alone does not affect c-fos expression. Embryos raised in $10^{-8}$M 17, 20P had 0-8 c-fos expressing cells per OE and those exposed at $10^{-10}$M 17, 20P had 0-6 c-fos expressing cells per epithelium (Table 4.1, Fig. 4.4D1). Exposure to 17, 20P resulted in no significant changes in c-fos expression for either concentration (Table 4.1, Fig.4.4 D1) when compared to EM or EM+EtOH (Fig. 4.4D1; Table 4.1). We next analyzed PGF$_{2\alpha}$, another putative pheromone in goldfish (Fig 4.4D2). Exposure to PGF$_{2\alpha}$ at $10^{-8}$M had 0-8 c-fos expressing cells per OE and those exposed at $10^{-10}$M had 0-6 c-fos expressing cells per OE (Fig. 4.4D, Table 4.1). The lower concentration ($10^{-10}$M) of PGF$_{2\alpha}$ resulted in no significant change ($p>0.1$) in c-fos expression (Table 4.1, Fig.4.4 D2) when compared to EM or EM+EtOH (Fig. 4.4D2; Table 4.1). In contrast to 17, 20P, exposure to PGF$_{2\alpha}$ resulted in a significant change ($p<0.005$) in c-fos expression for the higher concentration examined ($10^{-8}$M; Table 4.1).

Finally, we exposed embryos to taurocholic acid, an odorant we have previously shown to be attractive to zebrafish at three days post fertilization (Vitebsky et al., 2005). Exposure to taurocholic acid ($10^{-8}$M and $10^{-10}$M), resulted in a statistically significant reduction in olfactory epithelial c-fos gene expression at both $10^{-8}$M ($n= 102$ ; $p= <0.005$; Table 4.1) and $10^{-10}$M ($n= 130$ ; $p= <0.005$; Table 4.1) when compared to the distilled water (dH$_2$O) diluent control (Fig. 4.4D3). Importantly, there was no significant change in the number of c-fos cells between the EM and EM+dH$_2$O control groups ($p>0.1$, Table 4.1) demonstrating that the reduction in the number of c-fos cells we observe is a result of exposure to taurocholic acid.
Analysis of distribution of c-fos positive cells

When we analyzed the distribution of the c-fos expressing cells we found it to be a non-normal distribution. These data are represented in a frequency distribution of the number of c-fos expressing cells per OE (Fig. 4.5). Of all OE scored in the EM baseline group, 90% had between 0-2 c-fos expressing cells (Fig. 4.4 D1). Similarly, in the EtOH and dH2O controls the majority of the preparations had between 0-2 c-fos expressing cells (97% and 92% respectively, Fig. 4.4D). Thus control odorants embryos showed essentially the same pattern of gene response as that seen in the EM baseline (Fig. 4.4D, Table 4.1). The 17,20P exposed embryos also showed a response similar to that of the controls; 92-94% of 17,20P exposed OE contained 0-2 c-fos expressing cells (Fig. 4.4D1, Table 4.1).

Analysis of the OE of embryos exposed to PGF2α at 10^{-10}M showed that 92% of the OE scored had 0-2 c-fos expressing cells per OE making their distribution like that of the controls. In contrast, only 75% of the OE exposed to PGF2α at 10^{-8}M contained 0-2 c-fos positive cells (Fig. 4.4D2). In addition, the frequency of OE having 3+ c-fos expressing cells increased to 25% a significant increase over the 7% observed in the embryos exposed to PGF2α at 10^{-10}M (Fig. 4.4 D2; Table 4.1). Thus exposure to PGF2α at 10^{-8}M results in an increase in the number of embryos having an increased number of c-fos expressing cells in the OE.

Analysis of the OE of embryos exposed to taurocholic acid showed a down-regulation of c-fos expression. At both concentrations of taurocholic acid, 10^{-8}M and 10^{-10}M, most preparations showed no c-fos expression, (91% and 90% respectively). No OE contained 3 or more cells, and only a small percentage of OE had 1-2 cells expressing c-fos. Thus, for embryos
Figure 4.5. Distribution of frequency of observations of c-fos expressing cells changes with odor treatment. (A, B), y axis is frequency with which a given number of c-fos expressing cells was observed in an OE. x axis is the number of c-fos expressing cells in an OE. (A) PGF \(10^{-8}\)M (green) distribution is shifted to the right compared to that of EM or EtOH control (red and blue). 0 is not included in the distribution because the majority of observations in the controls were 0 (see Figure 4.4 D1-3), thus skewing the data and making it difficult to plot on a single graph. (B) Taurocholic acid (green \(10^{-8}\) and yellow \(10^{-10}\)) distribution is shifted far to the left compared with EM or dH\(_2\)O controls (red and blue). For data presented here between 40-50 olfactory epithelia were scored for each experiment.
A

