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

Cristian Calfún¹,², Calixto Domínguez¹,³, Tomás Pérez-Acle¹,⁴ and Kathleen E. Whitlock¹,²

¹Centro Interdisciplinario de Neurociencia de Valparaíso (CINV), Universidad de Valparaíso, Pasaje Harrington 287, Valparaíso 2360102, Chile, ²Instituto de Neurociencia, Facultad de Ciencias, Universidad de Valparaíso, Pasaje Harrington 269, Valparaíso 2360102, Chile, ³Center for Bioinformatics and Genome Biology, Fundación Ciencia & Vida, Av. Zañartu 1482, Santiago 7750000, Chile and ⁴Computational Biology Lab, Fundación Ciencia & Vida, Av. Zañartu 1482, Santiago 7750000, Chile

Correspondence to be sent to: Kathleen E. Whitlock, Instituto de Neurociencia, Universidad de Valparaíso, Pasaje Harrington 269, Valparaíso 2360102, Chile. e-mail: kathleen.whitlock@uv.cl

Accepted 30 December 2015.

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 isoforms of PEA showed that a subset of ORs containing both Otx2/Hnf3b binding sites were downregulated only in βPEA-exposed juveniles and this change persisted through adult life. Localization of OR expression by in situ hybridization indicates the downregulation 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.

Key words: *c-fos*, *otx2*, phenyl ethyl alcohol, RNA-seq

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), 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 on 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 RNA-seq 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, isoform-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 Valparaiso 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 (sv9; 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 (Hirotai and Mombaerts 2004; Kolterud et al. 2004), and Emy2 (McIntyre et al. 2008). DNA binding motifs in the zebrasfish 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://pge.unmass.edu/TFDBS/). By using MEME, and assuming conservation between orthologous transcription factors from Drosophila and zebrafish (Acampora et al. 2001; Boyle 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 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 αPEA (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 βPEA 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 pmol of each primer (Table 1), and GoTaq Green Master Mix (Promega), in a final volume 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), 1x 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 MCD-v2 software. The primer efficiency (Table 1) was calculated; any primer pair with efficiency lower than 95% and greater than 110% was 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 oPEA 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 paraformaldehyde 4% in PBS at 48 and 72 hours post-fertilization (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 posttest. 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 1x50bp 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.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Primer (5′–3′)</th>
<th>Amplicon length</th>
<th>Percentage of efficiency at 56 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>OR102-4</td>
<td>DQ306112</td>
<td>F) TGAGCCCTACTGTGCTTACAA R) TCAGGCGCCATGCTAATGTT</td>
<td>119 bp</td>
<td>102.7</td>
</tr>
<tr>
<td>OR103-1</td>
<td>DQ306104</td>
<td>F) CTTAATCCCTCGTGCGCTTTAT R) CGAGTGGGAGAAAGCTTCTACAG</td>
<td>101 bp</td>
<td>98.4</td>
</tr>
<tr>
<td>OR111-1</td>
<td>DQ306093</td>
<td>F) GTGGGCTTTGATGTTGCTTTAG R) CACTGCATGCAAACCTATACAGT</td>
<td>106 bp</td>
<td>99.2</td>
</tr>
<tr>
<td>OR115-5</td>
<td>DQ306037</td>
<td>F) ATTCCTGTGCGACCCCTCATA R) GATGGCTTTTGCTGTGGAGTG</td>
<td>110 bp</td>
<td>109.0</td>
</tr>
<tr>
<td>OR125-1</td>
<td>DQ306121</td>
<td>F) CTGTCGCGTCTGCAAGGCACAGGTCTT R) AGGTGGAAACAATCAACAGGCTCT</td>
<td>192 bp</td>
<td>96.3</td>
</tr>
<tr>
<td>OR136-1</td>
<td>DQ306047</td>
<td>F) TCAAAAGCGCCGCAGTCAGCCACAAAC R) TTTCTGGCCAGCGAAAGAGGACT</td>
<td>107 bp</td>
<td>106.0</td>
</tr>
<tr>
<td>OR137-3</td>
<td>DQ306051</td>
<td>F) TTGTCGATGCTGTTGCTGCTG R) AGAAGAAAGACGTGTCCTCA</td>
<td>102 bp</td>
<td>101.1</td>
</tr>
<tr>
<td>beta actin</td>
<td>AF057040</td>
<td>F) CGACGAGGAGGAGGGAAAACC R) CAACGGAAACGCTCATTGC</td>
<td>102 bp</td>
<td>95.0</td>
</tr>
</tbody>
</table>
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 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.
Stage for analysis of OR expression in response to PEA-induced changes in hpf for qPCR (see below). Because we have previously shown that mental stages analyzed by RT–PCR, but they were detected at 72 hpf for qPCR (see below). Our results show that 5 of the ORs onset of gene expression for the selected ORs, we isolated RNA from the developmental transcriptase–PCR (RT–PCR). Our results show that 5 of the ORs are expressed during early development in the zebra fish. We assayed Otx2, Hnf3b, Emx2, and Lhx2. The motifs are represented graphically by a logo where the height of each nucleotide indicates the level of conservation. (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, OR123-1 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.

