Genomic Plasticity in the Olfactory Epithelium mediated by Odorant Exposure in Zebrafish (*Danio rerio*)

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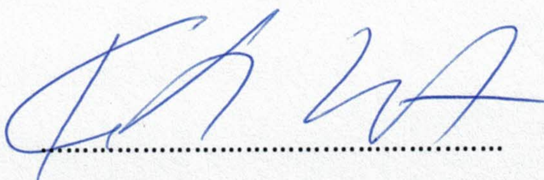

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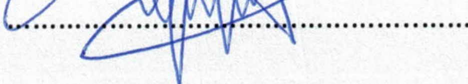

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Abbreviation

Bp: Base pair

CNS: Central nervous system

Ct: Threshold cycle

DNA: Deoxyribonucleic acid

Dpf: Days post fertilization

FDR: False discovery rate

GR: Glucocorticoid receptor

HPA: Hypothalamus–pituitary–adrenal

Hpf: Hours post fertilization

IEG: Immediate early gene

MAST: Motif Alignment and Search Tool

MEME: Multiple Expectation Maximization for Motif Elicitation software

MHC: major histocompatibility complex

OB: Olfactory bulb

OE: Olfactory epithelium

OMP: Olfactory marker protein

OR: Olfactory receptors

OS: Olfactory system

OSN: Olfactory sensory neurons

PCA: Principal component analysis

PCR: Polymerase chain reaction

PEA: Phenyl ethyl alcohol

PutBS: putative transcription factor binding sites

RNA: Ribonucleic acid

RPKM: Reads Per Kilo base Million

RT-PCR: Reverse transcription Polymerase chain reaction

SE: Standard error

TAAR: Trace amine receptors

TF: transcription factors

TFBS: Transcription factor binding sites

Hypothesis

"Odorant exposure can control olfactory receptor expression, changing the dynamics of OR expression within neurons."

General aims

·Analyze the effect of odorant exposure on the expression of ORs

·Analyze potential mechanisms that mediate odorant induced changes in gene expression

Specific aims

· Measure expression of OR genes in the olfactory epithelium to determine whether they are mediated by odorants

·Identify possible regulatory sequences of identified ORs

·Identify transcription factors involved in the control of the expression of ORs and in odorant modulation

·Analyze functionally the role of the regulatory elements identified

Introduction. Olfactory system of fish

Introduction

Olfactory System of fishes

The olfactory system detects odorants in the environment and these odors convey information about food, predators, conspecifics,and reproduction (reviewed in Yoshihara, 2009). Within the olfactory epithelium (OE), the olfactory sensory neurons (OSNs) interact directly with the environment, their dendrites are located within the mucosa where they bind olfactory stimuli (reviewed in Miyasaka et al, 2013). Furthermore, the OE generates new OSNs throughout the life of the animal, with a new neuron being generated every 30 days (Graziadei and Monti Graziadei, 1978; Mackay-Sim and Kittel, 1991). Because the olfactory system is plastic and in direct contact with the external environment, it is an excellent model to study potential environmental influences of the evironment on the nervous system.

The OE in the adult fish contains four populations of OSNs: the ciliated and microvilliar OSNs are the most abundant (reviewed in Hamdani and Døving, 2007) the crypt cells (Hansen and Finger, 2000), and the recently described kappe neurons (Ahuja et al, 2014). The OSNs extend their axons to the olfactory bulb where the axonal terminals of OSNs detecting the same odors form odor-specific olfactory glomeruli. Information is then further processed in the olfactory cortex, the amygdala (Shipley & Ennis 1996) and the habenula (Miyasaka et al, 2009).

Olfactory Receptor

The OSNs express olfactory receptors (OR) which belong to the family of 7 transmembrane G protein-coupled receptors (GPCRs: Buck and Axel, 1991) and transduce signals through an exclusive G protein , Golf, and the adenylate cyclase enzyme (Sklar et al 1986; Jones and Reed, 1989). In mouse there are approximately 1000 genes coding for ORs making this the largest gene family in vertebrate genome (Saraiva et al, 2015; Niimura et al, 2014). Genes coding ORs in vertebrates do not have introns (Buck and Axel 1991; Alioto and Ngai, 2005) and are arranged in clusters in the genome, where the genes with high homology are

contiguous within a cluster and are transcribed in the same direction (Rouquier et al, 1998; Niimura et al, 2014).

The number and variability of ORs in vertebrates are correlated with the environment in which the animal lives with terrestrial vertebrates having greater numbers of OR genes. In the mouse genome it has been shown that there are around 1100 genes coding for ORs (Saraiva et al, 2015; Niimura et al, 2014); however, despite the large number of genes there is little variation between their sequences, with the genes being classified in two large families: Class I ORs and Class II OR (Glusman et al 2000; Niimura et al, 2014). In the human genome around 1000 sequences of OR have been described with about fifty percent of them being pseudogenes (Menashe et al 2003; Niimura et al, 2014). A recent study has shown that elephant genome contains around 2000 functional ORs (Niimura et al, 2014). It has been shown that the number of OR genes in the teleost fish genome is around ten fold lower than mammals, with 140 sequences in zebrafish, 44 in fugu (*Takifugu rubripes*) and 42 in tetraodon (*Tetraodon nigroviridis*) (Alioto y Ngai, 2005; Azzouzi et al, 2014). However, fish ORs have more variability in their sequences, where the zebrafish ORs are classified in 40 families (Alioto y Ngai, 2005; Azzouzi et al, 2014).

Olfactory sensory neurons express one or a low number of ORs where each neuron expresses only one allele of a given OR gene (Chess et al, 1994). In zebrafish the OSNs expressing the same OR are located in a concentric pattern in the olfactory rosette (Weth et al., 1996). In contrast, in mouse OSNs expressing a specific OR are restricted to a limited area in the OE, however neurons are scattered within the area (Vassar et al., 1993; Ressler et al., 1994). In all vertebrates the axons of OSNs expressing the same OR converge on a single point in the olfactory bulb called glomerulus (Figure 1), with zebrafish having around 140 glomeruli per each OB (Braubach et al, 2012). Thus, information for a given odor is collected in different areas of the OE by widely distributed OSNs and is then integrated in the olfactory bulb. In addition to

spatial differences, it has been shown that ORs initiate expression at different times during development in zebrafish (Barth et al., 1996; Byrd et al., 1996) and mouse (Rodriguez-Gil et al, 2010). Therefore, the regulation of OR expression occurs both spatially and temporally in zebrafish and mouse.

Control of ORs expression

The mechanisms governing the control of ORs have not been fully elucidated, although crucial components have been identified such a specific genomic elements and TFs.

a. Genomic elements.

In mice genomic regions close to OR clusters have been shown to be important for the regulation of OR expression. A specific element, H element, has been identified on the chromosome 14. This 2 kb element is located 75kb upstream of the cluster where is located the MOR28 and has been shown to regulate OR expression within the cluster (Serizawa et al 2003; Lonvardas et al 2006; Fuss et al 2007; Nishizumi et al 2007). Mutations in the H element silence the expression of the ORs in the MOR28 cluster (Nishizumi et al, 2007), but there are no effects on the expression of receptors located in other OR clusters of the chromosome 14 (Fuss et al 2007). Additionally, it was shown that the H element could control the expression of ORs as a trans element on receptors located in chromosomes different than 14 (Lonvardas et al 2006). Other regulatory sequences have been identify, such as the P element, located on the chromosome 7, where mutations in this region affect the expression of ten ORs located in a cluster close to P element (Khan et al, 2011). These data show the presence of elements in the genome that act in the control of OR expression as *trans* and *cis* element

In zebrafish, the location of the OR gene has also been shown to be important in the control of expression. In two strains of transgenic zebrafish, in which the one OR gene was replaced by reporter gene (*OR103-1* by CFP and *OR111-7* by YFP), the CFP and YFP positive cells expressed an OR belonging to the same family as the replaced OR, which is located in the

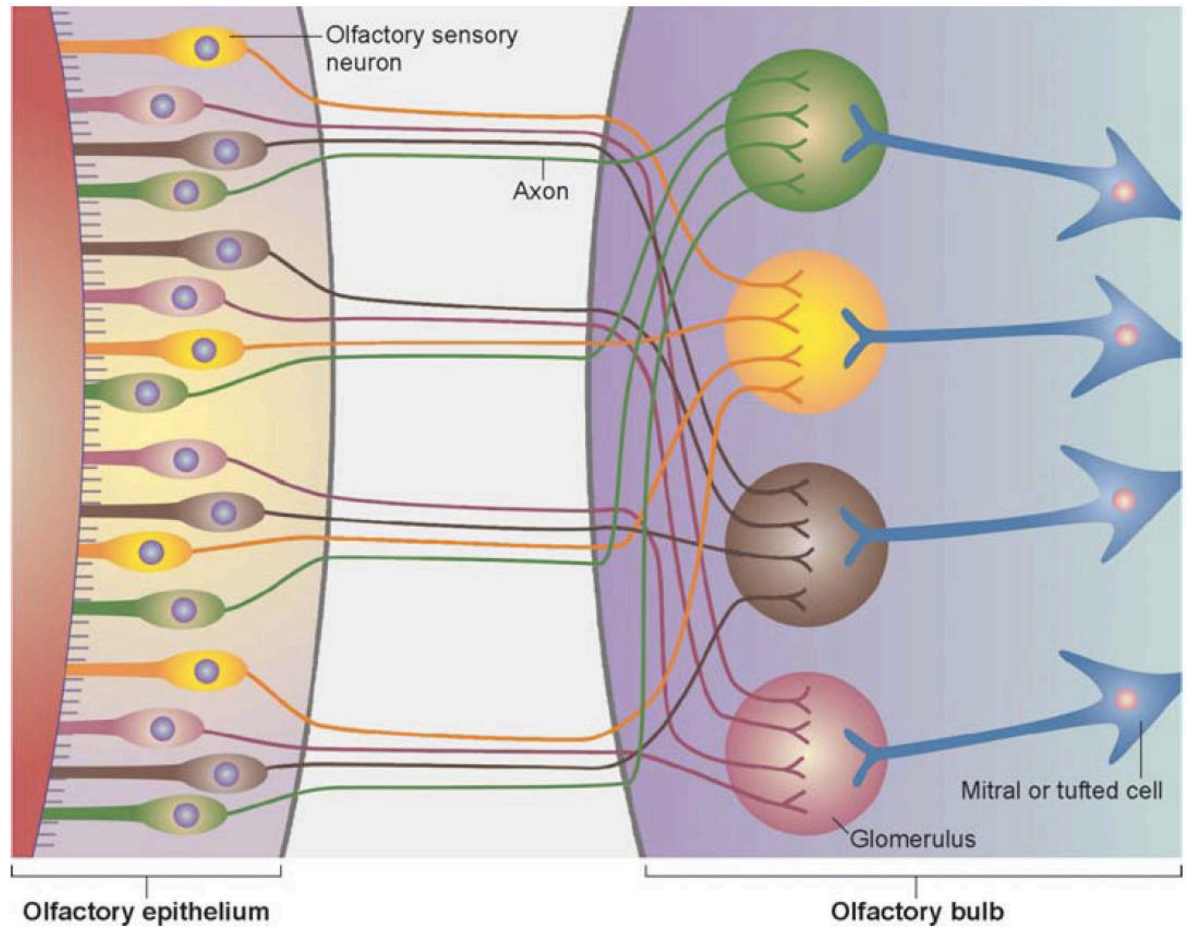


Figure 1. Organization of the olfactory system. On left the OE; the OSNs that express the same OR are represented in different colors, and send their axons to the same glomerulus in the OB (at right). In the OB, each OSN make synapses with a single mitral cell within the glomerulus (Mombaerts, 2006).

same cluster and downstream to this; thus when *OR103-1* is replaced by CFP the neuron express *OR103-2* or *OR103-5*, which are downstream than *OR103-1*; furthermore, when *OR111-7* is replaced by YFP, the neuron express receptor belonging to the *OR111* family (Sato et al 2007). Therefore, these studies show that the location of the OR genes in the cluster is important for the expression of an OR.

b TFs in the OR control expression

Homeodomain transcription factors (TFs) have been shown to regulate the expression of ORs. This class of TFs is involved in the formation and the development of structures including the nervous system (Kolterud et al, 2004; McIntyre et al 2008). In mouse it has been shown that the Lhx2 TF is necessary for OR expression, where *lhx2* mutant mice do not express the ORs *Olfr145, L45, M50* and *Olfr140*, as shown by *in situ* hybridization (Kolterud et al, 2004). Interestingly, in a recent study, a conditional mutant was generated for *lhx2*, where the gene was deleted selectively in OSN, showing a decrease in the expression of 965 ORs with no changes in the OSN number (Zhang et al, 2016). In addition, another homeodomain TF, Emx2, has also been linked with the control OR expression; *emx2* knockout mice show a reduction in the expression of 336 ORs according a microarray analysis, yet they form an OE and express OMP (an OSN marker) in reduced levels (McIntyre et al 2008).

Whole genome analysis and sequencing of all mouse ORs promoters have shown a high density of homeodomain TF binding sites (Young et al 2011; Plessy et al 2013). Interestingly, bioinformatic analysis has shown that regulatory elements such as the H element are enriched in homeodomain factor binding sites (Nishizumi et al, 2007) indicating a possible interaction between TFs of the homeodomain family and this regulatory sequence. Therefore, the evidence from analysis of mutants and bioinformatic data give insight to the potential role homeodomain TFs in the control OR expression.

Olfactory memory and plasticity in the OE

Animals can form memories of different environment stimuli; a specific type of memory formation is sensory imprinting which is a class of memory that is formed by an exposure to a sensory stimulus during early development, and this memory is maintained through life without reinforcement (Immelmann et al., 1975). Imprinting was initially described in birds by Lorenz (1935), where he demonstrated that juvenile geese recognize and follow their parents due to visual imprinting which is generated in the first hours after hatching (Lorenz, 1935). Olfactory imprinting has been described in different animal species. In mosquitoes it was shown that females exposed to odorant embryonic stage prefer to lay their eggs in water with those odorants (McCall and Eaton, 2001). In mammals, the European rabbit offspring prefer the food eaten by their mothers in the pre-natal stage, where this effect does not need post natal reinforcement (Hudson and Distel, 1998). The generation of behavioral imprinting in different animal species shows that early sensory memory is conserved and gives us an idea of the importance of this process in nature.

Olfactory imprinting has been described in fish and this process is correlated with plasticity in the OE. The most studied olfactory imprinting behavior is in salmon that are born in rivers, migrate to the sea where they pass their adult life and then return to the rivers where they were born. Through a variety of experiments using artificial odorants, it was shown that the salmon follow olfactory cues during the fresh water phase of the migration using memories of odor cues formed during early development (Hasler and Wisby, 1953). In these studies it was shown that fish exposed to the artificial odorant phenylethyl alcohol (PEA) early in development generate a memory of this odorant (Hasler and Scholz, 1983). More recently it was shown using patch clamp recording of single OSNs, that PEA memory in salmon is correlated with an increase in the activity of dissociated OSN to PEA (Nevitt et al, 1994). Various studies identified

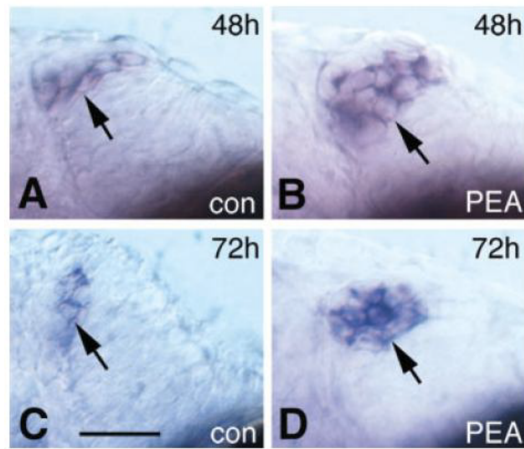
the hormonally controlled parr-smolt transformation as being the important window of development for olfactory imprinting in salmon (Dittman et al, 1996).More recently, it was shown that Pink salmon can form a memory of PEA by exposure in alevin stages, an early stage after hatching but before they have resorbed their yolk,, indicating that PEA memory can be formed very early in development prior to the parr-smolt transition (Bett et al, 2016). Studies using Chinook salmon have shown that in the alevin to fry transition stage (resorbed yolk and feeding), the OSNs have formed distinct, identifiable, glomeruli patterns within the OB, (Ochs et al, 2017). These results show that olfactory memory of PEA is correlated with both a behavioral and physiological response to PEA, and that exposure to PEA before yolk resorption plays an active role in the formation of PEA memory.

A transcriptomic analysis of the OE of chum salmon showed that fish captured in rivers (pre-spawning) have an increase in expression of seven ORs compared with fish caught in the sea (Palstra et al., 2015), which suggests a change in the sensibility to some odorants. Similar results were observed for the expression of N-methyl-d-aspartate receptors (NRs), olfactory receptors (ORs), and adrenocorticotropic hormone (ACTH) where salmon exposed to fresh water, had a higher level of expression of NCR, ORs and ACTH than fish exposed sea water (Kim et al, 2015). These results showed that changes in osmolarity and salinity of water are important to trigger changes in the olfactory system, which could be important to the reactivation of memory of odorants in the return migration

Olfactory imprinting has a role in conspecific recognition in zebrafish. Kin recognition is mediated by memory formation of chemical signals present in the odor of conspecific fish (Gerlach et al, 2008; Hinz et al 2012; Hinz et al 2013), where evidence suggests that memory formation occurs 24 hours after exposure to conspecific odors (Gerlach et al, 2008). Different studies have identified the peptides of the Major Histocompatibility Complex class II (MHC II) as an olfactory stimulus necessary for kin recognition (Gerlach et al, 2008; Hinz et al 2012; Hinz

et al 2013); these peptides are polymorphic and their composition depends on the genotype of each fish giving a signature to each them (Boehm and Zufall, 2006). Zebrafish larvae form olfactory memories of MHC peptides dependent on the genotype of the fish and this behavioral response was correlated with differences to the neuronal responses to MHC peptides in the OE of imprinted and non-imprinted fish (Hinz et al 2013). Recent evidence supports a potential role for crypt cells, a subpopulation of neurons in the OE, in the kin recognition, where 9 dpf larvae imprinted with conspecific odor showed activation in crypt cells to this stimuli compared what was not observed in non-imprinted larvae (Biechl et al, 2016). Therefore, these data showed that zebrafish can form an olfactory memory to social odors during early development.

Olfactory imprinting has been shown in zebrafish where fish exposed to PEA during the first three weeks of development remember the odorant when tested as adults. Furthermore this memory is correlated with increases in gene expression in the OE, including the TF *otx2*, as analyzed by microarray and whole mount *in situ* hybridization (Harden et al, 2006). Odorant exposure increased the number of the cells expressing *otx2* in the exposed embryos, an effect that was maintained in the adult (Figure 2) (Harden et al, 2006). Additionally, studies using the anemonefish (*Amphiprion percula*) showed that these fish imprint on reef water odors (Arvedlund et al, 1999) and the formation of this memory is correlated with increased expression of *otx2* in the OE (Veilleux et al, 2013). Thus, the correlation between odor memory with changes in responses of the OSNs and variations of gene expression in the OE suggests an active role for the peripheral nervous system in the memory formation.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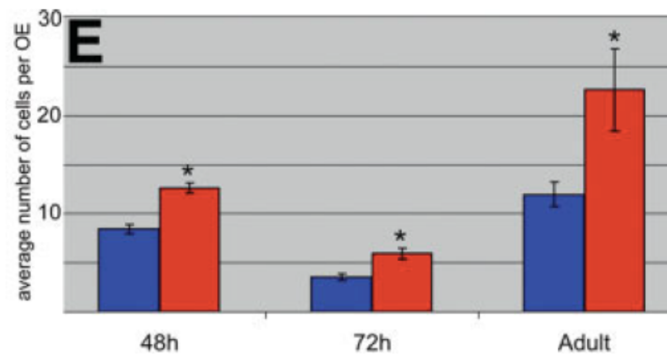
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30
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48h
72h
Adult
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*
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Figure 2. Fish exposed to PEA as juveniles show more cells expressing *otx2* in the OE. (A, C) Expression of *otx2* (arrow) in the ventral-anterior region of the OE in control fish at 48–72 h of development; (**B**, **D**) Expression of *otx2* (arrow) in the ventral- anterior region of the OE of PEA-exposed fish at 48–72 h, respectively. The number of cells expressing *otx2* was increased in PEA-exposed fish (**B**, **D**) compared with that in control fish (**A**, **C**). (**E**) There was a statistically significant increase in the number of cells expressing *otx2* in the OE of the PEA-exposed vs. control zebrafish at 48 h (control = 8.5 ± 0.48; PEA = 12.6 ± 0.51), 72 h (control = 3.6 ± 0.38; PEA = 5.9 ± 0.53), and as adults (control = 11.9 ± 1.25; PEA = 22.6 ± 4.16). Bars represent the average number of cells in control (blue) and PEA-exposed (red) fish. Data are plotted with S.E.M., asterisks indicate: $p < 0.0005$ for juveniles, $p < 0.05$ for adults. Scale bars: A–D = 40 μm. n = 40 OE for juveniles per treatment per time point; n = 20 OE for adults per treatment. (From: Harden et al., 2006).

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Chapter I. Changes in Olfactory Receptor Expression Are Correlated With Odor Exposure During Early Development in the zebrafish (*Danio rerio*)

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Abstract

We have previously shown that exposure to phenyl ethyl alcohol (PEA) causes an increase in the expression of the transcription factor *otx2* in the olfactory epithelium (OE) of juvenile zebrafish, and this change is correlated with the formation of an odor memory of PEA. Here, we show that the changes in *otx2* expression are specific to βPEA: exposure to αPEA did not affect *otx2* expression. We identified 34 olfactory receptors (ORs) representing 16 families on 4 different chromosomes as candidates for direct regulation of OR expression via Otx2. Subsequent *in silico* analysis uncovered Hnf3b binding sites closely associated with Otx2 binding sites in the regions flanking the ORs. Analysis by quantitative polymerase chain reaction and RNA-seq of OR expression in developing zebrafish exposed to different isomers of PEA showed that a subset of ORs containing both Otx2/Hnf3b binding sites were down regulated only in βPEA-exposed juveniles and this change persisted through adult life. Localization of OR expression by *in situ* hybridization indicates the down regulation occurs at the level of RNA and not the number of cells expressing a given receptor. Finally, analysis of immediate early gene expression in the OE did not reveal changes in *c-fos* expression in response to either αPEA or βPEA.