![Graph A](image)

B

![Graph B](image)
developing in the presence of taurocholic acid there was a significant
decrease in the number of c-fos expressing cells in the OE (Fig. 4.4 D3, Table
4.1). For the data where we observed significant changes in distributions of c-
fos expressing cells (PGF$_2\alpha$ and taurocholic acid) we plotted the frequency of
observations (1 observation is 1OE; Fig. 4.5, y-axis) against number of c-fos
expressing cells per observation (Fig. 4.5, x-axis). The embryos raised in
PGF$_2\alpha$ at 10$^{-8}$M show an increased number of OE containing high numbers of
c-fos expressing cells (Fig. 4.5A, green) relative to the OE of embryos
exposed to either EM+EtOH (Fig. 4.5A, blue) or EM (Fig. 4.5A, red). In
contrast, for embryos raised in taurocholic acid at either 10$^{-8}$M (Fig. 4.5B,
green) or 10$^{-10}$M (Fig. 4.5B, yellow), the number of OE with few c-fos
expressing cells is very large when compared to either EM+EtOH (Fig. 4.5B,
blue) or EM (Fig. 4.5B, red).

**Odorant exposure did not alter the expression pattern of two odorant
receptors**

We examined whether or not exposing juvenile zebrafish to behaviorally
relevant odorants could alter the expression of two receptors in the developing
OE. We cloned the odorant receptor (OR) OR11.1 (simultaneously cloned
and named OR101-1, GenBank accession number DQ306041; (Alioto and
Ngai, 2005)) and found that it is expressed as early as 24h (Fig. 4.6A, arrow)
and continues to be expressed at 48h (Fig. 4.6B, arrow) and 72h (Fig. 4.6C,
arrow). We examined OR11.1 after odorant exposure in part because of its
early expression in the OE (Fig. 4.6A) and also because it is expressed in
many cells compared to other ORs we have previously examined (avg. 6 cells
Figure 4.6. Expression of OR11.1 and V1R receptors in the developing olfactory epithelium does not change upon odorant exposure. (A-C) Expression of cells expressing OR11.1 (arrows) at 24h (A), 48h (B) and 72h (C). (E,F) Expression of cells expressing V1R (arrows) at 48h (E) and 72h (F). Expression patterns did not change upon odorant exposure (data not shown). (D) Schematic of the localization of cells expressing OR11.1 (green cells) and V1R (yellow cells) in 48h olfactory epithelium. Many of the cells expressing these receptors are located in an inner ring (gray) in the apical part of the developing epithelium. h= hours post fertilization, scale bar= 30µm. All photos are ventral views, anterior to the top of the page.
per OE in embryo medium; Harden and Whitlock, unpublished observations).
We exposed juvenile zebrafish to 17,20P, PGF$_{2\alpha}$, and taurocholic acid and the appropriate controls in the same manner as described for the c-fos studies. We did not see any significant difference in the expression of OR11.1 between control fish and those treated with the behaviorally relevant odorants for the first two days of development.

Vomeronasal receptors (V1Rs and V2Rs) have been proposed to bind pheromones in mammals (Dulac, 2000). Because the odorants being examined have been proposed to act as pheromones, their signals could be communicated through vomeronasal-like receptors. We examined the expression of the vomeronasal-like receptor in V1R (Pfister and Rodriguez, 2005) after exposure to the behaviorally relevant odorants. We found that V1R is not expressed at 24h but is expressed at 48h (Fig. 4.6E) and 72h (Fig. 4.6F) in the developing OE. V1R expression also did not change in response to odor treatment. We also examined the expression of a V2R vomeronasal-like receptor, which has been shown to be expressed in the adult OE but had not been examined during development (Pfister and Rodriguez, 2005). We found that the V2R-like receptor is not expressed during early development of the OE (data not shown).

In comparing the expression patterns of OR11.1 and V1R we found that OR11.1 is expressed throughout the developing OE (Fig. 4.6D) while V1R (Fig. 4.6D) is localized to the lateral portion of the developing OE at 48h. Cells expressing either of these receptors are not found basally. A high proportion of the cells expressing these ORs are located deep within the developing OE
(Fig. 4.6D, grey) in a region that contains mature OMP (olfactory marker protein) expressing olfactory sensory neurons (Harden et al., 2006).

