taken together, the high conservation of the ora gene repertoire across teleosts, in striking contrast to the frequent species-specific expansions observed in tetrapods, especially mammalian V1Rs, possibly reflects a major shift in gene regulation as well as gene function upon the transition to tetrapods. Humans have five intact V1R genes. It has been argued that although these five V1R genes have an open reading frame, they are not functional because a calcium channel gene (*TRPC2*) that is essential in the signal transduction pathway of the mouse VNO has become a pseudo gene in the lineage that leads to hominoids and Old world monkeys (Liman and Innan, 2003) However, at least one of the five V1R genes is expressed in the human olfactory mucosa ((Rodriguez et al., 2000). A recent study suggests that that these five genes can activate an OR-like signal transduction pathway in a heterologous expression system. It is therefore possible that the products of these genes function as pheromone or olfactory receptors. Adult humans do

not have a VNO but seem to be sensitive to pheromones (Shepherd, 2006). Another interesting observation is that chicken (Gallus gallus) have no functional or non-functional V1R and V2R genes(Grus and Zhang, 2008), while dog (Canis familiaris) have no functional V2R genes(Grus and Zhang, 2008), although birds use pheromones for mate choice and other behaviors (Bonadonna et al., 2009; Caro and Balthazart; Hirao et al., 2009; Zhang et al.). It is possible that some OR genes in the MOE are able to detect pheromones, as in humans (Keller et al., 2007).

1.4.4. Vomeronasal receptors family type2 (V2R)

Mammalian V2Rs are homologues of teleost OlfC. Teleost OlfC receptors belong to the class C metobotropic glutamate GPCRs, like the mammalian V2Rs. Humans do not have any functional V2R genes. OlfC are distinguished by their long extracellular NH2 terminals which are thought to be the binding domain for pheromones. The V2R genes in mammals are species specific and meticulous specificity has led to the loss of this family in several mammalian species (Young and Trask, 2007). Number of V2R genes varies from 0 (human, chimpanzee, macaque, dog and cow) to 121 (mouse) (Nei et al., 2008). All olfC subfamilies are present in zebrafish, but not in neoteleosts, and many indicate the species-specific gene expansions in zebrafish. OlfC repertoire size varies several folds between teleost species but stays in parallels range of mammalian homologue V2R. Zebrafish has the largest repertoire of all teleost OlfC repertoires (Alioto and Ngai, 2006; Hashiguchi and Nishida, 2006). Local gene duplication has also played a large role in the evolution of the OlfC family, as suggested by the arrangement of most OlfC genes in clusters of phylogenetically related genes (Alioto and Ngai, 2006; Hashiguchi and Nishida, 2006) .OlfC, unlike the other three olfactory receptor gene families, are not monophyletic. The three distinct clades fall together under the olfC heading (Alioto and Ngai, 2006). OlfC genes exhibit five conserved intron/exon borders that result in six exons in a characteristic short-short-long-short-short-long arrangement (Alioto and Ngai, 2006). Metabotropic glutamate receptors do not show these intron/exon borders. Negative selection is observed at distal ligand binding sites in OlfC and there is no evidence of positive selection (Alioto and Ngai, 2006). Although currently no ligands are known for any member of the largest group of OlfC genes (group 1), modeling suggests that many of them have amino acids as ligands like the one well investigated OlfC member from one of

the small groups, OlfC a1 (Luu et al., 2004). Thus, OlfC receptors may constitute the molecular basis to explain odor response studies, which predict many independent receptors for amino acids (Fuss and Korsching, 2001). V2R gene family has undergone an even more marked decline than the V1R gene family, with no functional genes remaining in the cow, dog, human, and chimpanzee or macaque genomes. Such decline demonstrates that V2Rs are no longer important for these species, either because other receptor families now detect pheromones or because pheromone-mediated signaling is now of lesser importance (Liman, 2006). By contrast, the large number of functional V2R genes and species-specific V2R gene family expansions in the mouse, rat and opossum genomes probably contribute to the ability of these species to detect large repertoires of pheromones (Young and Trask, 2007).

1.4.5. Formyl peptide receptor family (FPR)

FPRs are a new family of olfactory GPCRs in the vomeronasal organ, so for found in the mammalian species. FPRs are also expressed in the immune system, where they are believed to stimulate chemotaxis to sites of infection upon recognition of their ligands, such as formylated peptides from bacteria or mitochondria (Yang et al., 2002). FPRs are characterized by monogenic transcription and their expression patterns are remarkably similar to those of V1Rs and V2Rs. FPRs were reported to be expressed in diverse tissues (Migeotte et al., 2006; Panaro et al., 2006). Most recently, it has been shown that out of the seven murine FPR subtypes, some are predominantly expressed in a highly dispersed, small subset of neurons that bind with $G\alpha_{i2}$ or $G\alpha_{o,}$ in the VNO. Most recently FPRs have been identified as olfactory receptors expressed in the vomeronasal organ of mouse (Liberles et al., 2009; Riviere et al., 2009). Phylogenetic analyses indicate that genes encoding vomeronasal organ FPRs evolved recently in the rodent lineage, raising the possibility that these receptors impart a novel chemosensory function to rodents.

1.5. Olfactory signaling transduction

Olfactory perception is mediated by large, diverse family of G-protein-coupled receptors in both vertebrates and invertebrates. In the vertebrate zebrafish, 328 olfactory receptors have been discovered that are involved in olfaction (the detection of volatile compounds).

At the most basic level, the olfactory system in any animal must allow the brain to discern which olfactory receptors have encountered odorant at any given time. In mammals, olfaction is accomplished by approximately 1,000 diverse olfactory receptor genes (Mombaerts et al., 1996). Brain can determine which set of olfactory receptors are activated by identifying excited neurons, as each neuron expresses only one receptor. Mammalian olfactory neurons appear to use the same machinery for transducing signals from its odorant receptor molecules. The cell bodies of the set of neurons expressing a given olfactory receptor are distributed in specific zones of olfactory epithelium and intermingle with neurons expressing different receptors, but their projections converge to discrete loci in the olfactory bulb called glomeruli (Mombaerts et al., 1996). Thus, the brain could in principle determine which receptors have been activated by examining the spatial pattern of activity in the olfactory bulb; individual odorants are associated with specific spatial patterns. The *adaptation* of odorants is thought to derive from at least two different physiological mechanisms. First, the interaction of an odorant receptor with its ligand may be followed by inactivation, or *desensitization*, of the receptor due to phosphorylation of the receptor by a protein kinase. Second, the olfactory neuron may adapt to different concentrations of an odorant by adjusting the sensitivity of its cyclic nucleotide gated ion channels to cAMP, an effect conceptually analogous to light adaptation in the visual system, where light sensitivity is adjusted to match the intensity of light in the environment.

Olfactory signaling transduction is GTP-dependent, suggesting that olfactory transduction, like visual transduction, proceeds via a G protein-coupled mechanism. Olfactory receptors activate Golfa, Gsα-like G protein (Jones and Reed, 1989) upon perception of ligand. Golf-mediated activation of adenylate cyclase III then raises intracellular cAMP levels, causing a cyclic-nucleotide-gated channel to open (Fig. 4). The influx of cations through this channel ultimately leads to the formation of an action potential, which allows the primary neuron to signal to the brain. The axonal projections of the olfactory sensory neurons converge on defined glomeruli in the olfactory bulb. Olfactory receptors themselves play an instructive role in axon guidance and same olfactory receptor- initiated signal transduction machinery is used to mediate both olfactory perception and axon targeting (Belluscio et al., 1998; Wang et al., 1998).

Additional signal transduction cascades activated by odor binding include inositol 1,4,5-trisphosphate (IP3), cyclic GMP, and carbon monoxide, but their roles in transduction is not considered primary and is not currently understood completely. IP3 is also known as a second messenger and is involved in transmission of chemical signal (hormone, neurotransmitters, growth factors , Beta-adrenergic receptor agonists) received by the cell, to various signaling networks within the cell. IP3 is known to play a crucial role in initiating and broadcasting of chemical messages; however, the exact mechanism of how IP3 relates to the subsequent element in its signaling pathway, the calcium wave, remains highly controversial. Two essential signaling pathways have been identified that involve the intracellular signaling generation of IP3. The first signaling pathway is commenced by cytosolic soluble proteins PLC (Phospholipase-C). Neurotransmitters and hormones bind to GPCR and both the heterotrimeric G-AlphaQ/11, and G-Beta Gamma subunits regulate the function of PLC-Beta (Szlufcik et al., 2006). Release of second messengers DAG (1, 2-Diacylglycerol) and IP3 activation takes place as a results of the hydrolysis of PIP2 (Phosphatidylinositol-4, 5-Bisphosphate). ERK1/2 (Extracellular Signal Regulated Kinase-1/2) signaling pathway resulting in transcription factor activation and cell survival are activated by DAG, a physiological activator of PKC (Protein Kinase-C). The second IP3 signaling pathway is initiated by an enzyme PI3K (Phosphoinositide 3-Kinase) involved in phosphorylation of inositol lipids. The enzyme PI3K is also involved in generation of two signaling molecules, PIP2 (Phosphatidylinositol 3, 4-Bisphosphate) and PIP3 (Phosphatidylinositol 3, 4, 5-Trisphosphate). PI3K is activated by CD19, a co-receptor complex in B-cells. IP3, generated by PIP2 plays a vital role in the organization of cellular and physiological processes including fertilization, apoptosis, cell-division, cell proliferation, development, learning, memory and behavior (Futatsugi et al., 2005).

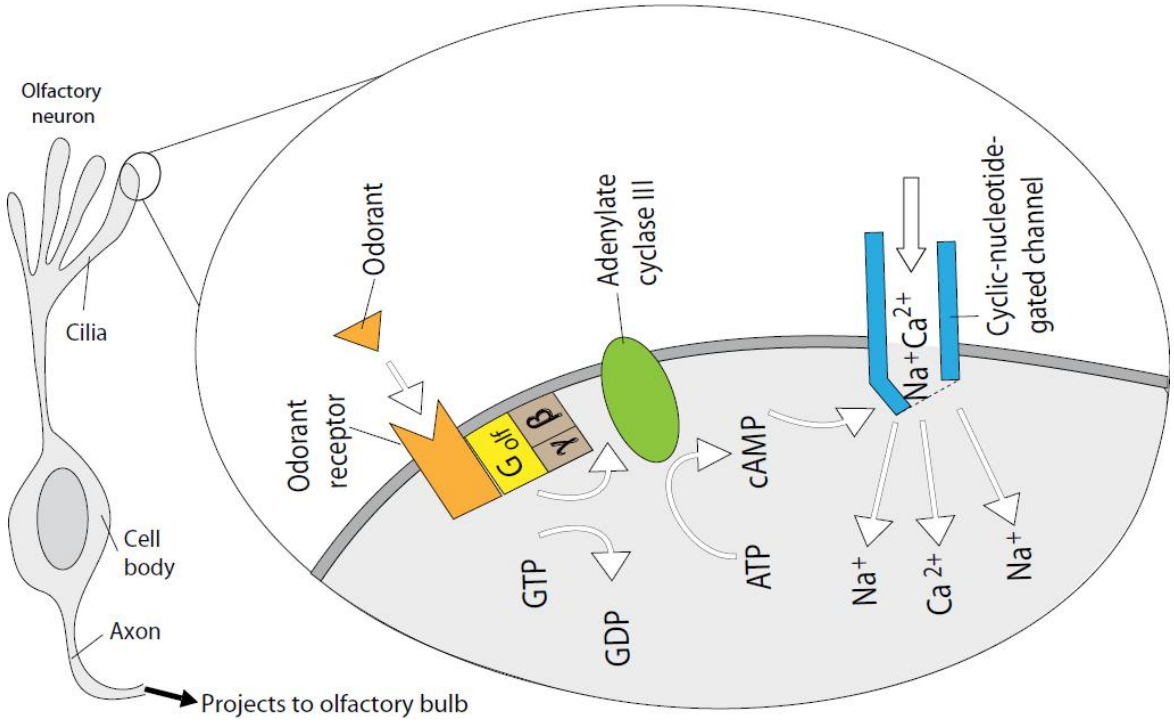

Fig. 4. the mouse olfactory signal transduction cascade. Odorant binding to the olfactory receptor is thought to activate G protein's GTP-coupled α-subunit, Gαolf. Activated Gαolf then dissociates from Gβγ and activates adenylate cyclase III, leading to an increase in the intracellular cAMP concentration. The increased cAMP leads to the opening of cyclic nucleotide gated cations channels, causing a depolarization that leads to the influx of cations and generation of action potentials in the sensory axon and the transmission of signals to the olfactory bulb.

1.6. Ligands for olfactory receptors

Olfactory receptor gene families vary between species. This lead to the hypothesis that olfactory receptor within the species may have their own characteristic set of chemical signals that are important for their survival and reproduction in a specific environment. Odorants/ligands for olfactory receptors are typically small organic molecules of less than 400 Da and can vary in size, shape, functional groups and charge (Malnic et al., 1999). Odorants include a set of various aliphatic acids, aldehydes, alcohols, ketones, esters and

amines; chemicals with aromatic, alicyclic, polycyclic or heterocyclic ring structures; and numerous substituted and combinations of these chemicals. Odorants generally bind to several receptors with diverse affinities and individual receptors generally bind more than one odorant (Buck, 2000; Kajiya et al., 2001), except some highly specific and unique receptors i.e. pheromones receptors (Friedrich and Korsching, 1998; Kajiya et al., 2001). The olfactory receptor genes are regard as the first centre of olfactory information processing. However, only few olfactory receptors genes are deorphanized in mammals ((Luu et al., 2004). The identification of ligand is a complex task due to the inefficient heterologous expression system for many olfactory receptors. Mammalians and to some extent teleost olfactory receptors GPCR including OR, TAAR, V1R, and V2R genes are expressed in a monogenic fashion (a particular receptor neuron expresses only a single gene from a single receptor family (Liberles and Buck, 2006; Mombaerts, 2004; Sato et al., 2007). The neurons expressing the same olfactory receptor converge into a single glomerulus in the olfactory bulb. Both genetic and imaging studies confers that each receptor gene designate a separate input channel of the olfactory system and the olfactory bulb comprises a receptotopic map of odor sensitivities, an odor map ((Friedrich and Korsching, 1998; Fuss and Korsching, 2001; Sato et al., 2005; Sato et al., 2007). In teleost, the only olfactory receptor with identified ligands is a member of the OlfC family, OlfCa1 (Alioto and Ngai, 2006). Interestingly, the optimal ligands for the goldfish receptor are basic amino acids, whereas the zebrafish receptor reacts most strongly to acidic amino acids. Odorant receptors expressed in heterologous cells couple to Gαolf that leads to odorant-induced increases in cAMP. The increases in cAMP can be monitored using a reporter gene assay (Liberles and Buck, 2006).

1.7. Fish behavior

Behavior is the function of the nervous system that biology seeks to explain and it is the initiation point of a biological investigation. Karl von Frisch (1941) first established that when the European minnow (*Phoxinus phoxinus*), a fresh water fish, is killed by a predator, damage to the skin releases an alarm substance ("Schreckstoff", or scary stuff) that elicits a fear reaction in conspecifics. Fish conspecifics run randomly as they first detect the “scary stuff”, and then they form a close school and retreat from the smell source. Initially, it was speculated that this reaction would be common among schooling

fishes (i.e zebrafish), as the combined defensive behavior would be most effective. The study for alarm reaction stayed confined to Ostariophysi until it was demonstrated that a similar alarm reaction in two darters species (Etheostoma exile and E. nigrum). The alarm reaction behavior study was later observed in Percidae, and recently in the gobies Brachygobius sabanus, Asterropteryx semipunctatus and for a sculpin. Large part of fish olfactory behavior has been restricted to Ostariophysan and Percid fish. Unfortunately, the chemistry of fish alarm pheromones is not well studied and no pure pheromone of fish has been isolated for detailed chemical analysis. It is demonstrated, however, that the pheromones of a species can be perceived by another species with alarm pheromone system, providing assumption that mechanism of alarm pheromone detection may be rather similar among species. The presence of an alarm system presents in species is an evolutionary dilemma and not yet fully discovered and understood. Fish does not release alarm substances if they just are stressed and threatened by predator, but mechanical damage to the skin releases the pheromone. Specialized alarm substance cells (ASCs, club cells), sensitive to minor mechanical damage, were identified in majority of fish skin. No other functions for these cells have been known yet. Several alarm substances have been examined for fish species, but details are restricted to the Ostariophysi and the Percidae. The alarm chemicals released from ASCs as a result of mechanical damage can induce fear response in conspecifics as well as in other species. Inter-specific alarm responses may be explained by phylogenetic relations of different species, which provide a selective advantage to avoid a common predator. Alarm reaction can vary from species to species based on their environment and experience and concentration of pheromone. The evolution of alarm system development is inadequately understood in fish. Odor signals are perceived and processed with high specificity by receptors. Fish ciliated neurons generally perceive bile acids, steroids and polyamines via ORs and TAARs, respectively, while microvillous olfactory receptor neurons generally perceive amino acids and nucleotides. Crypt cells of a have been shown to detect amino acids (Schmachtenberg, 2006; Vielma et al., 2008), although electrophysiological studies (Lastein et al., 2006) and backtracing experiments . A response to steroids by crypt cells in the olfactory bulb of crucian carp was shown (Hamdani el and Doving, 2006). In summary, the receptotopic map of fish olfactory bulb provides an opportunity to study functionally segregated responses of all olfactory receptor neurons to different stimuli. Odor responses in lateral, medial, and ventral glomeruli of zebrafish are measurable

(Friedrich and Korsching, 1998) and because of its small and semi-transparent olfactory, bulb zebrafish is suitable model to study the odor responses of all three olfactory receptor neuron populations simultaneously and possibly identify a spatial map of olfactory neural network. More recently, behavioral response of bees showing ability to discriminate the category of symmetrical images from that of asymmetrical ones and that of sequentially identifying pairs of 'same' objects from that of 'different' objects, even across modalities (Giurfa et al., 2001) paved the way for in-depth understanding of neurophysiological investigation on how the bee brain achieves that. In olfaction, complex behaviors such as how hamsters sense which over-mark is on top of another (Johnston and Bhorade, 1998) or the ability of dog able to find out the direction of a trail has been done very rarely. Most of the olfactory behavior research has focused on the relatively simple olfactory tasks of odor detection and discrimination.

There is a growing support for the differences in behavioral response among zebrafish populations. Strain and dose-dependent differences in perception of ethanol exposure was observed among EK, AB TU strains of zebrafish (Carvan et al., 2004; Loucks and Carvan, 2004). Polyamines have been identified as attractant olfactory cues in gold fish (Rolen et al., 2003) and are suggested to have a receptor-mediated transduction pathway, distinct from those used by amino acids or bile salts (Michel et al., 2003). Most importantly, behavioral results are reliant on degree of experimental interpretation, and this is perhaps the most difficult aspect to validate a behavior experiment.

1.8. Zebrafish as a model organism

Zebrafish are small tropical fish native to Southeast Asia. A unique combination of genetic and experimental embryologic advantages makes them ideal biological studies. Zebrafish is well apt for forward genetics because of large clutch size and relatively short generation time. The zebrafish lays hundreds of eggs at weekly intervals and these eggs are externally fertilized and can be biologically manipulated for large scale mutant screens. The nervous system of zebrafish is relatively less complex and is high similar to that of higher vertebrates. The olfactory bulb (OB) of zebrafish contains only ~80 glomeruli, compared to ~1800 in rodents (Baier and Korsching, 1994; Baier et al., 1994). The optical transparency and physical accessibility of zebrafish embryos make them an ideal system

to maximally utilize the advantages of transgenic animals, expressing fluorescent proteins such as green fluorescent protein (GFP). Axon guidance mechanisms can be studied in zebrafish during early development, by combining transgenesis with the use of GFP. It is also shown that axons dynamic behavior can be visualized in living embryos.

AIMS

Amines are basic olfactory cues for teleost in aquatic environment. The initial aim of this study was the identification and characterization of the complete repertoire of the trace amine-associated receptors (TAARs) family in lower vertebrates, which were expected to be good candidates for mediating amine detection in teleosts. This was carried out by use of extensive multidisciplinary approaches of *in silico* and *in vitro* biology, and resulted in fascinating answers about evolutionary history, intron dynamics, selectiion pressure and cellular localization of TAARs (Hussain et al., PNAS 2009).

So far all of teleost olfactory receptors are “orphans” (their ligands are not known) except one member of the OlfC family. Therefore our second objective was the identification of ligands for TAAR receptors. We could deorphanize a TAAR receptor that responds to aliphatic diamines and have characterized its chemical selectivity with respect to chain length and functional groups. Intriguingly we observed a clear behavioral response of zebrafish to these specific ligands with a similar chemical selectivity to that of the receptor itself. To investigate whether activation of this single TAAR receptor could be sufficient to generate the observed behavior we have characterized the activation of olfactory sensory neurons by the same ligands.

The results are consistent with the existence of at least two olfactory receptors for diamines, each of which may be sufficient to elicit a characteristic innate behavior upon activation by an ecologically relevant stimulus.

CHAPTER 2
RESULTS

VIII. RESULTS

1. Phylogenomics, selection pressure, intron dynamics and cellular expression of TAARs

1.1. TAAR genes encompass monophyletic origin distinct from aminergic GPCRs

Complete *taar* gene repertoire of 5 teleost fish species, a shark, frog, chicken, 4 placental, and 1 marsupial mammalian species (Table.2) was retrieved by using a recursive data mining search strategy including TblastN followed by BlastP algorithm, in protein and nucleotide databases of NCBI and Ensemble (see Methods for details). All retrieved *taar* genes were extensively analyzed by sequence alignment and were identified by the presence of eminent GPCRs and TAAR motifs. These genes were subdivided into 28 different subfamilies (Table.2, Supplementary Table1). Subfamilies 1 to 9 correspond to previously identified TAARs, with mostly mammalian members, whereas subfamilies 10 to 28 are fish-specific. The subfamilies segregate into 3 major clades (Fig. 5), which were designated into 3 classes in analogy to corresponding subdivisions in the odorant receptor (OR) gene family (Niimura and Nei, 2005). Class I (TAAR1, 10-11, 21, 27) contains mostly teleost genes, class II (TAAR 2-9, 12-13) comprises mostly tetrapod genes, and class III is restricted to teleosts (TAAR14-20, 22-26, 28), Class I (TAAR1, 10–11, 21, 27) and class II (TAAR2–9, 12–13) contain both tetrapod and teleost genes, but class III is restricted to teleosts (TAAR14–20, 22–26, 28).

All *taar* genes identified form a monophyletic group, clearly distinct from their close relatives, the aminergic neurotransmitter receptors (Fig. 5). The TAAR gene family also segregates with maximal bootstrap values from the ORs, which are less closely related, but belong to the same major family of GPCRs, the rhodopsin type GPCRs (Fredriksson et al., 2003). The appropriate choice of out-groups was especially accentuated in relevance to the proper delineation of the TAAR gene family. Representatives from all major aminergic receptor subtypes (cholinergic, dopaminergic, histaminergic, noradrenergic, and serotinergic receptors) were included in the phylogenetic analysis to avoid spurious results. The classical aminergic neurotransmitter receptors are relatively

close neighbors in the phylogenetic tree, but constitute a rather diverse group by themselves.

Species	Subfamilies		Number of taar genes	
	Number	Name	Intact (Class I, II & III)	Pseudo
Zebrafish	12	1, 10-20	112 (7, 18, 87)	4
Stickleback	7	21-27	48 (4, 0, 44)	0
Medaka	4	21-24	25 (6, 0, 19)	1
Opossum	7	1-6, 9	19 (1, 18, 0)	0
Takifugu rubripes	4	21, 22, 27, 28	18 (7, 0, 11)	0
Tetraodon nigroviridis	4	21, 22, 27, 28	18 (9, 0, 9)	0
Rat	9	1-9	17 (1, 16, 0)	2
Mouse	9	1-9	15 (1, 14, 0)	1
Cow	9	1-9	13 (1, 12, 0)	0
Human	6	1, 2, 5, 6, 8, 9	6 (1, 5, 0)	3
Frog	2	1, 4	3 (1, 2, 0)	0
Chicken	3	1, 2, 5	3 (1, 2, 0)	0
Elephant shark	2	1, 2	2 (1, 1, 0)	0
Sea lamprey	0	-	0	0

Table.2. Number of taar genes and subfamilies in all species analyzed. First column, name of species; second column, number of subfamilies per species; third column, subfamily names, e.g., 10-20 means TAAR10 to TAAR20; fourth column, number of intact *taar* genes per species, numbers for each class given in parentheses; fifth column, number of pseudogenes.

1.2. Rapid evolution of TAAR gene as a recurrent species-specific expansions in teleost

The teleost *taar* gene repertoires range from 112 for zebrafish (plus 4 pseudogenes) down to several fold smaller repertoires (stickleback 48, medaka 25, pufferfish each 18 genes). Mammalian families just reach minimal fish family size, while avian and amphibian families are minuscule, with only 3 genes each (Tables.2, Supplementary Table.1). Most

of these differences are caused by massive recent gene expansions in teleosts that led to 30 members within a single zebrafish-specific subfamily, TAAR20, and 28 genes in the stickleback-specific subfamily TAAR26. All but one zebrafish and one neoteleost TAAR subfamily (DrTAAR11 and TAAR24, respectively) have undergone recent gene duplications. In mammals gene expansions are less frequent and also much smaller those in teleosts (maximally to six genes, opossum *taar9*). No recent gene expansions were found for *taar* genes 1, 2, 3 and 5. No recent gene duplications have been observed in an amphibian (*Xenopus tropicalis*) nor in an avian species (*Gallus gallus*).

Individual teleost TAAR genes (except TAAR1) rarely possess any orthologs. Thirteen of nineteen subfamilies are restricted to a single species each (TAAR10-20, zebrafish; TAAR25-26, stickleback). Only two subfamilies contain genes from all four neoteleost species examined (TAAR21-22) and none are shared between zebrafish and neoteleosts, (Supplementary Tables 1). Even in the case of subfamilies containing orthologs, a gene expansion may occur in one species but not another, e.g. TAAR27 has expanded to seven genes in tetraodon, but remains a single gene in both stickleback and fugu (Supplementary Table.1). Thus most gene duplications have occurred rather recently, after the divergence of the teleost and neoteleost species analyzed here (Fig. 5), indeed even after the two pufferfish species diverged about 20-30 million years ago (Van de Peer, 2004).

In contrast, orthologs are readily identifiable between all mammalian species analyzed. Orthologs for all nine previously identified mammalian *taar* subfamilies are uncovered in another mammalian species, *Bos taurus* (Table.1, Supplementary Tables 1). In humans, all nine subfamilies are represented by one member each, three of them by pseudogenes (TAAR3, 4, and 7). Thus, *Homo sapiens* has a typical mammalian TAAR repertoire. Seven of the nine subfamilies (TAAR1-6, 9) are detected also in opossum, a marsupial mammal, i.e. should be present already in the MRCA of marsupials (Murphy et al., 2007) and modern mammals. Although very small, the amphibian and avian *taar* gene repertoires are not located at the base of the sarcopterygian tree and clearly belong to different mammalian subfamilies. Thus gene losses appear to have shaped the avian and amphibian gene families.

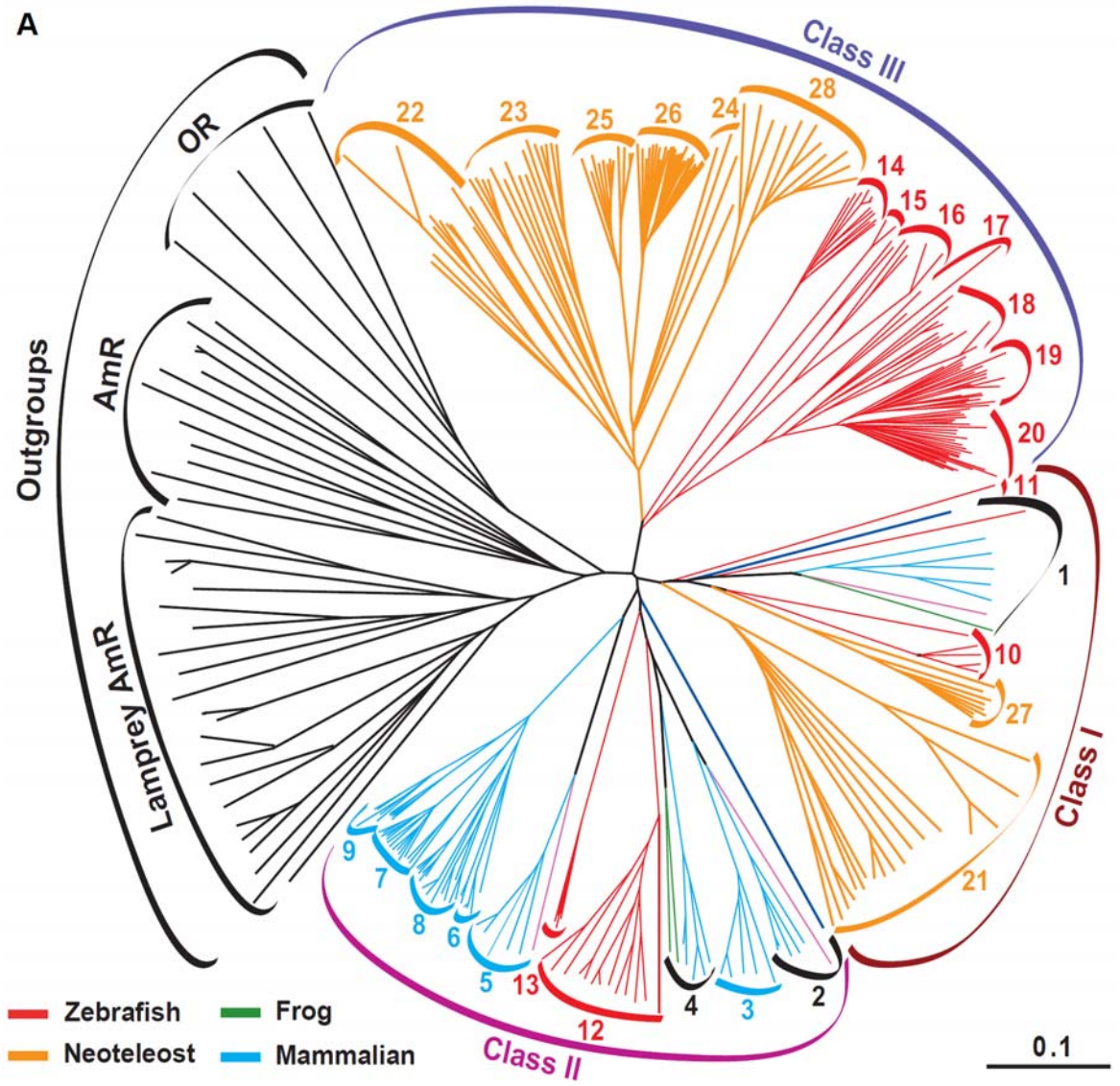

Fig. 5. Phylogenetic tree of TAAR family members and estimated minimal evolutionary age. (*A*) Radial tree of teleost and tetrapod TAARs, species groups are color-coded. We analyzed 5 teleost genomes (*Danio rerio*, zebrafish; *Gasterosteus aculeatus*, 3-spined stickleback; *Oryzias latipes*, medaka; *Takifugu rubripes*, fugu; *Tetraodon nigroviridis*, tetraodon), 5 mammalian genomes (*Monodelphis domestica*, opossum; *Bos taurus*, cow; *Mus musculus*, mouse; *Rattus norvegicus*; rat, *Homosapiens*, human), avian (*Gallus gallus*, chicken), amphibian (*Xenopus tropicalis*, clawed frog), lamprey (*Petromyzon marinus*), and elephant shark (*Callorhinchus milii*) genome. Zebrafish and mouse aminergic neurotransmitter receptors were used as outgroup together with a selection of ORs. (Scale bar, 10% divergence.) For accession numbers and/or gene Ids, see Supplementary Table 1.