Introduction

How the activation of olfactory receptors (ORs) leads to odor recognition is still unknown due to the large number of ORs and the wide variety of compounds that can form an odor (Mombaerts 1999; DeMaria and Ngai 2010), but clearly experience and neural plasticity play a role in odor perception and discrimination (for review, see Wilson and Stevenson 2003). Olfactory imprinting, a type of olfactory memory that is formed during early development and retained throughout life without reinforcement, has been described in invertebrates (McCall and Eaton 2001; Remy and Hobert 2005) and vertebrates (Hasler and Scholz 1983; Hudson and Distel 1998; Harden et al. 2006). This behavior is dependent upon local olfactory cues experienced during early development and has been studied extensively in pacific salmon, animals that retain a memory of home stream odors for life and use this memory to return and spawn (Hasler and Scholz 1983). Studies using phenyl ethyl alcohol (PEA) as an artificial odorant to imprint juveniles and bait the adults to PEA-marked sites (Hasler and Scholz 1983; Nevitt et al. 1994) demonstrated that the olfactory epithelia in PEA-imprinted fish showed a strong physiological response to PEA in comparison to the non-imprinted animals (Nevitt et al. 1994), supporting a role for peripheral nervous system in the formation of olfactory preferences. Similarly, we have shown that adult zebrafish make an olfactory memory of PEA and this behavior is correlated with changes in gene expression in the olfactory epithelium (OE; Harden et al. 2006; Whitlock 2006). Thus, the generation of long-term olfactory memory is correlated with both physiological and genetic changes in the peripheral nervous system.

The ability to discriminate among a wide spectrum of odorants is possible due to a multigene family ORs (Buck and Axel 1991), characterized by a common structure of 7

transmembrane G-protein-coupled receptors (Nei et al. 2008). Any olfactory sensory neuron (OSN) can express 1 or a very low number of ORs (Ngai et al. 1993; Chess et al. 1994; Malnic et al. 1999; Mombaerts 2004; Sato et al., 2005), and neurons expressing the same OR are scattered within the OE (Imai and Sakano 2007). The mechanism by which an OSN expresses a given OR from as many as 1000 OR genes (mouse) is still unknown (McClintock 2010), but studies in mouse suggest the presence of an upstream control element in the OR family MOR28, called H element, that is necessary for the expression of ORs within that family (Serizawa et al. 2003; Nishizumi et al. 2007). Other studies in mouse show that the onset of OR expression apparently depends of the chromosome location because ORs located on the same chromosome start to express at the same developmental stage (Rodriguez-Gil et al. 2010). These studies support a mechanism where the genomic location of the ORs is important for the selection of the OR genes to be expressed.

One potential mechanism for controlling the expression of ORs is through activity where OSNs are more likely to modulate an OR type that was stimulated early in development (memory) and often throughout life (food). Odor-induced activity can result in differential changes among types of ORs because blocking odor-induced activity by naris occlusion affects not only OSN cell density but also OR expression, and the changes are different depending on the specific OR (Coppola and Waggener 2012; Zhao et al. 2013). Furthermore, recent studies in postnatal mouse (Cadiou et al. 2014) show that some neurons expressing specific ORs chronically stimulated during early development decrease in neuronal density but increase RNA per neuron. Thus, odor environments can also regulate the expression of OR at distinct stages of development and this regulation can be in cell number and/or the concentration of RNA in a given cell.

We have previously shown that exposure to PEA during early development results in an increase in the number of cells expressing *otx2* in the OE in both juvenile and adult zebrafish, and this increase is correlated with the formation of an odor memory for PEA (Harden et al. 2006; Whitlock 2006). Thus, our previous findings suggest one potential mechanism for regulation of ORs important in detecting PEA is via the *otx2* transcription factor. The observed changes in *otx2* expression indicate that the peripheral sensory system may play a role in olfactory imprinting by modulating intracellular concentrations of OR RNA or the number of OSNs expressing a given OR. Increases in OR expression coupled with selective downregulation of other ORs may enhance the signal-to-noise ratio of the given receptor or receptors, thus prioritizing odor information experienced during early development. In zebrafish, 143 OR coding sequences have been predicted (Alioto and Ngai 2005; Hashiguchi et al. 2008), and as occurs in other vertebrates, these genes are arranged in clusters within the genome (Sullivan et al. 1996; Alioto and Ngai 2005). Here, we performed 2 *in silico* analyses to localize specific transcription factor binding sites (TFBS) suggested to play a role in the control of OR expression (Harden et al. 2006; McClintock 2010). We then used quantitative polymerase chain reaction (qPCR) and RNAseq analyses to correlate the expression of the ORs containing binding site motifs with changes associated with odor exposure. Through these analyses, we found developmentally regulated changes in OR expression but surprisingly found that exposure to PEA resulted in a lifelong, isomer-specific repression of OR expression for a specific set of ORs, supporting the role of genomic suppression in the fine tuning of OR expression.

Materials and Methods

Animals

Zebrafish from the new wild-type (NWT) and NWT/Cornell strains, derived from the AB line, were used for all experiments. The fish were maintained at 28 °C on a light–dark cycle of 14 and 10 h, respectively. The Institutional Animal Use and Care Committee of the Universidad de Valparaíso approved all animal procedures (#BEA 022-2013).

In silico *analysis*

To select ORs that could potentially be regulated by *otx2*, we used the zebrafish genome (zv9; http://www.ensembl.org/Danio_rerio/Info/Index) to search 3 kb upstream of all 143 OR coding sequences (Alioto and Ngai 2005). We used the Otx2 consensus sequence "TAATCC" (Briata et al. 1999; Kelley et al. 2000; Larder and Mellon 2009) and searched using Ensembl Genome Browser and Matcher (EMBL-EBI). In order to identify regulatory sequences associated with ORs, a whole genome search was performed for DNA binding motifs for the following transcription factors: Otx2, Hnf3b (known to interact with Otx2; Nakano et al. 2000), Lhx2 (Hirota and Mombaerts 2004; Kolterud et al. 2004), and Emx2 (McIntyre et al. 2008). DNA binding motifs in the zebrafish were identified using the Multiple Expectation Maximization for Motif Elicitation software (MEME suite; http://meme.nbcr.net/; Bailey and Elkan 1994). Because of the high-quality data, sequence representation of different TFBS was extracted from the database of Drosophila Transcription Factors DNA-Binding Specificities (http://pgfe.umassmed.edu/TFDBS/). By using MEME, and assuming conservation between orthologous transcription factors from *Droshophila* and zebrafish (Acampora et al. 2001; Boyl et

al. 2001), we used, as the input sequence, the TFBS obtained from Drosophila, and searched for TFBS statistically overrepresented in the zebrafish genome, considering a background sequence of intergenic regions extracted from the zebrafish genome. Once overrepresented TFBS were extracted, the localization on the zebrafish genome was identified by using the Motif Alignment and Search Tool (MAST; Bailey and Noble 2003). Finally, the results were visualized using the Artemis genome browser (Rutherford et al. 2000), obtained from the Sanger Institute Web site (http://www.sanger.ac.uk/resources/software/artemis/). Because the TFBS were identified through homologies, we refer to the sites identified as putative transcription factor binding sites (PutBS).

Odorant exposure

Embryos were collected and separated into experimental and control groups. For qPCR, developing fish were exposed every day for 3 days. For RNA-seq, 3 week and adult (6 months), fish were exposed every day for 3 weeks of development as described previously (Harden et al. 2006). Groups were exposed 1.0 × 10−6 M α PEA (Sigma-Aldrich, P-4277) by adding the appropriate amount of αPEA stock (10–3 M) prepared in distilled water, or 1.0 × 10−6 M βPEA (SAFC, W285811) by adding the appropriate amount of βPEA stock (10–3 M) prepared in distilled water. In a previous publication (Harden et al. 2006), the stock number for αPEA was mistakenly given (Sigma-Aldrich, P-4277) when β PEA was the form used in the imprinting studies. Fresh PEA was added every day and was not paired with feeding or cleaning. For *c-fos* expression analysis, the odorants were added 3 h before fixing the embryos. For the control group, only distilled water was added. All fish were maintained at 28 °C until sacrificed. For analysis by RNA-seq, the odorant exposure was made daily for 3 weeks, after which the fish were transferred to a recirculating system (AHAB-Pentair) without odorant. The PEA exposure experiment was performed in biological triplicates separated by a week.

RNA isolation and cDNA synthesis

RNA was isolated from each developmental stage: 3-day juveniles (100 juveniles/experimental group), 3 week (120 heads/experimental group), and 6 month (OE of 10 fish/experimental group). Juvenile and adult fish OE were dissected in RNA-later solution. Samples were processed with 0.7 mL of TRIZOL Reagent (Invitrogen). RNA was resuspended in 50 µL nuclease-free water (Applied Biosystems) and the concentration was measured using a Nanodrop (ND-1000, Thermo). RNA was treated with DNAse I Amplification grade (Invitrogen) and concentration was measured using Quant-it RNA Assay Kit (Invitrogen). RNA samples from juvenile and adult were precipitated, washed, and stored in 75% ethanol. For larvae, the cDNA was synthesized according to the manufacturer's instructions from 2 µg of total RNA using SuperScript II reverse transcriptase (Invitrogen), Oligo dT (Invitrogen), RNAse Out (Invitrogen), and dNTP mix, in a final volume of 20 µL.

Analysis of OR expression by PCR/qPCR

For PCR analysis, 2 µL of cDNA was used, 10 µmol of each primer (Table 1), and GoTaq Green Master Mix (Promega), in a final volumen 20 µL. Products were visualized using a 3% agarose gel in TAE buffer. Primers were designed for conventional and real-time PCR at equivalent parameters for each gene. qPCR analysis was performed on Mx3000p (Stratagen) thermocycler, using SYBR Green. Two µL of cDNA dilution (1:2) was used, 3 pmol of each primer (Table 1), 1× of Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) to a final volume of 10 µL. Relative quantification was done by ΔΔCt using beta actin as housekeeping gene (Table 1) and no RT and no template controls for each gene. The data were analyzed using the thermocycler software (MxPro-Mx3000P v4.10) and REST MC©-v2 software. The primer efficiency (Table 1) was calculated; any primer pair with efficiency lower than 95% and greater than 110% was

Table 1. Primers for OR genes analyzed by conventional and real-time PCR

Gene	Accession Number	Primer (5`-3`)	Amplicon length	Percent of efficiency at 56ºC
OR 102-4	DQ306112	F) TGAGCCCTACTGTGCTACAA R) TCAGGGCCCATGCTAATGTT	119 bp	102.7
OR 103-1	DQ306104	F) CTTACTCCCCTGGCCTTTAT R) CAGGTGGAGAAAGTCTTCAG	101 bp	98.4
OR 111-1	DQ306093	F) GTGGCCTTGATGGTGTCTTTGA R) CACTGCATGCCAACCTATACACTG	106 bp	99.2
OR 115-5	DQ306037	F) ATTCTGTGGCAGCCCTCATA R) GATGGCTTTGCTGTTGAGTG	110 bp	109.0
OR 125-1	DQ306121	F) CTGGTCGCTGCATGTAAAGCATCT R) AGGTGGAACAATCACCAGCTCT	192 bp	96.3
OR 136-1	DQ306047	F) TCAAAAGCCCACGATGACCACAAC R) TTTCTGGCAGCGAACAGGACT	107 bp	106.0
OR 137-3	DQ306051	F) TTGTCGAGTGCTGATTGCTG R) AGAGCAAGAGCTGTACTCCA	102 bp	101.1
beta actin	AF057040	F) CGAGCAGGAGATGGGAACC R) CAACGGAAACGCTCATTGC	102 bp	95.0

discarded. Analysis of the qPCR data was done using GraphPad Prism 5 software. qPCR results were compared using the Student's t-test and a false discovery rate analysis for multiple testing was performed using the Benjamini–Hochberg test (Benjamini and Hochberg 1995).

Whole mount in situ *hybridization*

To analyze the PEA effects on *otx2, c-fos*, and OR expression, juveniles were exposed daily to αPEA and βPEA following the protocol of Harden et al. (2006) and processed for whole mount *in situ* hybridization. Single-strand RNA probes was synthesized using *otx2, c-fos, OR103-1, OR111-1* (Sato et al. 2005), *OR115-5*, and *OR125-1* cDNA plasmids, and labeled with UTP digoxigenin with SP6/T7 Transcription Kit (Roche). Tissue was fixed with parafomaldehyde 4% in PBS at 48 and 72 hours postfertilization (hpf). For each treatment, the number of *otx2* positive cells in each OE (n = 20), *c-fos* positive cells (n = 22 OE), and *OR111-1* (n = 40) were counted and analyzed. No positive cells were reliably detected for ORs *OR103-1*, *OR115-5*, and *OR125-1* in spite of repeating the experiment twice (n = 40 each experiment) and running these ORs with *OR111-1*. The number of cells expressing *otx2* and *c-fos* was analyzed using 1-way analysis of variance (ANOVA) and Bonferroni's multiple comparison post-test. For all analyses, a P value < 0.05 was used and the number of cell expressing *OR111-1* was analyzed using the Student's t-test.

Transcriptomic analysis

The RNA-seq analysis was performed by GENEWIZ Inc. using an Illumina Highseq 2500 sequencer in a configuration of 1 × 50 bp Single End reads. Three biological replicates were run for all experiments. The reads obtained for each sample in the sequencing process were aligned to the zebrafish genome. The gene expression values for all samples were measured calculating

the RPKM values for all transcripts annotated in the zebrafish genome. Values were normalized by the reads distribution of any transcript in each sample, using a quartile transformation of the RPKM values. In order to compare the gene expression in control and βPEA-exposed groups, the fold change of all transcripts was calculated using normalized expression values.

Results

Analysis of TFBS

In order to localize Otx2 binding sites in the regions flanking the ORs, we initially searched regions lying within 3 kb directly upstream of 143 OR coding sequences (Alioto and Ngai 2005) in the zebrafish genome (zv9) using the Otx2 consensus sequence (TAATCC; Briata et al. 1999; Kelley et al. 2000; Larder and Mellon 2009) as the target sequences. Because the coding sequences for the ORs are arranged in tight clusters in the genome, regions greater than 3 kb often entered the coding sequences of adjacent ORs. Using this search paradigm, we located at least 1 binding site lying upstream of 34 of the 143 OR genes. These 34 ORs belong to 16 different families that localized to 4 different chromosomes, with the majority of these ORs being on chromosome 15 (Figure 1, red, light gray). Based on our previous experience with specific ORs and quality of genomic data, we selected 7 ORs from the original 34 ORs with putative Otx2 binding sites (PutBS). In order to further define regulatory sequences controlling ORs, we performed an analysis using open access bioinformatics tools (see Materials and methods) and searched the zebrafish genome for transcription factors associated with the ORs. Because the data are more complete, we used DNA binding site information obtained from the Drosophila genome to perform an approximation based on orthologous genes in Drosophila and zebrafish to look for PutBS of *otx2, lhx, emx2*, and *hnf3β* in the zebrafish genome (Figure 2). Our analyses showed that all PutBS were dispersed throughout the regions of OR clusters with some regions having multiple binding sites. We next focused our analysis on the 3 kb region upstream of 7 ORs from the initial binding site analysis and found DNA binding motifs for all PutBS upstream of the 7 ORs analyzed (Figure 3). There was no apparent pattern for the Emx2

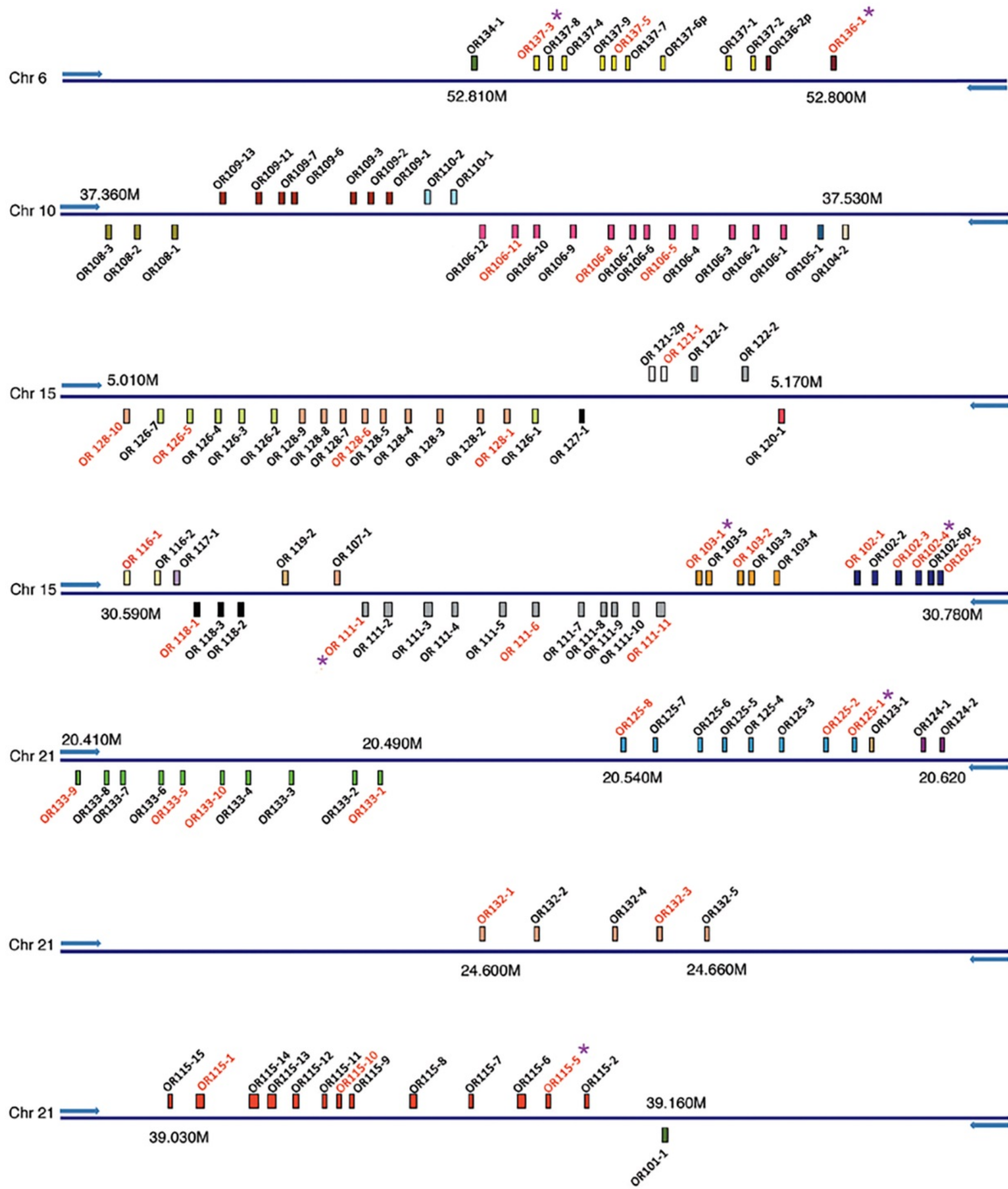


Figure 1. Identification of ORs with putative Otx2 binding sites. *In silico* analysis revealed 34 ORs containing the putative Otx2 binding site lying upstream of the coding sequences. These genes belong to 16 ORs subfamilies (indicated by numbers) located on chromosome 6 (Chr 6), chromosome 10 (Chr 10), chromosome 15 (Chr 15), and chromosome 21 (Chr 21) with plus and minus strands indicated (arrows). For expression analysis (Figures 4 and 5) 7 ORs, with putative Otx2 binding sites (asterisks), belonging to different subfamilies were initially selected: *OR137-5, OR136-1, OR102-4, OR103-1, OR111-1, OR115-5* and *OR125-1*.