**DISCUSSION**

We have found that the IEGs egr1, c-jun, and c-fos are expressed in the olfactory bulb of wild-type zebrafish embryos at 24, 48, and 72h. Of these IEGs, only c-fos and c-jun are expressed in the developing OE; c-jun uniformly throughout and c-fos in a small number of cells scattered throughout the OE. Furthermore, we were able to demonstrate that two odorants, PGF$_{2\alpha}$ (a putative pheromone) and taurocholic acid (a bile acid that is behaviorally significant in developing and adult zebrafish (Vitebsky et al., 2005)) can modulate c-fos expression in the developing OE; PGF$_{2\alpha}$ by increasing the frequency of OE with high numbers of c-fos expressing cells and taurocholic acid by increasing the frequency of OE with no c-fos expressing cells or very few c-fos expressing cells.

**Expression of IEGs relative to olfactory organ development**

The expression of the c-fos and c-jun IEGs in the OE are evident during a time period that coincides with the early differentiation and axongenesis of sensory neurons. The olfactory placode is first evident in the living embryo at 17-18h and by 24h axons of pioneer neurons extend from the olfactory placode into the developing bulb, (Hansen and Zeiske, 1993; Whitlock and Westerfield, 1998; Whitlock and Westerfield, 2000). 48 hours post fertilization begins a period of rapid neurogenesis (Mueller and Wullimann, 2003), and at this time, the olfactory pit has developed and microvillar receptor cells are observed (Hansen and Zeiske, 1993). By 72 hours post fertilization, the
olfactory system is mature enough to elicit a behavioral response to the amino acid odorant L-cysteine (Vitebsky et al., 2005).

**Activity of c-fos in the “absence” of odor**

The presence of c-fos activity in the embryo medium controls raises the possibility that c-fos induction may be possible with a wide range of odorants. In these studies we use exposure to embryos medium as a baseline for c-fos expression but it is far from an odor-less environment. Embryo medium contains various salts, pH buffers, and odors arising from the presence of other embryos. Clearly, there are many odorants present in embryo medium that may be eliciting c-fos expression. Additionally, pH effects are also a possibility. Furthermore, we performed preliminary experiments comparing c-fos expression in 48h and 72h embryos raised in embryo medium and water in which adult fish live and showed that “fish water” induced an increase in c-fos expression compared to embryo medium exposure (data not shown). One of the main differences between these two odor environments is presence of fish urine and other social cues in “fish water”. The expression of c-fos in embryo medium and “fish water” is highly suggestive that multiple environmental conditions and odors effect its expression.

**Pattern of c-fos expression and olfactory receptors**

Recent proposals of a stochastic mechanism of olfactory receptor gene choice suggest that odorants present in the environment may establish a positive feedback loop that can reinforce and maintain receptor choice. A deterministic model would predict that ORs are chosen through intrinsic signals that select specific olfactory receptor genes for expression in a given
olfactory sensory neuron (Shykind, 2005). In this model, particular olfactory receptors will appear in characteristic patterns across individuals in a species and olfactory epithelia within individuals. In contrast, the stochastic model predicts that a single transcriptional activating factor randomly selects one olfactory receptor gene for expression. In order for the selected gene to continue its expression, the activation and maintenance of a feedback loop is required (Shykind, 2005). This feedback loop may be established by the activation of the expressed receptor through the binding of its odorant ligand (Shykind, 2005). Our data show that numbers of c-fos expressing cells vary greatly across groups of OE (Table 4.1, Fig. 4.4), and furthermore that two OE of the same fish are not mirror images of one another (data not shown), which is reminiscent of OR expression in the developing OE.

**Early Neurogenesis**

It is likely that at 48 hours post fertilization, during a rapid period of neurogenesis (Mueller and Wullimann, 2003; Mueller and Wullimann, 2005), many olfactory receptor choices are being made. It has been shown in zebrafish that olfactory receptors can be expressed as early as 24h, placing the timing of feedback mechanisms well within the range of time points showing c-fos expression (Barth et al., 1996; Whitlock and Westerfield, 1998).

It is possible that c-fos activation could be non-instructive and instead be acting as a marker for neural activity. However, this is unlikely, as most olfactory sensory neurons present in the developing epithelium at these early time points do not yet have functional connections to the olfactory bulb. Most of the axons present have been established by a population of pioneer neurons (Whitlock and Westerfield, 1998). Thus, few sensory neurons in the
epithelium have functional connections to the central nervous system at 24h and 48h. However, because many of the sensory neurons in the epithelium do express olfactory receptors at this point, it may be that c-fos is being up-regulated by a signal transduction pathway downstream of odorant reception, and acting in a transcriptional capacity. Cells that are expressing olfactory receptors may be using c-fos to affect downstream maintenance of cell differentiation and balance of progenitor cells in response to odor environment.