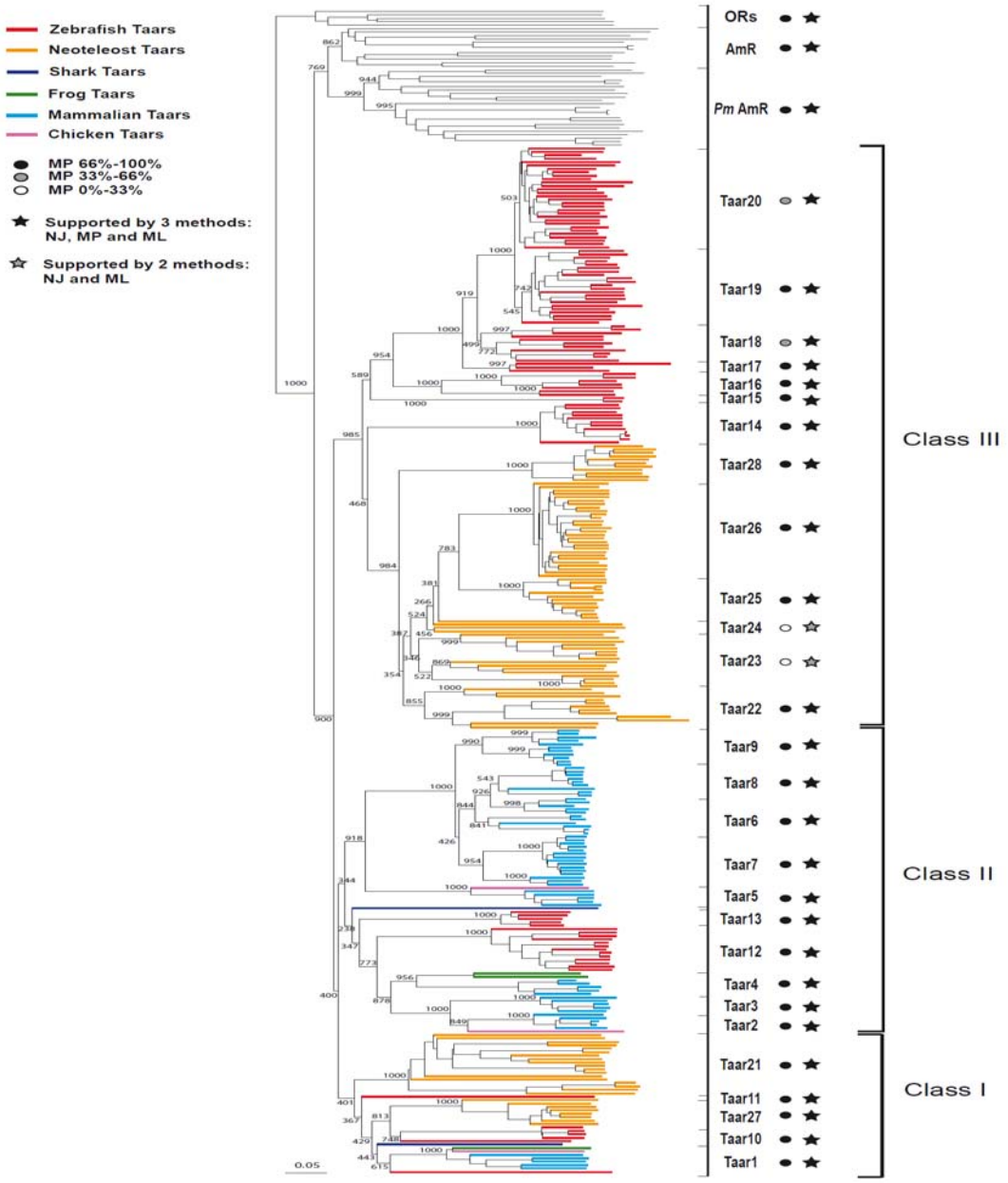

Fig. 6. Phylogenetic tree of the *taar* genes. The cladogram shown here corresponds to the unrooted tree in Fig. 5. The tree is constructed by using the neighbor-joining algorithm; bootstrap support at major nodes is indicated by numbers (1,000 cycles). All subfamilies are supported by all 3 tree algorithms used (neighbor joining; maximum parsimony, 100 bootstraps; maximum likelihood), except subfamilies 23 and 24 (supported by 2 methods). Red lines represent zebrafish *taar* genes; orange lines, neoteleost *taar* genes; dark blue, cartilaginous fish *taar* genes; green, amphibian *taar* genes; light-blue, mammalian *taar* genes; and black represents the outgroup (OR, odorant receptors; AmR, aminergic receptors; PmAmR, *Petromyzon marinus* (sea lamprey) aminergic receptors). Note the segregation in 3 clades, class I to III.

1.3. TAAR genes are an evolutionary young family

TAAR1 orthologs occur in both tetrapods and teleosts (Fig. 7), i.e., TAAR1 ought to have been present already in the most recent common ancestor (MRCA) of both lineages and is older than the actinopterygian/sarcopterygian split. To determine the evolutionary origin of the *taar* gene family, all currently available sequence information for cartilaginous fish and jawless fish were searched. Two *taar* genes, both with a perfectly conserved TAAR specific fingerprint motif (Lindemann and Hoener, 2005), were uncovered in the elephant shark, one of them an ortholog of TAAR1 (Fig. 5). Cartilaginous fish are considered basal to all jawed vertebrates (Venkatesh et al., 2001), so TAAR1 was present already in the MRCA of bony fish and cartilaginous fish and may be the ancestral member of class I. All tetrapod species analyzed contain a TAAR1 ortholog, as does the avian genome examined here. Interestingly, no orthologs for TAAR1 could be found in any of the neoteleost species analyzed, i.e., this ancestral gene appears to have been lost in neoteleosts. The other shark gene exhibits a basal location in class II (Fig. 5) and may thus correspond most to the ancestral class II *taar* gene. Despite an extensive search, no *taar* genes were uncovered in the genome of a jawless vertebrate (sea lamprey). Thus, the *taar* gene family appears to have originated in the MRCA of cartilaginous and bony fish as a pair of genes that later expanded into class I and II genes. No shark representative of class III was found, consistent with a later evolutionary origin of this class, after the segregation of the tetrapod from the ray-finned bony fish lineage.

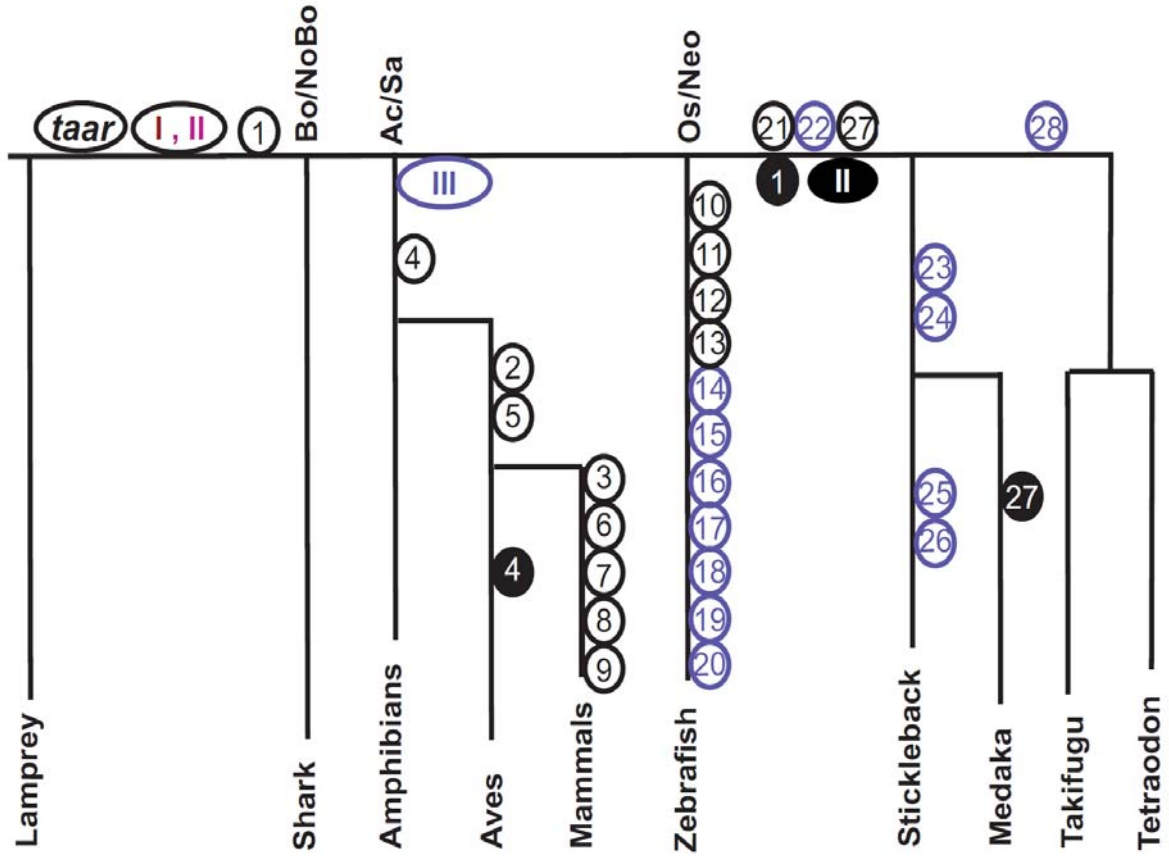

Fig. 7. The estimated minimal evolutionary age of TAAR subfamilies and genes. Open circles represent the gene gain events in each lineage, and filled circles represent the gene loss events. Inside each circle is the name of the respective gene or subfamilies. Emergence of the *taar* gene family and of the 3 classes of *taar* genes is indicated by ovals. The major phylogenetic transitions are indicated: bo/nobo, bony fish/cartilaginous fish; ac/sa, actinopterygian/sarcopterygian split, i.e., between the ray-finned bony fish (teleosts) and the lobe-finned fish giving rise to tetrapods; os/neo, ostariophysii/ neoteleostei segregation between less derived (zebrafish) and more modern fish (medaka, stickleback, pufferfish). The maximum-parsimony principle was followed, thus gene gains are depicted at the last possible stage before additional gains would become necessary for explanation but may in fact have occurred earlier. A gene gain implies preceding gene duplication on the same branch of the species tree that gave rise to the new subfamily. Subsequent gene duplications generate the extant members of the subfamily.

1.4. TAAR family is characterized by distinctive consensus motifs, despite the overall heterogeneity.

Taar genes frequently show low identity values_30% in pair wise comparisons. The retention of consensus motifs was analyzed to obtain a second line of evidence for proper delineation of the *taar* gene family. Of 48 amino acid positions absolutely conserved between human and rodent TAARs (1), the vast majority (41aa) are highly conserved in fish TAARs. Besides general GPCR motifs many TAAR-specific motifs are in these groups that are not present even in the closely related aminergic receptors (Fig. 8).

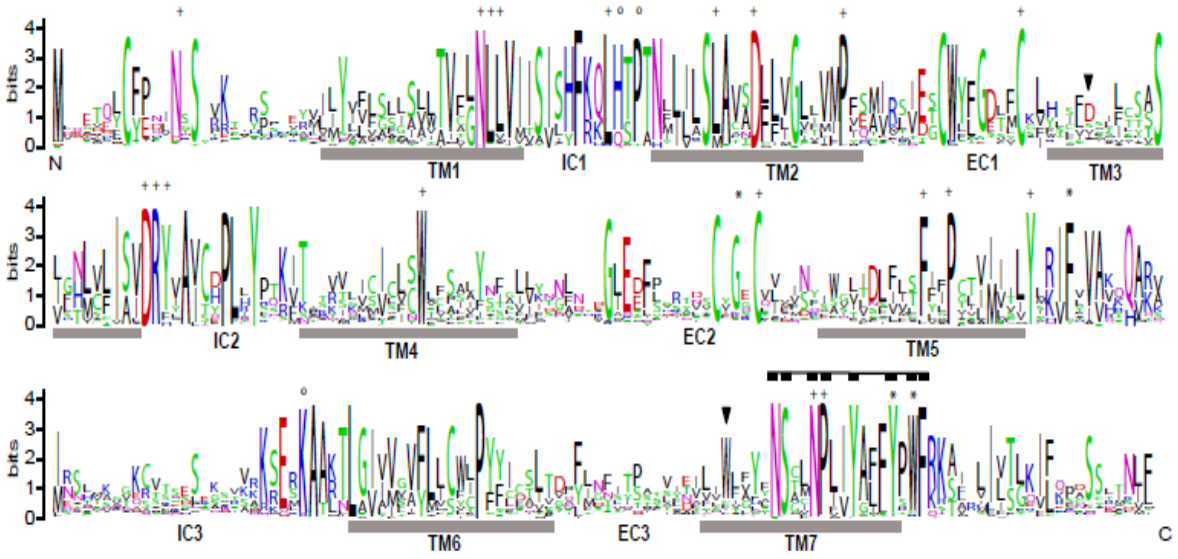

Fig. 8. Amino acid sequence conservation in the fish *taar* gene repertoire. Sequence logo representation of the alignment of all 223 fish full-length TAAR sequences, the height of the 1-letter amino acid code in the logo reflects the degree of conservation. Sequence logos were generated as described (32). TM, transmembrane region; IC, intracellular loop; EC, extracellular loop; plus signs, broadly conserved in rhodopsin type GPCRs; circles, conserved in some rhodopsin type GPCRs but not in aminergic receptors; asterisks, conserved in TAARs but not in other rhodopsin type GPCRs. Two triangles in TM 3 and TM 7 depict the aminergic ligand motif, filled rectangle motif in TM 7, the characteristic fingerprint for TAARs.

The characteristic TAAR fingerprint motif, described to be 100% sensitive and specific for mammalian TAARs (1), is strikingly conserved in all fish *taar* genes analyzed (Fig. 8). In contrast, 2 of the TAAR-specific amino acids from this motif are absent in the lamprey

receptors, and 2 others are only weakly conserved, further delineating the TAAR receptors from the group of aminergic receptors in general and from the lamprey aminergic receptor family in particular. As expected, amphibian and avian TAAR sequences share the great majority of conserved motifs as outlined above, supporting their assignment as *taar* genes. Some motifs distinguish the 3 classes of TAARs from one another, including the aminergic ligand motif (Huang, 2003), which is highly conserved in class I and II, but absent from all but one class III *taar* genes (Fig. 9).

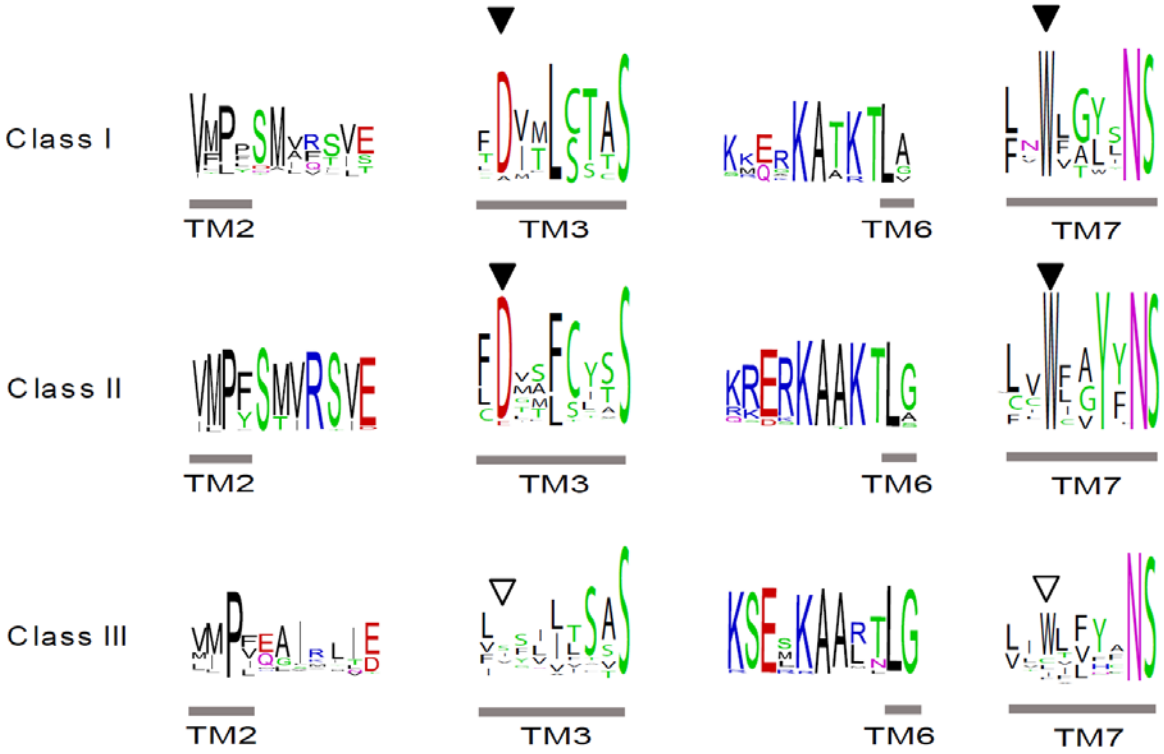

Fig. 9. Subclass-specific amino acid sequence conservation. Conservation is displayed as a sequence logo. Four motifs are shown (end of TM2, start of TM3, preceding TM6, and start of TM7, respectively) that distinguish among the 3 classes of TAARs. TM3 and TM7contain the 2 amino acids (filled triangle) constituting the aminergic ligand motif (1). Note the absence of the motif (open triangle) in class III genes.

1.5. Genomic arrangement of teleost TAAR genes pinpoints the evolutionary origin of class III.

Mammalian *taar* genes are found without exception in a single cluster in the genome (11). All newly identified mammalian, avian, and amphibian *taar* genes conform to this previously described pattern (Supplementary Table.1). In contrast, teleost *taar genes* are found in 2 large clusters and a few solitary genes (chromosomal allocation for zebrafish and medaka, large scaffolds for stickleback). Within the clusters, genes are organized mostly in accordance to phylogenetic relationship (Fig. 10), consistent with a genesis of the clusters by recurrent local gene duplication. A few exceptions to the colinearity of phylogenetic relationship and genomic location do occur (Supplementary Table.1), possibly caused by recent genomic rearrangements involving these genes. Interestingly, *taar*1 gene is always located at one end of the cluster in tetrapod and avian species, consistent with an asymmetric process being responsible for at least some of the repeated gene duplications. Average intergenic distance is 7.9 _ 0.5 kb (mean _ SEM, *n* _ 97) in the zebrafish gene clusters, with exception of a large intervening region at approximately the same relative position in both clusters (Supplementary Table.1). This similarity in cluster structure is consistent with the 2 clusters resulting from the whole genome duplication known to have occurred in early teleosts (12). Indeed, the cluster positions for zebrafish and medaka are syntenic not only within and between species, but also to the human cluster (see Supplementary Table.1) (12, 13). Class III *taar* genes are found in both genomic clusters and consequently, class III appears to be older than the whole genome duplication observed in early teleost evolution (Nakatani et al., 2007). Because, on the other hand, class III is restricted to teleosts, it appears to have originated shortly after the segregation of the teleost and tetrapod lineages.

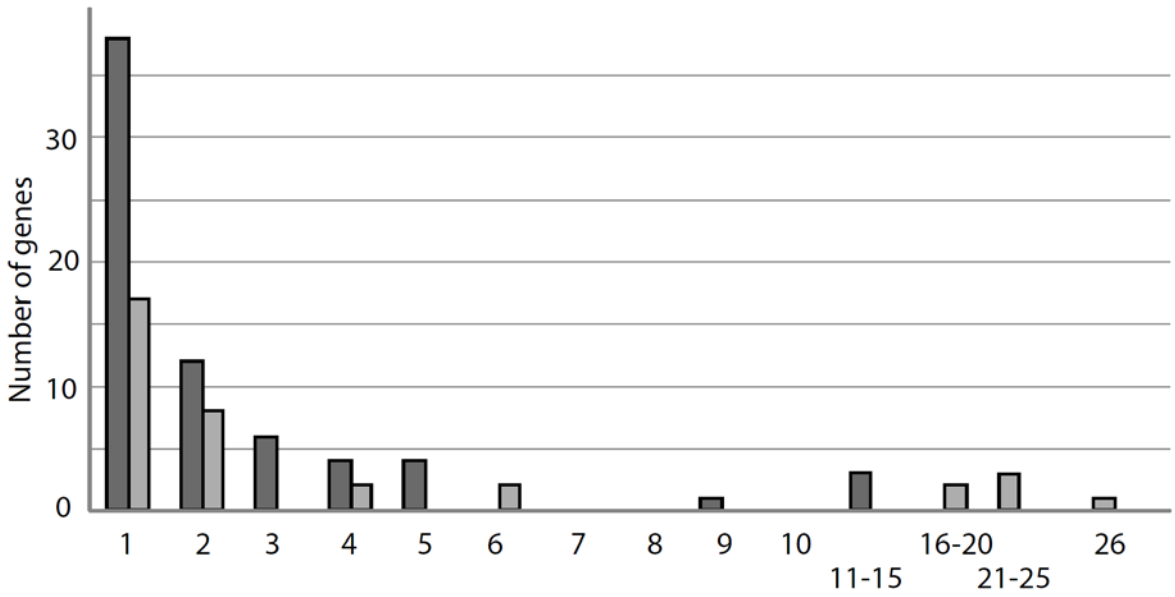

Fig. 10. Correlation of phylogenetic distance with physical distance in 2 zebrafish genomic clusters. For each gene within the clusters on chromosome 10 (dark gray bars) and chromosome 20 (light gray bars), the paralog with the highest homology was determined, and its position relative to the first gene was expressed as ordinal value, e.g., a value of 1 indicates a direct neighbor (most frequent case), and a value of 2 indicates 1 additional gene situated between the gene and its closest relative. Phylogenetic neighbors outside of the cluster occur only in 2 cases.

1.6. Gene duplication rate and gene divergence are much higher in teleost compared with mammalian species, suggesting a teleost- restricted rapid evolution of taar genes.

The teleost TAAR repertoires range from 112, 48, 25, to 18 genes (zebrafish, stickleback, medaka, and pufferfish, respectively), whereas mammalian families just reach minimal fish family size, and avian and amphibian families are minuscule, with only 3 genes each (see Table.2 and Supplementary Table.1). Most of these differences are caused by massive recent gene expansions in teleosts that led to 30 members within a single zebrafish-specific subfamily, TAAR20, and 28 genes in the stickleback- specific subfamily TAAR26. Only TAAR11 and TAAR24 have not undergone recent gene duplications. In contrast, mammalian gene expansions are less frequent, and also much smaller, maximally to 6 genes in opossum TAAR9. No recent gene expansions were found for TAAR1, 2, 3, and 5. No recent gene duplications have been observed in amphibian and

avian species (Fig. 5). Individual teleost TAAR genes rarely possess any orthologs. Thirteen of 19 subfamilies are restricted to a single species each, i.e., all gene duplications giving rise to these genes appear to have occurred after the respective species diverged from the other 4 (Fig. 6). Only 2 subfamilies contain genes from all 4 neoteleost species examined, and none contain genes from zebrafish and neoteleosts (see Table 2). Even in the case of subfamilies containing orthologs, a gene expansion may occur in one species but not another, e.g., TAAR27 has expanded to 7 genes in tetraodon but remains a single gene in both stickleback and fugu (see Table S2). Thus, most gene duplications have occurred rather recently, after the divergence of the teleost and neoteleost species analyzed here (Fig. 6) and many even after the 2 pufferfish species diverged 20–30 million years ago (Van de Peer, 2004). In contrast, orthologs are readily identifiable between all mammalian species analyzed. We uncovered bovine orthologs for all 9 previously identified mammalian *taar* subfamilies (Table. 2 and Supplementary Table.1). In humans, all 9 subfamilies are represented by 1 member each, albeit 3 of them by pseudogenes (Table.2). Seven of the 9 subfamilies are detected also in opossum, a marsupial mammal (Table.2), i.e., should be present already in the MRCA of marsupials (Murphy et al., 2007) and modern mammals. Although very small, with 3 genes each, the amphibian and avian *taar* gene repertoires are not located at the base of the tetrapod tree and clearly belong to different mammalian subfamilies. Thus, gene losses appear to have shaped the avian and amphibian gene families. We selected a mammalian and a fish species pair with approximately equal evolutionary distance for an initial comparison of evolutionary rates. Rat and mouse diverged _23 million years ago (Springer et al., 2003), very similar to the 18–30 million years given for *Tetraodon nigroviridis* and *Takifugu rubripes* (Van de Peer, 2004). For both pairs of species, many orthologs or ortholog subfamilies are observed. Differences between orthologs accumulate only after the separation of the respective species, thus larger divergence in 1 pair of species indicates a faster evolutionary rate. The maximal ortholog divergence is, without exception, higher for pufferfish than for rodent pairwise comparisons, maximally 68% for pufferfish, but only 16% for the rodents (Fig. 11). These data suggest a faster evolutionary rate in bony fish compared with tetrapods.

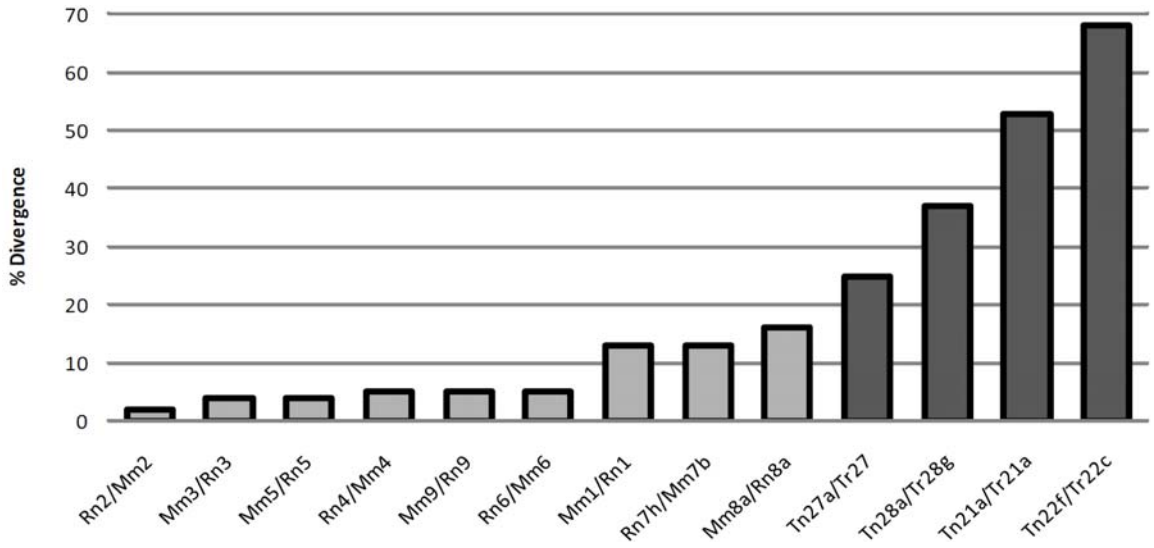

Fig. 11. Maximal divergence within rodent and pufferfish subfamilies. Maximal divergence between ortholog genes in rat *vs*. mouse and tetraodon vs. fugu comparison. Maximal divergence within the same subfamilies (paralog divergence) is also indicated. Values are based on amino acid comparisons and ordered by size. Note that even the largest value for rodent comparisons is below the smallest value for pufferfish comparisons.

1.7. Strong local positive selection in teleost taar genes is masked by global negative selection.

To better understand the evolutionary dynamics of the *taar* genes, the selective pressure on these genes was analyzed using both global and local analysis of substitution rates in synonymous vs. nonsynonymous base positions. The global dN/dS values calculated for each of the ortholog groups show that all of the gene groups are under negative selection (Fig. 12 and Supplementary Table.2), but the extent varies considerably, from 0.09 (pronounced negative selection) up to 0.8 (close to neutral selection). The average dN/dS value for the teleost-restricted class III is by far the highest, more than double the value for class II *taar* genes and significantly different from both class I and class II values (Fig. 12). The relaxed negative selection observed especially for class III TAAR subfamilies may result from an overall pronounced negative selection masking positive selection at some sites. To clarify this point, we analyzed the dN/dS values for each individual codon position for all genes of a particular *taar* subfamily. As predicted by the analysis of the previously calculated global dN/dS values, negatively selected sites were found without

exception throughout all of the *taar* gene families, with some preponderance in the transmembrane regions (Fig. 12). Consistent with the results of the global analysis, class III *taar* genes contain only approximately half as many negatively selected sites as the other 2 classes (Supplementary Table.1).