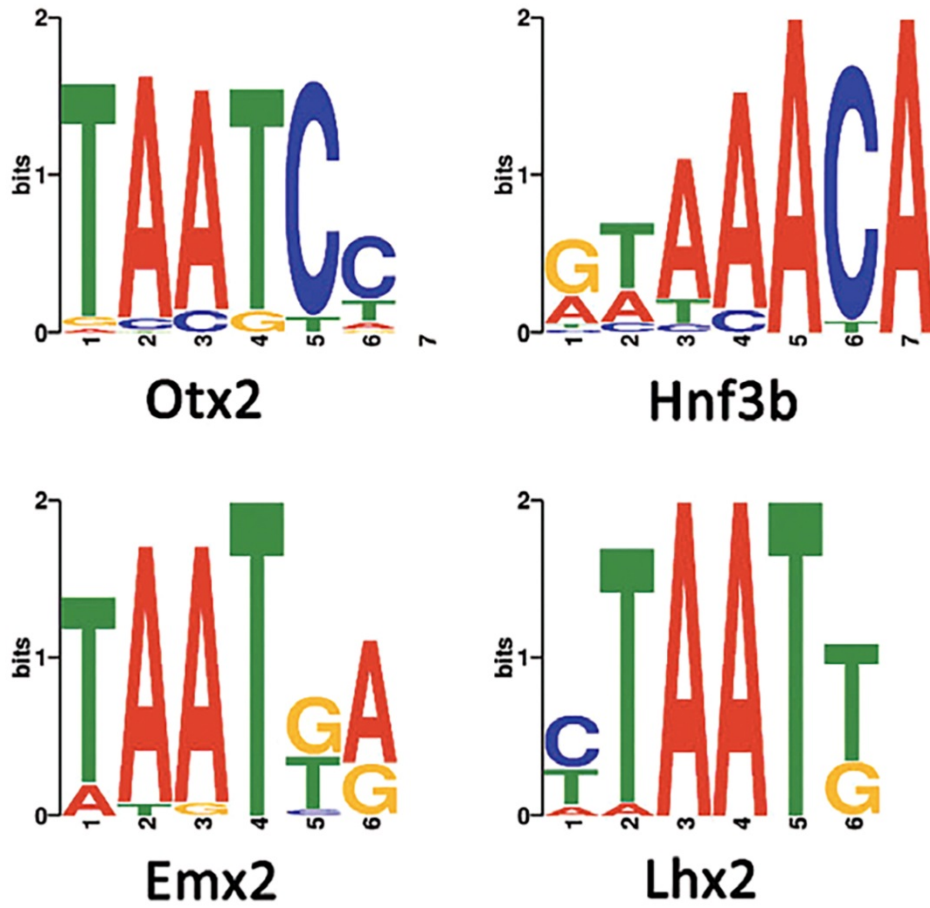


Figure 2. DNA binding motifs of conserved transcription factors Otx2, Hnf3b, Emx2, and Lhx2. The DNA binding motifs were created from aligned sequences of Drosophila orthologous TFs using MEME software and depict the consensus sequences for Otx2, Hnf3B, Emx2, and Lhx2. The motifs are represented graphically by a logo where the height of each nucleotide indicates the level of conservation**.**

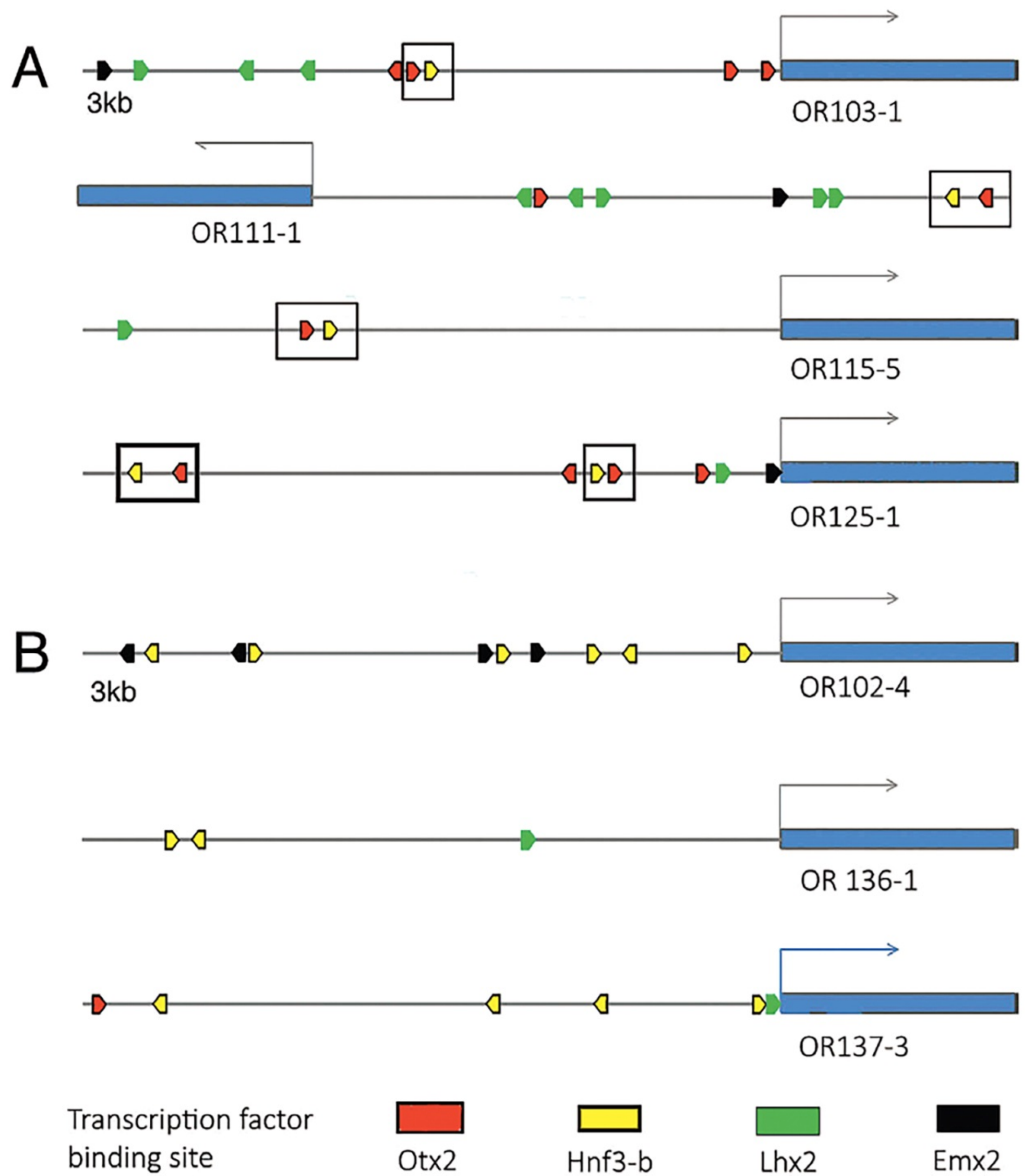

A
3kb
OR103-1
OR111-1
OR115-5
OR125-1
B
3kb
OR102-4
OR 136-1
OR137-3
Transcription factor binding site
Otx2
Hnf3-b
Lhx2
Emx2

Figure 3. Analysis of potential regulatory sequences controlling ORs. Binding sites for transcription factors Lhx2 (green/gray), Emx2 (black), Hnf3b (yellow/light gray box), and Otx2 (red/dark gray box) were all localized in the vicinity of ORs. (A) For 4 of the ORs chosen for expression analysis (Figures 4 and 5), Otx2 and Hnf3b motifs were located in close proximity (less than 150 bp, boxed in black). (B) For 3 of the ORs there was no association of Otx2 motifs with Hnf3b motifs. All sequences represent 3 kb upstream of the start site. DNA binding motifs for PTBS Emx2 and Lhx2 showed no apparent pattern.

and Lhx2 motifs (Figure 3, black, green/light gray). In contrast, 4 ORs contained both Otx2 and Hnf3b binding sites on the same strand, which were separated by less than 150 bp (Figure 3A, boxed area). The remaining 3 ORs had no Otx2 motifs in close association with Hnf3b (Figure 3B). Thus, we next tested whether there were differences in OR expression that correlated with the PutBS patterns observed by the bioinformatic analysis.

ORs expression in different developmental stages

Previous results from both our lab and that of others (Whitlock and Westerfield, 1998; Barth et al., 1996; Byrd et al., 1996) have shown that ORs are expressed during early development in the zebrafish starting at 24 hpf. In order to confirm and define the developmental onset of gene expression for the selected ORs, we isolated RNA from 24, 48, and 72 hpf embryos and analyzed their expression by reverse transcriptase–PCR (RT–PCR). Our results show that 5 of the ORs analyzed by RT–PCR initiated expression in the first 3 days of development but with slight differences in the time of onset: *OR102-4* and *OR111-1* were detected at 24 hpf, *OR125-1* was detected at 48 hpf, and *OR103-1* and *OR136-1* were detected at 72 hpf (Figure 4). *OR115-5* and *OR137-3* were not detected at any of the developmental stages analyzed by RT–PCR, but they were detected at 72 hpf for qPCR (see below). Because we have previously shown that PEA-induced changes in *otx2* expression are detectable at 48 and 72 hpf (Harden et al. 2006) and all 7 ORs chosen in this study are expressed at 72 hpf (Figures 4 and 5), we chose this developmental stage for analysis of OR expression in response to PEA.

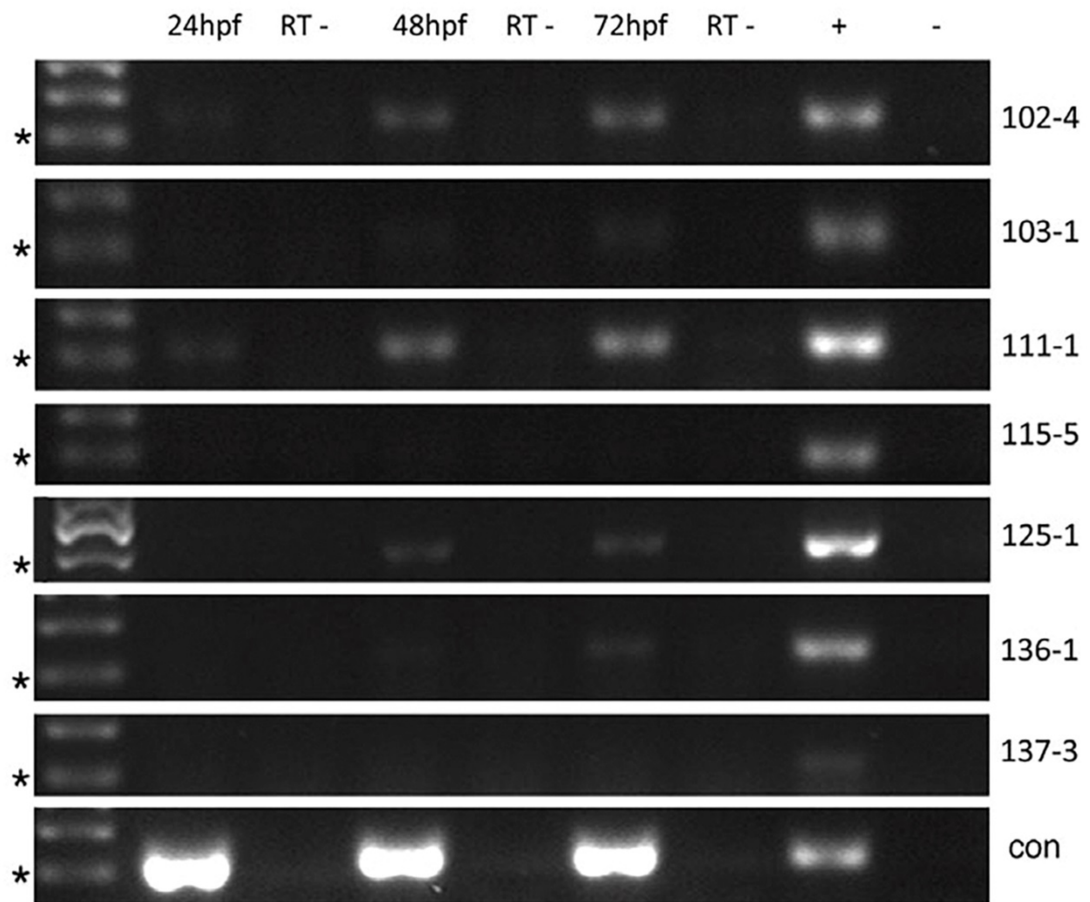
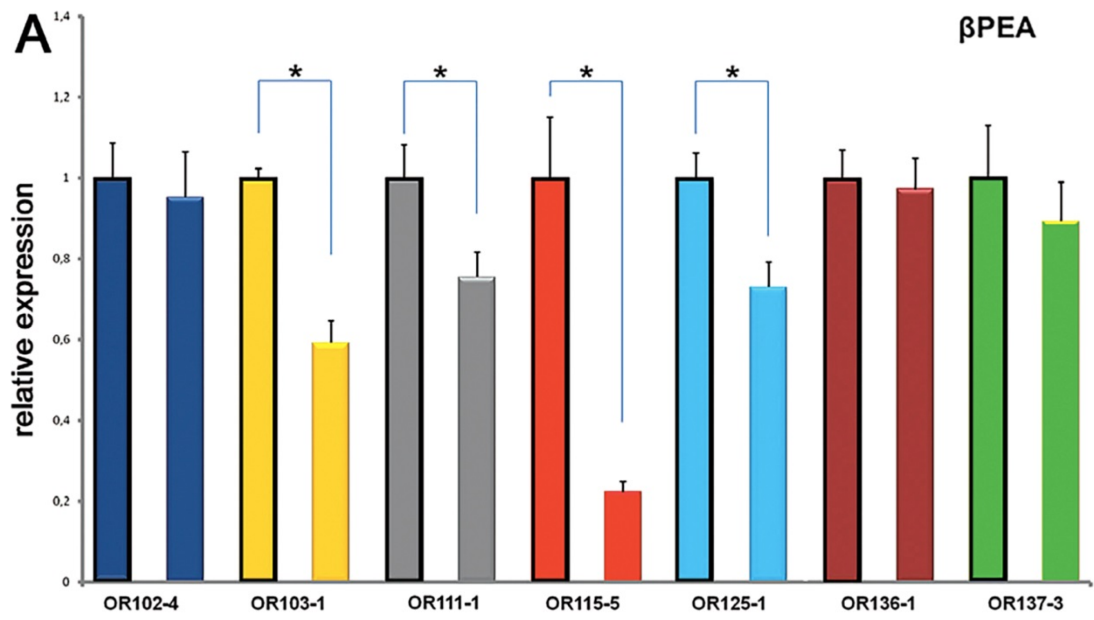


Figure 4. ORs expression increases during the first 3 days of development. RT–PCR analysis of 7 ORs with Otx2 binding sites within 3 kb upstream of coding region: at 24 hpf, only *OR102-4* (Ch 15) and *OR111-1* (Ch 15) were detected, at 48 hpf, *OR125-1* (Ch 21) was detected, and *OR103-1* (Ch 15) and *OR136-1* (Ch 6) were detected at 72 hpf. Positive controls were done with genomic DNA and negative controls correspond to no template controls. Asterisks indicate 100 bp; control is *β-actin*; 30 cycles per PCR run.

PEA-induced changes in gene expression

To determine whether the 7 selected ORs showed changes in expression in the presence of PEA, we performed an analysis of transcriptional response by qPCR at 72 hpf (Figure 5). We exposed embryos to the β isomer of PEA during the first 3 days of development and assayed by qPCR, differences in expression of the 7 representative ORs with Otx2 binding sites, in control and odorant-exposed groups. The βPEA exposure resulted in changes in OR expression. Surprisingly, 4 ORs analyzed showed significantly lower levels of expression in the presence of βPEA (Figure 5A). Specifically, *OR103-1* (ratio = 0.593 ± 0.06), *OR111-1* (ratio = 0.755 ± 0.06), *OR115-5* (ratio = 0.224 ± 0.03), and *OR125-1* (ratio = 0.731 ± 0.06) showed reduced expression. In contrast, the relative expression *OR102-4* (ratio = 0.95 ± 0.11), *OR136-1* (ratio = 0.97 ± 0.07), and *OR137-3* (ratio = 0.89 ± 0.09) showed no significant differences between the control and βPEA group. Of the 7 ORs analyzed by qRT–PCR, we observed that *OR115-5* showed the most dramatic difference in expression when the juveniles were raised in the presence of βPEA (Figure 5A; Supplementary Table 1). The 4 ORs that showed lower levels, as measured by qPCR contained Otx2 and Hnf3b motifs (Figure 3A) lying in close association (<150 bp). In contrast, the 3 ORs that showed no changes in expression have no Otx2 motifs in close association with Hnf3b (Figure 3B).

In order to determine whether the changes in the OR expression were specific to the PEA isomer, we exposed embryos from the same cohort to the α isomer of PEA (Figure 5B). Strikingly, in comparing the relative expression of the 7 ORs between control and αPEA exposed embryos, we observed that *OR102-4* (ratio = 1.12 ± 0.16), *OR103-1* (ratio = 1.01 ± 0.11), *OR111-1* (ratio = 0.99 ± 0.08), *OR115-5* (ratio = 0.91 ± 0.02), *OR125-1* (ratio = 1.01 ± 0.07), *OR136-1* (ratio = 0.99 ± 0.09), and *OR137-3* (ratio = 1.10 ± 0.09) showed no significant


A
βPEA
relative expression
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
*
*
*
OR102-4
OR103-1
OR111-1
OR115-5
OR125-1
OR136-1
OR137-3

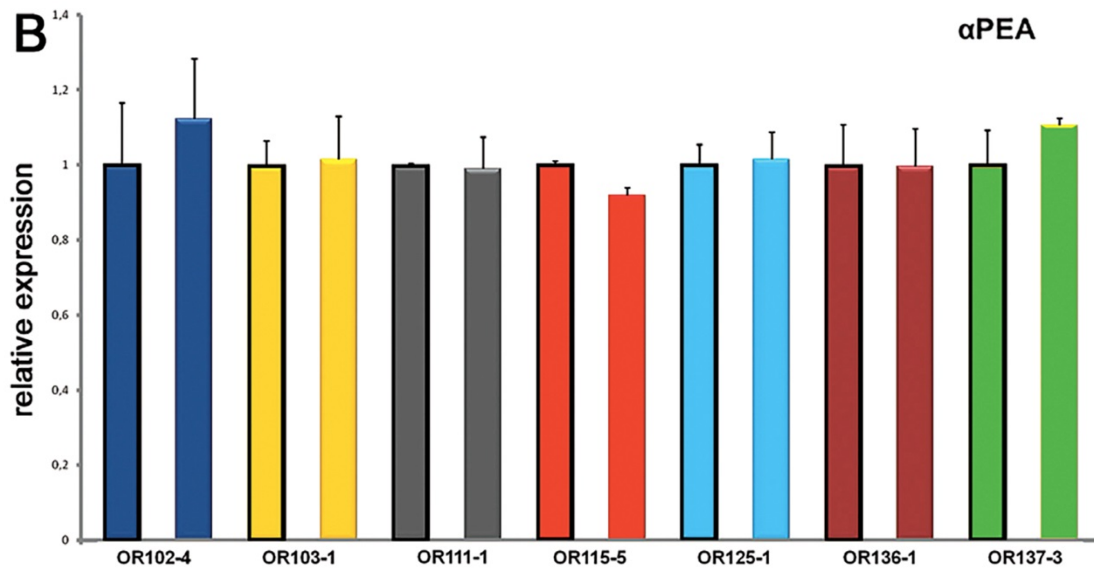
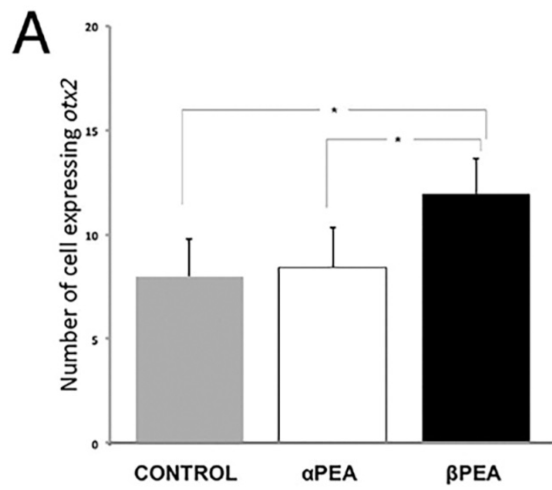

B
αPEA
relative expression
1,4
1,2
1
0,8
0,6
0,4
0,2
0
OR102-4
OR103-1
OR111-1
OR115-5
OR125-1
OR136-1
OR137-3

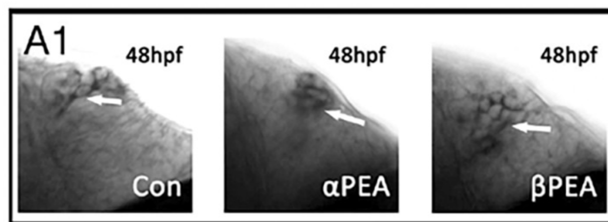
Figure 5. βPEA exposure is correlated with a decrease in OR expression when analyzed by qPCR. (**A**) Exposure to βPEA is associated with significant decreases in expression in 4 out of 7 ORs (*OR103-1; OR111-1; OR115-1; OR125-1*) in 3 dpf juveniles. For 3 ORs, *OR102-4, OR136-1*, and *OR137-3*, there was no difference in the expression ratio between control (bars with dark outline, left) and β PEA-exposed juveniles (Ct values Sup Table 1). (**B**) The αPEA-exposed groups showed no change in the expression ratio of the 7 ORs (*P < 0.05; analyzed by t-test; error bars represent standard error of the mean; Ct values Sup Table 2). Control for each OR on left with black outline. Data from 3 dpf juveniles (100/experimental group). 40 cycles per PCR run.

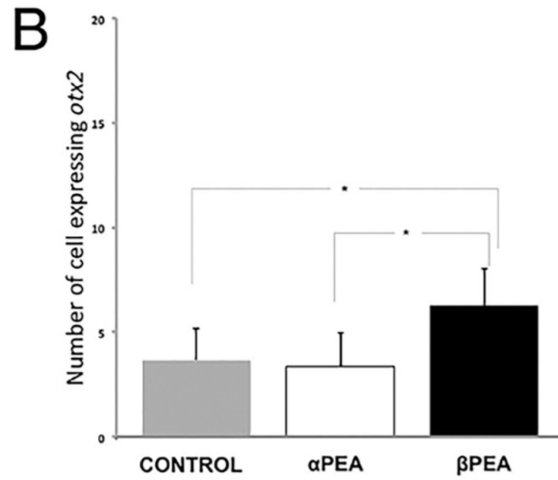
differences ($P < 0.05$) between experimental and control groups (Figure 5B; Supplementary Table 2).

PEA isomers have different effects on otx2 expression

To further analyze the differential effects of αPEA versus βPEA, we assayed *otx2* expression in the developing OE of 48 and 72 hpf larvae by whole mount *in situ* hybridization (Figure 6). Consistent with our previous results, βPEA-exposed 48 hpf juveniles showed an increase in the number of cells expressing *otx2* with an average of 11.9 ± 1.7 *otx2* positive cells/OE (n = 20) compared with control groups with 8.0 ± 1.8 otx2 positive cells/OE (n = 20). In 72 hpf juveniles, the increase in the number of *otx2* expressing cells was still apparent with the βPEA-exposed animals showing a significantly greater number of *otx2* positive cells/OE (6.3 ± 1.8 cells/OE; n = 20), compared with the control groups with (3.6 ± 1.5 cells/OE; n = 20). In contrast, exposure to the αPEA during development did not change the number of cells expressing *otx2*. For the 48 hpf juveniles, the average number of *otx2* expressing cells in the αPEA-exposed group (8.5 ± 1.9 cells/OE; n = 20) was not significantly different from the control group with (8.0 ± 1.8 cells/OE; n = 20). Likewise, for the 72 hpf juveniles (Figure 6B), there was no significant difference between the control (3.6 ± 1.6 positive cells/OE; n = 20), and αPEA-exposed animals (6.7 ± 1.1 cells; n = 22), revealing no statistically significant difference in the number of *otx2* positive cells/OE in the juveniles exposed to αPEA. In contrast, our analysis showed significant differences in the number of *otx2* expressing cells in the group exposed to βPEA, at both 48 and 72 hpf ($P < 0.05$, by 1-way ANOVA). Finally, we found significant differences ($P < 0.05$, by 1-way ANOVA) when comparing the number of *otx2* expressing cells in the αPEA- and βPEA-exposed groups.