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 isofrom 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 OR123-1 (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 isoform, we exposed embryos from the same cohort to the α isoform 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), OR123-1 (ratio = 1.01±0.07), OR136-1 (ratio = 0.99±0.09), and OR137-3 (ratio = 1.10±0.09) showed no significant 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 oPEA 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 oPEA-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 oPEA. 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.

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+ (OR111-1, OR125-1) and the third receptor on chromosome 15 (OR115-5). Two ORs, OR136-1 and OR137-3, that showed consistently detectable but variable changes across the developmental 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).

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 otz2 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 isoform. 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 programmed 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.

**Isoform-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 isoform 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 isoform that changes OR expression (βPEA) and an isoform (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 βPEA, 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

---

**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; OR115-1; OR125-1) in 3 dpf juveniles. For 3 ORs, OR136-1, and OR137-3, there was no difference in the expression ratio between control (bars with dark outline, left) and βPEA-exposed juveniles. **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). Control for each OR on left with black outline. Data from 3 dpf juveniles (100/experimental group).
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, OR123-1) may be those that are under the control of PEA via Otx2/Hnf3β regulation. Using transfection assays, it has been shown that the Otx2 homeodomain and C-terminal regions bind to HNF-3β resulting in HNF-3β repression of OTX2-directed gene expression (Nakano et al. 2000). We suggest a model (Figure 9) where interactions between Otx2 and HNF-3β may result in enhancement of the signal-to-noise ratio through repression of specific ORs and activation of others. In the presence of βPEA, the effects of Otx2 are mediated through Otx2/Hnf3β and Otx2 reflected by the potential expansion of cells expressing otx2. This expansion allows the decrease in ORs controlled by Otx2/Hnf3β on Ch 15 (OR103-1, OR111-1) and Ch 21 (OR115-1, OR123-1). 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.

**Figure 6.** Change in otx2 expression in OE of juvenile zebrafish depends on isoform 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 isoform-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.

### 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-elicted 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; Calfán C, Maturana C, McKenzie M, Harden M, and Whittle 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 PEAs 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 understood with gene regulation OR gene families occurring at the level of the TFBS and OR expression (Monahan et al. 2011; Santoro and Dulac, 2012) 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 odor-ant 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). 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 underlying control elements active at the level of the chromosome has yet to be determined.

Supplementary material
Supplementary material can be found at http://www.chemse.oxfordjournals.org/

Funding
This work was supported by FONDECYT-1111046 (K.E.W.); ICM-ECONOMIA Instituto Milenio Centro Interdisciplinario de Neurociencias de Valparaíso PQ9-022-F (K.E.W., T.P.-A.); PFB16 (T.P.-A.); CONICYT Doctoral Fellowship 21120793 (C.C.); FONDECYT Postdoctoral Fellowship 3140007 (C.D.).

Acknowledgments
We would like to thank the Whitlock lab, especially the Zebrafish Facility for their help in the maintenance of all the fish required for this study. We thank J. Ewer for careful reading of the manuscript and the lab of Dr. Yoshihara Yoshihiro for providing the following plasmids containing ORs: OR103-1; OR111-1 probes (Sato et al., JNS, 2007); OR115-5 and OR 125-1.

References