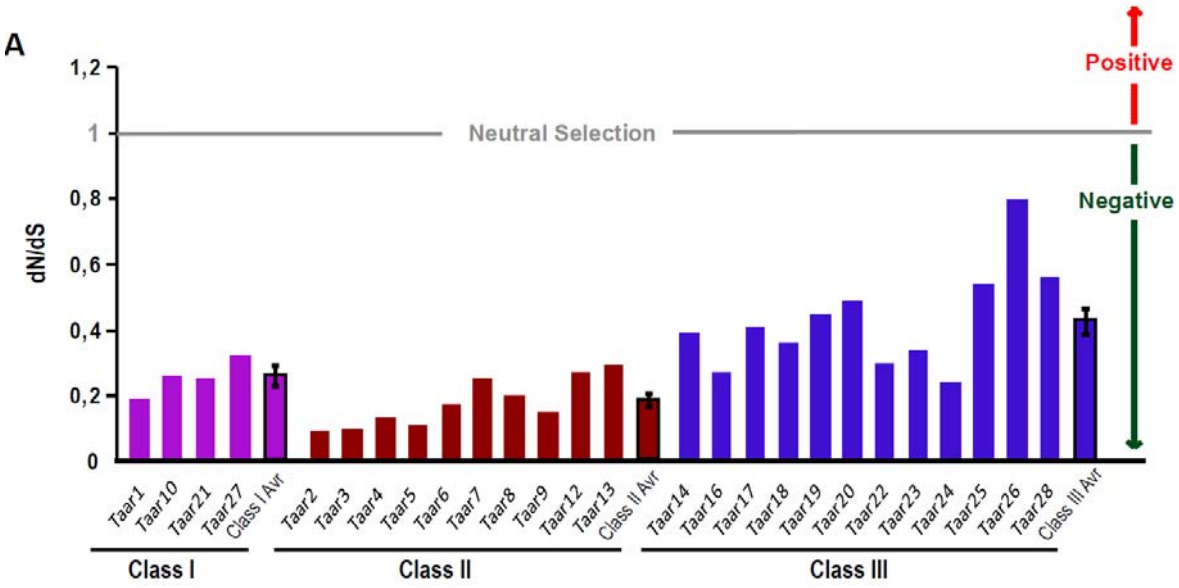

Fig. 12. Evolutionary distances and selective pressure on *taar* genes. (*A*) dN/dS ratios of the TAAR ortholog groups in which this analysis was possible (more than 2 genes per group). Genes are arranged by class, the class average is indicated by background shading.

Excitingly, the site-by-site analysis suggested a significant number of sites under positive Darwinian selection that were masked by the predominance of negative selection in the global analysis. Although there are few such sites in class I and II *taar* genes (0–2 sites per gene), several genes in class III show much higher values of up to 20 sites per gene (Fig. 13 and Supplementary Table.3). The values for class I and II *taar* genes are comparable with those reported for other olfactory receptor gene families (1–2 sites), (Alioto and Ngai, 2005; Alioto and Ngai, 2006). The analysis was repeated for zebrafish OR genes (Niimura and Nei, 2005) using the identical algorithm and obtained a range of 0–5 sites, on average 1 site per gene (see Table S5). To the best of our knowledge, the much larger number of such sites in class III *taar* genes is without precedent in olfactory receptor gene families. We conclude that the teleost-restricted class III, which is evolutionary much younger than class I and class II, is likely to have undergone extensive

positive selection. The more rapid evolution of class III has resulted in massive expansion of gene families beyond that observed in the older classes I and II.

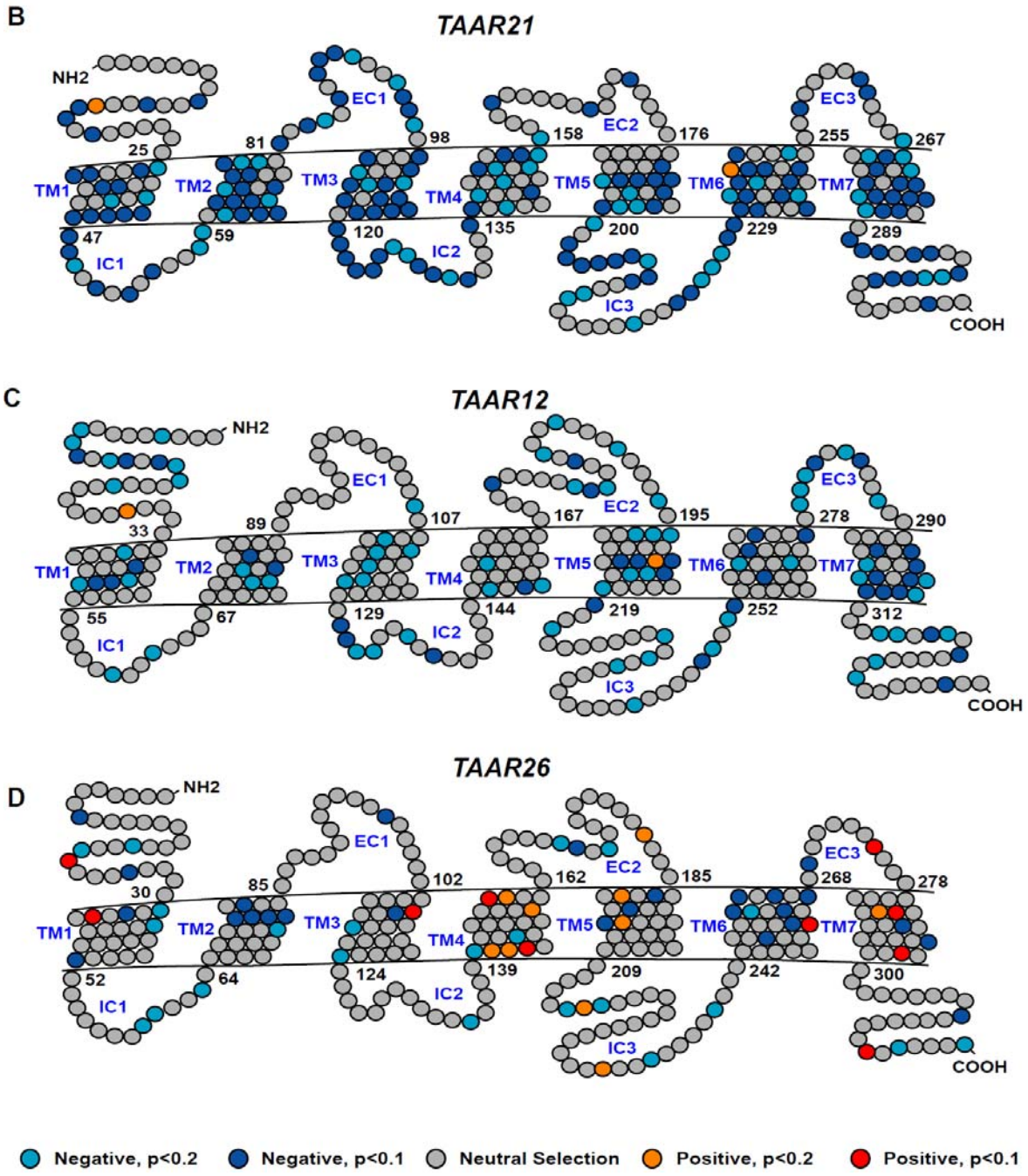

Fig. 13. (*B, C and D*) A representation of site-by-site selective pressure is shown for 3 TAAR sequences. (negative selection in light blue, *P* _ 0.2 or blue, *P* _ 0.1, neutral selection in gray, positive selection in orange, *P* _ 0.2, and red, *P*_0.1). (*B*) Results for

TAAR21, a class I subfamily, which includes ortholog genes of all 4 neoteleost species. (C) Results for TAAR12, a zebrafish specific classII subfamily. (*D*) Results for stickleback-specific TAAR26, a class III subfamily.

1.8. Dynamic loss and gain of introns restricted to the class III of neoteleost taar genes.

Generally *taar* genes are monoexonic, like the related ORs (Hashiguchi and Nishida, 2007). We report that, without exception, all class I, class II, and class III zebrafish *taar* genes are monoexonic. However, from class III, all *taar* genes of neoteleost subfamilies 23–26 and some genes from subfamily 28 contain an intron between TM1 and TM2 (Fig. 14). The intron is rather short, in the range of 76 to 373 nucleotide, with an average value of 155 nucleotide. Homologies between introns parallel those of the corresponding coding regions. The intron/exon border is strictly conserved (Ol*_taar23d* and Tr*_taar28f* show a slightly extended first exon), consistent with a single phylogenetic event early in the neoteleost lineage subsequent to the segregation from the more basal ostariophysan fish (Fig. 14). Consequently, the most parsimonious explanation for the absence of this intron in subfamily 22 and some genes of subfamily 28 is a secondary loss, which must have happened at least 2 times independently. The intron loss in subfamily 28 occurred very late, after the segregation of the 2 pufferfish species (Fig. 14), indicative of the unusually high intron dynamics in the *taar* gene family compared with the tiny average frequency of intron losses after the divergence of fugu and tetraodon (Loh et al., 2007). Another intron gain is predicted in an individual stickleback gene (*Ga_taar22a*, class III), but not in its pufferfish or medaka orthologs, i.e., late in the neoteleost evolution (Fig. 14). It is caused by insertion of a short repeat that leads to the expansion of a short, conserved poly CV stretch (see Fig. 8) into much of TM4. In total, at least 4 independent intron gain/loss events have occurred after the neoteleosts emerged. Because genome-wide searches so far have failed to identify a single intron gain in vertebrates (Loh et al., 2007), the 2 gain events documented here appear to be an extremely rare case and may be related to the selection for divergence of class III *taar* genes.

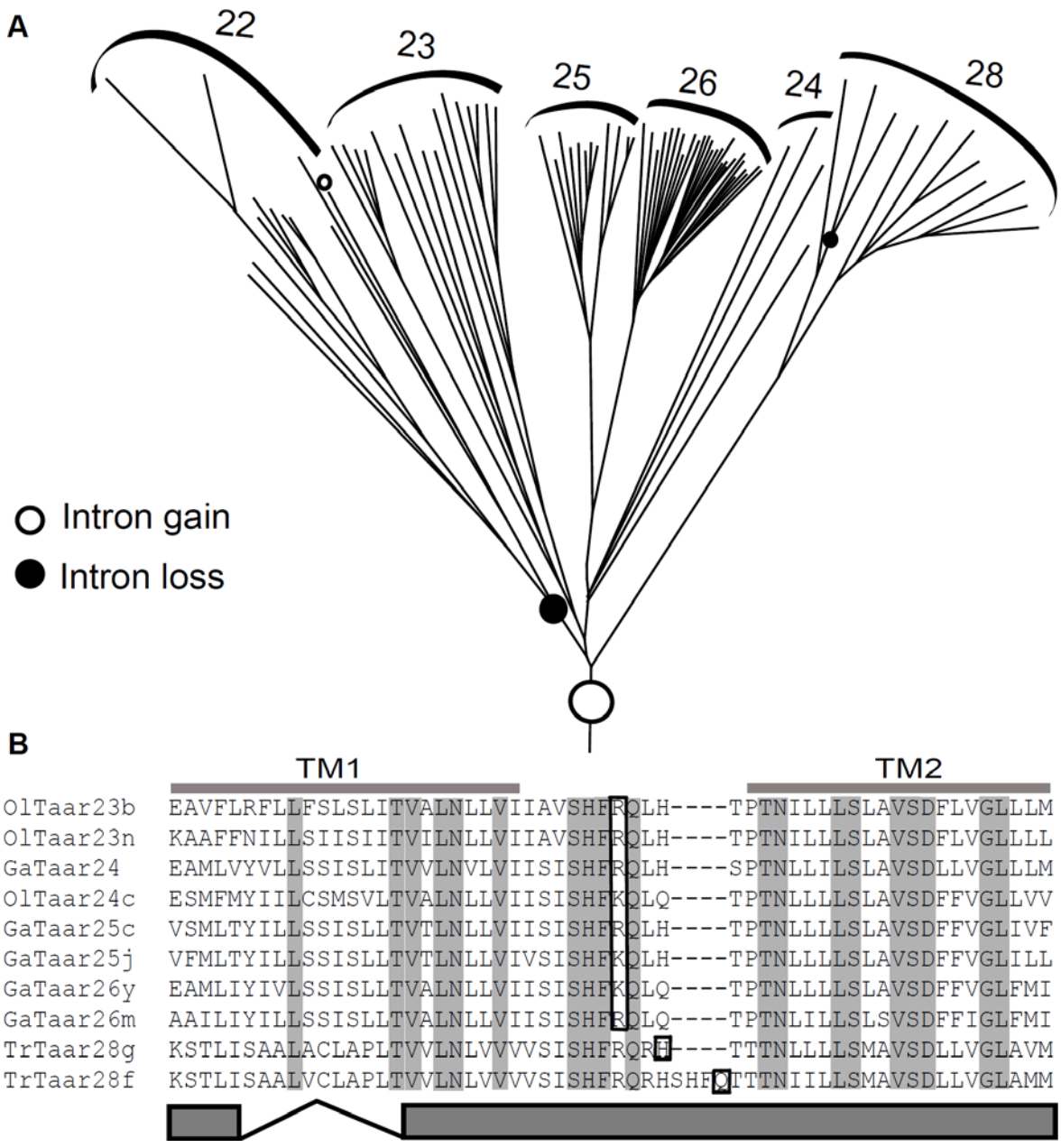

Fig. 14. Intron dynamics in class III neoteleost *taar* genes. (*A*) By using maximum parsimony, predictions for all independent events of intron gain or loss are depicted in the phylogenetic tree detail. (*B*) A representative subset of *taar* genes sharing an early intron gain exhibits a strictly conserved intron/ exon border (boxed). The intron interrupts a loop between TM 1 and TM 2.

1.9. Most taar genes are expressed in sparse olfactory sensory neurons.

The rapid evolution and positive selection observed in the *taar* gene family in teleosts are consistent with expectations for olfactory receptor genes (Alioto and Ngai, 2005), because efficient adaptation to changing environmental stimuli may require high evolutionary rates. Another requirement for olfactory receptor genes is an expression in the olfactory epithelium. This was analyzed by in situ hybridization using a representative subset of 8 *taar* genes from all 3 classes (class I, TAAR1, 10; class II, 12f, 13c; class III, 14d, 15a, 19l, 20t). Probes were chosen to minimize cross-reactivity with related *taar* genes as far as possible. All genes tested were expressed in the adult zebrafish olfactory epithelium (Fig. 15), except TAAR1. Labeled cells were sparsely distributed within the sensory area of the olfactory epithelium. A higher density of labeled cells for genes in TAAR19l and 20t (Fig. 15) is presumably caused by unavoidable cross-reactivity in these large and highly homologous subfamilies. No expression was observed in the outer, non-sensory ring of the nasal epithelium.

Within the sensory surface individual *taar* genes are expressed in overlapping, but clearly distinct, concentric expression domains (Fig. 16). *Taar* genes 19l and 20t occupy the most distal positions, with peak expression frequencies rather close to the border between sensory and non-sensory epithelium, and show a correspondingly skewed distribution, whereas *taar* genes 10 and 12f show more medial and more symmetrical radial distributions (Fig. 15, Fig. 16). These spatial patterns are reminiscent of the ring-like expression domains observed for zebrafish ORs (Saraiva and Korsching, 2007; Sato et al., 2005; Weth et al., 1996). Thus, the spatial expression patterns observed for TAARs support an expression in olfactory sensory neurons, consistent with an expression of most or all *taar* genes in these neurons. Furthermore, the frequency of labeled cells [10–50 per section, without *taar (Loh et al., 2007; Weth et al., 1996)* is within the range observed for ORs and the V1R-related ORAs (Saraiva and Korsching, 2007; Weth et al., 1996).

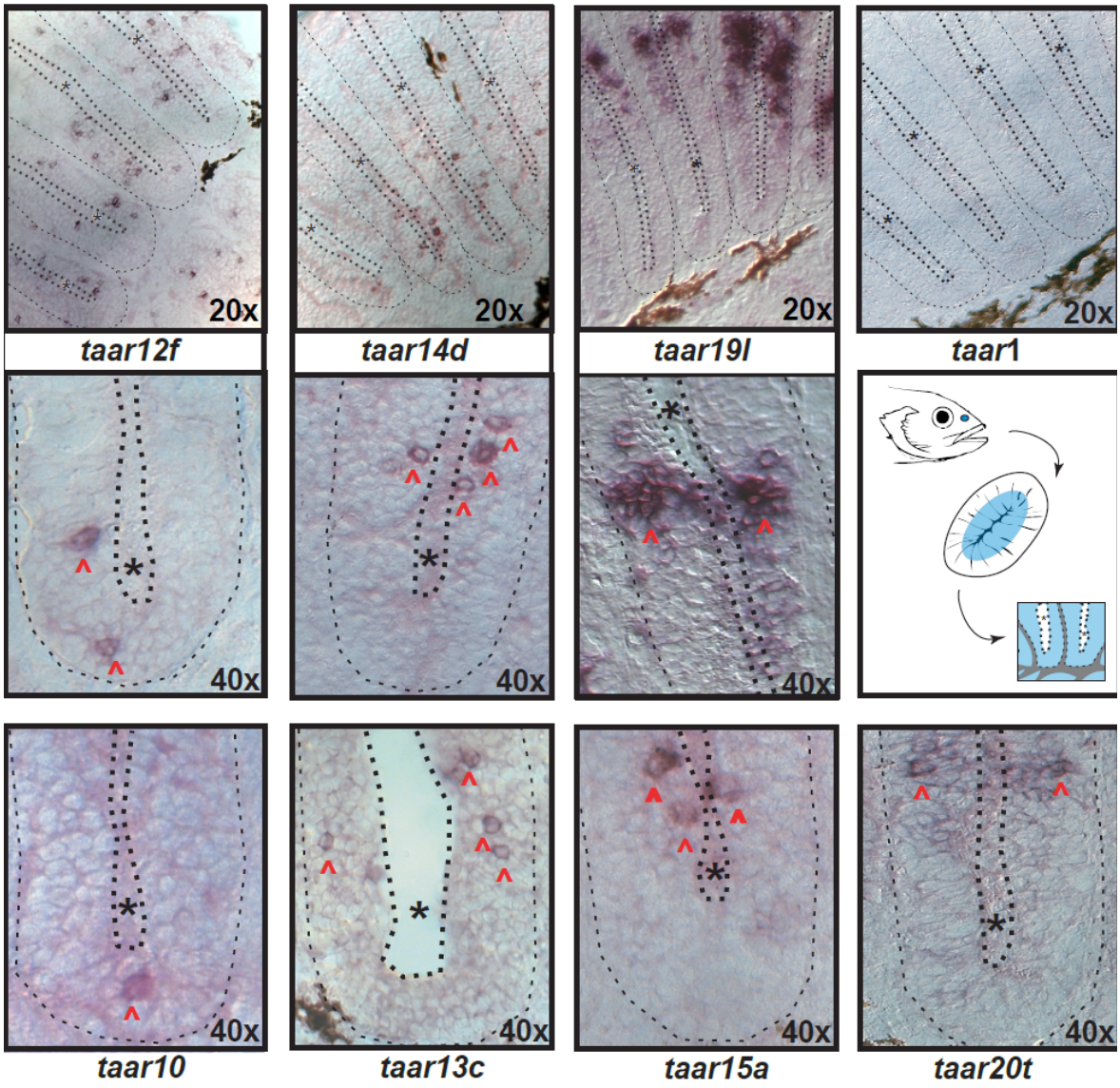

Fig. 15. Expression of *taar* genes in the zebrafish olfactory epithelium (OE). A schematic representation shows the approximate position of the olfactory epithelium in the zebrafish, the morphology of a horizontal section (lamellae are cut perpendicular to their flat face) and finally an enlargement of 2 lamellae. The central blue-colored area in the lamellae indicates the location of the sensory neuroepithelium (see ref. 20); gray areas and thin dotted line, basal lamina; black dots and asterisk, lumen. In situ hybridization was performed in horizontal sections with antisense RNA probes. The top row depicts the sensory region of several lamellae, whereas the other 2 rows show enlargements of 1 lamella, corresponding approximately to one-half of the schematical representation (*Center Right*). Red arrowheads point to labeled neurons, other symbols as above. *Taar* genes 10, 12f, 13c, 14d, and 15a are expressed in sparse cells, whereas *taar* 19l and 20t label a somewhat larger subset of cells within the sensory surface, probably because of cross hybridization in the large and closely related subfamilies *taar* 19 and *taar* 20.

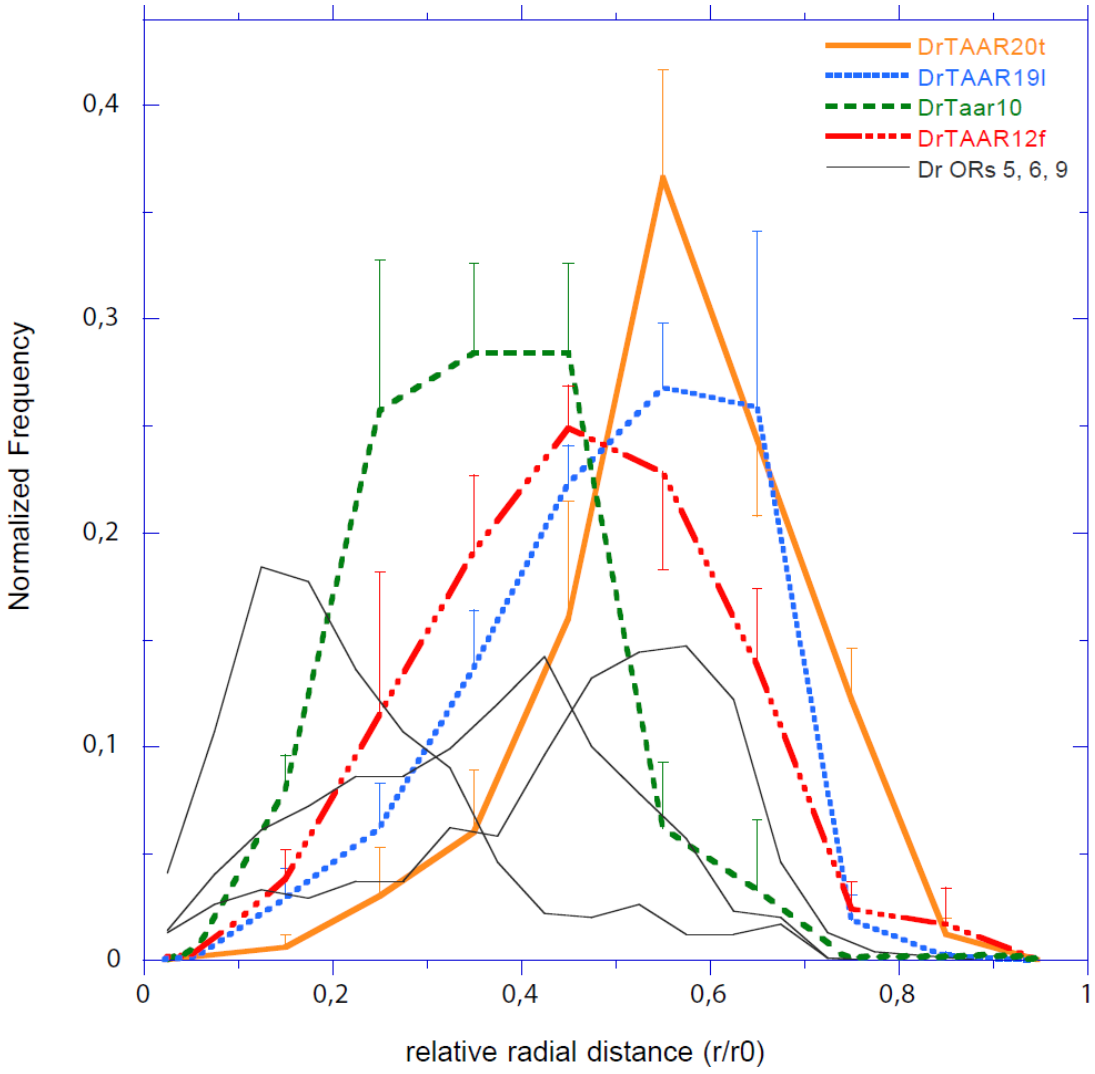

Fig. 16. Radial distribution of 4 TAAR genes. Positions of cells expressing particular TAAR genes were identified in horizontal sections of olfactory epithelia in the microscope and manually marked on printouts. Relative radial distance (*r*/*r*0) of labeled cells was measured for each lamella separately as distance from the nadir of the sensory layer, closest to the median raphe, divided by the total length of the corresponding lamella. For each section, a histogram of the radial distribution was calculated for 10 equidistant bins, frequency values obtained for each bin were normalized and averaged for several sections. Values given represent mean _ SEM. Thick lines, TAAR genes; thin black lines, reference curves from left to right (peak values) for OR genes *zor6*, *zor9*, and *zor5*, respectively (data taken from ref. 2). Note the skewness of histogram curves for TAAR12f, 19l, 20t, similar to the skewness observed for zOR6 and zOR5. Peaks for TAAR distribution are found medially and distally, similar to the proximally, medially, and distally centered distributions described for ORs.

2. Ligands for zebrafish TAARs

Ligands have only been identified for a handful of olfactory receptors of mammals (Krautwurst et al., 1998; Liberles and Buck, 2006; Mombaerts, 2004; Spehr et al., 2003) and insects (Asahina et al., 2008; Dahanukar et al., 2005; Ditzen et al., 2008; Syed and Leal, 2009). While only a single teleost olfactory receptor have been deorphanized, a member of the OlfC family, OlfC a1 (Alioto and Ngai, 2006). Therefore, an immense capacity of research exists for the identification of ligands for teleost olfactory receptors. Zebrafish TAAR family is a good candidate for deorphanization because aminergic ligand binding motifs, predictive of amine ligands, were found conserved in all of 25 TAAR genes of class I and II (Fig. 9). Technically, the identification of specific ligands for olfactory receptors is difficult because of the inefficient heterologous system, complexity of the task and species specific rapid evolution of genes repertoire. However, some recent modifications in the heterologous assays (Durocher et al., 2000; Liberles and Buck, 2006) make identification of olfactory ligands an amenable task.

2.1. DrTAAR13c recognize volatile diamines

Zebrafish TAARs genes were embedded with an amino-terminal addition of the first 20 amino acids of bovine rhodopsin (a 'rho tag') and were cloned in pcDNA3.1 vector (Liberles and Buck, 2006). The rho-tag modification helps the cell-surface expression of some odorant receptors in HEK293 cells (Krautwurst et al., 1998). TAARs were cotransfected in HEK293 cells with the cAMP reporter gene CRE-SEAP. CRE (cyclic AMP response element) is a pivotal target in many signaling pathways. An elevation of intracellular cAMP in response to activation of receptor by ligand binding is known to trigger protein kinase A, which translocates in the nucleus to phosphorylate CRE binding protein (CREB) transcription factors. CREB binds to CRE elements on the gene reporter to dose-dependently induce the translation of SEAP (Durocher et al., 2000; Montminy, 1997). The activity elicited by potential ligands applied (10μM) on HEK293 cells transfected with taar gene and reporter CRE-SEAP plasmid was assayed for SEAP activity using the fluorigenic SEAP substrate 4-methylumbelliferyl phosphate (MUP) (Clipstone and Crabtree, 1992; Liberles and Buck, 2006) (see methods for detail).

To identify the specific ligands for zebrafish olfactory receptors TAARs, 95 different chemicals (Supplementary Table.5) were used in the heterologous functional assay (Clipstone and Crabtree, 1992; Liberles and Buck, 2006). The chemicals used were mostly monoamines, diamines and polyamines but also included amino acids, mono and di-alcohols and few other compounds (see Supplementary Table. 5 for details). Eleven different zebrafish taar genes (DrTAAR1, 10, 11, 12f, 13a, 13b, 13c, 13d, 15a, 16c, 20t1) belonging to all three classes (classI, II and III) were examined. One olfactory receptor DrTAAR13c was activated exclusively by diamines and some polyamines (Fig. 17). The four other TAAR13 subfamily members (DrTAAR13a, DrTAAR13b, DrTAAR13d and DrTAAR13e) did not respond to diamines or any of the other chemicals examined.

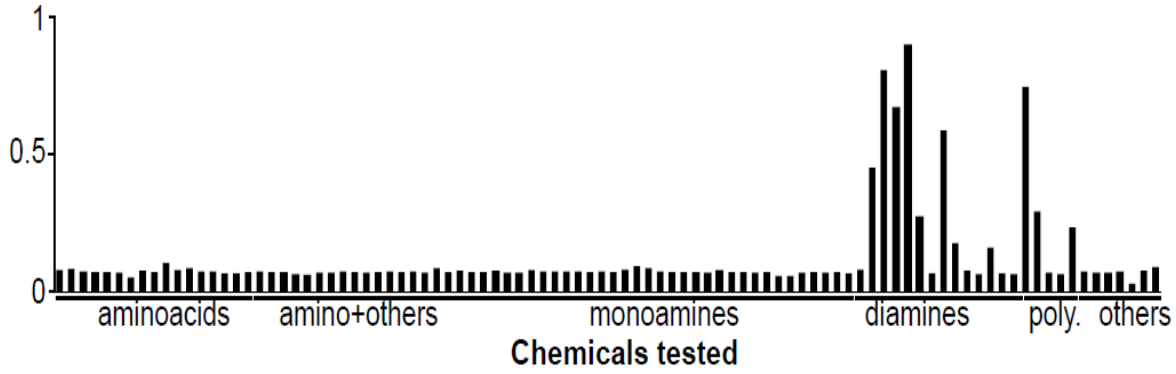

Fig.17. CRE-SEAP assay for 95 chemicals show activity for diamines and polyamines.

The diamines of various carbon chain length ranging from C3 to C10 (1,3 diaminopropane; 1,4 Putrescine; 1,5 Cadaverine; 1,6 Hexamethylenediamine; 1,7 Diaminoheptane; 1,8 Diaminooctane and 1,10 Diaminodecane) were tested in CRE-SEAP heterologous system. DrTAAR13c showed activity for diamines with carbon chain length four to eight albeit with different affinity (Fig. 18), but did not respond to short (diaminopropane) and very long (diaminodecane) aliphatic diamines. A dose response curve (0-1000 μM diamines) was determine to estimate the half maximal effective concentration (EC50) of these newly identified ligands (Fig. 18). The individual experiments were performed in triplicate and up to 7 independent experiments were done per stimulus.