A
Number of cell expressing otx2
20
15
10
5
0
*
*
CONTROL
αPEA
βPEA


A1
48hpf
48hpf
48hpf
Con
αPEA
βPEA


B
Number of cell expressing otx2
20
15
10
5
0
*
*
CONTROL
αPEA
βPEA

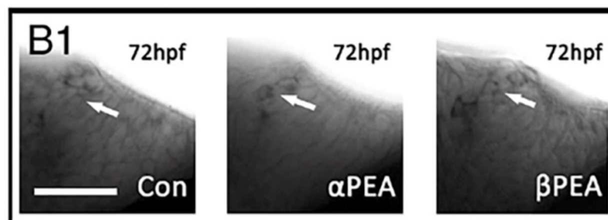

B1
72hpf
72hpf
72hpf
Con
αPEA
βPEA

Figure 6. Change in *otx2* expression in OE of juvenile zebrafish depends on isomer of PEA used. Number of cells expressing *otx2* at 48 hpf (**A**) and 72 hpf (**B**), detected by whole mount *in situ* hybridization (**A1**, **B1**, arrows). (**A**) At 48 hpf, the mean number of *otx2* positive cells was 8 ± 1.8 cells (n = 20) in controls, 8.5 ± 1.9 cells (n = 20) in the αPEA group, and 11.9 ± 1.7 cells (n = 20) in the βPEA group. No significant differences were observed between control and αPEA groups. (**B**) At 72 hpf, the same isomer-specific increase in *otx2* positive cells was observed in juveniles: 6.3 ± 1.8 (n = 20) in βPEA exposed versus 3.7 ± 1.5 (n = 20) for controls. αPEA-exposed juveniles showed 3.4 ± 1.6 (n = 20) *otx2* positive cells, thus not different from that of the controls. *P < 0.05 by 1-way ANOVA and Bonferroni's multiple comparison posttest; error bars represent standard deviation.

Analysis of c-fos expression

In order to determine whether PEA was eliciting a physiological response, we exposed fish to PEA (see Materials and methods) and analyzed the expression of *c-fos*, an immediate early gene (IEG) known to mount a transcriptional response to neuronal activity. We exposed zebrafish at 2 and 3 dpf to αPEA and βPEA to determine whether they elicit a genome response to potential neural activity caused by the PEA odorants. The number of *c-fos* expressing cells was analyzed at 48 and 72 hpf (Supplementary Figure 1). At 48 hpf, there was no difference in the mean of *c-fos* positive cells in the αPEA (6.9 ± 1.5 cells; n = 22) and βPEA (6.9 ± 1.4 cells; n = 22) when compared with the control group (6.6 ± 1.2 cells; n = 22). Likewise, at 72 hpf, no significant differences were observed between control (6.6 ± 1.1 cells; n = 22), αPEA (6.7 ± 1.1 cells; n = 22), and βPEA (6.6 ± 1.0 cells; n = 22) groups.

Genome-wide transcriptome analysis by RNA-seq

In order to determine whether the changes observed in the qPCR analysis persisted in time, we performed RNA-seq on βPEA exposed 3-week-old juveniles and adult OE. Our analysis revealed changes in a variety of ORs at 3 weeks (Supplementary Table 3) and in the adult (Supplementary Table 4) including the original 7 ORs analyzed by bioinformatics and qPCR. When comparing the qPCR and RNA-seq data (Figure 7), 2 receptors (*OR102-4, OR103-1*) were not consistently detectable across the developmental analysis (Figure 7B, asterisk, #; Figure 7C, asterisk). In contrast, in the presence of βPEA, the expression of 3 ORs assayed by RNA-seq was lower than in controls and this pattern of decreased expression continued through adulthood (Figure 7, boxed area). Of the 3 ORs with consistently detectable and decreased expression across the developmental analysis, 2 of these receptors are located on chromosome 21+ (OR115-5, OR125-1) and the third receptor on chromosome 15 (OR111-1). Two ORs, OR136-1 and OR137-3, that showed consistently detectable but variable changes across the developmental

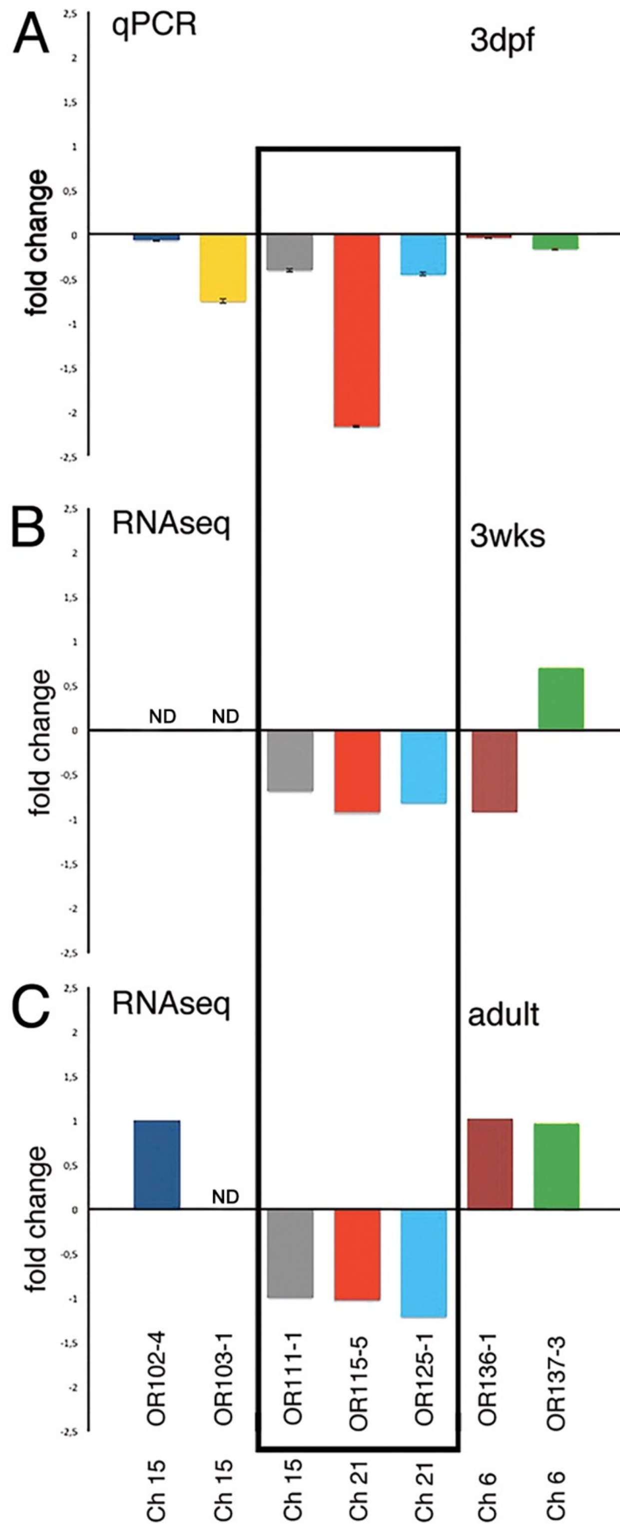
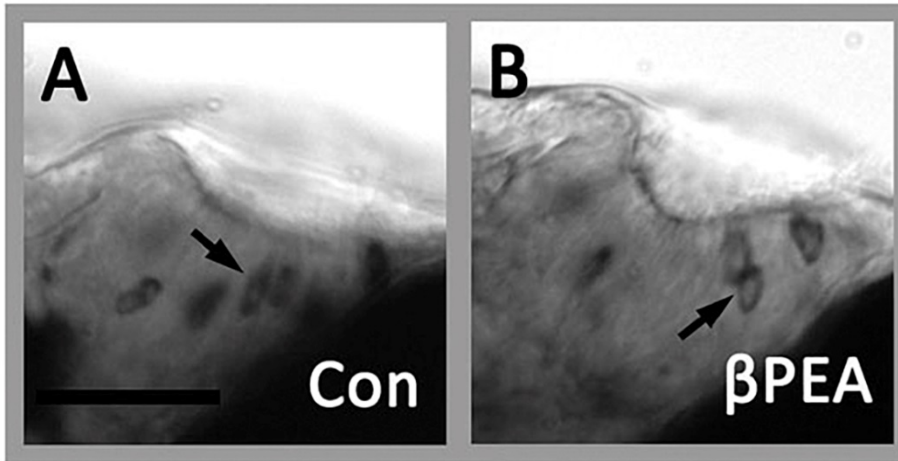

A
qPCR
3dpf
fold change
B
RNAseq
3wks
fold change
ND
ND
C
RNAseq
adult
fold change
ND
OR102-4
OR103-1
OR111-1
OR115-5
OR125-1
OR136-1
OR137-3
Ch 15
Ch 15
Ch 15
Ch 21
Ch 21
Ch 6
Ch 6

Figure 7. Changes in OR expression as assayed by qPCR and RNA-seq. Changes in OR expression assayed in PEA-exposed animals at 72 hpf (A, qPCR), 3-week juveniles (B, RNA-seq), and adult zebrafish (C, RNA-seq). (A) Abundance measured by qPCR (Figure 5A) transformed to fold change expression (*OR102-4*= -0.07 ± 0.01; *OR103-1*= -0.75 ± 0.02; *OR111-1*=-0.41 ± 0.01; *OR115-5*= -2.161 ± 0.01; *OR125-1*= -0.452 ± 0.02; *OR136-1*= -0.04 ± 0.00; *OR137-3*= -0.163 ± 0.01). (B) Fold change expression plot of the 7 ORs in 3-week juveniles measured by RNA-seq with lower expression for 5 ORs and higher expression for *OR137-3. OR102-4* and *OR103-1* were not detected at this stage (ND). (C) Expression of *OR111-1, OR115-5*, and *OR125-1* (boxed area) remains lower in PEA treated versus controls, consistent with results obtained at earlier developmental stages (A, B). In contrast, *OR102-4*, *OR136-1*, and *OR137-3* showed greater expression. *OR103-1* was not detected (ND).

analysis are both located on chromosome 6 (Ch 6). Thus, 3 of the 4 ORs originally shown by qPCR to have reduced expression in the presence of βPEA at 3 dpf consistently maintained the reduced expression pattern at 3 weeks and as adults when assayed by RNA-seq. In contrast, the fourth receptor (OR103-1) was not detected in the RNA-seq analysis.

Analysis of OR expression by in situ hybridization

In order to determine whether the changes observed in OR expression were due to changes in the number of OSNs expressing a given OR, or to changes in levels of OR expression within the cell, we performed *in situ* hybridization to visualize OR expression. We examined expression of ORs *OR111-1, OR103-1, OR115-5* and *OR125-1* because they showed decreases in expression in response to PEA (Figure 5) with only *OR111-1, OR115-5* and *OR125-1* showing sustained downregulation at 3 weeks and in adult animals. Using digoxigenin-labeled RNA probes generated against *OR111-1, OR103-1, OR115-1*, and *OR125-1*, we used 3 dpf embryos with βPEA to look for difference in OR expression. Of the 4 probes, only the probe recognizing *OR111-1* gave consistent results in 3 dpf juvenile fish (Figure 8A,B). Quantification of the results showed no difference in the number of *OR111-1* positive cells in control with a mean of 6.9 ± 1.9 cells (n = 40) and βPEA-exposed animals with 6.8 ± 2.8 cells (n = 40; Figure 8C).


A
Con
B
βPEA

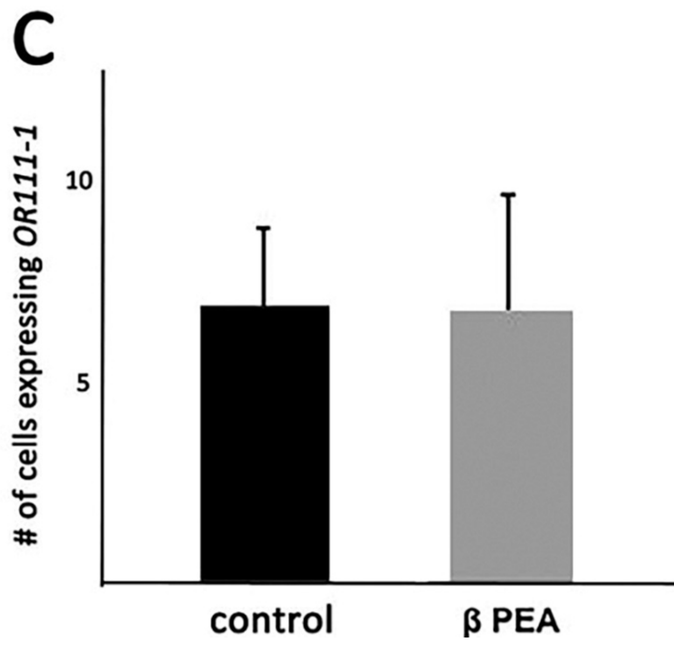

C
# of cells expressing OR111-1
10
5
control
β PEA

Figure 8. Number of *OR111-1* expressing cell in OE of zebrafish larvae does not change in the presence of βPEA. (**A**, **B**) Olfactory organ at 72 hpf processed for *in situ* hybridization for *OR111-1*. Individual cells expressing the receptor (arrow) are identifiable in the whole mount preparations in control animals (**A**) and βPEA-exposed animals (**B**). (**C**) The mean of *OR111-1* positive cells was 6.9 ± 1.9 (n = 40) in the control group and 6.8 ± 2.8 cells (n = 40) in the βPEA group. No significant differences were observed between control and βPEA groups. Scale bar = 30 μm. Error bars represent standard deviation.

Discussion

We have previously shown that zebrafish, like salmon (Hasler and Scholz 1983), can make and maintain memories of odorants experienced during early development and that exposure to PEA results in correlated changes in gene expression within the developing OE (Harden et al. 2006; Whitlock 2006). Here, we analyzed potential downstream targets of *otx2* transcription factor specifically ORs and found that 34 OR genes contained PutBS for Otx2 as well as other TFs, most importantly Hnf3b. Analysis of a specific subset these 34 ORs showed they were all expressed by the third day of development in control animals, and that 4 ORs are downregulated in response to βPEA. In agreement with previous behavioral studies showing that βPEA elicits an imprinting response (Hasler and Scholz 1983; Nevitt et al. 1994; Harden et al. 2006), here we show that downregulation of OR expression was specific to the βPEA isomer. Analysis of OR downregulation using *in situ* hybridization supports a model where modulation of OR expression occurs through changes in levels of RNA expression and not number of cells expressing a given RNA. The decreases in OR expression were detected at 3 dpf, 3 weeks, and in adults, thus supporting a model where PEA induced long-term changes in gene expression.

OR expressed during early development

The repertoire of chemosensory receptor gene expression can be variable during development in both vertebrate and invertebrate animals. In *Drosophila melanogaster*, the repertoire of chemoreceptor genes changes during development, in the adult is dependent upon mating, and is highly sexually dimorphic (Zhou et al. 2009). Likewise in rat, specific OR genes are expressed more highly in juveniles, yet overall OR gene expression does not decrease with age (Rimbault

et al. 2009). More relevant to this study, in salmon, the expression of ORs changes during development and these changes are different among specific anadromous populations (Johnstone et al. 2011). Furthermore, there is a 50-fold increase in ORs at the parr–smolt transition, the metamorphic-like change that prepares the fish to leave fresh water for the open sea (Dukes et al. 2004). Thus, the changes we have observed may be a mix of developmentally programed expression of ORs on which the environment induces life history–induced variation.

In situ hybridization analysis of OR expression in developing zebrafish embryos showed that OR mRNA can be detected as early as 24 hpf for some receptors, whereas for others expression was not detected until 120 hpf (Barth et al. 1996; Byrd et al. 1996), thus supporting a model of asynchronous expression of ORs. Data obtained in mouse support a model where developmental onset is correlated with chromosomal location such that ORs initiating expression at the same developmental stage are located on the same chromosome (Rodriguez-Gil et al. 2010). Our data suggest a potential relationship between chromosomal location and onset of OR expression where ORs on chromosome 15 and chromosome 21 were detected earlier than those located on chromosome 6 with the exception being *OR115-5* located on chromosome 21 and not expressed until 72 hpf. Thus, the data presented here support the idea that onset of OR expression is correlated with chromosomal location of the OR, although more data are needed to verify this potential trend.

Isomer-dependent effect of PEA on gene expression

Here, we showed for the first time that the previously reported effects of PEA on *otx2* expression as well as the current report of changes in OR expression are isomer specific where only exposure to the β-form of PEA elicits a decrease in OR expression that persists throughout the life of the animal. Although it has been shown that the olfactory system is capable of discriminating between compounds as similar as enantiomers (Laska and Teubner 1999; Joshi et al. 2006), there are currently no data of isomers causing differential effects on OE gene

expression. However, there are diverse data showing differential isomer effects on gene expression. For example, neuroblasts respond differentially in vitro to retinoic acid isomers, changing the expression levels of different nuclear receptors dependent on the isomers to which they are exposed (Lovat et al. 1999). Similarly, crotonitrile isomers cause different neurotoxic effects in vivo, where cis-crotonitrile causes degenerative damage, but trans-crotonitrile does not have appreciable effects (Balbuena and Llorens 2003). These studies suggest that the molecular structure is important in triggering a genomic and physiological response, as in the case of PEA where there is only a hydroxyl group position change in the PEA isomers, yet this subtle difference results in an isomer that changes OR expression (βPEA) and an isomer (αPEA) that does not.

PEA exposure is correlated with a reduction in OR expression

The consistent decrease in the OR expression levels of ORs *111-1, 115-5*, and *125-1* at 6 months post-fertilization is striking because the adult fish were exposed for only the first 3 weeks of development to β PEA, a period that correlates with metamorphosis in Danios as judged by pigment pattern (McClure 1999; Parichy and Turner 2003). Thus, the decrease in OR expression was maintained in the absence of βPEA, an observation correlated with our previous finding showing that the increase in *otx2* expression in βPEA-imprinted zebrafish is maintained in the adult OE (Harden et al. 2006). The ORs that show lower levels of expression throughout life in zebrafish exposed to βPEA (Figure 7; *OR111-1, OR115-5, OR125-1*) may be those that are under the control of PEA via Otx2/Hnf3b regulation. Using transfection assays, it has been shown that the Otx2 homeodomain and C-terminal regions bind to HNF-3beta resulting in HNF-3beta repression of OTX2- directed gene expression (Nakano et al. 2000). We suggest a model (Figure 9) where interactions between Otx2 and HNF-3beta may result in enhancement of the signal-to-noise ratio through repression of specific ORs and activation of others. In the presence of β PEA, the effects of Otx2 are mediated through Otx2/Hnf3b and Otx2 reflected by the

potential expansion of cells expressing *otx2*. This expansion allows the decrease in ORs controlled by Otx2/Hnf3b on Ch 15 (*OR103-1, OR111-1*) and Ch 21 (*OR115-1, OR125-1*). The expansion in the number of cells expressing *otx2* may reflect positive regulation of ORs sensitive to PEA. This may reflect activity-regulated high- and low-pass filters needed to prioritize sensory information (Monahan and Lomvardas 2012; Santoro and Dulac, 2012) where the first step in olfactory processing is to filter information before it arrives in the CNS. Thus, a constant, stochastic (Lyons and Lomvardas 2014) expression of receptors could be "edited" by the activity where, in response to odor stimulation, OSNs with activated ORs expand and/or repress lineages and maintain them.

PEA does not elicit IEG activity in the OE

IEGs are a rapid and transient response to a wide variety of cellular stimuli including neural activity. IEGs expression, such as *c-fos* and *c-jun*, can be elicited by neural activity including the perception of odors where specific loci (olfactory glomeruli) show foci of activity correlated with physiological responses to odors (Guthrie et al. 1993). More recently, *c-fos* expression has been reported in the OE and vomeronasal organ in mouse (Norlin et al. 2005; Haga- Yamanaka and Touhara 2013). Here, we showed βPEA does not elicit a genomic response as reflected by changes in *c-fos* expression, although there are little data on odor-elicited changes in IEGs in the peripheral nervous system including the OE. In zebrafish, the pattern of expression of the *c-fos* does change with some odorants such as amino acids and social odors, and the cells are generally located in distinct odor-dependent regions of the OE (Maturana 2010; Calfún C, Maturana C, McKenzie M, Harden M, and Whitlock K, unpublished data). Because no changes in *c-fos* expression were observed, the PEA response maybe be transmitted through other IEGs, of which over 30 have been identified or alternatively PEA may have a yet to be described mechanisms of action for eliciting a response.

TFBS and OR expression

The localization of multiple Otx2 PutBS binding sites in close proximity to OR gene clusters within the zebrafish genome suggests a potential role in odor-induced changes of OR expression during development and in the adult zebrafish. The mechanisms controlling olfactory gene expression are complex and poorly understand with gene regulation OR gene families occurring at the level of the clusters (Serizawa et al. 2003; Lomvardas et al. 2006; Fuss et al. 2007; Nishizumi et al. 2007) as well as the chromatin level where the open versus closed of the chromatin can regulate expression (Magklara et al. 2011; Santoro and Dulac 2012; Lyons et al. 2014) not only in mouse but potentially in zebrafish. Studies of OR gene regulation in mouse have uncovered zones rich in homeodomain binding sites (Lane et al. 2001; Vassalli et al. 2011) and mutations in the homeodomain sequences eliminate the expression of the OR gene family (Vassalli et al. 2011) and cause a reduction in the levels of DNA methylation (Lyons and Lomvardas 2014). The Emx2 homeodomain PutBS is interesting because microarray analysis of Emx2 mutants shows decreased mRNA levels in 365 ORs (McIntyre et al. 2008; McClintock 2010); however, these decreases in expression may result from loss of olfactory tissues because Emx2 is necessary for olfactory system formation. The roles of the transcription factors Emx2 and Lhx2 cannot provide a mechanism for the precise expression of only 1 allele of 1 odorant receptor gene in each OSN (Hirota and Mombaerts 2004; McIntyre et al. 2008); rather these TFs appear to act on whole clusters. Furthermore, ORs within a cluster can be modulated by a common regulatory element with a proximal to distal gradation of repression (Fuss et al. 2007). These studies indicate the potential importance in OR expression of homeodomain containing TFs, such as *otx2* (Acampora et al. 1995). Additionally, we observed PutBS for Otx2 and Hnf3b upstream of the downregulated ORs what were located in a short distance (lower than 150bp), which shows the higher probability for interaction for both TFs according previous study for different 27 TFs in *Drosophila* genome (Kazemian et al, 2013). Our data demonstrate that ORs

can be suppressed by odor exposure during early development, but whether they are being suppressed as individual ORs or as clusters is difficult to ascertain because we examined only representative ORs from distinct families.

Conclusions

In this study, we show a correlation between higher *otx2* expression and lower OR expression in the presence of βPEA and suggest a model of where downregulation of OR expression may enhance the signal-to-noise ratio in olfactory processing (Figure 9). Genes normally expressed and contain Otx2/Hnf3b motifs (Figure 9A, control) are further repressed in the presence of βPEA (Figure 9B, arrows, βPEA). Whether this model has overlying control elements active at the level of the chromosome has yet to be determined.

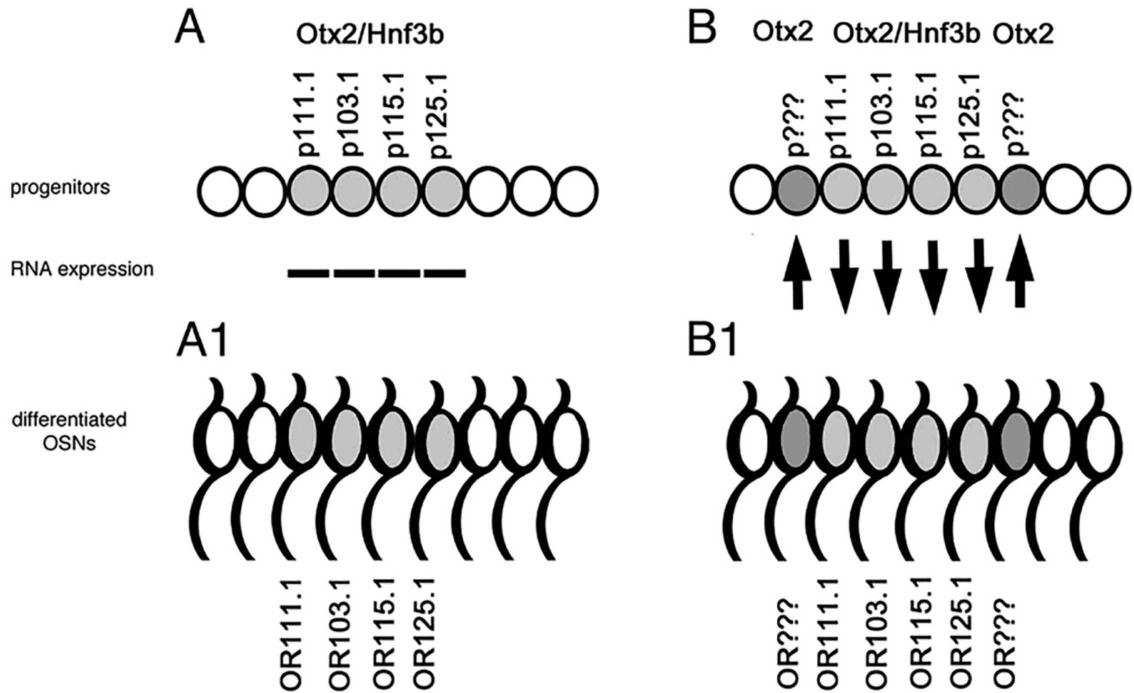


Figure 9. Regulation of OR expression may enhance signal-to-noise ratio though altering levels of OR RNA. (**A**) In control animals, progenitors of OSNs express *OR111-1, OR103-1* (chromosome 15), *OR115-1*, and *OR125-1* (chromosome 21) at a baseline level (dashed lines) in differentiated OSNs. (**A1**) The same ORs in animals exposed to βPEA (**B**) are repressed by the Otx2/Hnf3b transcription factors and show decreased levels of OR RNA (arrows) in differentiated OSNs (**B1**). Otx2 alone may mediate the up-regulation of ORs responding to βPEA (**B**, **B1**, OR???), yet to be identified**.**

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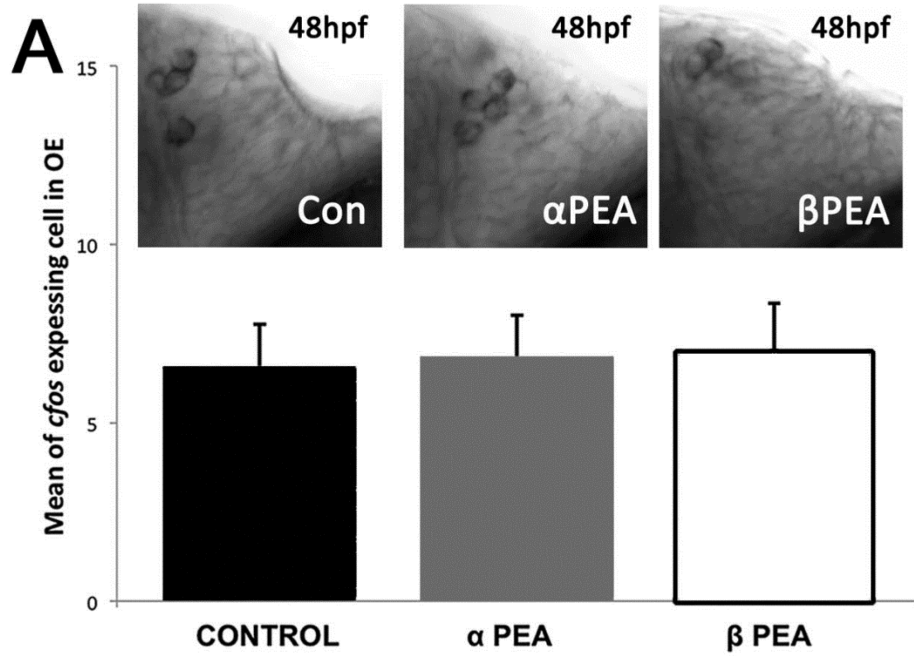
Supplemental data

Supplementary Table 1

Olfactory Receptor	Expression ratio Control Group	Mean of Ct value	Expression ratio β PEA Exposed Group	Mean of Ct value	% of change of gene expression ratio
OR 102-4	1 ±0.043	28.57	0.95 ±0.086	28.54	-5
OR 103-1	1 ±0.023	29.8	0.59 ±0.055	30.6	-41
OR 111-1	1 ±0.029	26.95	0.75 ±0.023	27.26	-25
OR 115-5	1 ±0.138	31.75	0.22 ±0.023	33.98	-78
OR 125-1	1 ±0.055	28.93	0.72 ±0.047	29.47	-28
OR 136-1	1 ±0.060	28.71	0.95 ±0.070	28.92	-5
OR 137-3	1 ±0.1290	28.33	0.89 ±0.096	28.68	-11

Supplementary Table 2

Olfactory Receptor	Expression ratio Control Group	Mean of Ct value	Expression ratio α PEA Exposed Group	Mean of Ct value	% of change of gene expression ratio
OR 102-4	1 ±0.164	30.07	1.12 ±0.158	29.87	12
OR 103-1	1 ±0.064	29.84	1.01 ±0.114	29.78	1
OR 111-1	1 ±0.004	27.74	0.99 ±0.083	27.71	-1
OR 115-5	1 ±0.010	34.46	0.91 ±0.021	34.55	-9
OR 125-1	1 ±0.054	26.70	1.01 ±0.0725	26.54	1
OR 136-1	1 ±0.099	29.82	0.99 ±0.0922	29.69	-1
OR 137-3	1 ±0.018	29.57	1.10 ±0.0871	29.29	10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Mean of cfos expessing cell in OE
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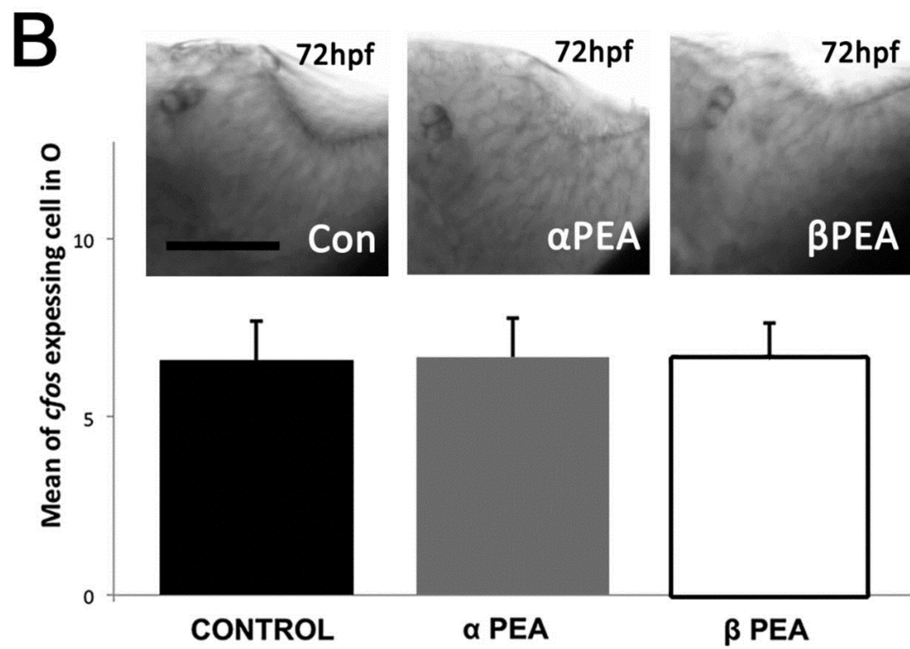
β PEA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72hpf
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10
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Mean of cfos expessing cell in O
CONTROL
α PEA
β PEA

Supplemental Figure 1. Expression of *c-fos* in OE of juvenile zebrafish does not change in the presence of PEA. Number of cells expressing *c-fos* at 48 hpf (**A**) and 72hpf (**B**) detected by whole mount *in situ* hybridization. (**A**) At 48 hpf, the mean of *c-fos* positive cells was 6.6±1.2 cells (n=22) in the control group, 6.9±1.5 cells (n=22) in the αPEA group, and 6.9±1.4 cells (n=22) in the βPEA group. (**B**) At 72 hpf the mean of *c-fos* positive cells was 6.6±1.1 cells (n=22) in the control group, 6.7±1.1 cells (n=22) in the αPEA group, and 6.6±1.0 cells (n=22) in the βPEA group. No significant differences were observed between control, αPEA and βPEA groups. Scale bar= 30 μm, data were analyzed by one way ANOVA and Bonferroni's Multiple Comparison post test; error bars represent SD**.**

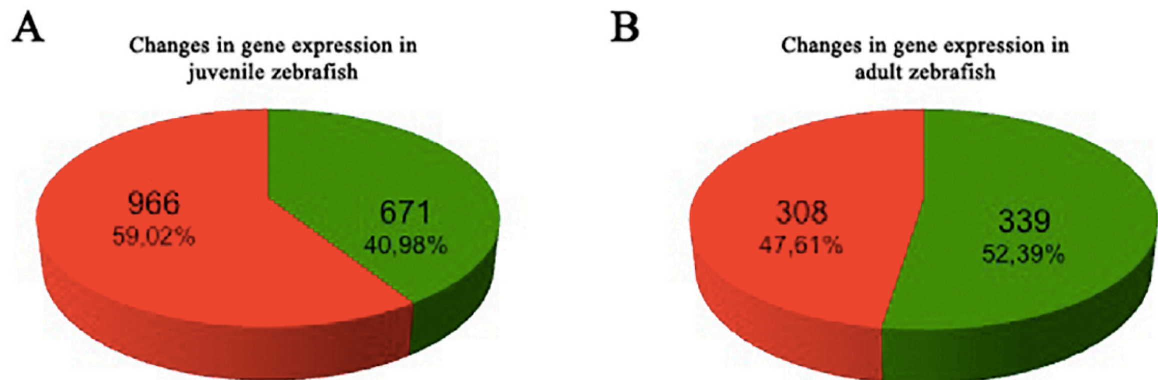
Appendix I. Additional supplemental results

Changes in gene expression in PEA exposure zebrafish

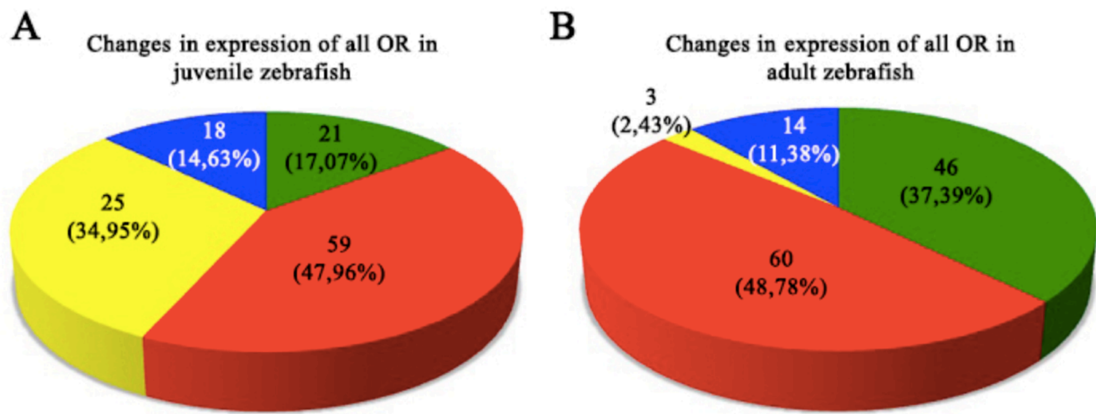
In order to measure changes in the gene expression levels in OE of zebrafish exposed to PEA, we exposed developing zebrafish to PEA for the first three weeks of life and then,performed RNA-seq analysis using RNA from juveniles (3 weeks) and adult OE (six months) fish. For samples from juvenile fish, we obtained a mean of 61,716,663 reads per samples in the sequencing process. The control and PEA exposed group were compared using normalized gene expression values obtaining the fold change for all transcripts. Significant differences were observed in 1637 transcripts, which correspond to 5.2% of the total transcripts measured; from these genes 671 increased their expression and 966 decrease their expression (Figure 1A). For adult OE a mean of 79,800,439 reads per sample was obtained. After calculating the gene expression values and comparing the control and PEA exposed group, significant differences were observed in the expression of 647 transcripts that correspond to 2.1%of the total transcripts measured, of which 339 increase their expression and 308 decrease their expression (Figure 1B). Therefore, the exposure to PEA was correlated with changes in the expression of a low number of genes in juvenile and adult OE.

Changes in expression of ORs repertoire in presence of PEA

In order to measure the effect of the PEA exposure on the OR expression for the 7 ORs analyzed in our original study, the RNA-seq data were filtered with focus on the OR genes. In this analysis 123 ORs were analyzed. In juvenile fishes, expression of ORs was very low with a mean of 0.2 RPKM for the control group and 0.9 RPKM for the PEA exposed group. *OR137-1* with 0.024 RPKM showed the lowest expression and *OR106-10* the highest expression (1.73



Appendix I, Figure 1. Changes in the gene expression in juvenile and adult zebrafish exposed to PEA. Summary of changes in gene expression measured by RNA-seq for genes showing significant changes when comparing control and PEA exposed groups; juvenile tissue (**A**) and adult OE (**B**). **A**, in juvenile zebrafish significant changes were observed in the expression of 1637 genes (5.2% of the total transcripts), which 966 genes (59.02%) decrease their expression (red) and 671 genes (40.98%) increased their expression (green). **B**, in adult OE changes were observed in 647 genes (2.05% of the total transcripts), of which 308 (47.61%) decreased their expression (red) and 339 genes (52.39%) increased their expression (green).



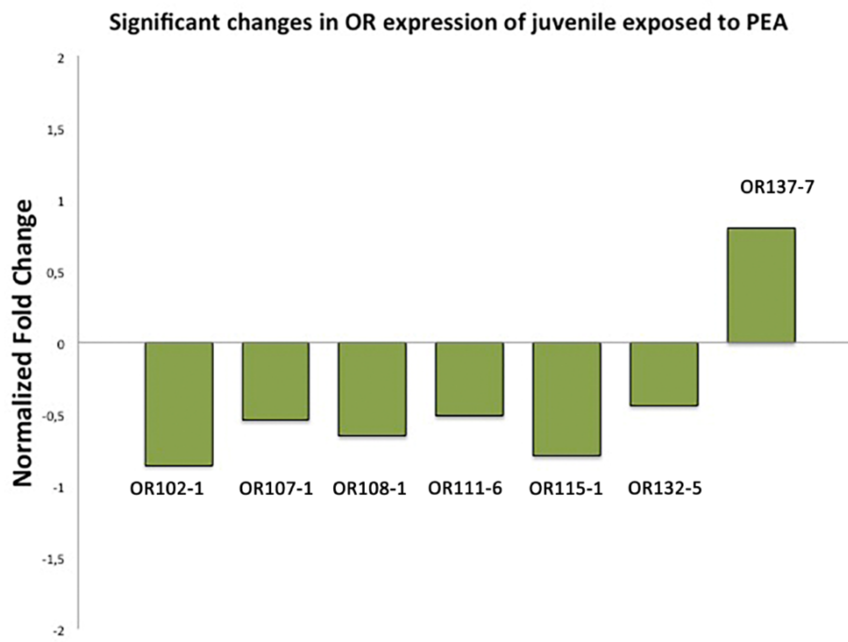
Appendix I, Figure 2. Changes in the expression of the complete repertoire of ORs in PEA-exposed juvenile and adult zebrafish. RNA-seq analysis of 123 ORs genes in juvenile (**A**) and adult (**B**) zebrafish exposed to PEA relative to controls. (**A**), in juvenile zebrafish 59 OR genes (47.96%) showed a decrease in expression (red), 21 receptors (17.07%) showed an increase in expression (green), 18 receptors (14.63%) did not change their expression (blue), and expression of 25 ORs (34.95%; yellow) where not detected. (**B**), in adult OE 60 OR genes (48.78%) showed a decrease in expression (red), 46 receptors (37.39%) showed an increase in expression (green), 14 receptors (11.38%) did not change their expression (blue) and 3 OR (2.43%) were not detected (yellow).

RPKM). The comparison between control and PEA group showed that 59 ORs decreased their expression and 21 increased their expression correlated with PEA exposure. In contrast 18 ORs did not change their expression and 25 ORs were not detected (Figure 2A). The statistical t-test performed showed significant differences in the expression of 7 ORs (Figure 3A) comparing the control and PEA exposed group, which six of them showed a decrease in their expression and only *137-7* increased their expression. Three ORs that decreased heir expression (*OR102-1, 107-1* and *111-6*) are located in the chromosome 15, while *115-1* and *132-5* decreased their expression and are located in the chromosome 21. *OR137-7* was the only receptor that increased expression, and is located in chromosome 6. For adult OE, the mean of expression level was 7.7 RPKM in the control group and 7.5 RPKM for PEA exposed group. A great variability in the OR expression level was observed, with the lowest expression for *OR123-1* with 0.2 RPKM and the highest for *OR108-3* with 67.7 RPKM. In comparing expression between control and PEA groups 60 ORs decreased their expression, 46 increased their expression, 14 receptors did not change their expression and 3 were not detected (Fig 2B). The statistical t test showed significant differences in the expression of 7 ORs (Fig. 3B) comparing the control and PEA exposed group. Two receptors increased their expression (*OR111-9* and *133-5*) and are located in chromosome 15 and 21 respectively, while five decreased their expression and are located in three chromosomes, *OR111-3* and *127-1* in chromosome 15, *OR123-1* and *124-4* in chromosome 21, finally *OR137-2* is located in chromosome 6.