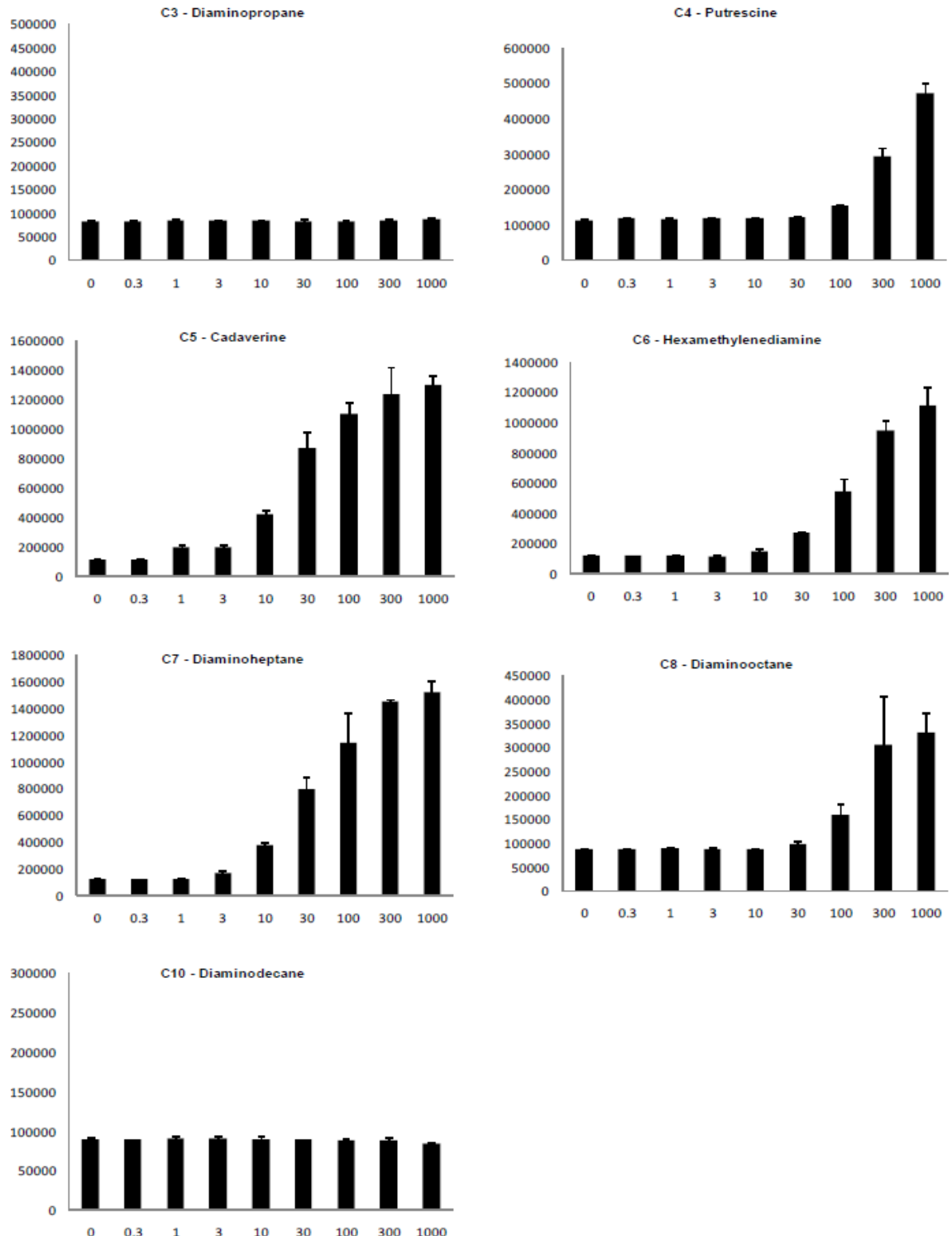

Fig.18. CRE-SEAP concentration dependence activity induced by exposure of DrTAARs13c to diamines. X-axis shows the concentration of chemicals (μM), Y-axis shows the level of CRE-SEAP activity (arbitrary units). (Data reproduced by David Ferrero, Harvard Medical School USA).

The dose response assay shows that a lower stimulus concentration is required for CRE-SEAP activity elicited by cadaverine (EC50=22+/-4) and diaminoheptane (EC50= 28+/-2), while a higher stimulus concentration is required for putrescine (EC50= 266+/-12), diaminohexane (EC50= 108+/-6) and diaminooctane (EC50= 87+/-3). A high stimulus concentration was also required for Cysteamine (EC50= 100), agmatine (EC50= 300) and histamine (EC50= >300) activity (Fig. 19).

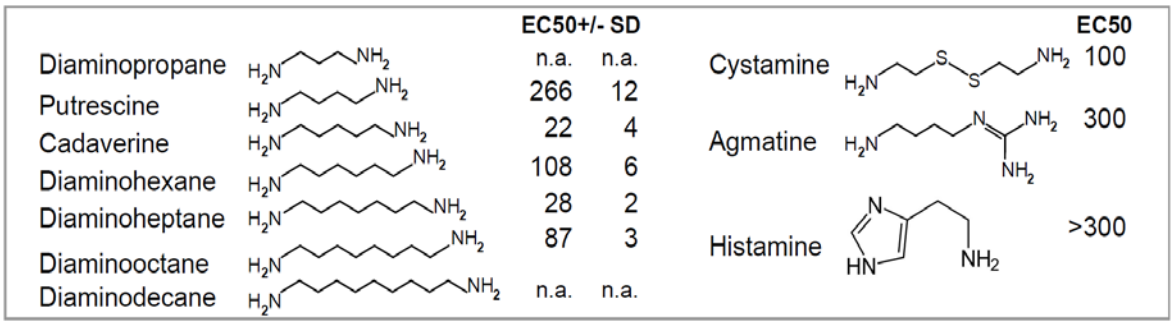

Fig. 19. Chemical structures and EC50 values (μM) of ligands identified for DrTAAR13c.

This data shows that DrTAAR13c can be activated by diamines of specific carbon chain lengths. Both smaller, diaminopropane, and longer, diaminodecane, carbon chain length diamines are not effective. Interestingly odd numbered carbon chain length diamines (cadaverine, diaminoheptane) are more effective that even numbered carbon chain length diamines (putrescine, hexamethylenediamine and diaminoctane).

2.2. DrTAAR13c activation requires at least 2 amino groups

Cadaverine is a 5-carbon diamine and is one of the potent activators of DrTAAR13c olfactory receptor (fig18, 19). To examine which molecular features of this ligand are required for activation of DrTAAR13, I tested CRE-SEAP activity of monoamines, monoalcohols, and amino-alcohols, initially at 10μM concentration. No activity of DrTAAR13 was observed at this concentration. The stimulus concentration of pentylamine, a 5-carbon monoamine, and 5-amino 1-pentanol, a 5-carbon monoalcohols, was gradually increased to 1000μM, but DrTAAR13c did not show any signal of activation also at this high concentration (Fig. 20). This suggests that DrTAAR13c is a receptor for

diamines and two remote positive charges (amino groups) are required for activation of DrTAAR13c. Olfactory receptor sites for diamines are highly specific for polyamines and not for structurally related compounds (Rolen et al., 2003).

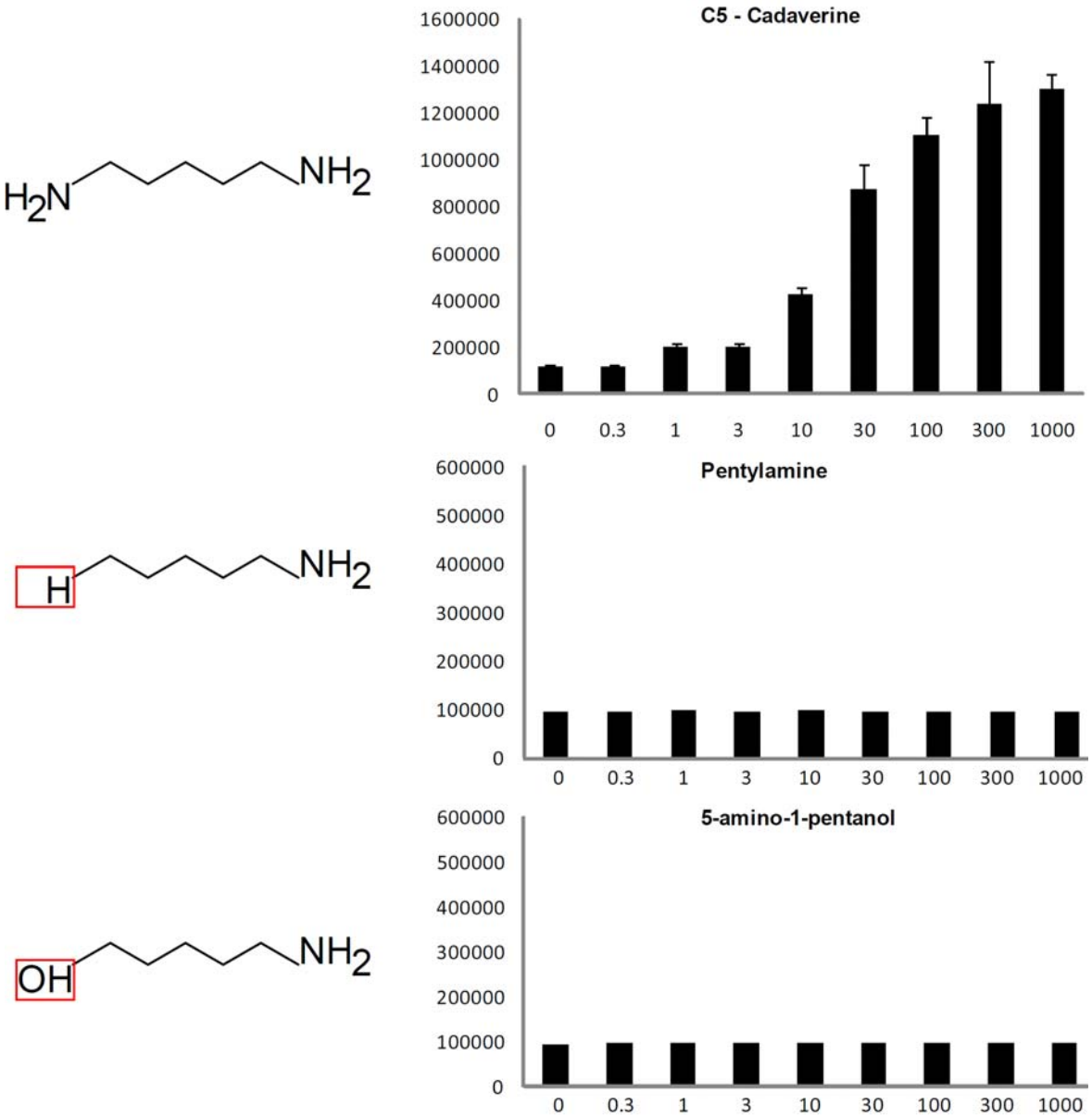

Fig. 20. DrTAAR13c is exclusively activated by diamines (cadaverine in the above figurer). No activity is shown for monoamines (pentylamine) and monoalcohols (5-amino-1-pentanol).

2.3. DrTAAR13c is activated by polyamines

A standard concentration (10μM) of polyamines (Agmatine Sulfate, Spermidine, Spermine, Adenine and Histamine dihydrochloride) was tested in CRE-SEAP assay. No activity was elicited by any of these polyamines on DrTAAR13c. The concentration of these polyamines was increased to 1000 μM and CRE-SEAP activity elicited by Agmatine Sulfate, Spermidine, and Histamine dihydrochloride was observed (Fig. 21). The EC50 for Agmatine Sulfate induced activity was high (300μM) while the EC50 for Histamine dihydrochloride was even higher (>300μM) compared to diamines (Fig. 19). The mechanism by which an increase in polyamine level leads to increase in olfactory sensitivity is still not clear. The possible explanation could be that in addition to having an independent receptor DrTAAR13c for diamines in zebrafish that does not recognize structurally relevant odorants (Fig. 20), there are also possibly relatively independent olfactory receptor sites among the polyamines themselves that recognize different polyamines with different affinity.

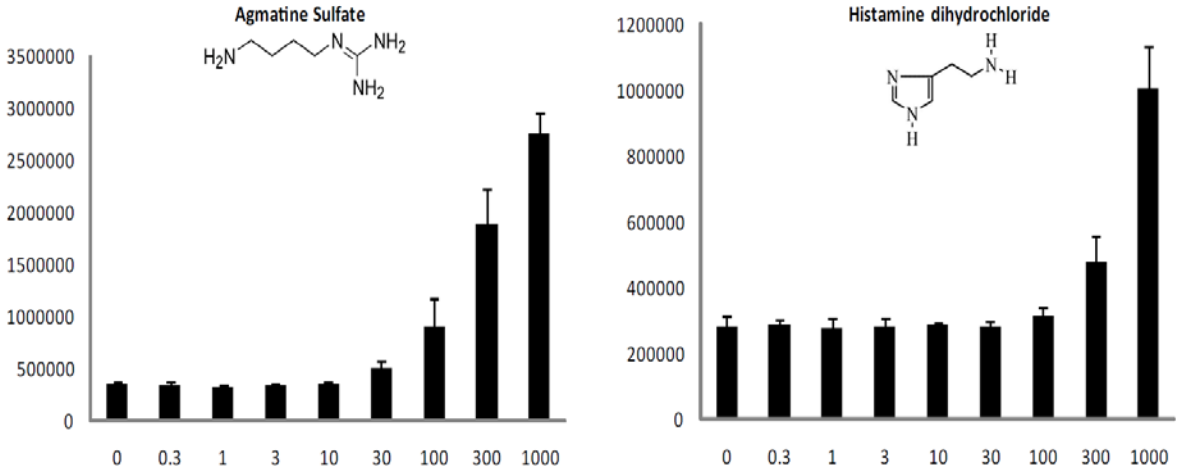

Fig. 21. DrTAAR13c is activated by polyamines at higher concentration. No activity is shown for monoamines (pentylamine) and monoalcohols (5-amino-1-pentanol).

2.4. DrTAAR13c recognizes natural activators

Putrescine and cadaverine are foul-smelling compounds that occur naturally as bacterial decarboxylation products of amino acids, lysine and arginine, respectively (Molenaar et al., 1993; Pessione et al., 2005; Vidal-Carou, 2005). In aquatic environment cadaverine may be generated as a result of putrefication of the dead fish over a period of time. To validate this supposition, I tested both fresh and rotten fish homogenate in CRE-SEAP assay of DRTAAR13c. Freshly prepared zebrafish homogenates were applied at different dilutions (100.000:1 - 10:1), no activity of DrTAAR13c was observed at any dilution. Next, zebrafish homogenate was left to rot in 1X PBS for 1 week, and then applied at different dilutions (100.000:1 - 10:1) in CRE-SEAP assay. Notably, DrTAAR13c show a higher response for rotten zebrafish homogenate (Fig. 21). The activity of taar gene increases with increased rotten fish dilutions but to a certain threshold. Probably, cadaverine was generated in the rotten fish homogenate bacterial decarboxylation over a period of 1 week.

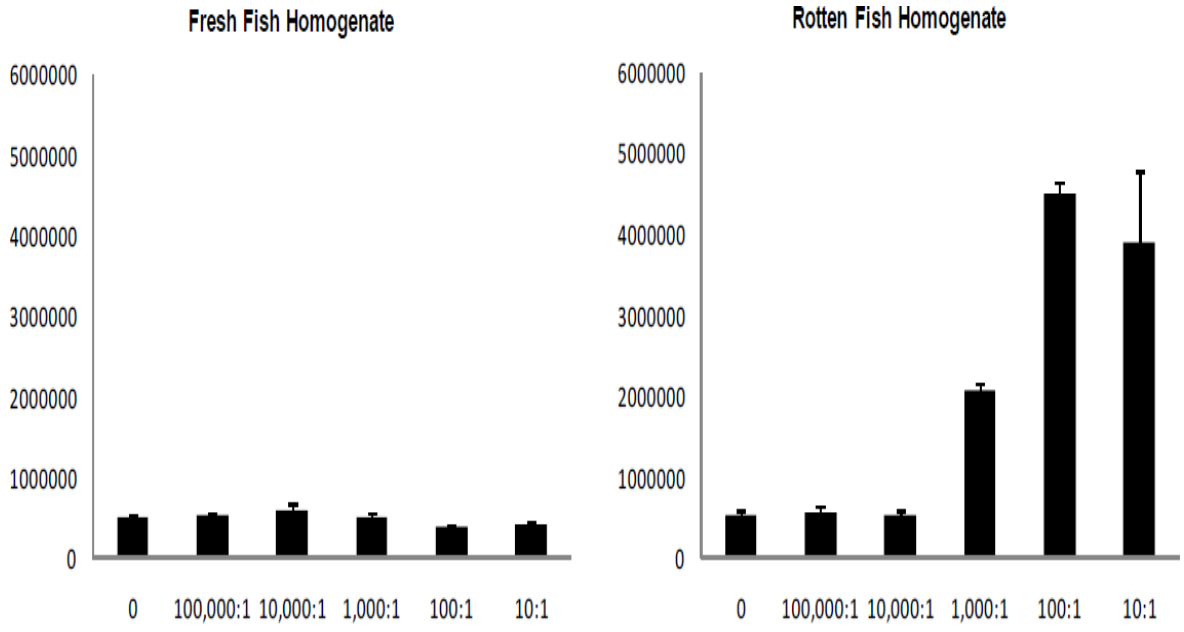

Fig. 21. DrTAAR13c show activity for different dilutions of 1 week old rotten fish homogenate (right panel). No activity was observed for fresh fish homogenate. (Data kindly provided by our collaborator David Ferrero, Harvard medical school, USA).

The HPLC purification of the rotten zebrafish homogenate was carried out to verify the possible cadaverine development. The HPLC analysis shows that cadaverine is the most abundant diamines found in rotten zebrafish homogenate, with smaller quantities of putrescine and histamine also present (Fig. 22). Thus the activation of DrTAAR13c by rotten fish homogenate is mainly caused by cadaverine.

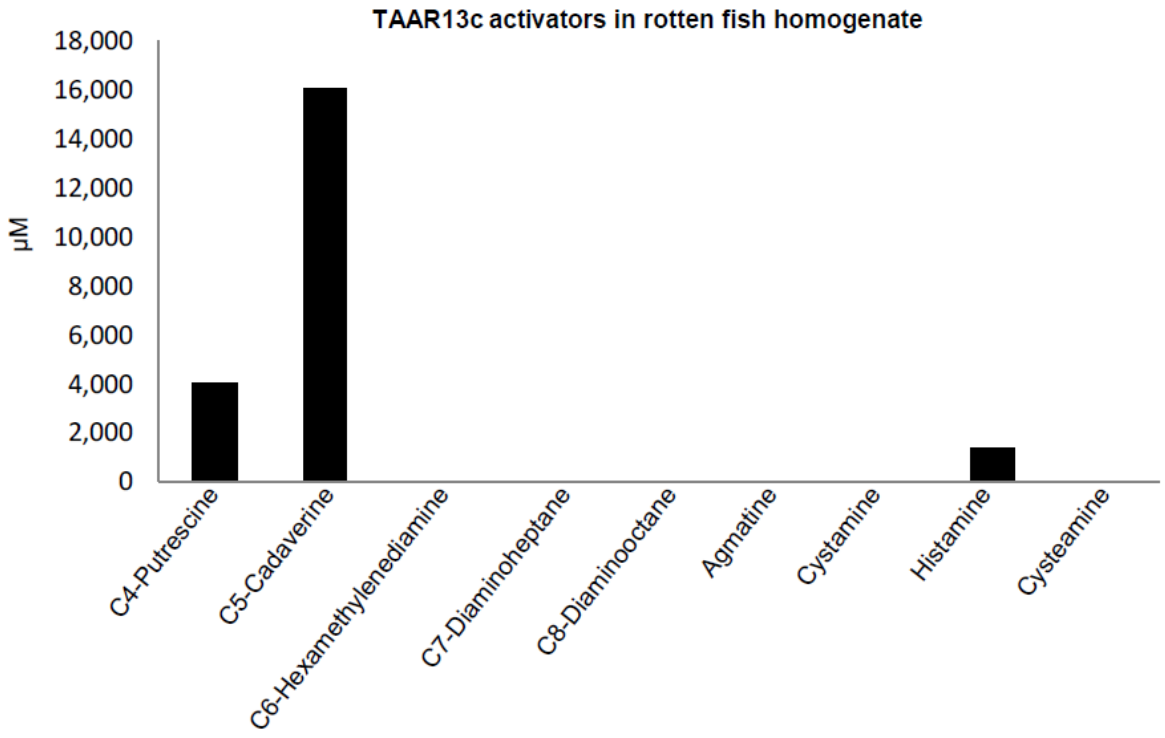

Fig. 22. HPLC analysis of rotten fish homogenate. Cadaverine is the main natural activator of DrTAAR13c as indicated by HPLC analysis. (HPLC analysis was carried out by our collaborator David Ferrero at Harvard Medical School, USA)

3. Behavioral response of zebrafish to diamines

Diamines (putrescine and cadaverine) are naturally occurring aliphatic polycations in the aquatic environment with their concentrations correlated to the degree of decomposition of certain aquatic animals (Mietz and Karmas, 1978). Since diamines concentrations vary with degradation, and they are distributed ubiquitously, teleosts are likely to encounter them in an aquatic environment and may sense them as signal of danger. A previous investigation tested putrescine as a possible olfactory stimulus in zebrafish, but the results was negative (Fuss and Korsching, 2001). The identification of putrescine and cadaverine as a ligand for zebrafish olfactory receptor (Fig. 18) and the existence of cadaverine in natural environment released from the dead conspecifics, as observed in the rotten zebrafish homogenate (Fig. 22), leads to the speculation that cadaverine may act as a physiological source that may signal danger (Pinel et al., 1981) and is perceived by one or many olfactory receptor(s). How does the zebrafish behave when it encounters the diamines in its aquatic environment? A behavioral assay was established to answer this question. Zebrafish was placed in an odorless, transparent glass tank ($100^{X}10^{X}20$ cm) extensively cleaned under deionizer running water (Fig. 23). Fish was allowed to acclimatize in 9 liters of fresh clean water for 45 minutes to 1 hour. The behavioral assay was performed in two stages; the pre-stimulus stage where no stimulus was applied and post-stimulus stage where stimulus was present (see methods for details). Fish movements were recorded by high definition (HD) video camera mounted above the behavioral tank (Fig. 23). The movies were analyzed by WINANALYZE automatic motion tracker to obtain the zebrafish movement tracks and coordinates (see methods for detail). Over 15 adult zebrafish were used in the behavioral assays. 6 random adult zebrafish (3 male and 3 female) with average motility were used to perform an analysis of chain length dependency of odor induced behavior. The behavioral assay was conducted in maximum silence in a dedicated room.

Fig. 23. Behavioral assay setup. (right picture): Complete behavioral assay with glass tank, stimulus application setup (gray box) and high definition video camera for recording zebrafish movement. (left picture): Fish tank (100^{X} 10^{X} 20 cm) with stimulus application tube on right side.

3.1. Zebrafish does not show specific behavior for water, a mock stimulus.

Zebrafish swims freely without any explicit preference for any area of the behavioral tank (Fig. 24A) in pre-stimulus condition. When freely swimming zebrafish encounter mock stimulus, water, in the post-stimulus stage, they do not show any specific behavioral response of attraction, avoidance or freezing (Fig. 24B). Thus I could conclude that no olfactory behavior is induced by non-olfactory components. The swimming pattern of zebrafish stays the same as pre-stimulus. The quantification of zebrafish movement tracks obtained by WINANALYZE show the presence of zebrafish all over the tank in pre and post-stimulus stages, without any reproducible inclination for a preferred place in the behavioral tank (Fig. 24C). While there were sometimes slight differences in the swimming patter, these differences were not reproducible and thus represent most likely the inherent variability of zebrafish swimming pattern. The results were same for all 6 zebrafish tested in the behavioral assay under the same conditions.

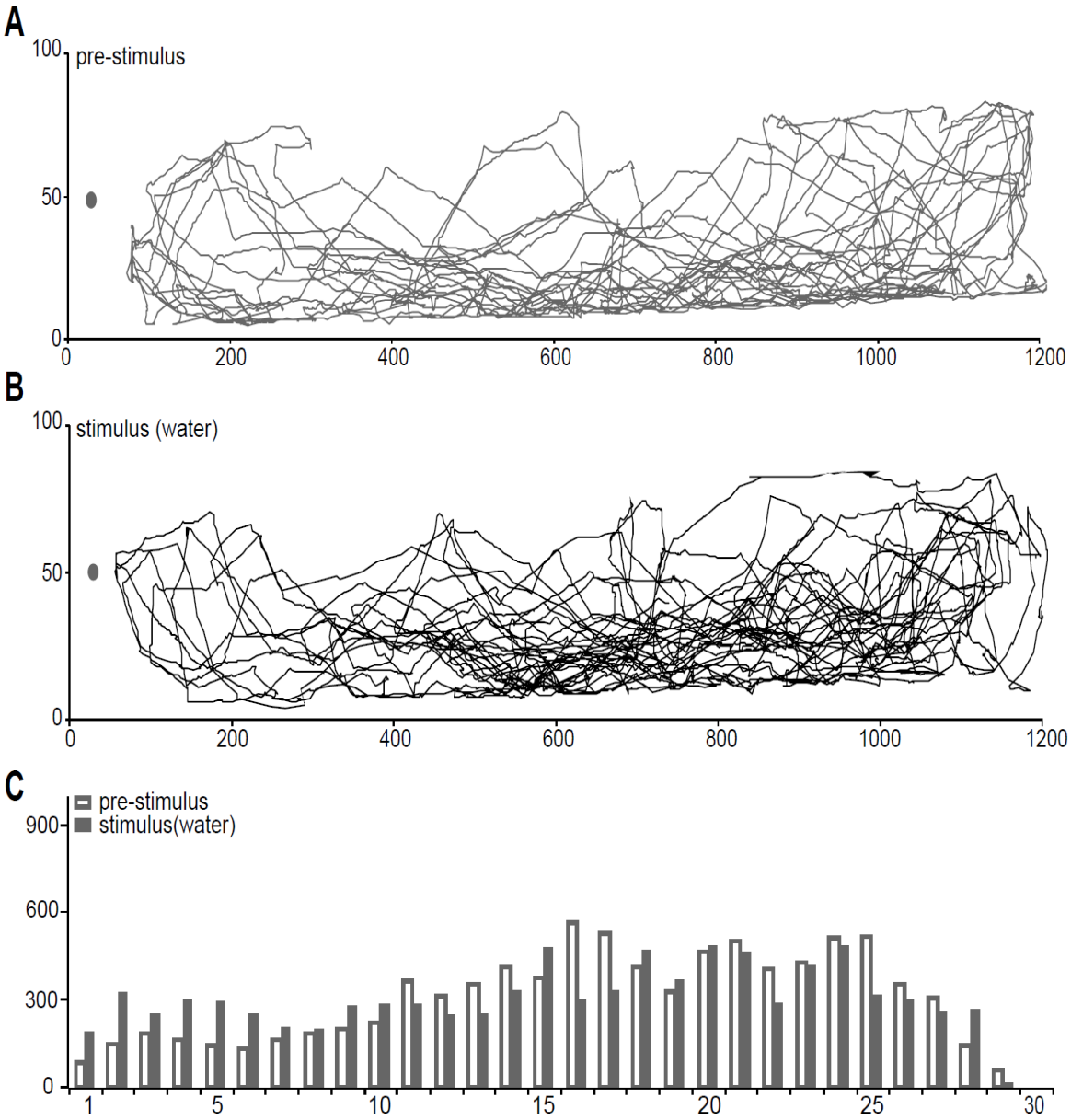

Fig. 24. Behavioral response of zebrafish to water. Tracks represent the motion of the zebrafish. The x-axis shows the length of behavioral tank (pixels), y-axis shows the width of the tank (pixels). The gray filled circle on the left-centre of the tank shows application point of stimulus. (A) pre-stimulus stage (no stimulus applied), the tracks show that zebrafish is moving freely all over the tank. (B) Water was applied as mock stimulus. No obvious difference was observed in pre and post stimulus tracks. (C) Quantification of pre-stimulus (empty bars) and post-stimulus (filled bars) tracks. Distance of zebrafish to site of stimulus application was measured. Data shown as histogram with 30 bin intervals of 40 pixels each. Y-axis shows the total time that the fish spends at that position, given as number of video frame. No recognizable preference behavior was observed and fish movement is equally present all over the tank in pre and post-stimulus stages.

3.2. Food induce attractive behavior in adult zebrafish

As before, zebrafish swims without preference in the pre-stimulus stage, mostly in an elongated circular pathway (Fig. 25A). When fish food extract was applied as a stimulus, zebrafish moved quickly towards the food within the first minute of the post-stimulus stage, an indication of olfactory stimulus, and investigates the stimulus by swimming upwards to the stimulus application points. Zebrafish prefers to stay there and spend >¾ of the post-stimulus time near the application area (Fig. 25B). Analysis of the tracks shows the preference of zebrafish for food stimulus (Fig. 25C).

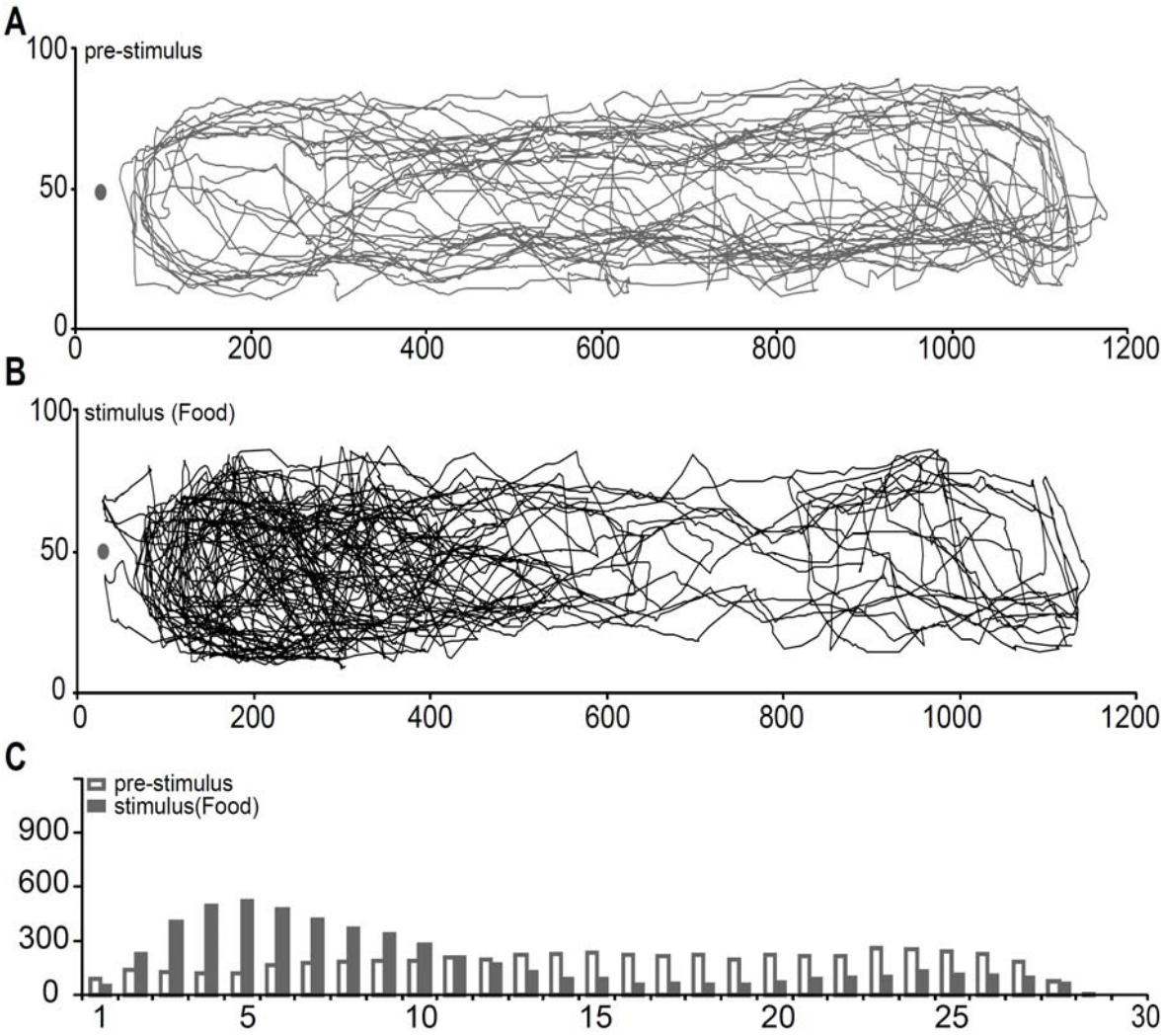

Fig. 25. Behavioral response of zebrafish to fish-food. (A) Zebrafish movement tracks in pre-stimulus state (no stimulus applied). The tracks show that fish is moving freely all over

the tank. (B) Zebrafish movement tracks in the post-stimulus state (fish food applied). Zebrafish shows clear attraction towards the stimulus. (C) Quantification of pre-stimulus (empty bars) and post-stimulus (filled bars) tracks. As expected, no specific behavior was observed in pre-stimulus and strong attraction towards the food stimulus is noted.

3.3. Diamines induce avoidance behavior in adult zebrafish

As expected, Zebrafish swim without any place preference in the tank during pre-stimulus stage (Fig. 26A). When 200µl of 1mM cadaverine solution in water was applied, fish moved slowly from its present position to the stimulus for investigation. Within seconds, fish swims back towards the opposite corner of the tank and stays there for a longer period of time (Fig. 26B). Zebrafish significantly shows such aversive behavior towards cadaverine, with rare forays into it, presumably for investigation purposes (Fig. 26B). Some events of freezing behavior were also observed (data not shown(Egan et al., 2009; Levin et al., 2007; Maximino et al., 2010). Zebrafish spends most of the post-stimulus time away from the stimulus application point as indicated by quantification of pre- (empty gray bars) and post-stimulus (dark bars) positions (Fig. 26C).

Zebrafish, generally portray an innate shoaling behavior, which commences soon after hatching (Engeszer et al., 2007; Whitlock, 2006). Shoaling behavior can increase the ability of an individual zebrafish to detect and avoid predators (Spence et al., 2008). A similar innate behavior expressed by zebrafish is “predator inspection behavior”, when an individual fish briefly leaves a shoal to approach a predator. These two traits are partly genetically determined in zebrafish (Wright et al., 2003). Putrescine and cadaverine are toxic products of dead animal´s putrefaction (Molenaar et al., 1993; Pessione et al., 2005; Vidal-Carou, 2005). The initial movement of the zebrafish towards cadaverine and other diamines is a form of “innate predator inspection behavior”. Zebrafish quickly leaves that vicinity upon sensing the danger portrayed by toxic smell of cadaverine and other diamines. The robust physiological aversive response to diamines (putrescine to diaminooctane) is possibly due to zebrafish´s well-developed corticosteroid stress axis (Alsop and Vijayan, 2009).

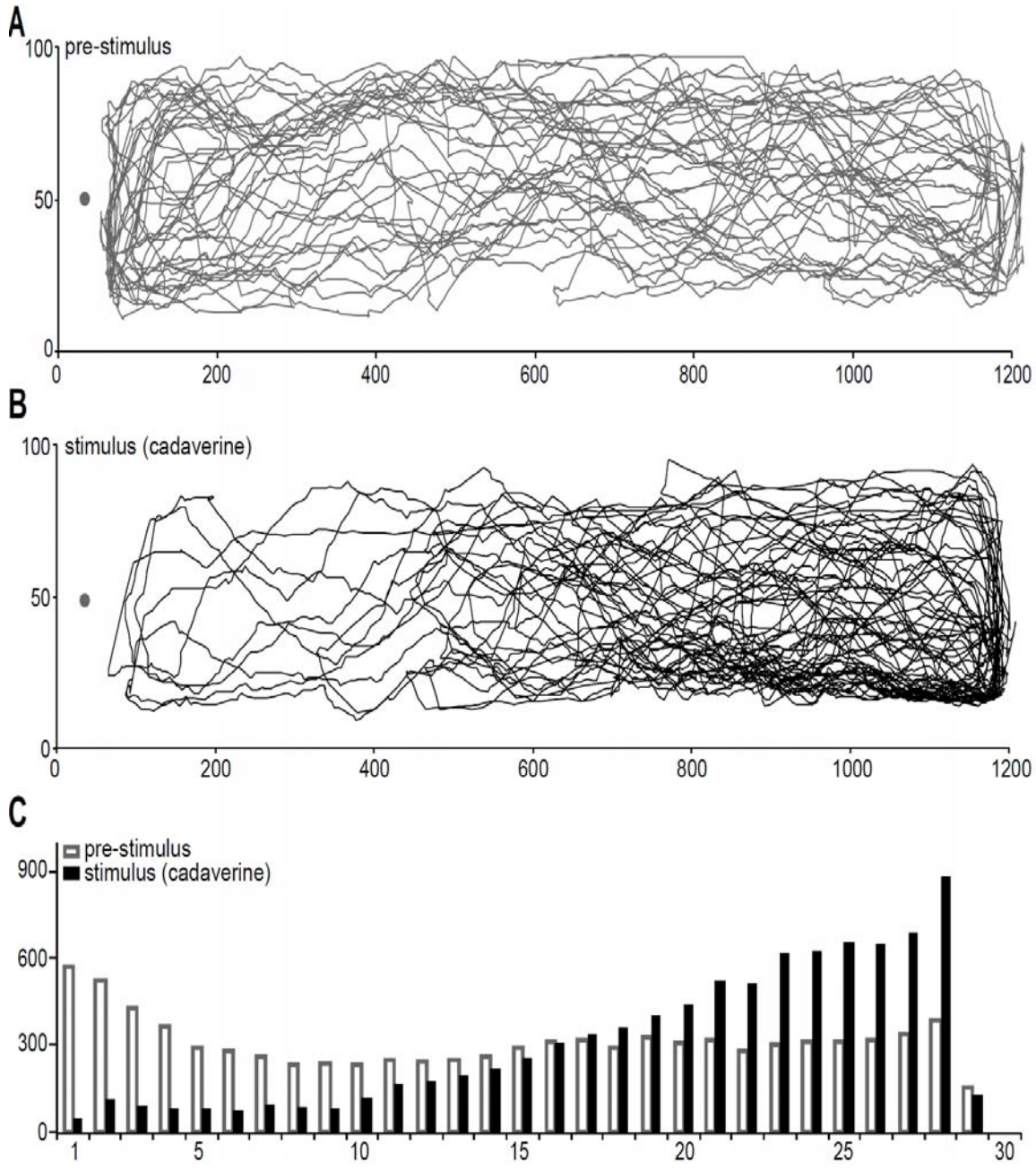

Fig. 26. Behavioral response of zebrafish towards cadaverine. (A) Zebrafish movement tracks in the pre-stimulus state. No specific behavior was observed. (B) Zebrafish movement tracks in post-stimulus state (cadaverine applied). Zebrafish investigate the stimulus as indicated by few track near application point (filled gray circle in the left-centre of the behavioral tank). There is a clear avoidance from the application point after initial investigation as shown by dense track on opposite side of application point. (C) Quantification of pre-stimulus (empty bars) and post-stimulus behavior (filled bars) also exhibit a strong avoidance in post-stimulus state.

In a series of zebrafish behavioral assays, each stimulus including diamines (diaminopropane, putrescine, cadaverine, diaminohexane, diaminoheptane, diaminooctane and diaminodecane) and two control stimulus (fish-food and water) was tested 6 times separately in the behavioral assay. 6 different adult zebrafish (3 males, 3 females) from Ab/Tü strain were used. The distance of zebrafish from the stimulus application point in pre-stimulus and post-stimulus stages was measured for each of this one stimulus - one zebrafish behavioral assays. The difference of post-stimulus distance minus pre-stimulus distance was taken as main activity position of zebrafish. No behavioral response was observed for water while zebrafish showed clear attraction towards food. Diaminopropane and diaminodecane also do not produce considerable behavioral response, their response spectrum fall into the range of behavioral response shown for water (Fig. 27). Significant aversive behavior was observed for putrescine, cadaverine, hexamethylenediamine, diaminoheptane and diaminooctane (Fig. 27). Periods of freezing behavior ((Jesuthasan and Mathuru, 2008; Speedie and Gerlai, 2008) and increase bottom dwelling (Egan et al., 2009; Maximino et al., 2010) were also observed, mostly for putrescine and cadaverine. Surprisingly, the aversive response for cadaverine was higher than other diamines, similar to high receptors activity by cadaverine in CRE-SEAP assay (Fig. 18).

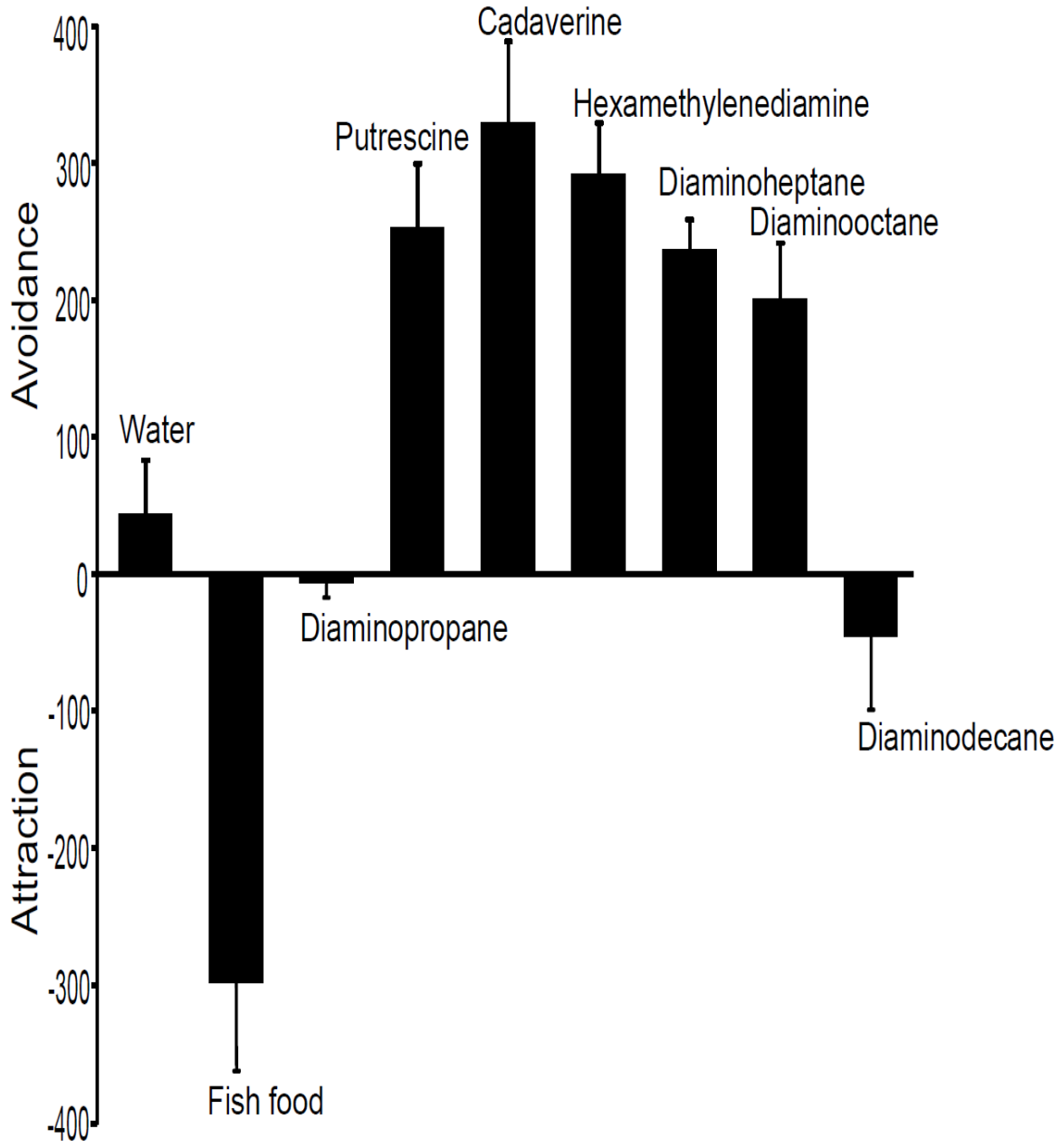

Fig. 27. summary graph of the average position of zebrafish in the behavioral tank. The x-axis contains bar graph for average of 6 experiments for each stimulus. A name of the stimulus is given above their respective bar. Y-axis contains values for the average position of the zebrafish in the behavioral assay based on the difference of post-stimulus distance minus pre-stimulus distance from the application point. Positive values represent avoidance and negative values represent attraction. No behavioral response was observed for water, diaminopropane and diaminodecane. Zebrafish shows attraction towards food. A strong avoidance behavior was observed for diamines (putrescine to diaminoctane).

3.4. Zebrafish aversive behavior towards diamines is concentration dependent

The intensity of avoidance behavior in response to diamines increases with the increased concentration of diamines. In a series of dose response experiments, cadaverine and diaminoheptane was applied as stimulus with a concentration range of 0.001-1000 mM, on 3 different adult zebrafish (male and female). Zebrafish exposed to even low concentration of these diamines show dramatic, measurable aversion (Fig. 28). There is a gradual increase in receptor sensitivity with increased concentration of diamines until it reaches a sustainable threshold with slight decrease afterwards (Fig. 28). The minor decrease in avoidance behavior could be due to possible deterioration of olfactory epithelium in response to higher concentration of diamines.

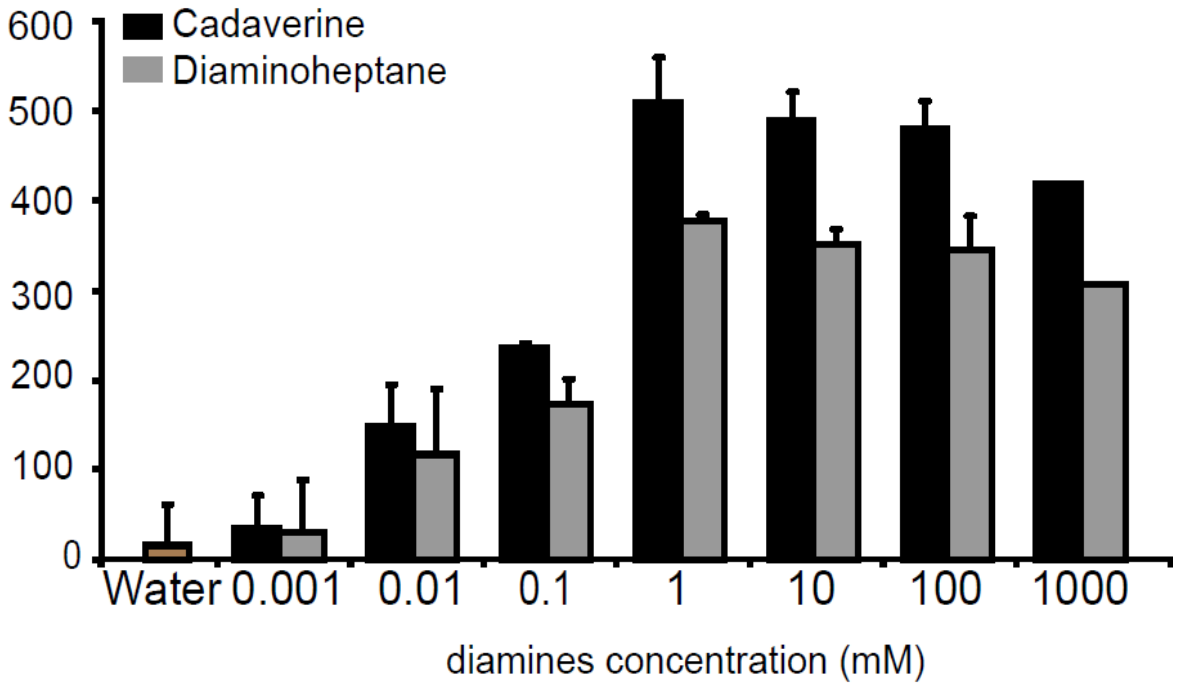

Fig. 28. The dose-response of zebrafish to cadaverine (dark bars) and diaminoheptane (gray bars). X-axis shows the concentration of stimulus applied and y-axis shows avoidance activity.

4. Diamines activate a sparse subset of olfactory sensory neurons in zebrafish olfactory epithelium

There is a stimulating possibility that the zebrafish behavior in response to diamines is induced by activation of DrTAAR13c, although there are many levels of olfactory signal processing between the receptor and the behavior that needs to be understood. As a first step to bridge the gap, activation of olfactory sensory neurons (OSNs) in the olfactory epithelium of zebrafish exposed to diamines with carbon chain length ranging between 3 to 10 (diaminopropane, putrescine, cadaverine, diaminohexane, diaminoheptane, diaminoctane, diaminodecane) and control stimulus (water, food) was analyzed by c-Fos immunostaining. All zebrafish were exposed to 5mM of diaminopropane-cadaverine and 2mM of diaminohexane-diaminodecane under the same conditions. Zebrafish exposed to >2mM of diaminohexane-diaminodecane do not survive for 1 hour, the time required for accumulation of c-Fos antigen in OSNs. c-Fos is a member of immediate early gene (IEG) family of transcription factors and is a neural activity marker of external stimuli, such as metabolic stress, neuronal activation and cellular trauma. c-Fos immunostaining is useful indicators of cellular activation including the identification of neurons activated by specific ligands and correlated changes in behavioral or physiological states.

The c-Fos immunostaining of olfactory sensory neurons measured for water, food and a series of aliphatic diamines shows that beyond putrescine (n=4) and cadaverine (n=5), olfactory sensory neurons (OSNs) are also significantly activated by somewhat longer carbon chain length diamines (n=6, 7, 8), but negligibly by shorter or much longer diamines (n=3, 10, respectively). The very few olfactory sensory neurons (OSNs) activated by water are possibly due to stress induced while transferring the fish into experimental setup. An increased number of olfactory sensory neurons (OSNs) were activated by food. The c-Fos labeled olfactory sensory neurons (OSNs) are sparsely distributed in a pattern similar to expression of *taar* genes in the olfactory epithelium of zebrafish (Fig. 29).

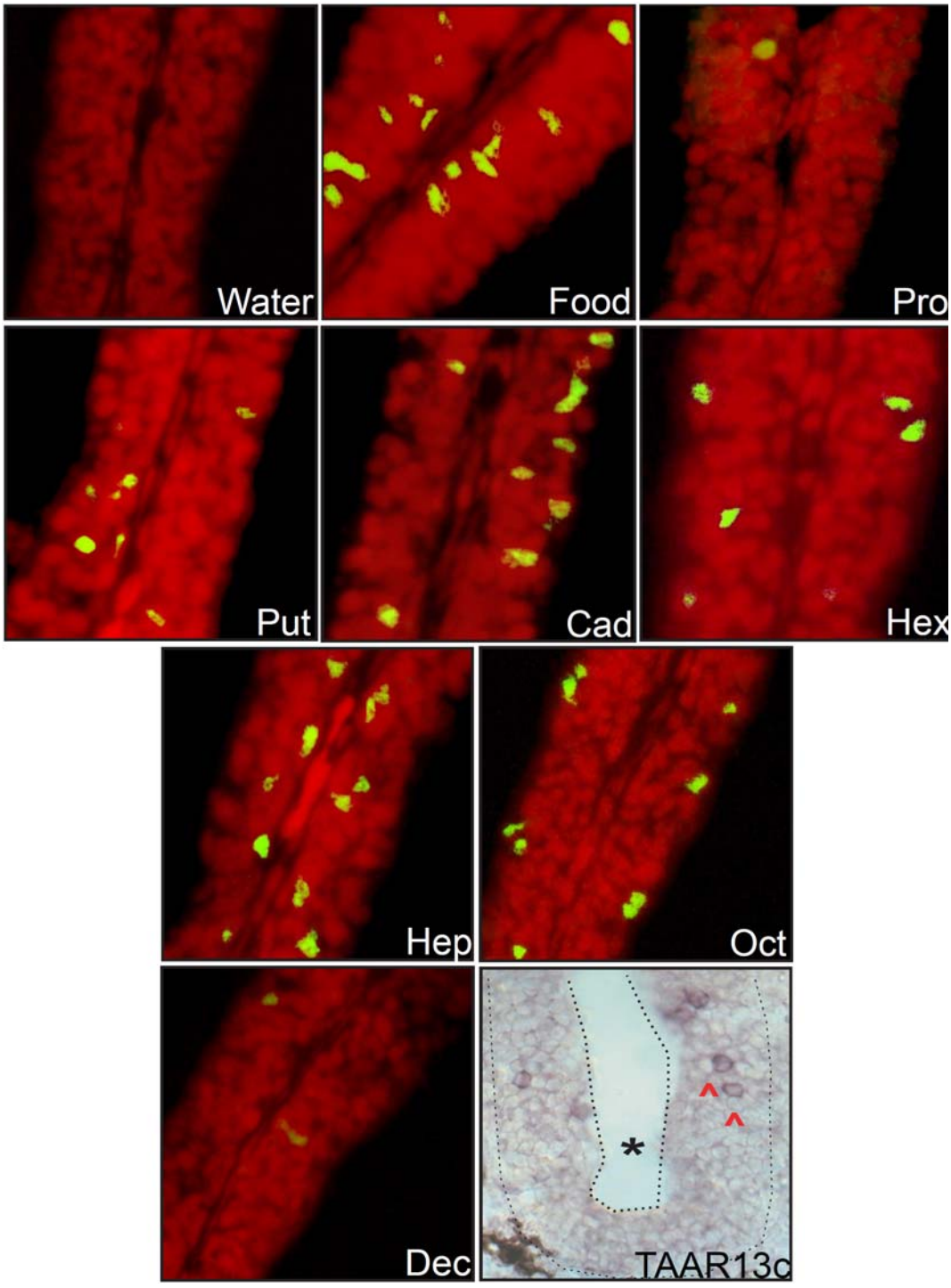

Fig. 29. c-Fos immunostaining of OSNs in the olfactory epithelium of zebrafish exposed to stimulus. OSNs are sparsely labeled for diamines (putrescine-diaminodecane). No or very few OSN was labeled for water while negligible numbers of OSNs were labeled for diaminopropane and diaminodecane. In-situ hybridization of DrTAAR13c shows sparsely labeled TAARs in the olfactory epithelium (bottom right panel), similar to c-Fos immunostaining.

The quantification of the c-Fos immunostained cells expressed in the olfactory epithelium exposed to water, food and diamines illustrates that cadaverine and diaminoheptane have the highest number of labeled OSNs while putrescine, diaminohexane and diaminooctane have relatively lower number of labeled OSNs (Fig. 30). Numbers of labeled cells in response to mock stimulus water are scant. Diaminopropane and diaminodenace have negligible number of labeled cells. Intriguingly, the chain length dependency of the c-Fos labeling (Fig. 30) closely parallels to that of the receptor activation both with respect to maximal signal size and EC50 estimates (Fig. 19).

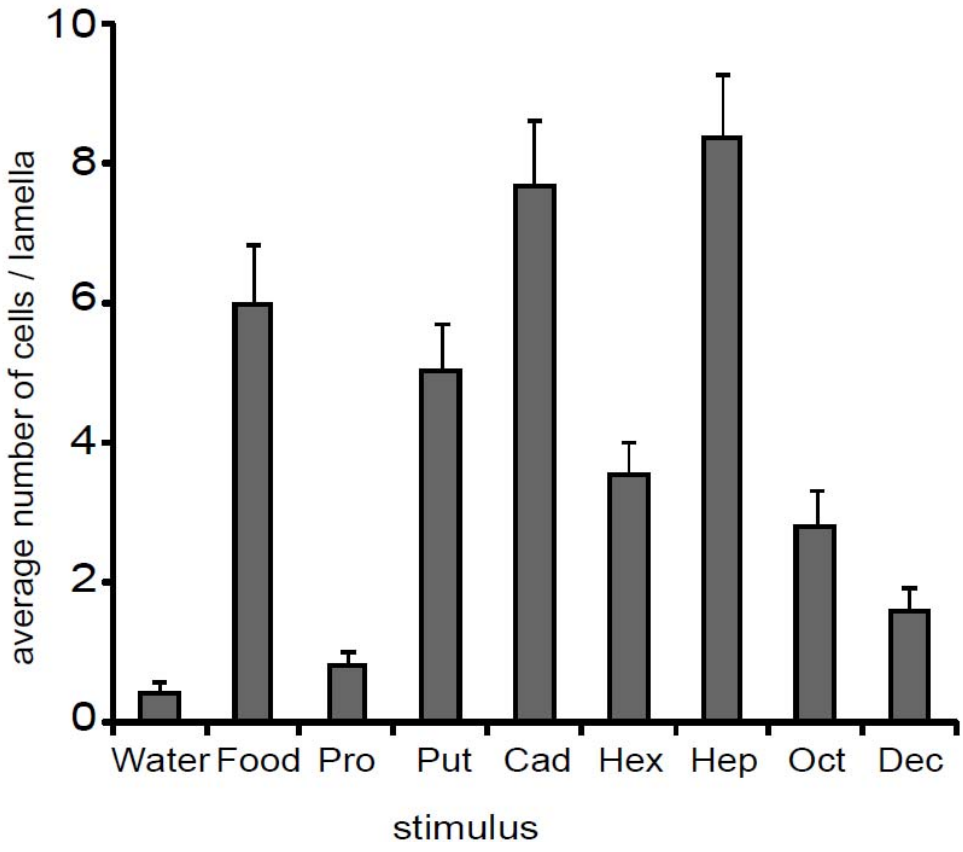

Fig. 30. Average number of c-Fos labeled cells/lamella in stimulus exposed zebrafish olfactory epithelium.

The dose response analysis of OSNs expression in response to cadaverine (0.05 - 5mM) and diaminoheptane (0.02 - 2mM) shows that number of c-Fos labeled OSNs do not increase with increased in stimulus concentration (Fig. 31). In fact, the number of c-Fos

labeled OSNs slightly decreases with increase stimulus concentration, possibly due to deterioration of olfactory epithelium.

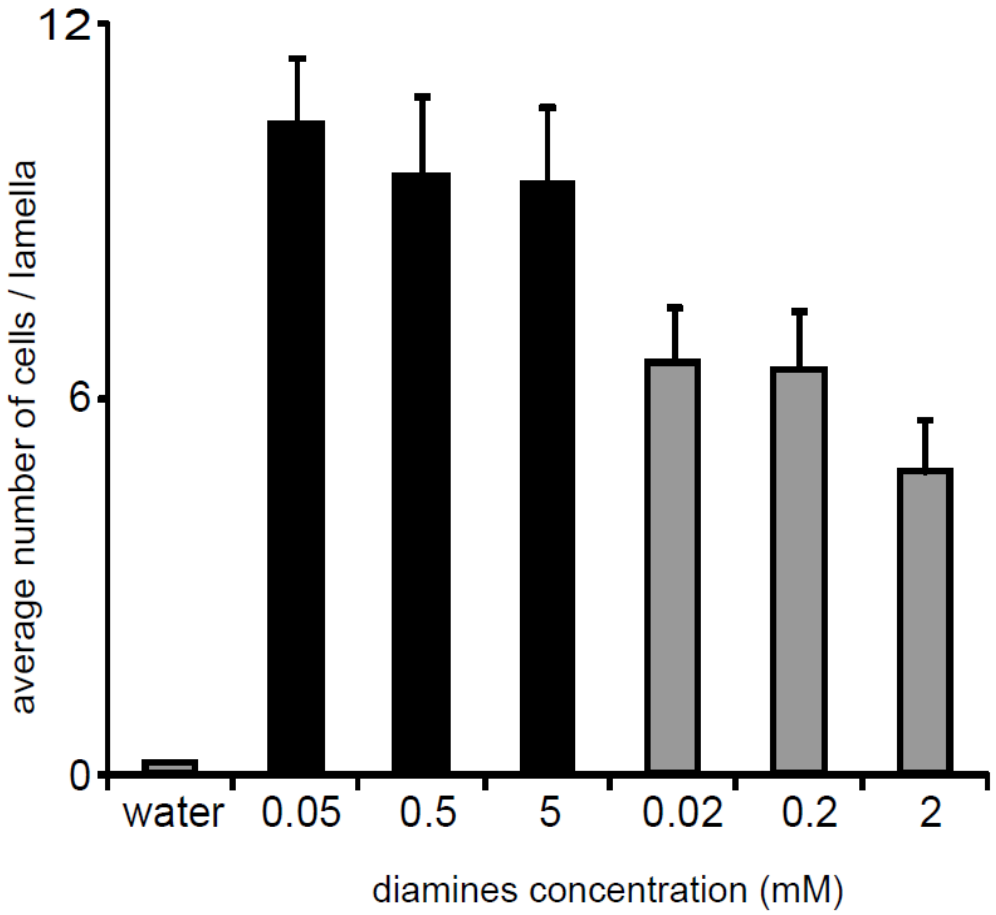

Fig. 31. dose-response analysis for cadaverine (black bars) and diaminoheptane (gray bars).

DrTAAR13c exhibit significant activity for odd numbered carbon chain length diamines (cadaverine and diaminoheptane) in CRE-SEAP heterologous system, similarly cadaverine and diaminoheptane activate higher number of OSNs in c-Fos immunostaining and also show strong behavioral phenomenon. Relatively lower activity was observed for even numbered carbon-chain length diamines (putrescine, hexamethylenediamine and diaminoctane) in the above given assays (Fig. 32). This leads to the possible hypothesis that DrTAAR13c is a receptor for odd numbered carbon chain diamines (C5, C7) and probably there is another receptors for perception of even number carbon chain diamines (C4, C6, C8).