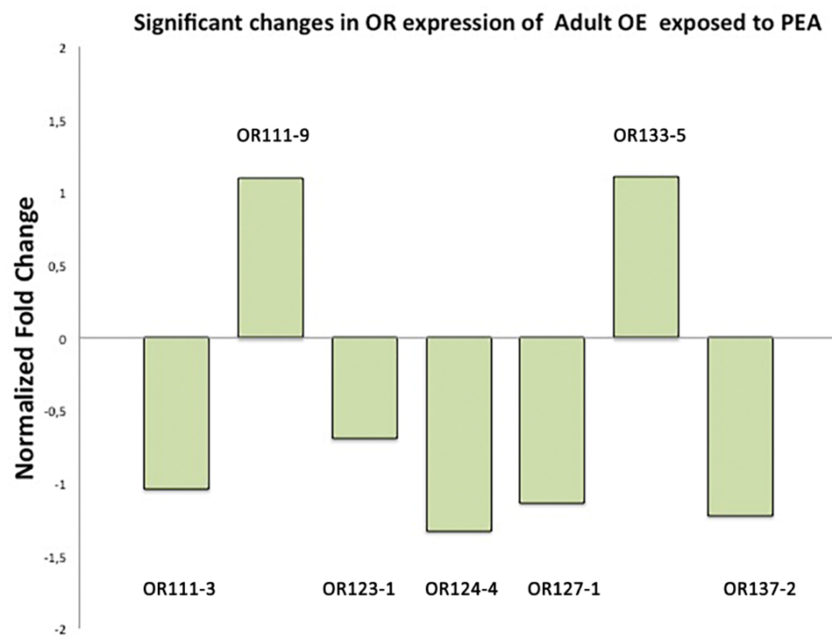
Changes in Chemoreceptors

Since the whole transcriptome of the zebrafish was analyzed in the RNAseq experiments the effect of the odorant exposure on the expression of non ORs G protein coupled receptor, the

A

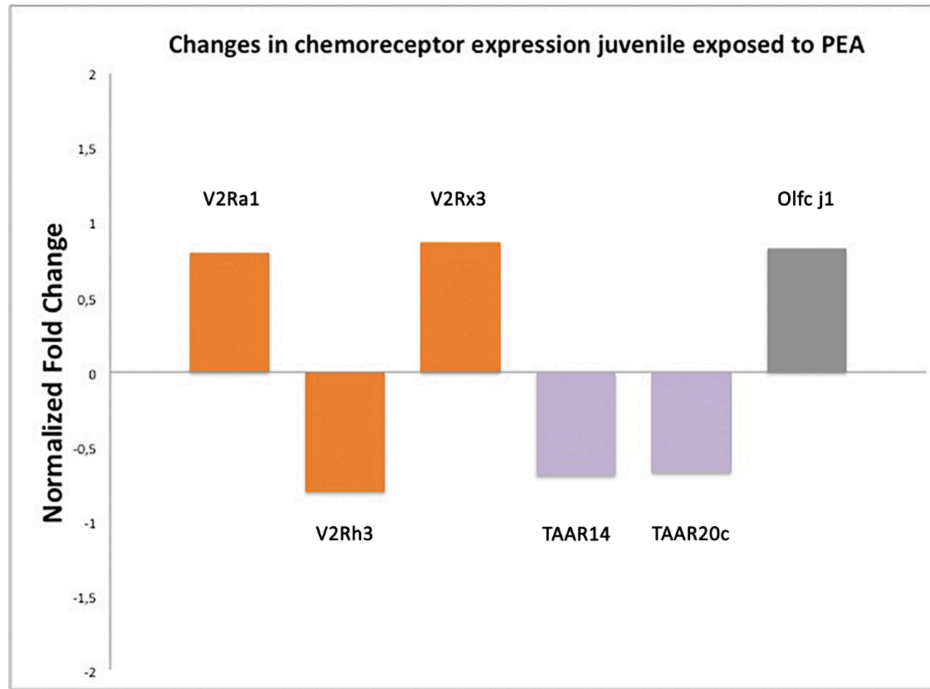


B

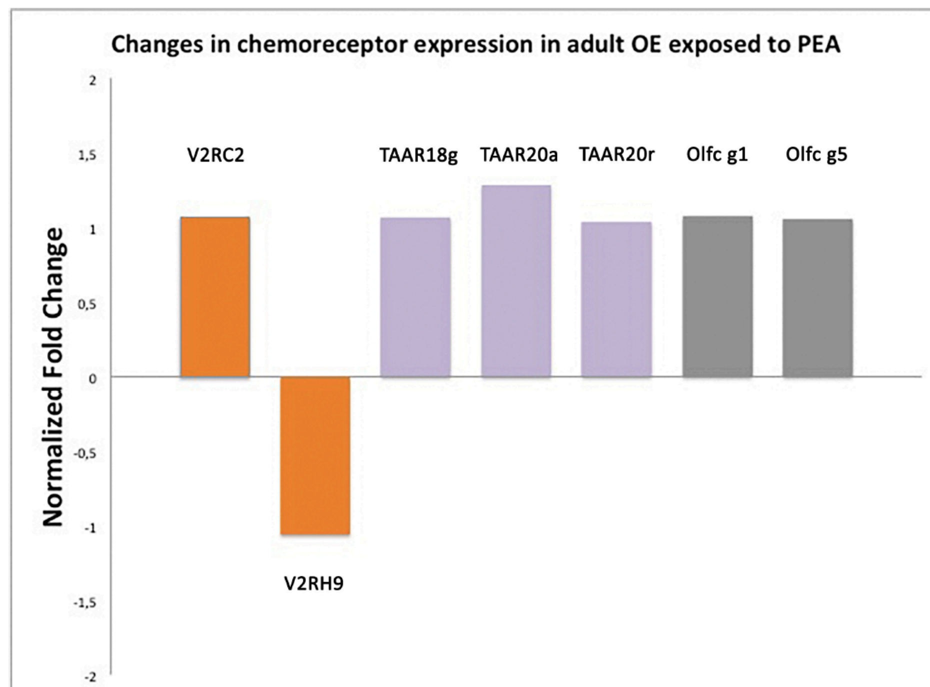


Appendix I, Figure 3. A subset of olfactory receptors show changes in expression patterns in the presence of PEA. Juvenile (**A**) and adult (**B**), show different changes in OR expression measured by RNA-seq (**A**), juvenile fishes showed a decrease in the expression of *OR102-1, OR107-1, OR108-1, OR111-6, OR115-13* and *OR132-5*, and just *OR137-7* increased their expression. (**B**), adult OE, showed decreased expression of *OR111-3, OR123-1, OR124-4, OR127-1* and *OR137-2*, and an increase in expression of *OR111-9* and *OR133-5*. Changes according t-test (pvalue < 0,1).

A



B



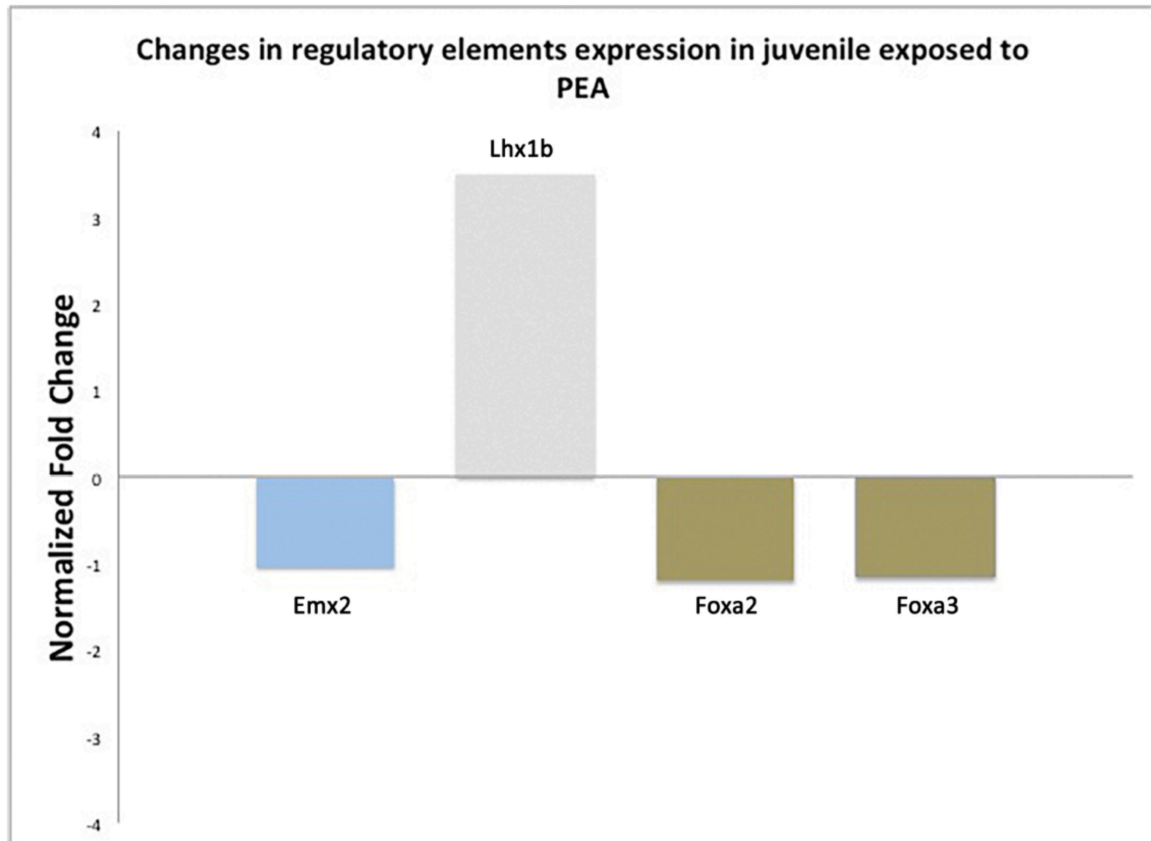
Appendix I, Figure 4. Changes in the expression of non-OR chemoreceptors in PEA-exposed animals. RNA-seq analysis of expression of vomeronasal receptors (VRs) (orange bars), Trace Amino Acid Receptors (TAARs) (purple bars) and Olf C receptors (grey bars) in juvenile (**A**) and adult (**B**) zebrafish. (**A**), in juvenile zebrafish showed an increase in the expression of *V2Ra1, V2Rx3* and *OlfC j1*, and a decrease in expression of *V2Rh3, TAAR14h* and *V2R20c.* (**B**), in adult OE showed an increase in expression of *V2Rc3, TAAR18g, TAAR20a, TAAR20r, OlfC g1*, and *OlfC g5*, and a decrease in the expression of *V2Rh*. Significant differences according t-test (p-value < 0,1)

chemoreceptor gene families of vomeronasal receptors (VR), Trace Amine-associated receptors (TAAR) and Olfactory receptor of family C (OlfC) was also included in the data set. In juveniles, the expression *V2Ra1, V2Rx3*, and *Olfc j1* increased and the expression of *V2Rh32, TAAR14h* and TAAR20c decreased (Figure 4A). In adult OE: *V2Rc2, TAAR18g, TAAR20a, TAAR20r, Olfc g1* and *Olfc g5* increased their expression and *V2Rh9* showed a decrease in expression (Figure 4B). Thus, the changes in the expression of other non-OR type chemoreceptors suggest a potential role for these receptors in PEA induced modulation different families of receptors in the OE.

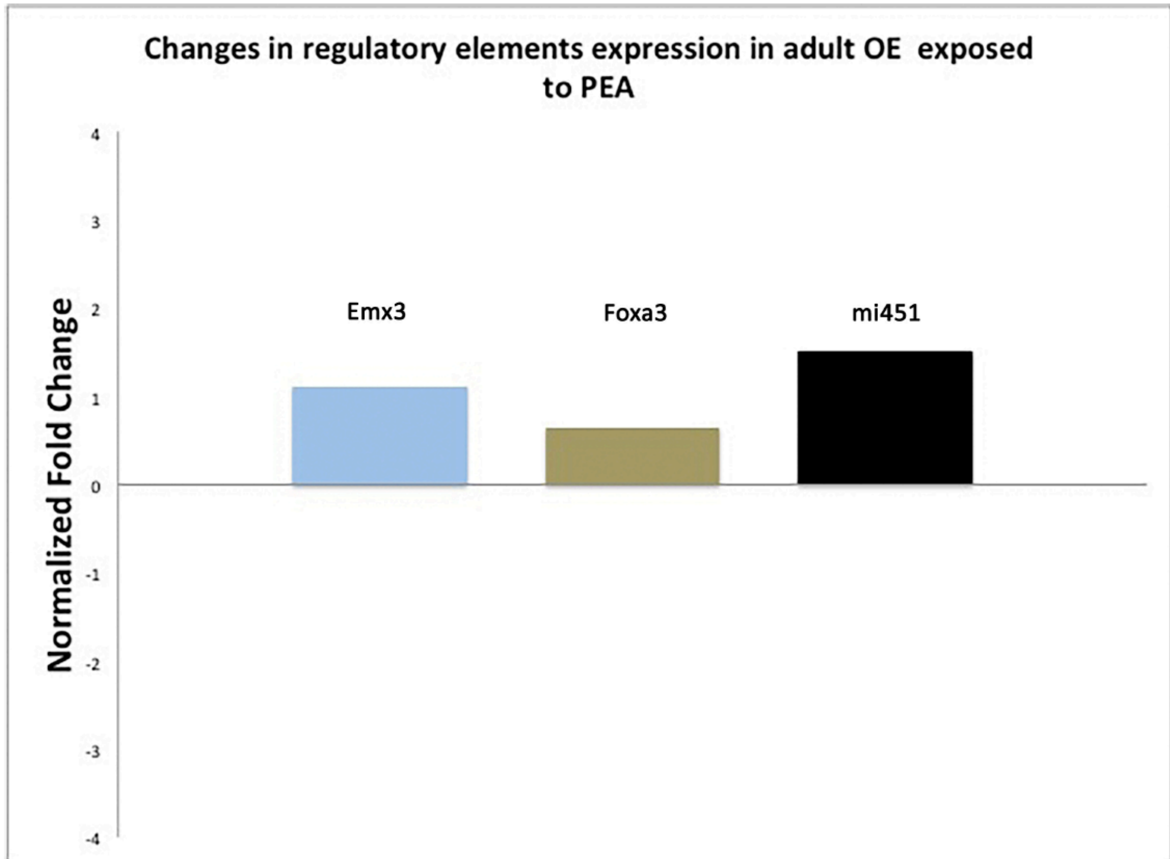
Changes in expression of TFs in PEA exposed zebrafish

In order to look for regulatory elements that could the changes in the expression of ORs by PEA exposure, we examined the TFs expressed in the OE and miRNA of juvenile and adult OE in our transcriptomic analysis. We observed changes in the expression of TF of the homeodomain and Foxa family, which are involved in the formation of the nervous system. In juvenile, significant decreases were observed in the expression of *emx2, foxa2* and *foxa3*, furthermore we observed an increase in the expression of *lhx1b*, a TF belonging to the homeobox family (Figure 5). In adult OE significant changes were observed in the expression of *emx3* and *foxa3*, which increased their expression (Fig. 6). Unlike juvenile fish, *foxa2* was not detected in adult OE.

The differences in expression of TFs could suggest a possible role for these genes in odorant induced modulation of gene expression in the OE. In addition to the changes in developmentally relevant TFs, an increase in the expression of miRNA *mi451a* (Figure 6) was observed, suggesting the possible role of this miRNA in the interaction of the PEA and the changes in the gene expression in the OE including the OR genes.



Appendix I, Figure 5. Changes in expression of TFs in PEA exposed juvenile fishes. Changes in the expression of TFs belonging to the *emx* family (blue bars), *lhx* family (grey bars), and *foxa* family (brown bars) in juvenile fishes measured by RNA-seq. The decrease in the expression was observed for *emx2, foxa2 and foxa3*, also was detected in the expression of *lhx1b*.



Appendix I, Figure 6. Changes in expression of TFs in PEA exposed adult OE. Changes in the expression of TFs belonging to the *emx* family (blue bars), *lhx* family (grey bars), *foxa* family (brown bars) and miRNA (black bars) in adult fishes measured by RNA-seq. In adult were observed increased in the expression of *emx3* and *foxa3* TFs, additionally was observed the increase in the expression of a miRNA called *mi451a*.

Chapter II. Analysis of olfactory marker protein (OMP) isoforms

Introduction

Olfactory Marker Protein (OMP) is a classical marker for ciliated OSNs in mouse, is a globular protein located in the cytoplasm of olfactory neurons (Keller and Margolis, 1975; Dibattista and Reisert; 2016). Behavioral studies in mice showed homozygous *omp* mutants have altered odorant discrimination and sensitivity (Youngentob and Margolis, 1999; Youngentob et al., 2001, 2004), while electrophysiological studies showed the *omp* mutant mice have altered odorant detection kinetics with a slower response to stimulus (Buiakova et al., 1996; Ivic et al., 2000; Reisert et al., 2007; Kwon et al., 2009). Additionally, the absence of *omp* was correlated with altered innervation of the olfactory bulb (St John & Key, 2005). A recent study showed OMP controls the cAMP in the OSN in presence and absence of odorants (Dibattista and Reisert; 2016). Therefore, this evidence suggests that *omp* is important for the maturation of OSNs and the regulation of their responses in differentiated neurons.

The *omp* gene was described originally in rodents (Keller and Margolis, 1975; Hartman and Margolis, 1975; Keller and Margolis, 1976), but has been detected in the genome of different vertebrates (Mombaerts et al, 1996; Celik et al, 2002; Yoshida et al, 2002, (Rössler et al, 1998). Additionally, different transgenic strains have been developed using the *omp* promoter in order to study the olfactory system (Mombaerts et al, 1996; Celik et al, 2002; Yoshida et al, 2002).

A bioinformatic study found the *omp* gene in different vertebrates genomes, where the protein sequences showed a homology higher than 50 percent; teleost fish, including zebrafish, and *Xenopus* have two isoforms of *omp* (Suzuki et al, 2015). In *Xenopus leavis*, two isoforms of *omp* were described, which showed different expression pattern (Rössler et al, 1998). Additionally, genomic analysis showed the presence of two isoforms of *omp* in the OE of medaka (Yasuoka et al, 1999) and salmon (Kudo et al, 2009). Zebrafish *omp* was originally

cloned based in homology with mouse sequence (Celik et al, 2002; Yoshida et al, 2002). However, in the current version of the zebrafish genome there are two annotated isoforms for *omp* (Suzuki et al, 2015): isoform b, which is the described *omp* form, and the isoform a, which was computationally predicted and annotated in the zebrafish genome and was not described when our study started, but was recently described (Suzuki et al, 2015).

We performed a transcriptominc analysis on juvenile fish and OE from adult fish in order to analyze the effect of the PEA exposure on the gene expression (Chapter I), where we found that both *omp* isoforms, were unaffected by PEA exposure, however the *ompa* isoform (not characterized) was expressed around one hundred fold more than *ompb* isoform. Therefore, in the current study we performed an analysis in order to analyze and describe the *ompa* expression and to determine whether the cells expressing this isoform correspond to a different neuron population.

Materials and Methods

Animals

Zebrafish from the new wild-type (NWT) and NWT/Cornell strains, derived from the AB line, were used for all experiments. The fish were maintained at 28 °C on a light–dark cycle of 14 and 10 h, respectively. The Institutional Animal Use and Care Committee of the Universidad de Valparaíso approved all animal procedures (#BEA 022-2013).

Transcriptomic analysis

A RNA-seq analysis was performed as described in Calfún et al., 2016 (Chapter I).

qPCR analysis of the omp isoforms expression

The qPCR analysis was performed as described in Calfún et al., 2016. The expression analysis of *ompa* and *ompb* was performed using adult OE from 10 fish. The primers used for the analysis are listed in Table 1.

Whole mount in situ hybridization

We analyzed *ompa* expression using whole mount *in situ* hybridization as described in Thisse et al., 1993 with changes in the proteinase K permeabilization as described in Harden et al, 2006. Single-strand RNA probe was designed using primer for *ompa* listed in Table 1. The *ompa* sequence was cloned using the pGEMT-easy vector (Promega), according manufacturer instruction.

Table 1. Primer used in *omp* isoforms analysis

Gene	Primer	Amplicon length	Analysis
ompa	PF TTCACCGTCACCGGCATCT PR GCATTTGATGGGCGAGTCCT	129 bp	qPCR
ompa	PF GGGTTCAGAAATGGAGCTGACA PR CTTTGGTCGCTCCTTCCTTGAATG	440 bp	*In situ* hybridization
ompb	PF CGCACAGGAGTTTGGTGAAAGGAT PR TGGAGGTCTGGAATTCAATGGAGC	128 bp	qPCR

Genome localization analysis

In order to examine syntenic relation of the zebrafish *omp* genes to *omp* genes of other vertebrates we analyzed genome localization of zebrafish *omp* isoforms in mouse and human, using the zebrafish genome browser (GRCz10; http://www.ensembl.org/Danio_rerio/Info/Index), the mouse genome browser (GRCm38.p5; http://www.ensembl.org/Mus_musculus/Info/Index) and the human genome browser (BRCA2; http://grch37.ensembl.org/Homo_sapiens/Info/Index) of the Sanger Institute.

Results

omp expression in transcriptomic analysis

Our RNAseq results revealed no differences in the expression of *omp* isoforms in PEA exposed juvenile and adult zebrafish. However, when we compared both *omp* isoforms, we observed significant differences in the reads measured for them in juvenile and adult fish. In juveniles we measured 1.80 RPKM for *ompa* and 0.01 RPKM for *ompb* in the control group, which is 180 fold higher in juveniles (Figure 1). Similar differences were observed adult OE, where we measured 19.28 RPKM for *ompa* and 0.07 RPKM for *ompb*, which is a difference around of 250 fold (Figure 1). These differences in the read number suggest that *ompa* could be expressed in higher levels than *ompb.*

qPCR analysis

In order to analyze the potential differences in *omp* isoform expression, we performed qPCR analysis using OE dissected from adult zebrafish (one year old). We compared the expression of *ompb* relative to *ompa* expression, using beta actin as housekeeping genes. For *ompa* we measured a relative expression of 1 +/- 0.01 (SE) and for *ompb* was 12.43 +/- 0.27 (SE) (Figure 2), when we calculated the fold change we observed that *ompb* is 3.64 +/- 0.08 (SE) more expressed than *ompa*.

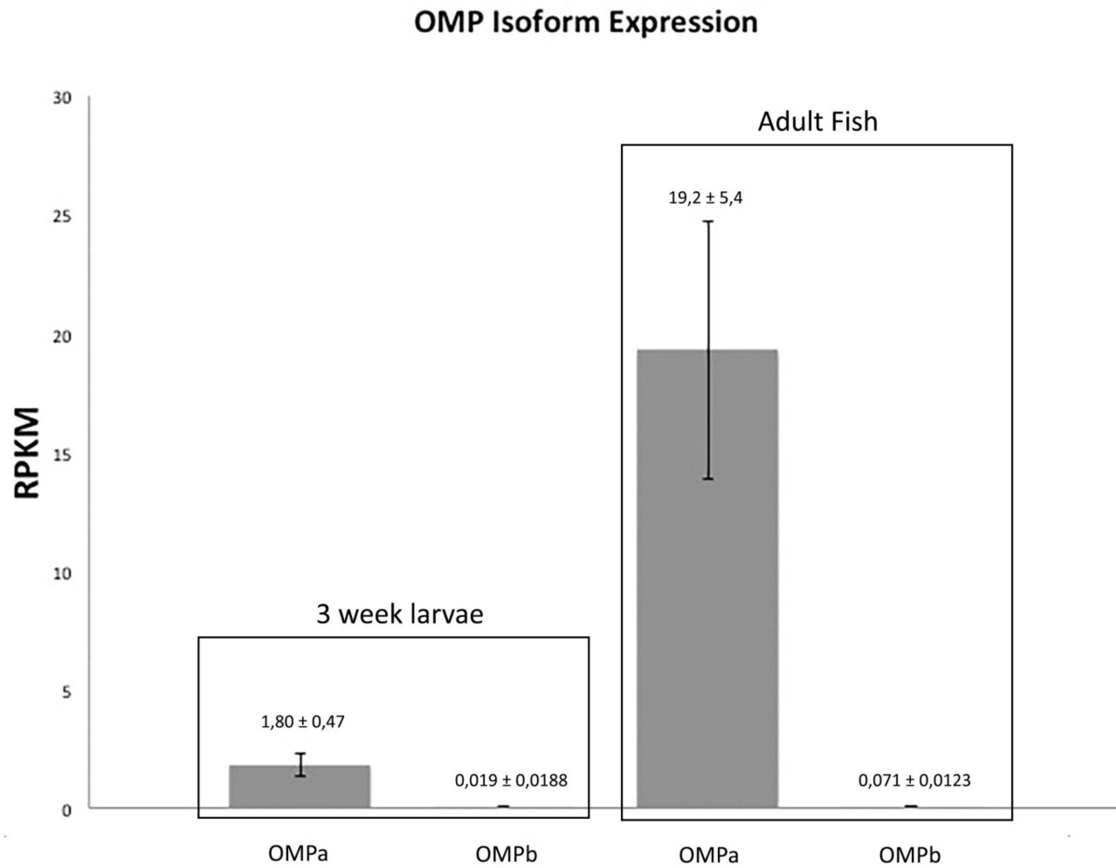


Figure 1. Expression of the two *omp* isoforms of zebrafish. RNA-seq analysis differences of expression levels of *ompa* and *ompb* (described isoform), results correspond to control group at 3 weeks and adult OE. In juvenile zebrafish *ompa* expression is around 100 fold higher than *ompb* (left columns). In adult OE were expression levels of *ompa* were 200 fold higher than *ompb*.

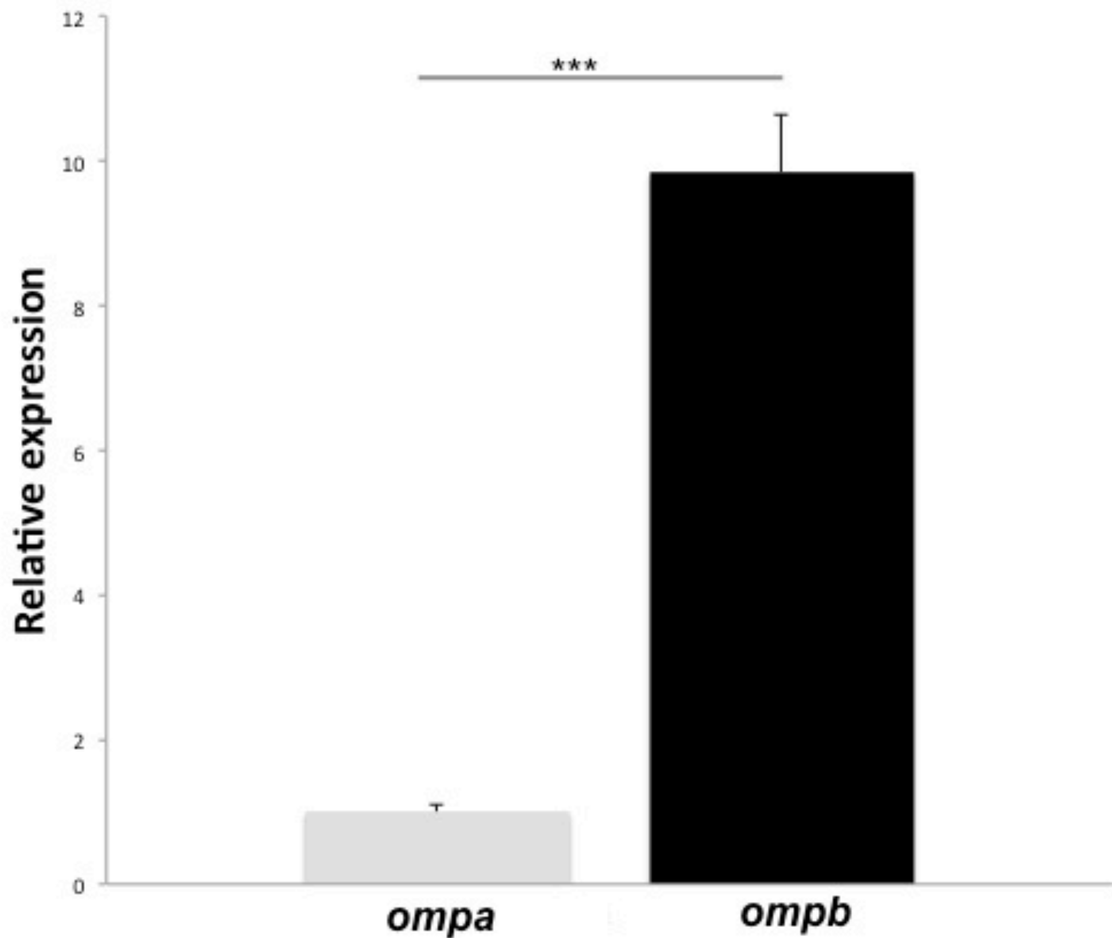


Figure 2. Differences in the expression of two *omp* isoforms of zebrafish. qPCR analysis of *omp* isoforms expression in adult OE, comparing *ompb* expression relative to *ompa*. The *ompa* relative expression was 1 +/- 0.01 (SE) and *ompb* was 12.43 +/- 0.27 (SE). (T student test, ***: $p < 0.005$).

In situ *hybridization analysis*

Since *ompa* had not been carefully described at the time we were doing these experiments, we performed whole mount *in situ* hybridization in zebrafish embryos in order to visualize the expression pattern of *ompa*. We observed *ompa* expressing cells in adult OE and in larvae at 36, 48 and 72 hpf (Figure 3), with no *ompa* expressing cell before 36 hpf. When we compared the *ompa* with *ompb*, we observed a number of *ompa* positive cells with around 2 cells at 36 hpf and 8 cells at 48 hpf, in contrast at these stages the number of *ompb* positive cells was higher and it was not possible to count these cells in juveniles. The expression patterns we observed for *ompb* are in agreement with previous reports (Celik et al, 2002; Yoshida et al, 2002) and the expression of *ompa* observed in adult OE is in agreement with the results recently described for Suzuki et al. (2015).

Genome localization analysis

We performed a bioinformatic analysis *ompa* and *ompb* the zebrafish genome (GRCz10) of *ompa* (Figure 4A, B) mouse genome (Figure 4C) and human genome (Figure 4D) to look for syntenic regions around the *omp* genes in these vertebrates. In all genomes analyzed the *omp* gene is located in an intron of the *calpain 5* gene (*capn5*), in zebrafish *ompa* is located in a intron of the *capn5a* gene and *ompb* in a intron of *capn5b*; both isoforms are oriented in different direction on the DNA relative to their respective *capn5* genes (Figure 4A and 4B). In contrast the mammalian *omp* genes are oriented in the same direction than their respective *capn5* genes (Figure 4A, B). When we looked at the genes located around *omp* and *cap5n* genes, we observed conservation in the genes downstream of mouse and human *cap5n* genes (*myo7A, gdpd4* and *pak1*), with the only exception of a predicted and unidentified gene in mouse (Figure 4 C, D); for zebrafish *ompa*, the downstream genes are present but are oriented in a different order (Figure 4

A); in contrast, the genes downstream of zebrafish *ompb* are conserved just two genes (*myo7A* and *gdpd4*), which are oriented in the same order as mouse and human (Figure 4B). For the region upstream of *omp* and *capn5*, we observed conserved genes for mouse and human (*acer3* and *b3gnt6*) (Figure 4C, D), however for zebrafish we did not observe common genes between *ompa* and *ompb* for moue and human *omp* in the region upstream (Figure 4A and 4B).

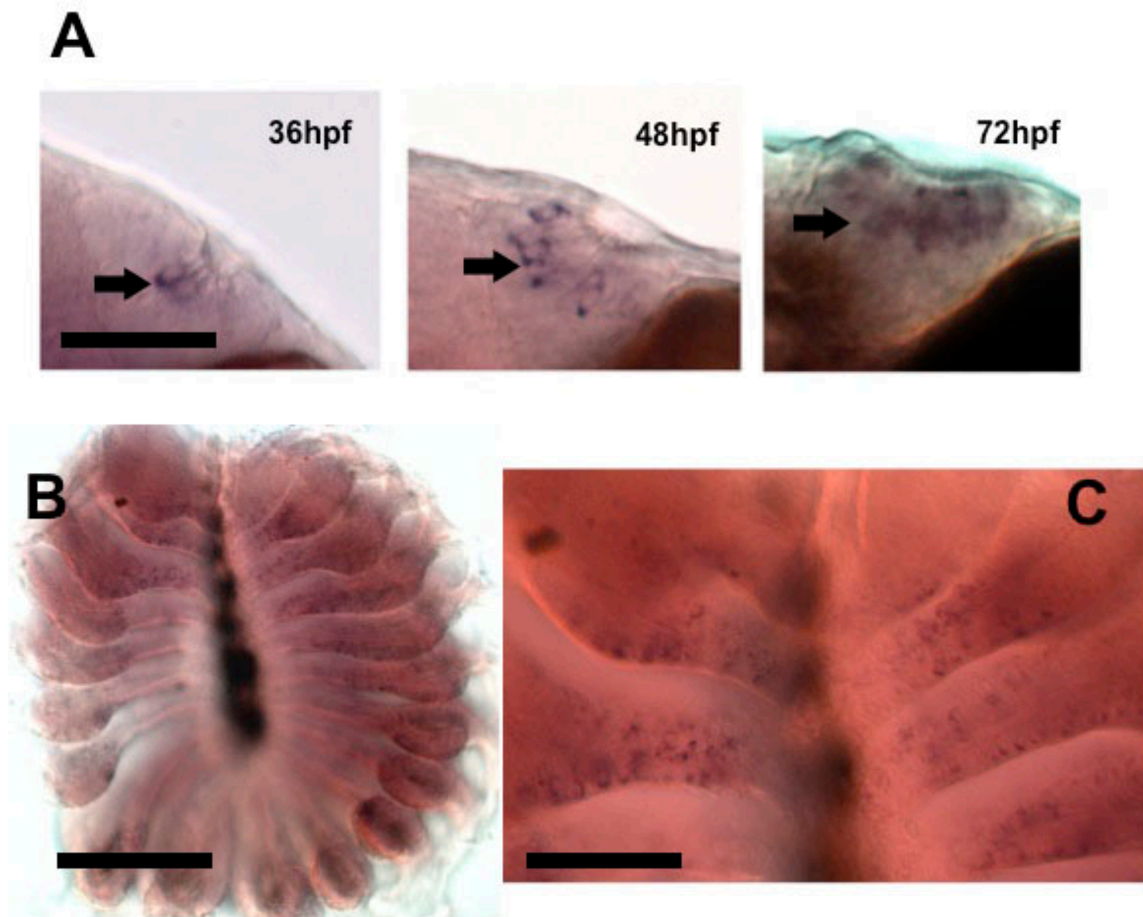


Figure 3. Expression pattern of *ompa* by whole mount in situ hybridization. We analyzed *ompa* expression at 36 hpf (**A**), 48 hpf (**A1**), 72 hpf (**A**) and adult OE (**B** and **C**). (**A**), In zebrafish embryos the *ompa* expressing cells (arrow heads) are restricted to an apical position in the developing OE, and the number of positive cells increased in time. (**B**), *ompa* expression in adult OE; the labeled cells (arrow heads) are located in the center of olfactory rosette, which is observed in the detailed picture (**C**). Scale bar **A** and **C** = 30 μm and for **B** = 60 μm

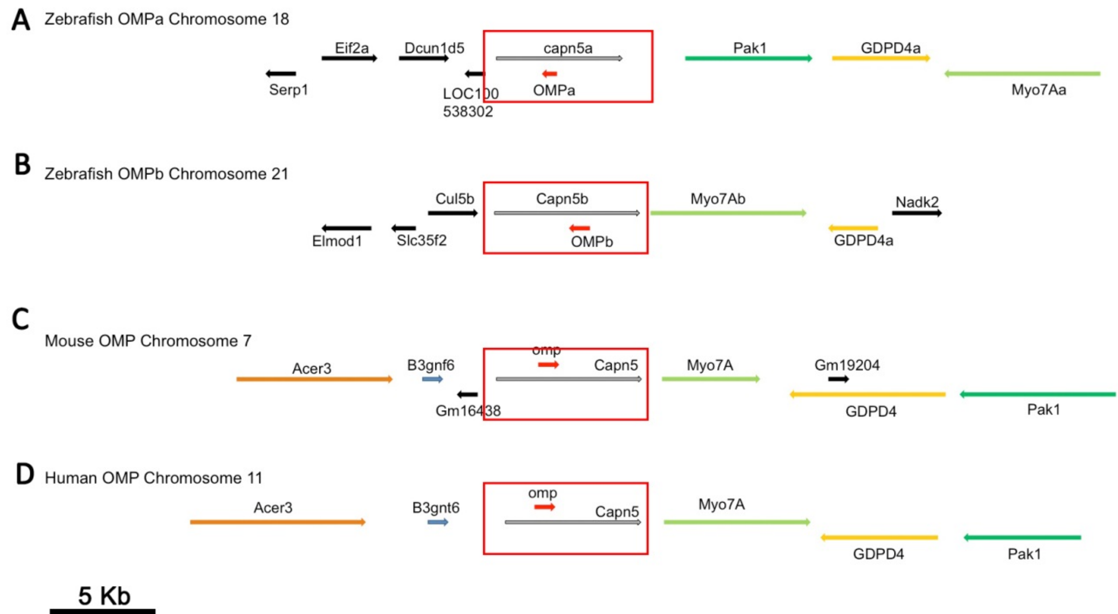


Figure 4. Localization of *omp* genes in genome of four vertebrates. Map of the genomic localization of zebrafish *ompa* (**A**), zebrafish *ompb* (**B**), mouse *omp* (**C**) and human *omp* (**D**), where every gene is represented as an arrow. The *omp* genes are located in an intron of their respective *capn5* gene (red squares); both zebrafish *omp* genes are transcribed in an opposite direction of their *capn5* genes (**A** and **B**, red square), but mouse and human are transcribed in the same direction than *capn5* genes (**C** and **D**, red square). In mouse and human the neighboring genes of *omp* are conserved (**C** and **D**), and for zebrafish *omp* genes two genes in the down stream region are conserved (**A** and **B**). Scale bar = 5Kb.

Discussion

Zebrafish have two isoforms of *omp: ompa*, that has only been described recently (Suzuki et al, 2015) and *ompb*, which was used to generate transgenic strains expressing reporter genes in the OSNs (Celick et al, 2002; Yoshida et al 2002; Sato et al 2002). Through RNAseq, and qPCR we found that the *ompa* isoform has lower expression than *ompb* in larval fish and adult OE. In confirming the pattern of *ompa* gene expression by *in situ* hybridization, it was evident that *ompa* expressing cells are located in the apical zone of the olfactory organ of zebrafish larvae and are lower cell number compared to *ompb*; in the adult OE *ompa* showed a different pattern than *ompb*, with a lower number of *ompa* expressing cells what were located in a central region of the olfactory rosette. These results are in agreement with the data of Suzuki et al (2015), who showed the *omp* isoforms are expressed in different cell population.

Suzuki described the expression of both *omp* isoforms in adult OE using double *in situ* hybridization and re-named these genes as *omp1* for *ompb* (previously described isoform) and *omp2* for *ompa* (not described) (Suzuki et al, 2015). They showed that the expression of *ompa* and *ompb* was restricted to different areas of the OE, with a few *ompa* expressing cells restricted to an apical region of the OE. In contrast there were more *ompb* expressing cells and they were located in the basal region of the adult OE (Suzuki et al, 2015). Both the *ompa* cells and *ompb* cells co-express neuron markers, which indicates that these cells are neurons (Suzuki et al, 2015). Interestingly very few cells were double labeled, supporting the hypothesis that *ompa* and *ompb* are different cells population

Our initial RNA-seq analysis showed *ompa* (isoform not previously described) was highly expressed compared with *ompb*, however we performed a second analysis on our RNA-seq data, in collaboration with the Whitehead Institute (Boston, US), where we observed the reverse results with a higher expression of *ompb* compared with *ompa*. The results of the second

analysis were in agreement with the data of our qPCR study, were we measured a higher expression of *ompb* compared with *ompa*, which suggests there were mistakes in the identification and RNA-seq reads assignment for *omp* isoforms in our first analysis. Additionally, we performed whole mount *in situ* hybridization for *ompa* and observed a low number of cells expressing the gene in the apical region of developing olfactory organ of embryos and *ompa* positive cells located in the sensitive region of adult OE, which is in agreement with the described by Suzuki et al (2015) for the expression of this gene. The number of *ompa* expressing cells was lower than *ompb* in larvae and adult, which is in agreement with our qPCR analysis and with Suzuki et al (2015). Therefore, our results of the expression pattern observed for *ompa* suggest that these cells are a different population than *ompb*, supporting the described by Suzuki et al (2015).

In mammalians, *omp* is a single gene located on an intron of the *calpain 5* gene; in mouse the cell expressing *omp* are located in the main OE (Mombaerts et al, 1996; Potter et al, 2001) but the expression of these gene has been observed in low levels in other tissues (Kang et al, 2015). In contrast, the presence of two forms of *omp* has been observed in the genome of different teleost fishes (Yasuoka et al, 1999, Kudo et al 2009; Suzuki et al, 2015); the analysis of the expression of these genes in medaka and salmon (Yasuoka et al, 1999, Kudo et al 2009) showed that the *omp* isoforms are expressed in different pattern in the OE. When we analyzed the genomic location of zebrafish *omp* isofoms and compared with the mouse and human genome, we observed that *calpain 5* gen and other neighbor genes are duplicated too, showing this region has been duplicated in the zebrafish genome, as has been reported in zebrafish and other teleost (Suzuki et al, 2015). Moreover, phylogenetic analysis of *omp* protein sequence from different vertebrates showed that *omp* genes of teleost are in a different clade than mammals (Suzuki et al, 2015). Interestingly, two isoforms of *omp* were described in *Xenopus leavis* (Rössler et al, 1998), with apparent functional segmentation in their expression, since one *omp*

isofom is expressed in cells exposed to water and the other protein is expressed in cells exposed to air (Rössler et al, 1998). Despite the presence of two *omp* isoforms in the *Xenopus leavis* genome, these have no phylogenetic relationship with teleost forms and are located in the tetrapod clade a group that arose from sarcopterigian fishes. (Suzuki et al, 2015). The presence of two *omp* forms in teleost; which are diploid with a genomic duplication, and *Xenopus laevis*, polyploid animals (Session et al, 2016), is not observed in the *Xenopus tropicalis*, which is diploid (Reviewed in Grainger, 2012), which suggest the presence of two *omp* forms was produced in the genome duplication observed in the teleost evolution and duplication events in the lineage leading to amphibians.

Hence, our results and the described by Suzuki et al (2015) show the potential presence of a novel OSN population in the OE of zebrafish, which have a different location in the OE than the *ompb* expressing cells.

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Chapter III. Secondary analysis of RNA-seq data uncovers novel genes in olfactory imprinting

Introduction

The early exposure to the artificial odorant PEA is correlated with changes in the expression of TF and ORs in zebrafish (Harden et al 2006; Calfún et al, 2016). In order to study the effect of the PEA exposure on the expression of the whole transcriptome of the OE we did a RNA-seq analysis (GeneWiz Inc., US). In the results of the analysis done by GeneWiz we observed differences in the expression of different genes including some ORs (Chapter I, Calfún et al, 2016). However, due to the different types of bioinformatic analysis of RNA-seq data, we performed a second analysis of our data (Chapter I, Calfún et al, 2016) in collaboration with the lab of Hazel Sive, Ph.D. of the Whitehead Institute, MIT (Boston, USA), which have more experience with RNA-seq data from zebrafish, looking for significant changes in gene expression of PEA exposed OE of zebrafish,

Our second analysis showed significant differences in the gene expression of adult OE exposed to PEA compared to control. In particular we observed a significant increase in expression of genes related to immune and stress response in adult OE. From the up-regulated genes we have confirmed by qPCR that the *fkbp5* gene, has increase expression in larvae (one and three weeks post fertilization) as well as in adult OE. The *fkbp5* gene is a co-chaperone of the Heat Shock protein 90 (Hsp90) (Smith et al, 1990; Reviewed in Rein 2016), what participate in many cellular process as protein folding but the role of this gene in the control of the traffic to the nucleus of the steroid receptors have been widely described (Zannas and Binder, 2014; Reviewed in Rein 2016) In contrast to the *fkbp5* results, in our qPCR analysis we were not able to confirm the changes in expression observed for the immune genes. Our results show the increased expression of gene what this is correlated with PEA exposure in zebrafish, this change is maintained in adult OE despite the odorant exposure was just for three weeks.

Material and Methods

Animals

Zebrafish from the new wild-type (NWT) and NWT/Cornell strains, derived from the AB line, were used for all experiments. The fish were maintained at 28 °C on a light–dark cycle of 14 and 10 h, respectively. The Institutional Animal Use and Care Committee of the Universidad de Valparaíso approved all animal procedures (#BEA 022-2013).

RNA isolation

The RNA isolation and cDNA synthesis were performed as described in Calfún et al., 2016

Transcriptomic analysis

An initial analysis was performed as described in Calfún et al., 2016, and a second analysis of the RNA-seq data was realized in collaboration with the lab of Hazel Sive, Ph.D. of the Whitehead Institute (Boston, USA). In order to analyze the data distribution, Principal component analysis (PCA) was performed on the replicates for each group. The data from different replicates were paired by groups (Control-PEA). Using the paired analyses were calculated Log_2 of fold change (FC), the P values and FDR for these. Then the genes were filtered using the following criteria: genes with zero counts were discarded and just were considered genes with Log2FC -1 or smaller and 1 or bigger, which also have p values and FDR (false discovery rate) lower than 0.05.

qPCR analysis of the ORs expression

The expression analysis of major histocompatibility complex class I UBA (*mhc1ua*) and FK506 binding protein 5 (*fkbp5*) was performed using the same RNA samples from adult OE previously

used in the RNA-seq analysis, for 3 weeks and one week post fertilization, 100 fish were dissected per experimental group. The analysis was performed by qPCR according as Calfún et al., 2016, using the primers listed in Table 1.

Table 1. Primer used in qPCR analysis

Gene	Primer	Amplicon length
mhc1uba	PF GGGATCAGACAACTCATCTCGT PR GCAGGAGAAGGATGGATGTCACT	101 bp
fkbp5	PF GCCCGTTCTCGACTTGAGTCAT PR GGATCTTTCCCAGCGTTTGTCTGA	109 bp

Results

Post analysis of RNA-seq data

In order to take advantage of the RNA seq data obtained in our PEA imprinting experiments , we performed a new analysis of our original RNA-seq data (Calfún et al, 2016) in collaboration with the lab of Hazel Sive, Ph.D. and make use of the Whitehead Institute Bioinformatics Center, MIT B (Boston, USA), which is a premier bioinformatics group with ample experience in RNA-seq analysis using zebrafish. The RNA-seq analysis was performed using three biological replicates, where three experiments were performed in parallel. Initially a Principal component analysis (PCA) was performed on the raw data for all replicates; this analysis showed a batch effect in the data distribution of different groups, since a similar distribution for all replicates for the control groups was expected and other similar distribution for all replicates for PEA exposed groups. However the PCA analysis showed a data distribution by experiment, where control and PEA exposed group for the first experiment showed similar distribution, observing the same effect for the other experiments. Therefore it was necessary correct this effect using a paired analysis of the data.

Using the paired corrected data, Control and PEA exposed group were compared and statistical analysis were performed (Material and Methods) (Figure 1), then the results were filtered selecting for genes that showed significant changes in expression (p_value<0.05 and FDR<0.05) (Figure 1 red dots) at least 2 times.