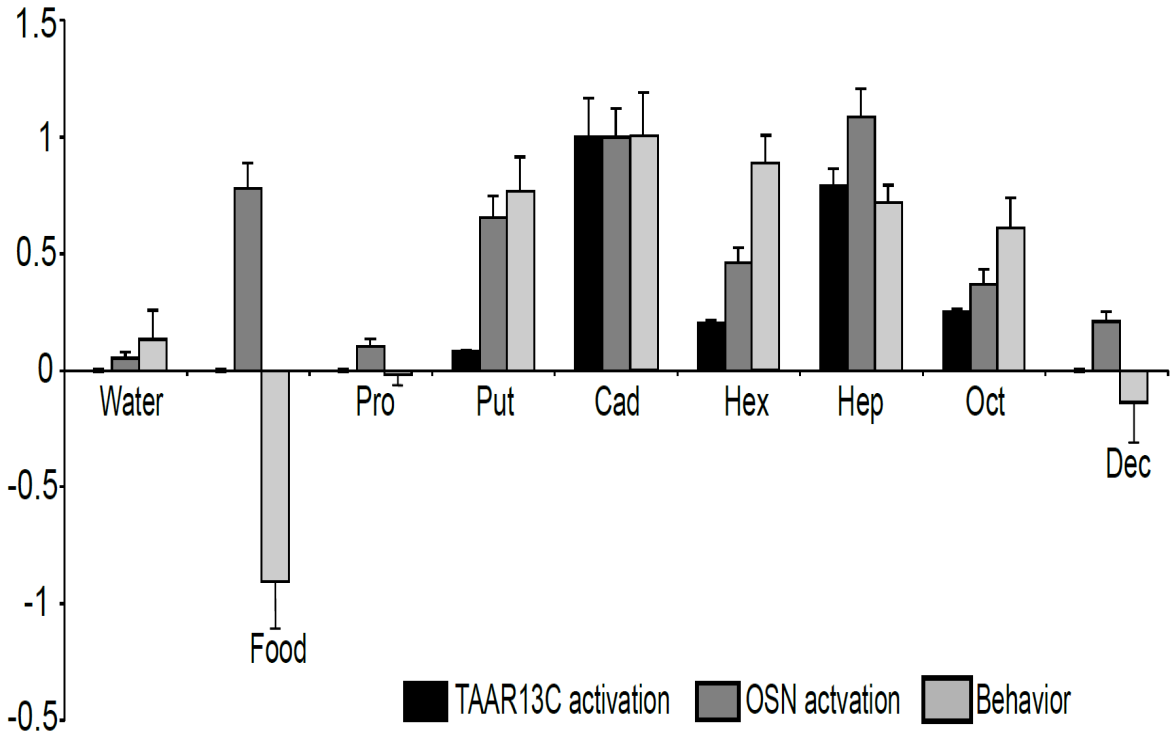

Fig. 32. Summary graph for Ligand efficiency, c-Fos and behavioral assay. The odors are indicated below the x-axis. Y-axis represents the activity values. Values are normalized to cadaverine responses. For the ligand efficiency the inverse of the Ec50 was used(a lower EC value equals a high efficiency).The activity of DrTAAR13c is higher for odd numbered carbon-chain length diamines (cadaverine and diaminoheptane) and lowers for even numbered carbon-chain length diamines (Putrescine, diaminohexane and diaminooctane).

CHAPTER 3
DISCUSSION

IX. Discussion

TAARs, unlike the other 3 families of olfactory receptor genes (OR, V1R, V2R), have not undergone major radiation in mammals. Initial aim of this study was to define the characteristic properties of the family responsible for the extensive ramification observed in teleosts. Currently, rather completely sequenced genomes are available for several teleost species, and this study takes advantage of this large improvement in data bank quality to establish the complete *taar* gene repertoire in 5 teleost fish species. Previous estimates of family size have been either too low (Gloriam et al., 2005), presumably because of incomplete databases or too high because of inadequate delineation of the *taar* gene family from the related aminergic neurotransmitter receptors (Hashiguchi and Nishida, 2005). In our experience, it is necessary to include representatives from all major aminergic receptor families to obtain a proper delineation of the *taar* gene family, which is supported by the presence of the characteristic TAAR fingerprint motif (Lindemann and Hoener, 2005). In this analysis, all lamprey receptors previously considered TAARs (Hashiguchi and Nishida, 2005) clearly segregate with teleost and tetrapod aminergic receptors and not with teleost or tetrapod *taar* genes. Despite an extensive search, no further lamprey *taar* genes were found. Consequently, the origin of the TAAR family appears to be more recent than previously thought. The discovery of shark *taar* genes allows us to place the origin within the MRCA of cartilaginous and bony fish. Unexpectedly, the major clade of *taar* genes, class III, emerged even later, within the teleost lineage of bony fishes, i.e., after the segregation from the tetrapod lineage. This clade shows several exceptional properties that stand out from class I and II *taar* genes (and, incidentally, from all other known olfactory receptor gene families). Class III contains three-fourths of all teleost *taar* genes and exhibits no evidence of gene loss, in contrast to the loss of class II and TAAR1 in neoteleosts.

A hallmark of class III *taar* genes is the strong positive selection suggested by the unusually high dN/dS ratios observed in this clade. Three species-specific subfamilies of class III show dN/dS ratios _1 at many individual sites, 10-fold above the maximal number determined for class I and II genes, which are comparable with ORs and V2R-like OlfC genes in this respect (Alioto and Ngai, 2005; Alioto and Ngai, 2006). Not a single positively selected site was found in another group of olfactory receptor genes, the V1R-

like ORAs (Saraiva and Korsching, 2007). Positive selection is a rare event genome wide (Bakewell et al., 2007) ; thus, its large frequency in class III *taar* genes high above that found in other olfactory receptor genes is very significant. A high dN/dS ratio is usually taken as evidence for a selective pressure on sequence divergence. However, because of several confounding influences, among them saturation of mutations and nucleotide bias, calculated dN/dS ratios may not accurately reflect the factual selective pressure. Nevertheless, with the possible exception of very closely (_90% amino acid homology; (Yokoyama et al., 2008)) or very distantly related genes, high dN/dS ratios appear to be a reliable indicator of positive selection (see refs. 23 and 25). The average homology for groups of *taar* genes analyzed here was nearly always in the range between 90% and 60%, predominantly _80%. Thus, the dN/dS ratios _1 obtained for several class III *taar* genes appear likely to reflect positive Darwinian selection. Once ligands become available for class III TAARs, it will be informative to directly examine the adaptive value of the divergence observed in class III taar genes. For ORs, positive selection has been argued as a mechanism to maximize the odor space recognizable by the receptor repertoire. The likely presence of extensive positive selection in the teleost *taar* gene family supports a role as olfactory receptor genes.

Two independent intron gains and 2 independent intron losses, all exclusively in the neoteleost *taar* genes of class III, underscore an evolutionary dynamics unprecedented for olfactory receptors (Niimura and Nei, 2005) and beyond. Although there has been some controversy surrounding intron gains in higher eukaryotes (Carmel et al., 2007), it is now commonly thought that very few, if any, intron gains occurred during vertebrate evolution (Coulombe-Huntington and Majewski, 2007; Loh et al., 2007). Thus, the independent gain of 2 introns in a single subclade of a single gene family constitutes an extraordinary finding. Intron retainment may be favored by the selective pressure toward divergence as evidenced by dN/dS ratios _1. Taken together, the accelerated evolution of class III teleost taar genes conceivably might mark the birth of another olfactory receptor gene family.

Teleost *taar* genes from all 3 classes are expressed in generally sparse olfactory receptor neurons. The frequency of expression appears to lie in the range of that described for ORs (Weth et al., 1996) and would be consistent with monogenic expression, which

already has been demonstrated for mammalian TAARs (Liberles and Buck, 2006). The mostly intermediate position of labeled neurons in the apical–basal dimension of each lamella is consistent with an expression in ciliated receptor neurons (Sato et al., 2005), which again would be analogous to the mammalian situation. TAARs are expressed in ring-like domains similar to those described for teleost ORs ((Weth et al., 1996), possibly suggesting some similarity in regulation of expression of ORs and TAARs. The ligands of teleost TAARs from class I and class II may include amines (Liberles and Buck, 2006; Lindemann et al., 2005) for mammalian TAARs, consistent with the presence of the aminergic ligand motif (9) and the detection of amines by the fish olfactory system (Rolen et al., 2003). A comprehensive analysis of ligand spectra for a representative subset of *taar* genes will be required to obtain a robust understanding of olfactory representation of the amine group of odors at the peripheral level. The absence of the aminergic ligand motif in class III genes suggests an evolutionary shift in ligands, away from amines, for this largest class of teleost TAARs. An understanding to what extent the rapid evolution of class III *taar* genes may enable rapid adaptation to changing ecologies both within and between species will have to await the identification of ligands for these receptors. The genesis of class III appears to be already the second shift in function in the evolution of the TAAR family. The earlier shift occurred during the genesis of the class I and class II genes, because the most ancient of all extant *taar* genes found in teleosts and tetrapods, TAAR1, is not an olfactory receptor and not detected in either zebrafish or mouse olfactory epithelium (Liberles and Buck, 2006). Thus, the TAAR family appears to have begun its existence with a function different from the one currently emphasized.

The olfactory receptors of teleosts including zebrafish are orphans (without known ligands) except one member of OlfC, OlfCa1 (Alioto and Ngai, 2006). OlfCa1 perceives amino acids with different affinity in a heterologous expression system. It is also possible that most OlfC receptors will turn out to bind amino acids, since they share a predicted amino acid-binding motif (Alioto and Ngai, 2006). Polyamines have been recognized as olfactory stimuli for an actinopterygii, goldfish *Carassius auratus* (Rolen et al., 2003). Interestingly, the optimal ligands for the goldfish receptor are basic amino acids, whereas the zebrafish receptor perceive most strongly to acidic amino acids. The ligands response spectrum for few mammalian olfactory receptors is known (Krautwurst et al., 1998; Liberles et al., 2009). Although there is an observation for relaxed specificity of

ligand i.e., structurally related compounds can excite a particular receptor, yet there can be drastic differences based on the physicochemical nature of the ligands i.e TAARs recognize hydrophobic, volatile amines. Teleost and tetrapod V1R and V2R could in principle have similar sets of ligands, because their ligands are expected to be hydrophilic and are transported through mucosa. However, the available data do not hold up in favor of this hypothesis.

DrTAAR13c responded specifically to diamines in a ligand spectra of 95 different chemicals including amino acids, amino + structurally related compounds, monoamines, diamines, polyamines and others (see Supplementary Table. 5 for details). The four other members of the TAAR13 subfamily did not respond to diamines or any of the other chemicals possibly because every olfactory receptor has its specific set of ligands that that can activate it. High activity of DrTAAR13c was observed for cadaverine and diaminoheptane (odd number diamines) and relatively low activity was observed for putrescine, diaminohexane and diaminoctane (even numbered diamines) suggesting that DrTAAR13c is receptor for cadaverine and diaminoheptane (odd number diamines) and possibly there is also another receptors for putrescine, diaminohexane and diaminoctane (even numbered diamines). No response was observed for compounds similar to diamines like monoalcohols and monoamines. This advocates that the ligand binding pocket of DrTAAR 13c require two remote positive charges for activation. Putrescine and cadaverine are bacterial decarboxylation products of amino acids. A physiologically natural source of diamine odors might be dead conspecifics, whose presence presumably would signal danger. Indeed, rotten but not fresh fish extract does activate TAAR13c (Fig. 22) and a HPLC purification of the extract from rotten zebrafish shows cadaverine as most abundant diamine, with smaller quantities of putrescine and histamine also present. Polyamines usually induce activation of DrTAAR13c at a higher concentration. There is evidence of a novel transduction pathway mediating detection of polyamines by the zebrafish olfactory system. The mechanism by which an increase in polyamine level leads to increase in olfactory sensitivity is still not clear. A possible explanation could involve action of polyamines on ion channels. This strengthen the idea that cadaverine may be perceived as an indicator of danger and plays a major role in avoiding the predator in the aquatic environment.

Behavior is delicate both in the form of conducting the experiment and also for concluding the results (Bally-Cuif, 2006). The main concerns of this study were to design a suitable behavioral assay, conduct behavioral experiments and demonstrate that the results are a valid measure of the behavior under consideration. Behavior study needs adequate controls, in order to ensure that the results are not due to unrelated artefacts (Bally-Cuif, 2006; Ninkovic and Bally-Cuif, 2006). A minor difference in the experimental set-up can generate different results. Precision of measurement is required to determine the specific behavior. In this behavioral assay, a great care was devoted to avoid all possible artefects including outside disturbance (visual or auditory), acclimatization stress, temperature variations, water impurity and general handling of the zebrafish. Behavior can vary according to time of day at which it is performed especially mating and feeding behavior. All behavior experiments in this study were carried out at the same time of days. Food and water were used as controls in this study.

Zebrafish moved freely in all parts of the tank but did not show any response to water (control) in pre and post-stimulus while showed a strong attraction to food (control) and spent approximately ¾ of the post-stimulus time near application point. This shows the stability of olfactory assay in a sense that behavioral response of zebrafish is induced by olfactory stimuli only. No behavioral response was observed for diaminopropane and diaminodecane, similar to no activity shown by DrTAAR13c for diaminopropane and diaminodecane in CRE-SEAP heterologous system. Significant avoidance behavior was observed for C4-C8 diamines (putrescine, cadaverine, diaminohexane, diaminoheptane and diaminoctane) although avoidance was higher for cadaverine (Fig. 27) similar to high activity of DrTAAR13c for cadaverine in heterologous system (Fig 18, 19). This leads to the assumption that DrTAAR13c may be the possible olfactory receptor involved in perception and generating behavioral response to putrescine and cadaverine. A knockout of DrTAAR13c will give a solid answer of this assumption.

One more evidence in this regards comes from c-Fos immunostaining of the OSNs. No activation of OSNs was observed for water, diaminopropane and diaminodecane while putrescine (n=4) and cadaverine (n=5), and somewhat longer carbon chain length diamines (n=6, 7, 8) showed activation of OSNs (Fig. 29). Intriguingly, the chain length dependency of the c-Fos labeling closely parallels that of the receptor activation both with

respect to maximal signal size and EC50 estimates (Fig. 18, 19). The ligand spectrum of the DrTAAR13 olfactory receptor closely parallels the behavioral effectiveness of these diamines. The chain length dependence of the behavioral response is highly similar to that of receptor and olfactory sensory neuron activation. The behavioral response to cadaverine may be fully explained by a singular TAAR receptor, whereas the behavioral response to putrescine appears to be predominantly via another, so far unidentified receptor. This data is consistent with the existence of a defined neuronal circuit in vertebrates that elicits a characteristic innate behavior upon activation of a single olfactory receptor by an ecologically relevant stimulus.

CHAPTER 4
MATERIALS and METHODS

X. MATERIAL AND METHODS

1. Experimental Materials

1.1. Animals

Wild-type zebrafish of the Ab/Tü strain (mix between the Oregon and Tubingen strains) were used for insitu hybridization, c-Fos immunostaining and for behavioral assay. Adult zebrafish (Danio rerio) were kept in an aquaria filled with a one-to-one mixture of desalted water and tap water. Zebrafish were kept in groups, at a day/night rhythm of 14/10 hours at a water temperature of 28°C and fed daily with dry flake foods and brine shrimp (artemia; Brustmann, Oestrich-Winkel).

In order to bring out controlled reproductivity, selected females and males fish were put into the same tank separated by transparent wall, a day before mating. Early in the following morning, fish were then put in another tank without separation, to mate freely. Fertilized eggs were collected. Zebrafish embryos and larvae were kept in petri dishes at a density of about 50 embryos/petri dish in embryo medium (E3: 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 MgSO4, Methylenblue 5-10%) at 28°C without feeding for the first five days of post fertilization (dpf).The embryos were then raised and collected at 24h intervals for histological and immunohistochemical processing. Embryos fixed at a stage older than 24 h postfertilization (hpf) were raised in 2 mM 1-phenyl-2-thiourea (PTU) in embryo medium after the epiboly stage (about 12 h) to prevent pigmentation. The embryonic and larval stages used for all investigations reported here ranged between one and 21 days postfertilization (dpf).

1.2. Chemicals suppliers

Chemicals used for this TAARs study were from Amersham Pharmacia Biotech (Freiburg), Applichem (Darmstadt), Ambion (Austin, USA), JTBaker supplied by Fisher Scientific (Schwerte), Biozym (Hessisch Oldendorf), Calbiochem (Darmstadt), GIBCO/Invitrogen (Karlsruhe), Clontech (USA), Difco (Detroit, USA), Fluka (Neu-Ulm), Merck (Darmstadt), Molecular Probes (Leiden, NL), Roth (Karlsruhe), Serva (Heidelberg) and from Sigma (Deisenhofen) .

1.3. Plastic ware

The disposable plastic ware like 15 ml and 50 ml Falcon tubes, 6-, 24-, 48-, 96-well plates, petridishes in various sizes were from BD or Castor, purchased from Fisher Scientific or BD biosciences. 0.2 ml PCR tubes and sterile pipette tips were from M_P supplied by Fisher Scientific. Sterile pipette tips were also purchased from ratiolabs and nerbe plus (Germany). Gloves (white and blue) were purchased VWR (Germany). Non-sterile pipette tips were supplied by LaFontaine (Forst/Bruchsal) and Labomedic (Bonn).

1.4. Preparation of solutions

Solutions were prepared with distilled water from milli-Q (Millipore). Solutions were autoclaved for 20 min at 121 bar or filter sterilized (0.2-0.45 µm pore diameter). Glassware was autoclaved and oven baked for 2 h at 180°C. For RNA-work, solutions and water were treated with 0.1% diethylpyrocarbonate (DEPC), shaked vigorously and mixed for about 20 min on a magnetic stirrer (IKAMAG-RET) to bring the DEPC into solution. The solutions were then autoclaved to remove any trace of DEPC. Tris buffers cannot be treated with DEPC because it reacts with primary amines. DEPC decomposes rapidly into CO2 and ethanol in the presence of Tris buffers. Therefore, Tris buffers were prepared by using water that has been treated with DEPC first. Most of the standard stock solutions like EDTA, Tris, TAE, TBE, TE, PBS, SDS, SSC, NaOAc, and culture media like LB and SOC were prepared as described in (Sambrook J 1989).

1.5. Laboratory equipment

General lab equipments were used for the molecular and cell biology techniques, including – balances, centrifuges, electrophoresis equipment, electroporation pulser, heating blocks and plates, hybridization and incubation ovens, micropipettes, PCR and gradient thermocyclers, pH meter, shakers, sterile hood, UV transilluminator, vortexes and waterbaths. Fresh frozen sections were obtained using the Cryostat CM 1900, Leica. A Nikon SMZ-U binocular microscope equipped with Nikon CoolPix 950 digital camera attached was used to document whole mount images. A Zeiss AxioVert microscope with an attached Diagnostic Instruments Spot-RT camera was used to document non-fluorescent images. A fluorescent microscope Zeiss Axioplan I Imaging equipped with Apotome and HRm AxioCam (Zeiss, Germany) was used to document fluorescent images of tissue in sections.

1.6. Nucleotides

Nucleotides for PCR, in situ-PCR, reverse transcription, and for in vitro transcription were purchased from Invitrogen Life Technologies (Karlsruhe).

1.7. Bacterial strain

Escherichia coli XL1 Blue MRF' (Stratagene, Heidelberg) bacterial strain was used for DNA amplification. CERTOMAT BS-1 from B.Braun biotech international (Germany) was used to inoculate bacteria @ $37C^0$.

1.8. Enzymes

Restriction enzymes used were either from New England Biolabs (Schwalbach, Taunus) or from Amersham Pharmacia Biotech (Freiburg). T3, T7, and SP6 RNA Polymerase, T4 DNA Polymerase, Taq DNA Polymerase, Expand High Fidelity Taq Polymerase, Expand Long Template Taq Polymerase, T4 DNA ligase, were purchased from Roche Biochemicals (Mannheim). Reverse Transcriptase Superscript II was purchased from Invitrogen Life Technologies (Karlsruhe) or from Bioline (Luckenwalde). RNase-free DNase RQ1 was from Promega (Mannheim), RNaseA and Proteinase K was purchased from Sigma or Roche Biochemicals (Mannheim).

1.9. Plasmids and vectors/properties

The plasmids used were the following: pGEM-T, Promega 3 kb; B/W; T vector; ampicillin resistance pBluescript II KS(+) ,Stratagene, 2.96 kb; B/W; ampicillin resistance ,pDrive , Qiagen; 3.85 kb; B/W; ampicillin and kanamycin resistance B/W: blue/white selection possibleDescription

1.10. Primary antibodies

1:200 c-Fos (K-25) rabbit polyclonal (Santa Cruz), 1:500 Anti-DIG sheep Fab fragment coupled with alkaline phospatase, Roche, 1:500-1000 Anti-Flu sheep Fab fragment coupled with alkaline phospatase, Roche, 1:500-1000.

1.11. Secondary antibodies

Donkey Y-rabbit, Alexa Fluor 488 coupled, Molecular Probes, 1:200 Donkey Y-rabbit, Alexa Fluor 594 coupled, Molecular Probes, 1:200

1.12. Dyes, substrates, embedding media and counter stains

1.12.1. Alkaline phosphatase substrates

NBT/BCIP (Roche Biochemicals) blue/violet chromogenic precipitate, HNPP/Fast Red (Roche Biochemicals) red chromogenic and fluorescent precipitate.

1.12.2. Horseradish peroxidase substrates

Diaminobenzidine (DAB) (Roche Biochemicals) brown chromogenic precipitate Alexa Fluor 488 and 594 tyramide from the TSA kit with HRP-Streptavidine (Molecular Probes, Invitrogen detection technologies).

1.12.3. Embedding media

Vectamount (Vector) embedding medium for chromogenic substrates Vectashield (Vector) embedding medium for fluorescent substrates and dyes; good bleaching retardant.

1.12.4. Dyes and counterstains

Vectashield contains DAPI that is used as counterstaining for the nuclei.

1.13. Oligonucleotide primers

Oligonucleotide primers were purchased from Invitrogen Life Technologies. The primers were delivered or dissolved at a standard concentration of 100 mM. Working dilutions were prepared at a concentration of 10 mM and stored at -20°C. Primers were used for different purposes like sequencing, cloning, and for preparation of in situprobes, by addition of T3-RNA Polymerase binding site (TATTAACCCTCACTAAAGGGAA). All used primers are listed below:

Table.3 Primer sequences for cloning TAAR genes

Primer name	Primer sequences
DrTaar1-Fw	ATGGATCTCTGTTATGAGGCG
DrTaar1-Rev	GATGTAGAAGGAAAACACAGAGGTG
DrTaar10-Fw	ATGGACCTAAGCAATTCA
DrTaar10-Rev	TACCATCGCAAATCCAACAA
DrTaar11-Fw	T C A G A G T C A T C A G T G G T C T G C
DrTaar11-Rv	T C C A A C A A A A G T T T G G A T T T A T C T C
DrTaar12f-Fw	ATGAAGCCTTCAAATGAGAC
DrTaar12f-Rev	GTCACAAATGGCCCAGTACC
DrTaar12l-Fw	TGACTTCAAATGAGACTCAAACTG
DrTaar12l-RV	TCAAGGTGCTTGAGTTACCAAA
DrTAAR13c-Fw	ATGGATTTATCATCACAAG
DrTAAR13c-Rev	AACTGACCACAAGGCATTGAA
DrTaar14d-Fw	ATGAATCTTACAGCAGTGA
DrTaar14d-Rev	AATGGCAAAACACACTGCTG
DrTaar14e-Fw	CAGCAGTGAACCAAACTGATATG
DrTaar14e-Rv	TCACATTCATCAGCGAGGAG
DrTaar15a-Fw	ATGGAATTTCAAGAGC
DrTaar15a-Rev	TGGTGCAATAAATGTAACTATTAAGTC
DrTaar16c-Fw	TGGACAATCGATCACTCCAG
DrTaar16c-Rv	CATGTGTGCTTCTGGGAACA
DrTaar17b-Fw	A T G A A A G G A C A G A A A G G A G A
DrTaar17b-Rv	T C A T G A A T T A T T T G T A A A A
DrTaar18a-Fw	A T G A A A G G A C A G A A A G G A G A
DrTaar18a-Rv	T C A T G A A T T A T C T T T A A A A
DrTaar19l-Fw	ATGAAAGGACGGAAAGGAGAGC
DrTaar19l-Rev	ACACATGTCTGTTCTGTTTGAAGTG
DrTaar19p-Fw	A T G A A A G G A C A G A A A G G A G A A
DrTaar19p-Rv	T T A C A G T T C A T G T A C T G T A A A
DrTaar20c1-Fw	GAAAGGACAGAAAGGAGAGCA
DrTaar20c1-Rv	TCAGAGAGGACGCAAAGTGA

DrTaar20t-Fw	ATGAAAGGACAGAAAGGAG
DrTaar20t-Rev	CTCTCCATAACATTCATCTGTTCC

Table.4 Primer sequences for in situ hybridization probe

Primer name	Primer sequences
DrTaar1-Fw	ATGGATCTCTGTTATGAGGCG
DrTaar1-Rev	GATGTAGAAGGAAAACACAGAGGTG
DrTaar10-Fw	ATGGACCTAAGCAATTCA
DrTaar10-Rev	TACCATCGCAAATCCAACAA
DrTaar12f-Fw	ATGAAGCCTTCAAATGAGAC
DrTaar12f-Rev	GTCACAAATGGCCCAGTACC
DrTAAR13c-Fw	ATGGATTTATCATCACAAG
DrTAAR13c-Rev	AACTGACCACAAGGCATTGAA
DrTaar14d-Fw	ATGAATCTTACAGCAGTGA
DrTaar14d-Rev	AATGGCAAAACACACTGCTG
DrTaar15a-Fw	ATGGAATTTCAAGAGC
DrTaar15a-Rev	TGGTGCAATAAATGTAACTATTAAGTC
DrTaar19l-Fw	ATGAAAGGACGGAAAGGAGAGC
DrTaar19l-Rev	ACACATGTCTGTTCTGTTTGAAGTG
DrTaar20t-Fw	ATGAAAGGACAGAAAGGAG
DrTaar20t-Rev	CTCTCCATAACATTCATCTGTTCC

Forward and reverse primers are shown, the latter only with their gene-specific sequence (a T3-specific promoter site is added in 5' position). PCR was performed using the following conditions: 5 min at 96°C, followed by 35 cycles of 30 sec at 96°C, 30 sec at Tm (°C), and 60 sec at 72°C, and a final extension of 10 min at 72°C. Tm was 60ºC for Taar1, Taar10, Taar12f, Taar19l and Taar20t; 50ºC for Taar13c, Taar14d and Taar15a. The templates for the RNA probes were amplified from the cloned DNA using the same forward primers as above and reverse primers with the described T3 promoter site (TATTAACCCTCACTAAAGGGAA) attached to their 5’ end.

2. Molecular biological techniques

Standard molecular biology techniques such as genomic DNA extraction, PCR, Colony PCR, DNA amplification by small and large scale plasmid DNA preparations, quantification of DNA and RNA, agarose gel electrophoresis, restriction enzyme digestion, isolation of DNA fragments, ethanol precipitations, filling up reactions of 3' and 5' overhangs, dephosphorylation of 5'ends, ligation of DNA fragments, preparation and transformation of competent cells were essentially performed as described in (Sambrook J 1989).

2.1. Isolation, purification and quantification of DNA and RNA

2.1.1. Isolation of genomic DNA

Genomic DNA from the whole adult zebrafish was isolated according to Hogan et al., 1986. Adult zebrafish were decapitated and internal organs were removed. The tissue was frozen in liquid 91 nitrogen and pulverized. After addition of lysis buffer (0.1 M Tris/HCl, 0.2 M NaCl, 5 mM EDTA, 0.2% w/v SDS, pH 8.5) and proteinase K (150 µg/ml) the tissue was incubated under continuous rotation at 55°C overnight. Undissolved material was pelleted. After a phenol/chloroform extraction the DNA was precipitated using 1/10 volume of 3 M NaOAc (pH5.2) and 2 volumes of 100% ethanol. The DNA was washed two times with 70% ethanol, dried and dissolved in 100-500 µl H2O overnight at 4°C.

2.1.2. Genomic DNA PCR

Genomic PCR was carried out using 0.5 ug of genomic DNA. Genomic PCR for TAAR genes was carried out under these conditions.

96 C^0 5:00[**96 C^0** 1:00; **48 C^0** 1:00; **72 C^0** 1:30]40x; **72 C^0** 10:00; **4 C^0** infinite

Which means an initial denaturing step of five minutes at 96 **C^0** followed by 40 cycles of 94 **C^0** for 1minute, **48 C^0** for 1minute and **72 C^0** for 1minute and 30 seconds minutes and then a final extension at 72oC for 10 minutes.

2.1.3. Quantitation of DNA and RNA

The concentration of DNA and RNA in solution was estimated using agarose gel electrophoresis by comparing the intensity of the bands of interest with the 1 kb band of a 10kb ladder (DNA-Hyperladder, Bioline) of known concentration. RNA samples were denatured in 50% formamide for 3 min at 100°C before loading.

2.1.4. Agarose gel electrophoresis

DNA and RNA were loaded on 1% agarose gels containing 0.5 µg/ml ethidium bromide in 1 x TAE Buffer and run at 5-10 V/cm. Genomic DNA, was loaded on low concentration agarose gels (1%) gel and run slowly (1-2 V/cm) to ensure better separation and to avoid smearing the DNA. The loading dye used was purchased from Bioline. The DNAHyperLadder I (Bioline) was used for estimation of molecular weight.

2.1.5. Isolation of DNA fragments from PCR products or agarose gels

DNA fragments were isolated from agarose gels according to the manufacturer's instructions. In general QIAquick Gel Extraction Kit (Qiagen), QIAquick PCR Purification Kit (Qiagen) or Roche High Pure PCR Product Purification Kit (Roche Biochemicals) was used. All these kits make use of a column filled with a silica-gel membrane. DNA adsorbs to the silica-membrane in the presence of high salt while contaminants pass through the column. Impurities are washed away and the pure DNA is eluted with Tris buffer (low salt condition).