In this new analysis we observed the same differences in ORs expression as the first analysis (Chapter I), however with the exclusion criteria of p_value<0.05 and FDR<0.05, the changes previously observed for ORs were not significant, where was used a t_test in the statistical analysis for comparing Control and PEA exposed groups.

When we analyzed just the filtered genes a decrease was observed in three genes

(*gnpda1, fbxo15, myhc4*), which are not in the same gene family and have no apparent relationship; however we observed a significant increase in the expression of 12 genes of which 7 are related with adaptive immunity and *fkbp5* gene, a immunophilin family member (Table 2).

Expression of adaptive immunity and stress response genes

In order to analyze the expression of the genes identified by our post-analysis (Table 2) of the RNA-seq data, we performed a qPCR analysis of PEA exposed fish at 1 week and 3 weeks fish (using dissected heads) and adult OE (using the RNA from our original RNA-seq analysis).

We did not analyze the expression of five of the seven genes (Table1) because they code for immunoglobulin peptides, which can be transcribed in several transcripts by recombination process (reviewed in Proudhon et al, 2015), making it difficult to generate primers for their detection. Thus we focused in other two genes: *mhc1uba* (major histocompatibility complex class I UBA) expressed in the antigen presenting cells participating in the immune system (reviewed in Dirscherl et al, 2014) and *fkbp5*, which participates in stress responses (reviewed in Madox et al, 2013).

We compared the *mhc1uba* expression between control and PEA exposed group, where no significant changes were observed at 1 week, 3 weeks and adult OE (used in RNA-seq) (Figure 2, Table 3). In contrast, significant changes in expression of *fkbp5* were observed in PEA exposed fish at one week, 3 weeks and adult fishes (used in RNA-seq) (Figure 3, Table 4), where an increase in the expression was maintained at the three stages analyzed

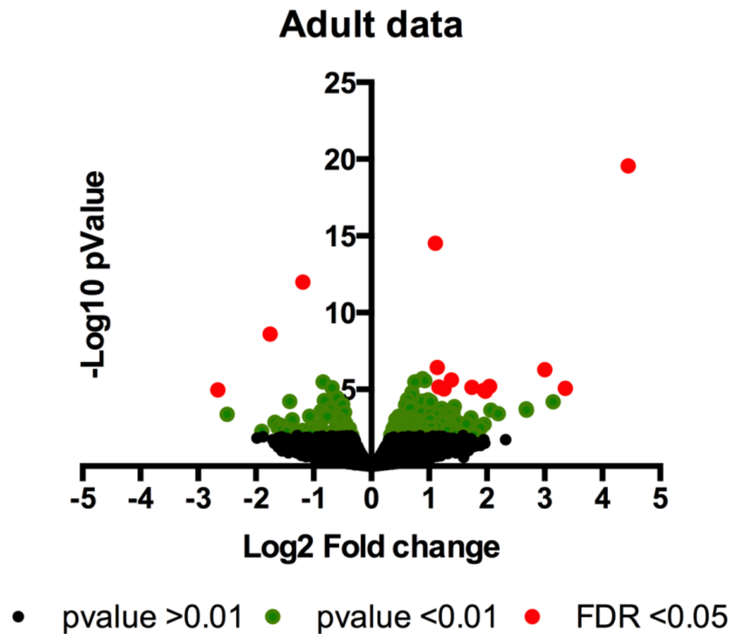

Figure 1. Plot of gene distribution according the changes in their expression and their statistical significance as measured by RNAseq. The x axis represent the changes in the gene expression (log 2 fold changes), where the genes with values > 1 increase their expression more than 2 fold, in contrast the genes with values < 1 decrease their expression more than 2 folds. The y axis represents the significance of the expression ($-\log_{10}$ p_value), where the genes located in positions > 2 have p_values <0.01 (green dots). The genes with significant differences according two statistical test (p_values < 0.01 and FDR < 0.01) are highlighted in red.

Table 2. Genes with highest significant increase in their expression in adult PEA exposed OE according RNA-seq analysis. *fkbp5*: immunophilin family gene *mhc1ub*a, *igl3c3*, *ighv2-1, ighv1-4* and *ighv4*: adaptive immunity

Gene Name	log_2FC(PE/control)	log_2(averageCPM)	p_value	FDR
fkbp5	1.17	9.25	7.17E-06	0.011
mhc1uba	1.26	9.62	9.33E-06	0.012
igl3c3	1.74	0.49	7.26E-06	0.011
ighv2-1	3.36	-1.75	8.48E-06	0.012
ighv1-4	1.38	4.47	2.45E-06	0.006
igl3v4	2.05	-1.56	6.50E-06	0.011

FC: Fold Change
CPM: Copies per million

These results obtained by RNA-seq and qPCR analyses show an increase in *fkbp5* expression in the adult OE, as well as an increase in the expression during other developmental stages.

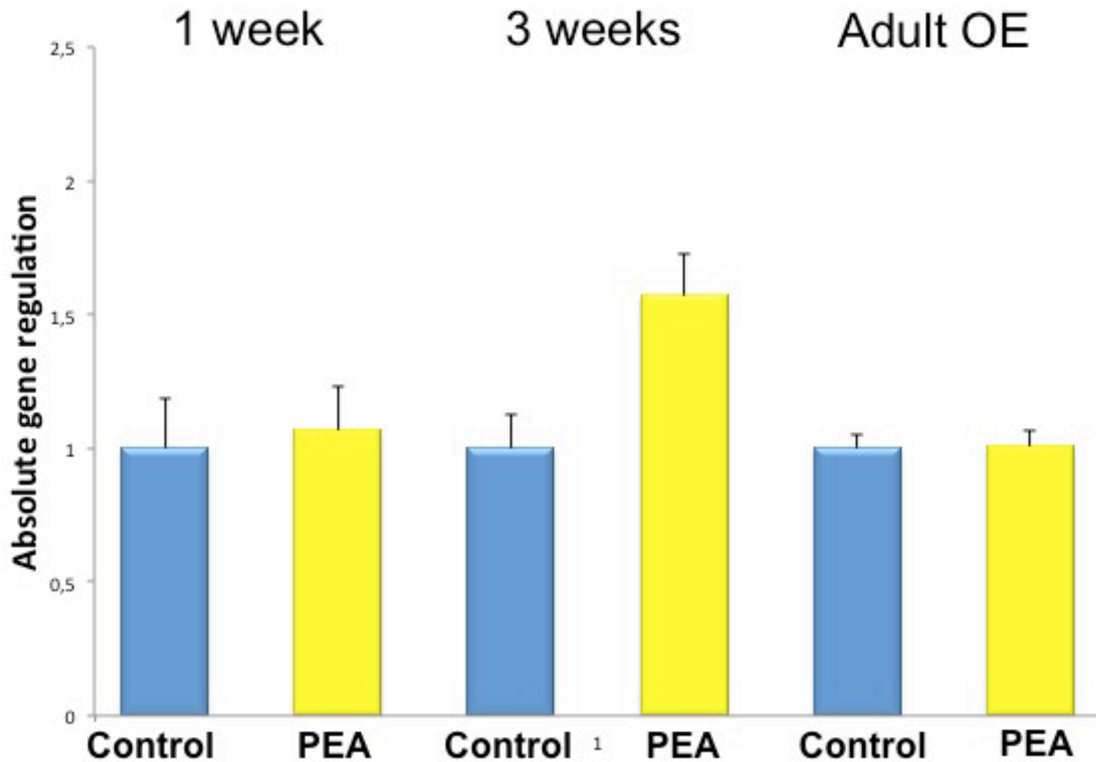

Figure 2. Expression of *mhc1uba* analyzed by qPCR. Exposure to PEA is not associated with changes for *mhc1uba* in 1 week larvae and adult OE (from original RNA-seq analysis), comparing PEA exposed group (yellow bars) vs control group (blue bars), while in 3 week juveniles show a tendency to increase the expression in PEA exposed fish, however it is not significant (*P < 0.05; analyzed by t-test; error bars represent standard error of the mean).

Table 3. Differential expression of *mhc1uba* measured by qPCR.

Experimental Group	Control	PEA exposed
1 week	1 ±0.2	1.1 ±0.2
3 weeks	1 ±0.1	1.6 ±0.2
Adult OE	1 ±0.1	1.0 ±0.1

Mean ± SE

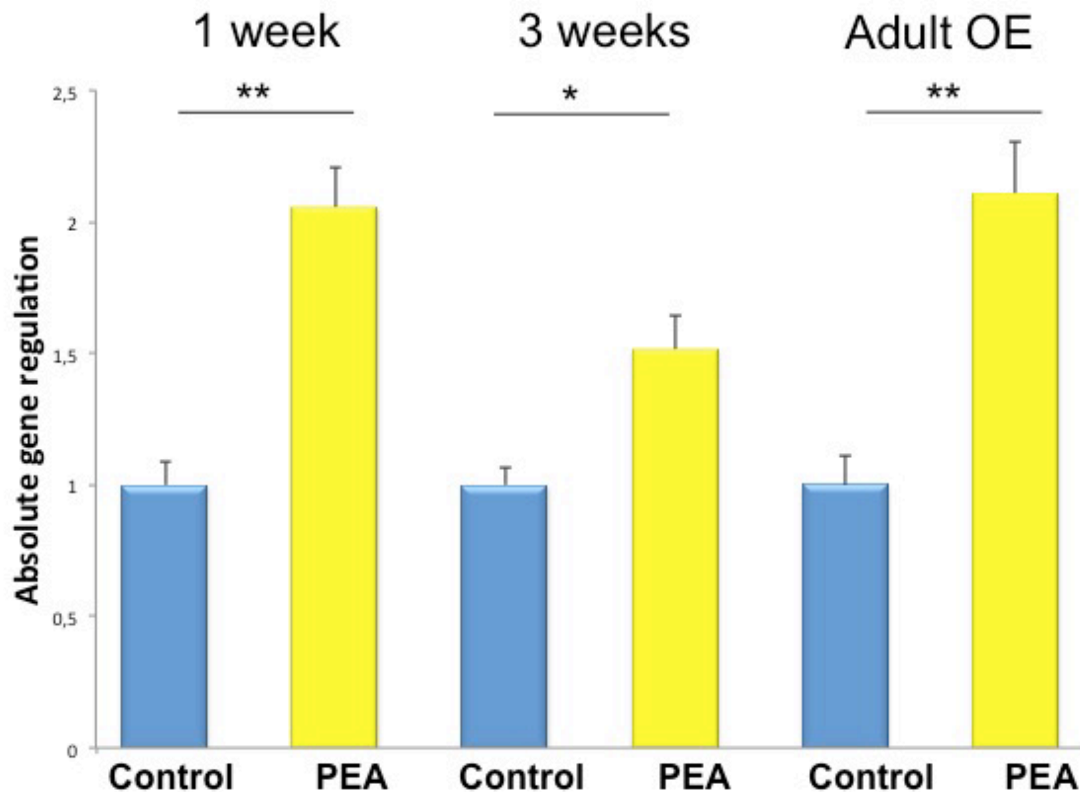

Figure 3. Expression of *fkbp5* in PEA-exposed animals increases in qPCR analysis. Exposure to PEA is associated with changes for *fkbp5* at 1 week, 3 weeks and adult OE (from original RNA-seq analysis) comparing PEA exposed group (yellow bars) vs. control group (blue bars). For 1 week the absolute gene regulation was 1 ±0.1 for control and 2.1 ±0.2 for PEA exposed; for 3 weeks was 1 ±0.1 for control group and 1.5 ±0.1 for PEA exposed; finally for adult OE was 1 ±0.1 for control group and 2.1 ±0.2 PEA exposed (*$p < 0.05$ and **$p < 0.01$; analyzed by t-test; error bars represent standard error of the mean).

Table 4. Differential expression of *fkbp5* measured by qPCR**.**

Experimental Group	Control	PEA exposed
1 week	1 ±0.1	2.1 ±0.2
3 weeks	1 ±0.1	1.5 ±0.1
Adult OE	1 ±0.1	2.1 ±0.2

Mean ± SE

Discussion

In recent years the next generation sequencing (NGS) technologies, which are high throughput sequencing techniques, have allowed us to analyze a great numbers of genes including whole genomes at the same time (Reviewed in Schuster, 2008 Reviewed in Wang et al 2009). As a result currently RNA-seq is the best method to measure changes in the expression of a whole transcriptome exceeding other techniques as microarrays, which are limited by the number of genes represented in the chips (Reviewed in Wang et al 2009). The ability to collect data on the genome wide level, using techniques such as RNA-seq, creates new challenges for developing methodologies to analyze the data.

In the present study we performed a second analysis of our original RNA-seq data (Calfun et al, 2016) to further analyze the effect PEA of exposure on the OE, where the data were paired by groups (Control-PEA) in order to avoid a batch effect observed in the data distribution. This second, distinct, analysis allowed us to perform two statistical tests to filter the data. With this approach we observed different results than the observed in our first analysis, however the differences were observed in the value of fold change for the genes; the tendency to increase or decrease gene expression observed in our first analysis was maintained.

Strikingly we observed significant increases in the expression of genes involved in adaptive immunity and a chaperone protein in the adult OE. When we analyzed the expression of two differentially expressed genes by qPCR, we observed no changes in the expression of *mhc1uba* (major histocompatibility complex peptide), in contrast we confirmed the increase of *fkbp5*, which is a chaperone of the inmunophilin family (Zannas and Binder, 2014) in adult OE, differences that are maintained at one and three weeks PEA exposed zebrafish. Our results suggest the role of *fkbp5* in the PEA effect on the OE that we reported previously.

PEA exposure is possibly related with changes in immune system genes.

Odorant exposure can elicit an immune response. In mice, male exposed to female odor showed resistance to flu virus, with macrophage and neutrophil migration to upper respiratory tract (Litvinova et al, 2010), which is similar to the elicited by bacterial Lipopolysaccharide (LPS) present in the external membrane of Gram-negative bacteria (Moshkin et al, 2013). Interestingly, our transcriptomic analysis showed an increase in the expression of immune system genes such as immunoglobulins and *mhc* in PEA exposed animals; however we were unable to confirm these differences in *mhc* by qPCR analysis. Therefore, some our data suggest the PEA odorant exposure elicit a potential activation of the immune system.

We do not know the mechanism of activation of the immune system in the OE by odorant exposure; however, different evidences show the role of the GPCR, the same family of the ORs, in the modulation of the immune system. In an in vitro analysis was observed that human mast cells release the pro inflammatory cytokines IL-8 and TNF-α, which is dependent of the activation of G_o protein (Yu et al, 2016). In contrast, the membrane type progesterone receptor, which is a GPCR, have showed a anti inflammatory effect in different cell types; in breast cancer TNFα signaling induces the release of IL6, which is inhibited by the membrane progesterone receptor (Okamoto and Mizukami, 2016). In addition, a similar effect was observed in mouse macrophages, where the bacterial LPS induce the release of IL6 by macrophages, then the activation of the G-protein coupled estrogen receptor in macrophages suppress the cytokine release in presence of LPS (Okamoto et al, 2017). Therefore, the activation members of GPCR family can modulate the release of pro-inflammatory cytokine and the potential recruitment of the leukocytes, then we can think the activation of ORs could activate a signal pathway and modulate the activation of the immune system in the OE, however is necessary study this potential relationship in order to elucidate this mechanism.

fkbp5 functions

The product of the *fkbp5* gene FKBP 51, acts as co- chaperone for the heat shock 90 (Hsp90) regulating different cellular process, but it role in the steroid hormone receptor traffic to the nucleus have been the more studied (Smith et al, 1990; Reviewed in Rein 2016). The role of *fkbp5* has been widely described for the GR, which binds cortisol under stress condition; the GR translocation to the nucleus is controlled by heat shock 90 (Hsp90) and FKBP51, which act as chaperon proteins avoiding the translocation (Zannas and Binder, 2014). When cortisol binds to GR, the FKBP51 affinity for the receptor is lost and ligand-receptor complex translocate to the nucleus (Zannas and Binder, 2014). Additionally, *fkbp5* expression is activated by GR (Reynolds et al, 1999; Binder et al, 2009), therefore GR and *fkbp5* work in a negative feedback loop where the activation of the receptor increases *fkbp5* levels and this decrease de GR response (Reynolds et al, 1999; Binder et al, 2009). Interestingly, a polymorphism has been observed in *fkbp5* gene, which has been related with changes glucocorticoid response (Binder et al, 2004; Han et al, 2017), and additionally, have been correlated with a predisposition to psychiatric disorders in response to environment conditions as post traumatic stress disorders (Binder et al, 2004, Menke et al, 2012, Klengel and Binder, 2015; Han et al, 2017). Moreover, zebrafish larvae exposed to cortisol by five days shows upregulated glucocorticoid-responsive genes and an increase in the expression of *fkbp5*, effect measured by RNA-seq and qPCR (Harting et al 2016). On the other hand, the role of FKBP 51 and Hsp90 controlling the traffic of GR to the nucleus, has been observed with the androgen receptor and the progesterone receptor (Barent et al, 1998; Stechschulte and Sanchez, 2011), where as observed for GR, the expression of *fkbp5* increase with this hormones (Hubler and Scammell, 2004; Magee et al, 2005). Therefore, *fkbp5* also participates in mechanisms such as the trafficking of molecules to the nucleus.

The *fkbp5* expression has been related with pathways different than the steroid receptor traffic and stress response. The protein FKBP51 (codify by *fkbp5*) belongs to a family called

immunophylins, which mediates the role of immune suppressive drugs such as FK506 (Reviewed by Zannas and Binder, 2014), these drugs have an inhibitory effect over the antigen presentation mediated by Major histocompatibility complex class II (Imai et al, 2007). Additionally, FKBP 51 inhibits the calcineurin phosphatase activity, which block the transcription of the Nuclear Factor of Activated T cells (NFAT) in T lymphocytes (Baughman et al, 1995; Weiwad et al, 2006), where the *fkbp5* – calcineurin interaction is calcium independent (Li et al, 2002). Recent studies have shown that *fkbp5* participates in the autophagy process, where it acts organizing the promoter of autophagy Beclin 1 (Gassen et al 2014; Gassen et al 2015a), which is a protein complex that initiates the process of autophagy (Wang et al, 2012). Therefore, despite the interest for the role of *fkbp5* in the stress response, there is a variety of evidence indicating that this gene participates in different cellular process.

Increased levels of *fkbp5* expression have been observed in the CNS, where mice exposed to chronic stress expressed high levels of *fkbp5* mRNA in the paraventricular nucleus and central nucleus of the amygdala, areas related with emotional response (Scharf et al, 2011). The same effect was observed in hippocampus after dexamethasone administration what activates the GR (Scharf et al, 2011). Chronic administration of corticosterone has been correlated with increased levels of *fkbp5* in blood and hippocampus in mice (Lee et al, 2010). Intriguingly, our results showed increased expression of *fkbp5* in PEA exposed zebrafish which is correlated with the changes in expression of ORs and *otx2,* and also memory of this odorant (Harden et al, 2006; Calfún et al.; 2016), which showed the putative role of *fkbp5* in the PEA exposure effect in the OE.

We synthesized RNA probes in order to detect *fkbp5* by *in situ* hybridization, however were not successful in labeling the *fkbp5* expressing cells. To date our ability to understand the potential function of *fkbp5* have been hampered by the inability to visualize the gene expression

in the OE. But directed mutagenesis driven by an olfactory specific promoter such as that controlling olfactory marker protein (OMP) gene (Celik et al, 2012), would allow us to inactivate this gene specifically in the OSNs as a fist step toward understanding the role of *fkbp5* in olfactory imprinting.

The variations in the *fkbp5* expression correlated with changes in the environment have lead to some authors to propose *fkbp5* as a mediator in adaptation process (Reviewed in Rein, 2016) between other functions. Otherwise, *fkbp5* has been related with epigenetic changes in DNA, where *fkbp5* can reduce in the activity of DNA methyl transferase 1 (DNMT1) (Gassen et al, 2015b); interestingly the DNA methylation is a process involved in the olfactory neural progenitor differentiation (Colquitt et al, 2014), whereby, *fkbp5* could be a modulator for this process. Therefore, our results lead to propose a model where the PEA exposure increase the expression of TFs as *otx2* which change the ORs expression levels, then *fkbp5* increases expression in response to the changes in the environment modulating the adaptation of the OE (Figure 4).

Further analysis is necessary in order to test this model, since the work presented here represents a first insight of the relationship between *fkbp5* and memory formation identifying the first element for this possible model.

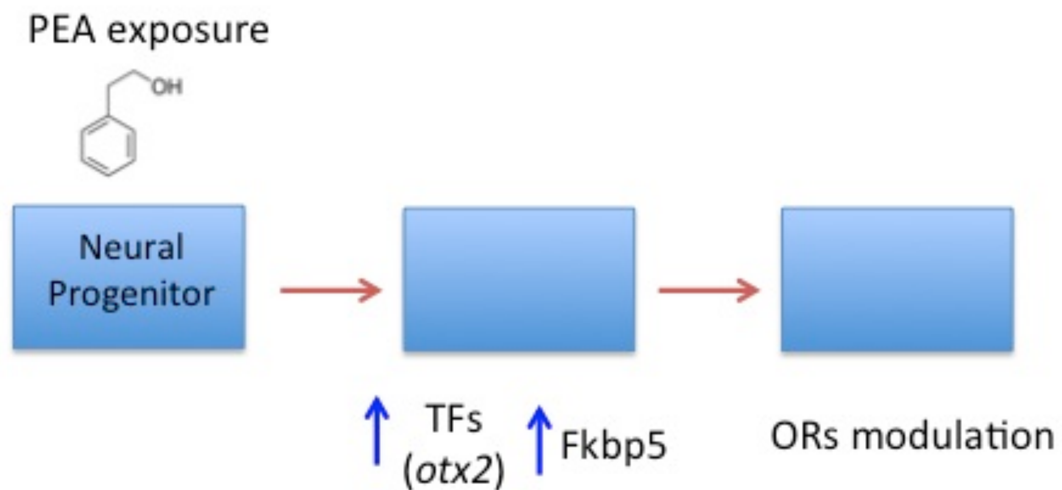

Figure 4. Model for PEA exposure and stress response in the OE. PEA exposure increases expression of TFs in neural progenitor cells in the OE as *otx2* and increases *fkbp5* expression, which acts in the response to environmental change (PEA odor). The TFs modulate the mRNA levels of ORs with binding sites for the TFs in the progenitor cells**.**

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