2.1.6. Ligation of DNA fragments and PCR products

Ligation reactions were used to combine vector and insert DNA. For this purpose purified insert DNA was ligated to dephosphorylated vector DNA using T4 DNA ligase (Roche Biochemicals) according to (Sambrook J 1989) et al., 1989 and the supplier’s instructions. PCR products (1-4 µl) were ligated directly after amplification into the pBluescript II SK+ (Stratagene), pDrive (Qiagen) or pGEM-T (Promega) vector according to the manufacturer's instructions.

2.1.7. Subcloning of DNA fragments by electroporation

For electroporation the bacterial suspension of XL1 Blue strain was thawed on ice, mixed with 1-2 µl of ligation mixture and after a 1 min incubation at room temperature transformed using 1 mm cuvettes and the GenePulser from BioRad at E=18 kV/cm, C=25 µF, R=200 W. After transformation 1 ml of pre-warmed LB medium was added to the bacteria and they were incubated for 1 h at 37°C before plating on ampicillin (50 µg/ml) and tetracyclin (150 µg/ml) plates. In cases where blue-white selection was possible X-gal (800 µg/ml) and IPTG (0.5 mM) were added to the plates. The bacteria were grown on agar plates overnight at 37°C.

2.1.8. Subcloning of DNA fragments by DH5α chemically competent E. coli

DH5α Chemically Competent E. coli is an effective method of subcloning mostly used in this study. The ligation reaction was briefly centrifuged and place on wet ice. The tube of DH5α cells was also thaw on ice.DH5α cells were gently mixed with pipette were made aliquot 50 or 100µl .1 to 5 µl (1-10 ng DNA) of ligation reaction was added directly into the competent cells and mix by tapping gently. Vials were incubated on ice for 30 minutes. Then vials were heat-shock for exactly 20 seconds in the 37°C and were placed on ice for 2 minutes.1Ml pre warmed Lb medium was added to each vial. Vials were shaked at 37°C for exactly 1 hour at 225 rpm in a shaking incubator. 100µl of media from each vial was spread on labeled LB agar plates. Plates were inverted and incubated at 37°C overnight.

2.1.9. Colony PCR for identification of positive clones and determination of insert length

Single bacterial colonies were picked and inoculated in LB medium containing the appropriate antibiotic in 96 well multititer plates. The bacteria were grown for one to three hours in an orbital rotator at 37°C. Five µl of bacterial suspension was used as a template in a PCR reaction. All PCR reactions were carried out in a final volume of 20 µl containing 1 x PCR buffer, 1.25 mM MgCl2, 10 pmoles of each primer, 0.1 mM of each dNTP, 1 U of Taq DNA polymerase (from Roche Biochemicals). Generally M13 primers or other vector primers like T3 and T7 primers were used. Clones that were positive in the PCR were used to inoculate 3 ml of LB medium. DNA was extracted using the small-scale preparation of DNA protocol and digested to confirm the positive result of the PCR. Single clones were then subjected to sequence analysis.

2.1.10. Small scale plasmid DNA preparation (Miniprep)

In cases where the recombinant E. coli clones had to be identified, plasmid DNA was isolated in small scale. Single colonies of interest were inoculated into LB-medium (10 g Tryptone, 5 g Yeast extract, 10 g NaCl; pH 7.0) containing the appropriate antibiotics (ampicillin [100 µg/ml], tetracycline HCl [50 µg/ml], or kanamycin [50 µg/ml]) in a volume of 3 ml and grown in an orbital shaker (~300 rpm) at 37°C overnight. In general, a miniprep kit (either from Qiagen or from Sigma) was used for this purpose. The plasmid purification protocols are based on a modified alkaline lysis procedure (Birnboim and Doly, 1979) followed by binding of plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins, and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation.

2.1.11. Phenol/chloroform extraction

Reaction mixtures that had a smaller volume than 200 µl were adjusted to this volume using H2O and phenol-chloroform extracted using an equal volume of phenol-chloroformisoamylalcohol (PCI = 25:24:1). This mixture was vortexed and centrifuged for 1 min. The aqueous phase was carefully transferred to a new reaction tube and extracted again using 92 200 µl of PCI. The aqueous phase was then extracted using 200 µl chloroform. The aqueous phase was then ethanol precipitated as described in the next section.

2.1.12. Ethanol precipitation

DNA was precipitated using 0.3 M sodium acetate and two to three volumes of ice-cold absolute ethanol. RNA was precipitated using 0.8 M lithium chloride and 2.5 volumes of icecold absolute ethanol. Precipitation was allowed at -20°C for 30 min or at -80°C for 10 min. After centrifugation at maximum speed for 30 min in case of DNA and 20 min in case of RNA at 4°C, the pellets were washed with 70% ethanol air-dried and re-suspended in the appropriate buffer.

2.1.13. Restriction enzyme digestion of DNA

Digestions for characterization of plasmid DNA were performed using about 200 ng of plasmid in 1 x restriction enzyme digestion buffer and 6 U of restriction enzyme in a total volume of 20 µl. Digestion mixtures were incubated for 1-2 h at the appropriate temperatures for each enzyme as suggested by the manufacturer.

2.1.14. Preparation of glycerol stocks

Glycerol stocks were prepared by adding 150 µl of sterile glycerol to 850 µl bacterial culture, vortexing to ensure even dispersion of the glycerol and freezing in liquid nitrogen. Afterwards, tubes were transferred to -80°C for long-term storage.

2.1.15. Sequencing of DNA

DNA sequencing was carried at the core facility of the Institute of Genetics by Rita Lange on an ABI Prism 3730 DNA Analyzer (Applied Biosystems, USA). For each cycle sequencing reaction, the following reagents were added into each tube: 2□l of BigDye terminator premix (ABI Prism), 3.2pmol primer, 100ng of purified plasmid DNA and autoclaved distilled water to a final volume of 10□l. Then, the mixture was mixed and briefly spun down. The sequencing profile used was as follows: 40 cycles at 950C for 20 sec, 500C for 15 sec and 600C for 4 min. The samples were ethanol precipitated and dried thoroughly. The dried samples were stored at -20°C in the dark until they were electrophoresed. Sequence analysis was carried out using BLAST (Basic Local Alignment Search Tool), accessed through the Internet (http://www.ncbi.nlm.nih.gov/). Alignments of the sequences with several closely related genes were carried out subsequently.

3. Histological studies

3.1. Preparation of cover slips

Cover slips were treated with Repel Silane (Amersham Pharmacia Biotech) to inhibit the binding of antibodies and probes to them. Cover slips were dipped into Repel Silane, acetone and absolute ethanol for 5 sec each and air-dried in a dust-free place on the back of a microtiter plate that was used as a rack.

3.2. Tissue preparation and sectioning

Adult zebrafish were decapitated with a sharp scalpel. The head was put immediately in a petridish containing ice-cold PBS, pH 7.4. Barbels attached with lips, olfactory epithelia, olfactory bulbs, whole brains, gills, hearts and livers were dissected out.

3.3. Cryosectioning

For cryostat sectioning, tissues were put in TissueTek (MILES, Elkhart, Indiana, USA), oriented and frozen at -20°C. Olfactory epithelia were sectioned at 10 µm. Sections were mounted on coated Superfrost plus slides and dried for 3 h at 55°C. Sections were used immediately, since it was found that storage impaired the signals in the in situ hybridization.

3.4. Immunohistochemistry (IHC)

3.4.1 Antibody Staining on Fresh Frozen Cryostat Sections

Sections were fixed in 4% PFA for 10-20 min at room temperature (or alternatively overnight at 4oC) and washed three times for 10 min each in PBS 1x (pH 7.5). Tissue in the slides was then dried by incubation in aceton for 15 min at -20oC. The slides were washed 3 x for 5 min in PBST (PBS + 0.1% triton-100). Blocking was done in 5% normal goat serum (NGS) in PBST for at least one hour at room temperature. The tissue was then incubated with the primary antibodies (c-Fos) in 5% NGS in PBST overnight at 4°C (or alternatively at room temperature for 2 hours). After extensive washing in PBST (3 x 10 min), the sections were incubated with the correspondent coupled Alexa-488 or -594 secondary antibodies in PBST for 2 hours at room temperature. The sections were mounted and embedded in Vectashield (Vector).

3.5. *In Situ* Hybridization (ISH)

In situ hybridization to cellular RNA was used to determine the cellular localization of specific TAAR genes within complex cell populations and tissues. Various methods were used for different purposes.

3.6. Labeling of RNA using Digoxigenin, Biotin or Fluorescent *in vitro* transcription

A range of probes can be used for the detection of mRNA in situ hybridization experiments. However, in vitro transcribed riboprobes are the best choice on tissue sections (Cox, 1984). These probes are single-stranded and may span hundreds of nucleotides, which results in specific antisense probes with high detection sensitivity. Moreover, in vitro transcription allows the synthesis of ideal control probes, as the sense probes have identical length and G + C content, defining similar properties of hybridization compared to the antisense probes. After synthesis of the probes they were not hydrolyzed into smaller pieces, as this treatment leads to elevated background signals. Sense and antisense RNA probes labeled with digoxigenin (DIG)-, fluorescein- or biotin-labeled UTP were generated by in vitro transcription according to the manufacturer's instructions (RocheBiochemicals). Before beginning the transcription reaction, the template DNA was generated either by PCR using insert specific primers that contained the T3 polymerase promoter sequence or by linearization with a restriction enzyme. For the latter, T7 or SP6 polymerase promoter sequences in the vector backbone were utilized for transctiption. The template DNA was then purified using a PCR purification kit (Qiagen). The labeling reaction was performed in a total volume of 20-40 µl. About 200-500 ng (for PCR template) or about 1000ng (for linearized plasmid template) of plasmid was used for labeling. Transcription buffer and DIG-, fluorescein- or biotin-labeling mixture were added to a final concentration of 1 x. 4 U of RNA polymerase (T3, T7 or SP6) and 20 U of RNAse inhibitor (Roche Biochemicals) were also added. The reaction was incubated at 38°C for 2 hours and terminated by addition of 2 µl of EDTA (200 mM, pH 8.0). The RNA transcript was ethanol precipitated and analyzed for size and integrity using agarose gel electrophoresis. Labeling efficiency was estimated using DIG quantification teststrips (Roche Biochemicals).

3.7. *In situ* hybridization on sections of olfactory epithelia

Sections (10 µm) were fixed in 4% paraformaldehyde for 10 min at room temperature. Hybridizations were performed overnight at 60°C using standard protocols as previously described (Weth et al., 1996). Anti-DIG primary antibody coupled to alkaline phosphatase (Roche Molecular Biochemicals) and NBT-BCIP (Roche Molecular Biochemicals) was used for signal detection.

4. Cell culture and CRE-SEAP functional assays using HEK 293 cells

A high-throughput assay to monitor the function of TAARs was used. The activated TAARs couple to cAMP pathways in HEK-293 cells, presumably through endogenous Gαs present in these cells. This allowed monitoring TAAR function using a cAMP-dependent reporter gene, CRE-SEAP, which contains secreted alkaline phosphatase (SEAP) downstream of five tandem cAMP response elements (CRE). Zebrafish TAAR genes embedded with an amino-terminal addition of the first 20 amino acids of bovine rhodopsin (a 'rho tag'), a modification that facilitates the cell-surface expression of some odorant receptors in HEK293 cells (Krautwurst et al., 1998) were used in CRE-SEAP assay. 11 zebrafish TAAR genes including DrTAAR1, 10, 11, 12f, 13a, 13b, 13c, 13d, 15a, 16c, 20t1 were examined for 95 different chemicals separately (Fig. 16). TAARs were cotransfected in HEK293 cells using lipofectamine. HEK293 cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified 7% CO_2 incubator. cells were split by adding 3.5ml Trypsin/EDTA solution (cover bottom of flask) an incubate at 39°C for 5 min. Cellls were to 50ml conical tube containing 21.5ml DMEM+ (500mL DMEM + 5mL Penincilin-Streptomycin+25mL Fetal Bovine Serum (all from GIBCO company). Cells containg tubes were spin tube at 1,000 rpm for 5 min at 4°C and medium was aspireated and resuspended supernatant in 1ml DMEM+ (using 1 ml pipette) and 19ml DMEM was added to re-suspension. 20µl dye (Trypan blue) was added in 20 µl of cells to be counted. Cells were counted and dilute to 250,000 cells / 1mL = 50,000 cells / 200ul (per well) using the following formula:

Count = total of 4 red boxes (16 squares on each)/2
Count * 10000 = cells/1ml _ split by 1000 = x cells/1µl
50,000/ x cells = x µl of cells to add per 200 µL DMEM+ per well or alternatively:
3,000,000/ x cells = x µl of cells to add per 12 mL DMEM+ per plate (60 wells)

200µl cell dilution was added to 96-well plate as needed for assay (Each plate fills 60 wells, borders filled with PBS, that is 60 wells x 200ul = total 12 mL of $DMEM^+$ + 3,000,000 cells) and incubated O/N at 39°C. Remaining cells were split (20ml total per flask) for further use as under:2:1 dilution for 2 days growth (10mL cells+10mL DMEM+),

5:1 dilution for 3 days growth (4mL cells+16mL DMEM+), 10:1 dilution for 4-5 days growth (2mL cells+18mL DMEM+). Co-transfection was performed the next day. Co-transfection mix consisted of 20 ng plasmid with receptor (stock is at 20 ng/ul) +20 ng Cre-SEAP plasmid (stock is at 250 ng/ul) + 9 ul DMEM+1 ul PLUS reagent, that makes total volume of 10ul per well. The mixture was let sit @ RT for 15 min. After 15 minutes 50 ul DMEM was added per well+10 ul of Lipofectamine (25 xs concentrated). Mix was left to stay for 3hours.If lipofectamine stays with the cells for more than 5 hours, they die. After 3 hours 70 ul media was aspirated from the wells and 200 ul of DMEM with initial dilutions of 10uM ligands per well was added. Imaging was performed the3rd day. Plates were plastic wrapped and incubated @ $68C^0$ for 2 hours. Plates were cooled down at RT.120 ul of 0.1M MUP(4-methylumbelliferyl phosphate) + 10 mL 2M Diethanolamine Bicarbonate pH10, adjust pH with dry ice buffer was added in each plate. cyclic AMP accumulation data and CRE-SEAP-reporter gene assay data was acquired at 1, 5 and 20 minutes after adding buffer, by Envision2 plate reader.

5. Behavioral assay

The behavioral assay was performed in a glass tank (Fig. 22) with dimension of (100_X10_X20 cm). The total water capacity of behavioral tank was 18 liters. Tank was half filled (9 liters) with clean, desalted water from fish room. The temperature of the water was maintained at 28 C^0. Adult zebrafish 8 months - 1 year old were used in behavioral experiments. A single zebrafish was put into the tank water and was given 45 minutes to 1 hour for acclimatization in the tank. The stimulus was applied through a glass pipette attached to the tank. There was a barrier between the tank and application of stimulus site to avoid visual influence on the experiments. The activity of the zebrafish was monitored by HD video camera (Fig. 22) that captured video at 30 frames/seconds. A room was dedicated for behavioral experiments and maximum silence was provided. A stimulus with stock concentration of 1mM was used in each experiment, except water and food. Each behavioral experiment was carried out in two stages. First pre-stimulus stage, in which no stimulus was applied to the fish and fish activity was recorder for 5 minutes in water. Generally zebrafish is an active fish and moves freely in water. In the next stage of post stimulus (that started with the 6^{th} minute), a stimulus was applied through the glass pipette, avoiding complete visibility of the researcher to the fish. The video camera keeps

recording the post-stimulus activity. The total experiment consisting of 5 minutes pre-stimulus and 5-minutes post-stimulus was recorded in a single video shot. The 10 minutes movie of fish behavioral movement was analyzed by WINANALYZE tracking software (http://www.winanalyze.com). WINANALYZE uses a virtual tracker to make tracks of fish movements (Fig. 23a, b) in addition to providing coordinates of fish moments in pixels. The data obtained from WINANALYZE was analyzed using multiple algorithms.

6. Data Mining

6.1. TAARs

All annotated TAAR sequences were compiled and used as query in TblastN searches in the NCBI and Ensembl databanks. Additionally blastP searches were performed in the NCBI databanks and automated ortholog prediction was used in the Ensembl databank (Hubbard et al., 2007). For shark, lamprey and zebrafish, also EST databanks were searched, in addition, for elephant shark WGS sequences with 1.4 fold genomic coverage were analyzed. Search was recursive until no new candidates were found. Validation of candidates as proper taar genes required: a) position within the TAAR clade in the phylogenetic analysis; b) application of the BLASTP algorithm in the NCBI nonredundant database should result in confirmed TAARs as first hits; c) presence of typical TAAR family motifs; d) CDS length between 800 and 1300 amino acids; e) presence of seven trans-membrane domains (regions assignment according to conserved position as described in (Lindemann et al., 2005; Lindemann and Hoener, 2005). For the accession numbers of the taar genes see (Hussain et al., 2009).

6.2. Phylogenetic analysis

MAFFT, version 5.8 (http://align.bmr.kyushu-u.ac.jp/ mafft/online/server/)6.3, was used for multiple protein alignments using the E-INS-i strategy with the default parameters. Phylogenetic trees were constructed by using neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods (30, 31). Subclades within the taar gene family were determined from the tree as the largest clades that fulfilled 2 criteria: the clade had _70% bootstrap support in the NJ analysis (except the closely related families 18–20), was supported in the MP and ML, and all members within the clade had at least 40% protein identity to each other (except taar23 and 24, which cannot be resolved well and

have to be considered provisional). Twenty-eight such subclades or subfamilies were identified, comprising both previously uncharacterized subfamilies and genes from previously known subfamilies.

6.3. dN/dS analysis

The global dN/dS ratios for the full-length ORF of the 223 fish TAARs receptor coding sequences were determined by using the Single Likelihood Ancestor Counting (SLAC) package (http://www.datamonkey.org), which implements the Suzuki-Gojobori method (Suzuki and Gojobori, 1999). The nucleotide alignments were manually edited to match the amino acid alignment used in the phylogenetic trees and sequence logo. To make inferences about selective pressure (positive and negative selection) on individual codons (sites) within the coding sequence of the teleost fish TAARs genes, the Single Likelihood Ancestor Counting (SLAC) package (http://www.datamonkey.org), which implements the Suzuki-Gojobori method (Suzuki and Gojobori, 1999), was used. The algorithm is briefly outlined. First, a best-fitting nucleotide substitution model was automatically selected by fitting several such substitution models to both the data and a neighbor-joining tree generated from the alignment described above. Taking the obtained substitution rates and branch lengths as constant, a codon model was employed to fit to the data and a global dN/dS ratio was calculated. Then a codon by codon reconstruction of the ancestral sequences was performed using maximum likelihood. Afterwards the expected normalized (ES) and observed numbers (EN) of synonymous (NS) and non-synonymous (NN) substitutions were calculated for each non-constant site. dN = NN/EN and dS = NS/ES were then computed, and if dN < dS (negative selection) or dN > dS (positive selection), a pvalue derived from a two-tailed extended binomial distribution was used to assess significance. Tests on simulated data (S.L.K. Pond and S.D.W. Frost, methods available at http://www.datamonkey.org) show that p values equal or smaller than 0.1 identify nearly all true positives with a false positive rate generally below the nominal p value; for actual data, the number of true positives at a given false positive rate is lower. In the present study, two thresholds for significance (0.1 and 0.2) were taken into account in order to identify residues potentially involved in odorant-binding activities.

CHAPTER 5
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XI.REFERENCES

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XII.SUPPLEMENTARY INFORMATION

Supplementary Table.1 - List of all taar and outgroup genes

Gene Name	Synonyms	Chromosome	Location	Accession number	EST
Dr_Taar1	zTA1b	20	54463291-54464289	ENSDART00000060779	OE & Brain
Dr_Taar10		20	54426459-54427487	ENSDART00000014396	OE
Dr_Taar10a		20	54432894-54433904	ENSDART00000060795	OE
Dr_Taar10b		20	54453770-54454780	ENSDART00000060783	OE
Dr_Taar10c		20	54436728-54437738	ENSDART00000060791	Embryo
Dr_Taar10d		20	54445152-54446162	ENSDART00000060787	OE
Dr_Taar11	zTA1a	20	54458998-54459981	ENSDART00000007567	OE
Dr_Taar12a		20	46086903-46088501		Brain & OE
Dr_Taar12b	zTA69	20	54490233-54491246	ENSDART00000060770	Brain & OE
Dr_Taar12c		20	54486762-54487778	ENSDART00000060773	Brain & OE
Dr_Taar12d	zTA71	20	46067894-46068859		Brain & OE
Dr_Taar12e		20	54530232-54531245	ENSDART00000060758	OE & Brain
Dr_Taar12f	zTA72	20	54517187-54518200	ENSDART00000060763	OE & Brain

Dr_Taar12g		20	54522272-54523282	ENSDART00000037777	OE & Brain
Dr_Taar12h		20	54538161-54539195	ENSDART00000060754	Brain & OE
Dr_Taar12i		20	54477413-54478435	ENSDART00000060778	OE
Dr_Taar12j		20	54545123-54546145	ENSDART00000060750	Brain & OE
Dr_Taar12k	zTA73	20	216108-217142		OE & Brain
Dr_Taar12l		20	14367272-14368479	ENSDART00000064810	OE
Dr_Taar12m		15	2549861-2551166	ENSDART00000063348	OE
Dr_Taar13a		10	54414291 - 54415313		Brain
Dr_Taar13b	zTA64	20	54407350-54408375	ENSDART00000060799	Brain
Dr_Taar13c	zTA65	20	54388665-54389690	ENSDART00000060803	Brain
Dr_Taar13d		20	54399225-54400250	ENSDART00000060800	Embryo
Dr_Taar13e	zTA66	20	54414300-54419192	ENSDART00000060797	Brain
Dr_Taar14a		20	54353917-54354879	ENSDART00000060806	Embryo
Dr_Taar14b		20	54893962-54894948	ENSDART00000060716	OE
Dr_Taar14c		7	77815446-77818549	ENSDART00000073522	OE
Dr_Taar14d	zTA70	20	54881175-54882161	ENSDART00000060717	OE

Dr_Taar14e		20	54858946-54859932	ENSDART00000060724	OE
Dr_Taar14f		20	54876107-54877093	ENSDART00000060719	OE
Dr_Taar14g		20	54864008-54871387	ENSDART00000060720	OE
Dr_Taar14h		20	54339800-54340762	ENSDART00000060811	OE
Dr_Taar14i	zTA68	20	54335864-54336823	ENSDART00000038379	OE
Dr_Taar14j		20	54330227-54331189	ENSDART00000060815	OE
Dr_Taar14k	zTA67	20	54350249-54351205	ENSDART00000060808	OE
Dr_Taar14l		20	54345473-54346429	ENSDART00000060810	OE
Dr_Taar15a		20	54836241-54837227	ENSDART00000074482	Brain & OE
Dr_Taar15b		20	54383896-54384882	ENSDART00000060804	Embryo
Dr_Taar16a		10	45611149-45612353	ENSDART00000076403	OE
Dr_Taar16b		10	45607700-45606742	ENSDART00000076404	Brain & OE
Dr_Taar16c	zTA63	13	291485-292459		Brain & OE
Dr_Taar16d		13	625049-626563	ENSDART00000082178	OE & Brain
Dr_Taar16e	zTA62	13	633903-639694	ENSDART00000082164	Brain & OE
Dr_Taar16f	zTA36	10	45630716-45629785		Brain & OE

Dr_Taar16g	zTA35	10	45635543-45634495	ENSDART00000076382	Brain & OE
Dr_Taar17a	zTA48	10	45616694-45617728		Brain & OE
Dr_Taar17b	zTA47	10	45624185-45626594	ENSDART00000062763	OE & Brain
Dr_Taar17c	zTA49	10	45625665-45626648		Brain & OE
Dr_Taar18a		10	45541637-45542587	ENSDART00000030565	OE
Dr_Taar18b		10	45575185 - 45574090	ENSDART00000085892	OE & Brain
Dr_Taar18c		10	45571055-45569948		OE & Brain
Dr_Taar18d	zTa28	10	45549056-45550027	ENSDART00000085900	Brain & OE
Dr_Taar18e		10	45554908-45553862		OE & Brain
Dr_Taar18f	zTA61	10	45561824-45560848	ENSDART00000076422	Brain & OE
Dr_Taar18g	zTA27	10	45545167-45546210		Brain & OE
Dr_Taar18h	zTA18	10	45579595-45580647		Brain & OE
Dr_Taar18i	zTA19	10	45596234-45597303	ENSDART00000049070	Brain & OE
Dr_Taar18j	zTA20	10	45601887-45602876		Brain & OE
Dr_Taar18k		10	45601833-45602894		OE & Brain
Dr_Taar19a		10	46047546-46048523	ENSDART00000062707	OE & Brain

Dr_Taar19b		10	46083909-46085495		Brain & OE
Dr_Taar19c	zTA54	10	46091169-46092376	ENSDART00000043020	Brain & OE
Dr_Taar19d	zTA34	10	46078311-46079177	ENSDART00000032932	Brain & OE
Dr_Taar19e		10	46052727-46053710	ENSDART00000076331	OE & Brain
Dr_Taar19f	zTA59	10	46066316-46067266		OE & Brain
Dr_Taar19g	zTA33	10	46072194-46073195		Brain & OE
Dr_Taar19h	zTA50	10	45994749-45995753	ENSDART00000080193	Brain & OE
Dr_Taar19i	zTA31	10	46041846-46042847	ENSDART00000062696	Brain & OE
Dr_Taar19j		10	46036393-46037382		Brain & OE
Dr_Taar19k		10	45652022-45652966	ENSDART00000062720	OE & Brain
Dr_Taar19l	zTA32	10	45647528-45648568	ENSDART00000040322	OE & Brain
Dr_Taar19m		10	46000405-46001397		Brain & OE
Dr_Taar19n		10	46010425-46011423	ENSDART00000062709	OE
Dr_Taar19o	zTA51	10	45987464-45988465	ENSDART00000076348	OE & Brain
Dr_Taar19p		13	12610683-12612441	ENSDART00000080187	OE
Dr_Taar19q	zTA29	10	46032382-46037292	ENSDART00000054504	OE & Brain

Dr_Taar19r		10	45670389-45671369	ENSDART00000058034	OE & Brain
Dr_Taar19s	zTA30	10	45656679-45657737		Brain & OE
Dr_Taar19t		10	45660522-45661511		Brain & OE
Dr_Taar19u	zTA16	10	45677535-45678581		Brain
Dr_Taar19v		10	45677589-45678581	-	Brain
Dr_Taar20a	zTA44	10	45350191-45351246		Brain & OE
Dr_Taar20a1	zTA23	10	45491713-45492579		Brain & OE
Dr_Taar20b	zTA39	10	45356183-45357130	ENSDART00000062778	OE & Brain
Dr_Taar20b1	zTA21	10	45479796-45480845		Brain & OE
Dr_Taar20c	zTA45	10	45397017-45398036	ENSDART00000076430	OE & Brain
Dr_Taar20c1		10	45487291-45486336		OE & Brain
Dr_Taar20d	zTA40	10	45405137-45406132	ENSDART00000085912	Brain & OE
Dr_Taar20d1		10	45497808-45496853		Brain & OE
Dr_Taar20e	zTA38	10	45406138-45405150		Brain & OE
Dr_Taar20f	zTA41	10	45369137-45370150	ENSDART00000046136	Brain & OE
Dr_Taar20g		10	45377510-45378535	ENSDART00000041600	OE & Brain

Dr_Taar20h		10	45366005-45365017		OE & Brain
Dr_Taar20i	zTA43	10	45383272-45384324		Brain & OE
Dr_Taar20j	zTA53	10	45432992-45433981		Brain & OE
Dr_Taar20k	zTA25	10	45516089-45517060		OE & Brain
Dr_Taar20l	zTA57	10	45423213-45424205		Brain & OE
Dr_Taar20m	zTA24	10	45525456-45526454		Brain & OE
Dr_Taar20n		10	45428757-45427769		Brain & OE
Dr_Taar20o	zTA42	10	45471587-45472636		Brain & OE
Dr_Taar20p	zTA90+	10	45466633-45465645		Brain & OE
Dr_Taar20q	zTA91+	10	45460333-45461919		Brain & OE
Dr_Taar20r	zTA37	10	45438081-45447488	ENSDART00000022615	Brain & OE
Dr_Taar20s		10	45532540-45531552		Brain & OE
Dr_Taar20t	zTA56	10	45419797-45420783	ENSDART00000038407	OE & Brain
Dr_Taar20u	zTA55	10	45414238-45415200	ENSDART00000085907	Brain & OE
Dr_Taar20v			BC093335		OE & Brain
Dr_Taar20w	zTA46	10	45437982-45438977		Brain & OE

Dr_Taar20x	zTa26	10	45507567-45508559		OE & Brain
Dr_Taar20y	zTA22	10	45502431-45501443		Brain & OE
Dr_Taar20z	zTA52	10	45501436-45502485		Brain & OE
Ga_Taar21a		groupXVIII	806192-807403	ENSGACT00000005640	
Ga_Taar21b		groupXVIII	849378-850334	ENSGACT00000005649	
Ga_Taar21c		groupXVIII	864851-865838	ENSGACT00000005661	
Ga_Taar22a		groupIX	13760495-13761558	ENSGACT00000024727	
Ga_Taar22b		group-I	22436982_22436003		
Ga_Taar23		groupXVI	15950422-15951745	ENSGACT00000010786	
Ga_Taar24		groupXV	16483483-16485039	ENSGACT00000011049	
Ga_Taar25a		groupXVI	17108838_17107707		
Ga_Taar25b		groupXVI	16450671-16452078	ENSGACT00000010998	
Ga_Taar25c		groupXVI	16978741-16979885	ENSGACT00000011316	
Ga_Taar25d		groupXVI	16460421-16461727	ENSGACT00000011007	
Ga_Taar25e		groupXVI	16467898-16469661	ENSGACT00000011022	
Ga_Taar25f		groupXVI	16974284-	ENSGACT000000	

			16975596	11311	
Ga_Taar25g		groupXVI	16946301-16948367	ENSGACT00000011304	
Ga_Taar25h		scaffold_37	867705-869195	ENSGACT00000001187	
Ga_Taar25i		scaffold_37	830907-832333	ENSGACT00000001173	
Ga_Taar25j		scaffold_37	845277-846439	ENSGACT00000001174	
Ga_Taar25k		scaffold_37	852505-855055	ENSGACT00000001178	
Ga_Taar25l		scaffold_37	880733-881869	ENSGACT00000001195	
Ga_Taar26a		scaffold_160	51365:52762:-1		
Ga_Taar26a1		groupXVI	16966117-16967188	ENSGACT00000011310	
Ga_Taar26b		scaffold_37	1893790-1894994	ENSGACT00000001272	
Ga_Taar26b1		scaffold_37	815923-817008	ENSGACT00000001171	
Ga_Taar26c		scaffold_160:	94133:95530:-1		
Ga_Taar26d		scaffold_37	1868618-1869815	ENSGACT00000001270	
Ga_Taar26e		groupXVI	17043139_17042020		
Ga_Taar26f		groupXVI	17028081-17029718	ENSGACT00000011318	
Ga_Taar26g		groupXVI	17077068_17075993		
Ga_Taar26h		groupXVI	4734246_47353		

			68		
Ga_Taar26i		groupXVI	4761489_4762619		
Ga_Taar26j		groupXVI	4753832-4754968	ENSGACT00000002929	
Ga_Taar26k		groupXVI	4855585-4856789	ENSGACT00000002944	
Ga_Taar26l		groupXVI	4814442-4815533	ENSGACT00000002940	
Ga_Taar26m		scaffold_37	1878555:187995 2:-1		
Ga_Taar26n		scaffold_56	1059527-1060737	ENSGACT00000002821	
Ga_Taar26o		groupXVI	17164416_17165546		
Ga_Taar26p		scaffold_160	139282:140697:1		
Ga_Taar26q		groupXVI	17057147-17058154	ENSGACT00000011320	
Ga_Taar26r		groupXVI	17085255:17086652:1		
Ga_Taar26s		groupXVI	17095712_17094581		
Ga_Taar26t		groupXVI	17020491_17021620		
Ga_Taar26u		groupXVI	17003486_17004615		
Ga_Taar26v		groupXVI	17175154_17176284		
Ga_Taar26w		groupXVI	17119616:17121019:-1		
Ga_Taar26x		groupXVI	17065776-	ENSGACT000000	

			17066988	11323	
Ga_Taar26y		groupXVI	17151885-17152878	ENSGACT00000011329	
Ga_Taar26z		groupXVI	16449795_16448676		
Ga_Taar27		groupI	27258576-27269505	ENSGACT00000020298	
Ol_Taar21a		24	10175766-10176731	ENSORLT00000019540	
Ol_Taar21b		24	10072258-10074154	ENSORLT00000019531	
Ol_Taar21c		24	10186887-10187888	ENSORLT00000019546	
Ol_Taar21d		24	10194348-10195725	ENSORLT00000019549	
Ol_Taar21e		24	10204903-10205901	ENSORLT00000019555	
Ol_Taar21f		24	10166806-10167735	ENSORLT00000019535	
Ol_Taar22		2	30183414-30184613	ENSORLT00000007813	
Ol_Taar23a		Scaffold691	13484-14893		
Ol_Taar23b		21	15078821-15080029	ENSORLT00000017413	
Ol_Taar23c		scaffold691	7458-8583	ENSORLT00000023953	
Ol_Taar23d		scaffold3620	1585-2740	ENSORLT00000023739	
Ol_Taar23e		scaffold4535	1771-2903	ENSORLT00000024697	

Ol_Taar23f		21	15066726-15068348	ENSORLT00000017409	
Ol_Taar23g		21	30824724-30825926	ENSORLT00000022830	
Ol_Taar23h		21	15113170-15114295	ENSORLT00000017425	
Ol_Taar23i		21	15246610-15248028		
Ol_Taar23j		scaffold2246	3196-4302	ENSORLT00000023320	
Ol_Taar23k		21	15257320_15256218		
Ol_Taar23l		21	15276889_15275788		
Ol_Taar23m		21	15334359_15333269		
Ol_Taar23n		21	15312452-15313546	ENSORLT00000017451	
Ol_Taar23o		21	15083894-15085278	ENSORLT00000017421	
Ol_Taar24a		21	30838329-30840233	ENSORLT00000022832	
Ol_Taar24b		21	30845621-30846947	ENSORLT00000022836	
Ol_Taar24c		21	30862260-30863356	ENSORLT00000022838	
Md_Taar1		2	407115274-407116293	ENSMODT00000030995	
Md_Taar2		2	407094705-407095724	ENSMODT00000022248	
Md_Taar3		2	407041839-	ENSMODT000000	

			407042870	30996	
Md_Taar4a		2	407018009-407016930		
Md_Taar4b		2	407016970-407029879	ENSMODT00000030998	
Md_Taar5		2	406983915-406984946	ENSMODT00000022251	
Md_Taar6a		2	406873483-406875111		
Md_Taar6b		2	406878994-406880013	ENSMODT00000031001	
Md_Taar6c		2	406861305-406862345	ENSMODT00000022260	
Md_Taar6d		2	406967819-406968859	ENSMODT00000030999	
Md_Taar6e		2	406933774-406934850	ENSMODT00000031000	
Md_Taar6f		2	406909541-406910617	ENSMODT00000022259	
Md_Taar9		2	406713166-406729709	ENSMODT00000022262	
Md_Taar9a		2	406798654-406799547	ENSMODT00000022263	
Md_Taar9b		2	406818556-406819593	ENSMODT00000031002	
Md_Taar9c		2	406834315-406835976		
Md_Taar9d		2	406852655-406854316		
Md_Taar9e		2	406777770-406778840	ENSMODT00000022265	
Md_Taar9f		2	406728666-		

			406729746		
Tr_Taar21a		scaffold_2286	7867-8688	SINFRUT00000131749	
Tr_Taar21b		scaffold_2618	2998-3949	SINFRUT00000181393	
Tr_Taar21c		scaffold_375	193436-194398	SINFRUT00000150779	
Tr_Taar21d		scaffold_2286	2710-3675	SINFRUT00000150777	
Tr_Taar21e		scaffold_682	7142-8116	SINFRUT00000181172	
Tr_Taar21f		scaffold_682	15028-15969	SINFRUT00000175284	
Tr_Taar22a		scaffold_3049	7460-8444	SINFRUT00000168032	
Tr_Taar22b		scaffold_36	1263458-1264444	SINFRUT00000174634	
Tr_Taar22c		scaffold_62	988428-989300	SINFRUT00000171815	
Tr_Taar27		scaffold_144	4933-5793	SINFRUT00000179744	
Tr_Taar28a		scaffold_2971	155-950		
Tr_Taar28b		scaffold_5473	768-1529	SINFRUT00000181876	
Tr_Taar28c		scaffold_347	234123-233001	SINFRUT00000178656	
Tr_Taar28d		scaffold_55	384-1178	SINFRUT00000183354	
Tr_Taar28e		scaffold_347	223711-222590		

Tr_Taar28f		scaffold_7591	430-1508	SINFRUT00000180900	
Tr_Taar28g		scaffold_347	183483-182389		
Tr_Taar28h		scaffold_347	190927-189849		
Tn_Taar21a		14	830088-830960	GSTENT00035509001	
Tn_Taar21b		14	869461-878434	GSTENT00035507001	
Tn_Taar21c		Un_random	124477975-124478871	GSTENT00011223001	
Tn_Taar22a		3	2466479-2465399		
Tn_Taar22b		3	2461859-2460719		
Tn_Taar22c		3	2455140-2454359		
Tn_Taar22d		3	2457239-2456279		
Tn_Taar22e		3	2468939-2467919	GSTENT00015819001	
Tn_Taar22f		18	5142103-5142852	GSTENT00035829001	
Tn_Taar27a		Un_random	126873732-126874616	GSTENT00011732001	
Tn_Taar27b		Un_random	126880750-126881709	GSTENT00011734001	
Tn_Taar27c		Un_random	126905659-126906669	GSTENT00011735001	
Tn_Taar27d		Un_rando	42642638-	GSTENT00009214	

		m	42643618	001	
Tn_Taar27e		Un_random	113714467-113715447	GSTENT00009350001	
Tn_Taar27f		Un_random	105520168-105521166	GSTENT00007835001	
Tn_Taar28a		Un_random	45834268-45835045	GSTENT00013017001	
Tn_Taar28b		Un_random	117442902-117443705	GSTENT00009988001	
Tn_Taar28c		Un_random	91619317-91620063	GSTENT00005517001	
RnTaar1		1	22045364-22046362	ENSRNOT00000021510	
RnTaar2		1	22027912-22028907	ENSRNOT00000035424	
RnTaar3		1	22018606-22019634	ENSRNOT00000035539	
RnTaar4		1	22008118-22009161	ENSRNOT00000047810	
RnTaar5		1	21996992-21998005	ENSRNOT00000061209	
RnTaar6		1	21984658-21985695	ENSRNOT00000021529	
RnTaar7a		1	21977118-21978194	ENSRNOT00000021545	
RnTaar7b		1	21967019-21968095	ENSRNOT00000021559	
RnTaar7c		1	21955553-21956629	ENSRNOT00000050763	
RnTaar7d		1	21934361-21935437	ENSRNOT00000051416	

RnTaar7e		1	21926752-21927828	ENSRNOT00000046379	
RnTaar7g		1	21912398-21913474	ENSRNOT00000044271	
RnTaar7h		1	21898531-21899607	ENSRNOT00000043436	
RnTaar8a		1	21857801-21858925	ENSRNOT00000044098	
RnTaar8b		1	21829913-21830947	ENSRNOT00000045563	
RnTaar8c		1	21814634-21815668	ENSRNOT00000043157	
RnTaar9		1	21799696-21800742	ENSRNOT00000038523	
Mm_Taar1		10	23609822-23610820	ENSMUST00000051532	
Mm_Taar2		10	23630004-23630999	ENSMUST00000079134	
Mm_Taar3		10	23638974-23640005	ENSMUST00000045152	
Mm_Taar4		10	23649910-23650953	ENSMUST00000092660	
Mm_Taar5		10	23660122-23661135	ENSMUST00000092659	
Mm_Taar6		10	23674025-23675062	ENSMUST00000057080	
Mm_Taar7a		10	23681821-23682897	ENSMUST00000078532	
Mm_Taar7b		10	23689355-23690431	ENSMUST00000092658	
Mm_Taar7d		10	23716638-	ENSMUST000000	

			23717714	92657	
Mm_Taar7e		10	23727030-23728106	ENSMUST00000092656	
Mm_Taar7f		10	23738926-23740002	ENSMUST00000071691	
Mm_Taar8a		10	23765916-23766950	ENSMUST00000051133	
Mm_Taar8b		10	23780676-23781710	ENSMUST00000092655	
Mm_Taar8c		10	23790294-23791328	ENSMUST00000092654	
Mm_Taar9		10	23797904-23798950	ENSMUST00000041180	
Bt_Taar1		9	63844020-63845624		
Bt_Taar2		9	63821486-63823081		
Bt_Taar3		9	63810823-63812451		
Bt_Taar4		9	63796757-63802538		
Bt_Taar5		9	63790900-63791913	ENSBTAT00000010332	
Bt_Taar6a		Un	263025242-263026315	ENSBTAT00000047909	
Bt_Taar6b		9	63472554-63473591	ENSBTAT00000046084	
Bt_Taar7a		Un	293821629-293822690	ENSBTAT00000039034	
Bt_Taar7b		9	63416496-63417569	ENSBTAT00000037774	

Bt_Taar7c		9	63450500-63452136	ENSBTAT00000011316	
Bt_Taar8a		9	63492912-63493937	ENSBTAT00000020857	
Bt_Taar8b		Un	262942450-262943484	ENSBTAT00000008724	
Bt_Taar9		9	63508158-63508949	ENSBTAT00000004932	
Hs_Taar1		6	133007816-133008835	ENsT00000275216	
Hs_Taar2		6	132979982-132987107	ENsT00000367931	
Hs_Taar5		6	132951505-132952518	ENsT00000258034	
Hs_Taar6		6	132933205-132934182	ENsT00000367934	
Hs_Taar8		6	132915525-132916553	ENsT00000275200	
Hs_Taar9		6	132901120-132902168	ENsT00000340640	
Xt_Taar1		scaffold_172	2058717-2059793	ENSXETT00000000206	
Xt_Taar4a		scaffold_172	2081286-2082349	ENSXETT00000000188	
Xt_Taar4b		scaffold_172	2072396-2073450	ENSXETT00000000192	
Gg_Taar1		3	58772958-58773950	ENSGALT00000022674	
Gg_Taar2		3	58790520-	ENSGALT000000	

			58791542	19656	
Gg_Taar5		3	58802258-58803280	ENSGALT00000022676	
Cm_Taar1		WGS		AAVX01005735	
Cm_Taar2*		WGS		AAVX01045569	
Outgroups:					
Aminergic R.tors:					
Dr_serotonin R. 2B	Htr2b			DQ864496	
Dr_ histamine R. H2	hrh2			NM_001045338	
Dr_ dopamine R. D2a	drd2a			NM_183068	
Mm_histamine R. H3	Hrh3			NM_133849	
Mm_dopamine R. 3	Drd3			NM_007877	
Mm_serotonin R. 5A	Htr5a			NM_008314	
Mm_dopamine R. D1A	Drd1a			NM_010076	
Mm_adrenergic R. beta 1	Adrb1			NM_007419	
Rn_serotonin R. 2A	Htr2a			NM_017254	
Rn_histamine R. H 2	Hrh2			NM_012965	
Rn_dopamine R. D3	Drd3			NM_017140	
Rn_adrenergic R. beta 2	Adrb2			NM_012492	
OR:					
Dr_OR131		15	29704040 - 29705023	ENSDART00000100030	

Dr_OR22		15	29659462-29660635	ENSDART00000009390	
Mm_OR121		17	37888801-37889766	ENSMUST00000074555	
Mm_OR446		6	42877232-42878158	ENSMUST00000101461	
Rn_ORi15		10	60267950-60268897	ENSRNOT00000040777	
Lamprey AmR:					
Contig11088:617:2278				GENSCAN00000071721	
Contig11088:12981:14642				GENSCAN00000145282	
Contig1988:18760:20379				GENSCAN00000086194	
Contig1988:36280:37854				GENSCAN00000080186	
Contig25386:1986:3629:				GENSCAN00000100832	
Contig2410:15193:16920				GENSCAN00000068085	
Contig32780:2648:4291				GENSCAN00000007566	
Contig29539:762:2393				GENSCAN00000093854	

Contig39824:7093:8748				GENSCAN00000010072	
Contig6569:10667:15719				GENSCAN00000142135	
Contig19989:8020:9663				GENSCAN00000098663	
Contig39552:7626:9284				GENSCAN00000077187	
Contig34843:480:2114				GENSCAN00000091849	
Contig58368:4024:5520				GENSCAN00000114105	
Contig8493:19657:21222				GENSCAN00000148282	
Contig8493:16826:18391				GENSCAN00000148281	
Contig56958:2841:4508				GENSCAN00000030920	
supercontig:PMAR3:Contig4553:9517:11127				GENSCAN00000087423	
				GENSCAN00000	

supercontig:PMAR3:Contig6110:8321:9970				01971	
supercontig:PMAR3:Contig17881:4845:6429				GENSCAN00000018535	
Contig32699:8482:9981				GENSCAN00000016801	
Contig17881:15313:16914				GENSCAN00000129481	
Contig6569:1523:3133				GENSCAN00000144047	
Pseudo genes:					
Zebrafish:					
		1	243399754-243399774		
		13	351799-352842		
		20	45965163-45966302		
		10	36614089-36614794		
Medaka:					
		7	5425949-5488965	ENSORLT00000003004	
Mouse:					

	Taar7c_P			AY702333	
Human:					
	TAAR3_P			AF112461	
	TAAR4_P			NG_004855	
	TAAR7_P			NG_004854	
Rat:					
	TAAR7i_P			AY702324	
	TAAR7f_P			AY702323	

Supplementary Table.2-Global dN/dS values of TAAR subfamilies

	ClassI				ClassII										ClassIII											
TAAR	1	10	21	27	2	3	4	5	6	7	8	9	12	13	14	16	17	18	19	20	22	23	24	25	26	28
dN/dS	0.19	0.25	0.25	0.32	0.09	0.10	0.13	0.11	0.17	0.25	0.20	0.15	0.27	0.29	0.39	0.27	0.41	0.35	0.45	0.49	0.30	0.34	0.24	0.54	0.80	0.56
Average	0.26				0.18										0.43											
n	4				10										12											
Std Error	0.03				0.02										0.04											

Global dN/dS values are shown for each TAAR subfamiliy. For each class of *taar* genes the average global value, *n*, and standard error are shown.

Supplementary Table.3-Number of positively and negatively selected sites in TAAR subfamilies

	Class I				Class II										Class III											
TAAR	1	10	21	27	2	3	4	5	6	7	8	9	12	13	14	16	17	18	19	20	22	23	24	25	26	28
Sig level	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2
# pos sites	0	2	0	2	0	2	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	2	0	0	0	0	0	2	0	0	0	3	3	10	11	19	0	1	0	4	0	0	0	3	10	29	0	1
Average	1.75				0.50										5.25											
n	4				10										12											
Std error	0.25				0.22										2.08											
# neg sites	80	127	4	44	124	177	27	58	29	114	12	83	36	108	43	130	67	133	51	103	20	68	98	145	39	102	7	45	23	54	20	69	1	19	36	77	31	62	57	91	46	95	81	127	0	42	13	30	26	47	15	35
Average	101.50				103.10										62.33											
n	4				10										12											
Std error	31.02				9.73										9.01											

Numbers of positively and negatively selected sites are given for each TAAR subfamily. For each class of taar genes the average number of positively and negatively selected sites, *n*, and standard error are shown.

Supplementary Table.4-Selective pressures in Danio rerio odorant receptor genes

Global dN/dS values of OR subfamilies							
OR subfamily	OR5	OR6	OR7	OR8	OR10	OR15	OR21
dN/dS	0.16	0.26	0.3	0.34	0.34	0.27	0.29

Number of positively and negatively selected sites in OR subfamilies														
OR subfamily	OR5		OR6		OR7		OR8		OR10		OR15		OR21	
Significant Level	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2
# pos. sites	0	0	0	0	0	1	0	0	0	1	1	1	2	5
# neg. sites	0	19	1	3	7	27	6	29	84	131	178	218	77	138

Supplementary Table.5-List of chemicals used in CRE-SEAP assay

	Chemical name	SEAP value 1mM	no ligand	Fold activation	chemical group	chemical group 2
1	4-(Dimethylamino) Butyric acid	307,081	275, 338	1.1	amino acid	amino acid
2	4-Aminobenzoic Acid in DMSO	324,424	277, 859	1.2	amino acid	amino acid
3	B-Alanine	425,128	414, 248	1.0	amino acid	amino acid
4	GABA	415,263	414, 248	1.0	amino acid	amino acid
5	L-Arginine monohydrochloride in DMSO	274,351	277, 124	1.0	amino acid	amino acid
6	L-Aspartic Acid in DMSO	264,884	277, 124	1.0	amino acid	amino acid
7	L-Glutamic Acid, non animal source in DMSO	203,826	277, 124	0.7	amino acid	amino acid
8	L-Histidine monohydrochloride monohidrate in DMSO	275,802	257, 336	1.1	amino acid	amino acid
9	L-Isoleucine in DMSO	245,754	242, 253	1.0	amino acid	amino acid
1	L-Leucine in DMSO	350,535	242,	1.4	amino acid	amino acid

0			253			
11	L-Lysine monohydrochloride in DMSO	304,949	277, 124	1.1	amino acid	amino acid
12	L-Methionine in DMSO	335,008	277, 124	1.2	amino acid	amino acid
13	L-Phenilalanine in DMSO	249,879	242, 253	1.0	amino acid	amino acid
14	L-Serine in DMSO	283,249	277, 124	1.0	amino acid	amino acid
15	L-Threonine in DMSO	255,710	277, 124	0.9	amino acid	amino acid
16	L-Tryptophan in DMSO	229,970	242, 253	0.9	amino acid	amino acid
17	L-Valine in DMSO	275,409	277, 124	1.0	amino acid	amino acid
18	N,N-Dimethylglycine Hydrochloride	288,118	277, 859	1.0	amino acid	amino acid
19	Taurine	273,491	269, 064	1.0	amino acid	amino acid
20	1-Dimethylamino-2-propanol	278,489	275, 338	1.0	aminoalcohol	aminoalcohol and related
21	2-(dimethylamino) Ethanethiol	243,316	269, 064	0.9	aminothiol	aminoalcohol and related
22	3-(Dimethylamino) Propiophenone Hydrochloride	223,186	257, 336	0.9	aminoketone	aminoalcohol and related
23	3,4-Dimethoxyphenethylamine	273,312	277, 859	1.0	aminoether	aminoalcohol and related
24	3-Methoxy Tyramine	270,488	277, 859	1.0	amino alcohol	aminoalcohol and related

25	4-methoxyphenethylamine	286,848	275,338	1.0	aminoether	aminoalcohol and related
26	5 amino 1 pentanol	94,835	93,305	1.0	aminoalcohol	aminoalcohol and related
27	Amino-2-propanol	275,103	279,704	1.0	aminoalcohol	aminoalcohol and related
28	Cysteamine Hydrochloride	2,360,000	279,704	8.4	aminothiol	aminoalcohol and related
29	Ethanolamine	412,669	400,880	1.0	aminoalcohol	aminoalcohol and related
30	N,N-dimethylethanolamine	276,240	277,859	1.0	aminoalcohol	aminoalcohol and related
31	Octopamine Hydrochloride	285,799	277,859	1.0	aminoalcohol	aminoalcohol and related
32	Tyramine Hydrochloride	267,373	279,704	1.0	aminoalcohol	aminoalcohol and related
33	2-Aminopentane	496,100	414,248	1.2	monoamine, primary	monoamine, primary
34	2-Methylbutylamine	283,920	279,704	1.0	monoamine, primary	monoamine, primary
35	3-(Methylthio)Propylamine	299,257	279,704	1.1	monoamine, primary	monoamine, primary
36	A-Naphthylamine in DMSO	563,713	269,064	2.1	monoamine, primary	monoamine, primary
37	Aniline Hydrochloride	271,096	269,064	1.0	monoamine, primary	monoamine, primary
38	Benzylamine	436,400	414,248	1.1	monoamine, primary	monoamine, primary
39	Butylamine	398,114	414,248	1.0	monoamine, primary	monoamine, primary
40	Cyclohexylamine	268,475	277,859	1.0	monoamine, primary	monoamine, primary

41	Cyclopentylamine	281,335	257, 336	1.1	monoamine, primary	monoamine, primary
42	Ethylamine	413,958	400, 880	1.0	monoamine, primary	monoamine, primary
43	Hexilamine in DMSO	420,966	400, 880	1.1	monoamine, primary	monoamine, primary
44	Isoamylamine	414,478	400, 880	1.0	monoamine, primary	monoamine, primary
45	Isobutylamine	416,687	400, 880	1.0	monoamine, primary	monoamine, primary
46	Isopropylamine	401,681	400, 880	1.0	monoamine, primary	monoamine, primary
47	Methylamine	419,458	408, 279	1.0	monoamine, primary	monoamine, primary
48	Pentylamine	94,996	95,3 67	1.0	monoamine, primary	monoamine, primary
49	Phenethylamine	317,468	279, 704	1.1	monoamine, primary	monoamine, primary
50	Quinaldine in DMSO	407,048	257, 336	1.6	monoamine, primary	monoamine, primary
51	Quinoline	321,201	269, 064	1.2	monoamine, primary	monoamine, primary
52	1-Methylindole in DMSO	416,355	408, 279	1.0	monoamine, tertiary	monoamine other than primary
53	1-Methylpiperidine	410,745	408, 279	1.0	monoamine, tertiary	monoamine other than primary
54	1-Methylpyrolidine	406,224	408, 279	1.0	monoamine, tertiary	monoamine other than primary
5	Dibutylamine in DMSO	409,128	414,	1.0	monoamine,	monoamine

5			248		secondary	other than primary
56	Dimethylamine	394,756	414, 248	1.0	monoamine, secondary	monoamine other than primary
57	Indole in DMSO	436,595	400, 880	1.1	monoamine, secondary	monoamine other than primary
58	N,N-Dimethyl Benzyl Amine	276,309	275, 338	1.0	monoamine, tertiary	monoamine other than primary
59	N,N-Dimethyl Isopropyl amine	273,902	275, 338	1.0	monoamine, tertiary	monoamine other than primary
60	N,N-Dimethyl-1-naphthylamine in DMSO	262,680	275, 338	1.0	monoamine, tertiary	monoamine other than primary
61	N,N-Dimethylaniline	277,248	275, 338	1.0	monoamine, tertiary	monoamine other than primary
62	N,N-Dimethylcyclohexylamine in DMSO	330,924	414, 248	0.8	monoamine, tertiary	monoamine other than primary
63	N,N-Dimethylphenethylamine in DMSO	225,268	275, 338	0.8	monoamine, tertiary	monoamine other than primary
64	Piperidine	251,228	257, 336	1.0	monoamine, secondary	monoamine other than primary
65	Pyrrolidine	403,929	408, 279	1.0	monoamine, secondary	monoamine other than primary
6	Tetramethyl Ammonium	266,490	277,	1.0	monoamine,	monoamine

6	Chloride		859		quarternary	other than primary
67	Trimethylamine, 25 wt. % in water	281,159	279,704	1.0	monoamine, tertiary	monoamine other than primary
68	1-(2-Aminoethyl) Pyrrolidine	254,720	269,064	0.9	diamine, aliphatic, cyclic	diamine, aliphatic, cyclic
69	Ethylene Diamine	455,323	400,880	1.1	diamine, aliphatic, linear	diamine, aliphatic, linear
70	1,4-Diaminobutane Dihydrochloride aka Putrescine	2,570,000	408,279	6.3	diamine, aliphatic, linear	diamine, aliphatic, linear
71	Cadaverine Dihydrochloride	4,500,000	400,880	11.2	diamine, aliphatic, linear	diamine, aliphatic, linear
72	Hexamethylene diamine	1,112,706	118,895	9.4	diamine, aliphatic, linear	diamine, aliphatic, linear
73	1-7 Diaminoheptane	1,520,000	121,267	12.5	diamine, aliphatic, linear	diamine, aliphatic, linear
74	1-8 Diaminooctane	332,091	87,018	3.8	diamine, aliphatic, linear	diamine, aliphatic, linear
75	1-10 Diaminodecane	83,980	90,163	0.9	diamine, aliphatic, linear	diamine, aliphatic, linear
76	Cystamine dihydrochloride	3,390,000	414,248	8.2	diamine, aliphatic, linear	diamine, aliphatic, linear
7	Tetramethyl-1,4-Butane	641,268	257,	2.5	diamine,	diamine,

7	Diamine		336		aliphatic, linear	aliphatic, linear
78	2,5-Dimethylpyrazine	283,890	269,064	1.1	diamine, aromatic	diamine, aromatic
79	5-aminoindole Hydrochloride	248,875	277,859	0.9	diamine, aromatic	diamine, aromatic
80	5-methoxytryptamine in DMSO	619,821	275,338	2.3	diamine, aromatic	diamine, aromatic
81	Gramine in DMSO	253,653	269,064	0.9	diamine, aromatic	diamine, aromatic
82	Tryptamine in DMSO	368,280	408,279	0.9	diamine, aromatic	diamine, aromatic
83	Agmatine Sulfate	2,900,000	279,704	10.4	polyamine, aliphatic, linear	polyamine, aliphatic, linear
84	Spermidine	1,050,000	257,336	4.1	polyamine, aliphatic, linear	polyamine, aliphatic, linear
85	Spermine	234,868	242,253	1.0	polyamine, aliphatic, linear	polyamine, aliphatic, linear
86	Adenine in DMSO	235,872	257,336	0.9	polyamine, aromatic	polyamine, aromatic
87	Histamine Dihydrochloride	911,958	279,704	3.3	polyamine, aromatic	polyamine, aromatic
88	1-5 pentanediole	265,532	257,336	1.0	alcohol	other
89	Creatinine Hydrochloride	262,294	269,064	1.0	amide	other
90	Ethyl Butyrate (not amine)	401,026	408,279	1.0	ester	other
9	Hexanal (not amine)	419,765	408,	1.0	aldehyde	other

1			279			
92	Riboflavin in DMSO	98,452	242, 253	0.4	riboflavin	other
93	Sucrose	257,931	242, 253	1.1	sugar	other
94	Uracil in DMSO	302,560	242, 253	1.2	amide	other

XIII. Appendix

Abbreviations

Ab/Tü	mix of the Oregon and Tubingen strains
Actinopterygii:	ray finned fish
AOB:	accessory olfactory bulb
AC:	Adenylyl cyclase
BSA:	bovine serum albumine
Bp:	base pairs
cDNA:	complementary DNA
CRE:	cyclic response element
DAB:	diaminobenzidine
DEPC:	diethylpyrocarbonate
DIG:	digoxigenin
Dpf:	days post fertilization
DNA:	desoxynucleic acid
Dr:	*Danio rerio* (zebrafish)
DNTP.	desoxynucleotide phosphate
EDTA:	ethylenediaminetetraacetic acid
GG	Grueneberg ganglion
GPCR	G protein-coupled receptor
HRP.	horse radish peroxidase
Kb:	kilo base
LOT:	lateral olfactory tract
M:	molar
mM:	millimolar
MCS	multiple cloning site
µg:	microgram
min:	minutes
MOB:	main olfactory bulb
MOE:	main olfactory epithelium
MOT:	medial olfactory tract
MYA	million years ago
Ng:	nanogram
NGS:	normal goat serum
OC:	olfactory cortex
OE:	olfactory epithelium
OB:	olfactory bulb
OBP:	odorant binding proteins
OMP:	olfactory marker protein
OR:	olfactory receptor
OSN:	olfactory sensory neuron
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PFA:	paraformaldehyde
RNA:	ribonucleic acid
RT:	room temperature

Sarcopterygii:	lobe finned fish
SEAP:	secreted alkaline phosphate
SO:	septal organ
SSC:	sodium citrate
TAARs:	Trace Amine-Associated Receptors
TE:	tris-EDTA
TM:	Trans-membrane
V1R:	vomeronasal receptors type 1
V2R:	vomeronasal receptors type 2
VNO:	vomeronasal organ
VR:	vomeronasal receptor
VSN:	vomeronasal sensory neurons
X-Gal:	5-Bromo-4-chlor-3-indoyl-D-galactopyranosid

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2008	**Harvard University, USA** Liberles lab, Harvard Medical School. Project: Identification of ligands for Trace Amine Associated Receptors (TAARs)
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Peer reviewed publications

Hussain, A., Saraiva, L.R., Korsching, S.I. (2009) Positive Darwinian selection and the birth of an olfactory receptor clade in teleosts. Proc. Natl. Acad. Sci. USA. 106(11):4313-4318

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Köln, den 04. Nov, 2010