**Olfactory bulb-epithelial correspondence?**

In mouse, it is well known that activation of particular odorant receptors (and their corresponding olfactory sensory neuron(s)) is reflected in the olfactory bulb by the activation of specific glomeruli (reviewed by Mombaerts, 1999). The activation of glomeruli is often identified by the activation of c-fos, yet, to date, whether specific activation patterns of c-fos in the bulb can be extended to specific and associated c-fos induction in the periphery has only been recently initiated (Halem et al., 2001; Norlin et al., 2005). The idea that olfactory sensory neuron IEG activation may relate to olfactory bulb IEG patterns is an intriguing possibility and our results provide a good basis on which to investigate this possibility in the future.

**Olfactory Receptor Expression**

We examined the expression of one OR and one vomeronasal-like receptor (V1R) in response to odorant exposure. While we did not see a difference in expression of these two receptors we have only examined one OR out of the potentially 143 intact OR genes in the zebrafish genome (Alioto and Ngai, 2005). The V1R receptor we examined is the only V1R-like receptor
identified in the zebrafish genome to date (Pfister and Rodriguez, 2005). The one V2R receptor we examined, which is not expressed during OE development (this work; data not shown), is one of a predicted ~70 V2R-like intact receptor genes in the zebrafish genome (Hashiguchi and Nishida, 2005). Therefore, it is important to point out that there are many more odorant receptors that could be examined in relation to exposure of these odorants. When considering the stochastic mechanism of OR selection (Shykind, 2005) it is likely that early receptor expression plays a role in the selection of the receptor that a cell will permanently express. The stochastic model proposes that OR expression is initially unstable and that a functional OR could activate a feedback mechanism, which would lead to OR commitment in that cell. It is possible that binding of a particular OR (or vomeronasal receptor) to odorants present in the environment during development might be one way of determining an OR’s functionality, thereby resulting in activation of the feedback pathway. OR binding could lead to a preference for OR expression in particular developmental environments. The increase we observe in c-fos expression in the developing OE may be involved in mediating the expression of ORs in response to environmental stimuli. Since doing this work the complete OR repertoire of zebrafish has been characterized (Alioto and Ngai, 2005), making it possible to examine expression (using expression microarrays) of all ORs in different environmental contexts during development. If expression changes are observed in particular ORs it would be interesting to determine whether or not their expression overlaps with expression of c-fos and if change in c-fos is necessary for the observed change in OR expression.
**c-fos expression in the OE**

Norlin and colleagues propose that *c-fos* induction in the mouse OE is associated with induction of apoptosis (Norlin et al., 2005), but this role in zebrafish is unlikely, as there is little apoptotic activity in the developing olfactory epithelium in zebrafish (Hansen and Zeiske, 1993; Sanders and Whitlock, 2003). A further study investigating IEG expression in the vomeronasal organ (VNO) and accessory olfactory bulb (AOB) of the mouse demonstrated a sexually dimorphic pattern of gene expression in this accessory olfactory system (Halem et al., 2001). Halem et al. (2001) observed activation of Egr-1 in the VNO and Fos in the AOB, which could suggest that activity in the periphery and CNS is reflected through different IEG signaling pathways.

**Conclusions**

We have found that *c-fos* expression is modulated in an odorant specific manner by two different odorants, a putative pheromone PGF$_2\alpha$ and a bile acid, taurocholic acid. The pattern, timing and specific odor-mediated modulation of *c-fos* expression identifies it as a potential regulator of olfactory epithelial development based on environmental conditions. *c-jun* is expressed throughout the epithelium, potentially providing a background on which *c-fos* may add specificity. *c-fos* alone or *c-fos* and *c-jun* could act in concert to regulate downstream genes, affect differentiation, and possibly maintain a feedback loop to destroy or reinforce receptor choice. With these data we will further investigate the influence of the environment on IEG expression in the developing zebrafish olfactory system.
METHODS

Animals

All animal care and maintenance was performed in accordance with Cornell University animal use and care guidelines. The wild-type strain developed in the Whitlock Lab, “New Wild Type” (NWT), was used for all experiments. Embryos were staged at time of fertilization as described by Kimmel et al. (1995). After odor treatments, embryos were collected and fixed in the chorion with 4% paraformaldehyde overnight at 4°C. Embryos were dechorionated the next day and stored in methanol at -20°C until used for in situ hybridization.

Cloning of IEGs

Reverse Transcriptase polymerase chain reaction (RT-PCR) was used to isolate cDNAs of the three IEGs. cDNA was made from RNA isolated from 1-3 day old wild type zebrafish embryos using SuperScript II reverse transcriptase (Invitrogen). IEGs were amplified by using Platinum ® Taq DNA polymerase High Fidelity (Invitrogen). PCR products were ligated into pGEM®-TEasy vector (Promega). To amplify egr1 (NM 131248) primers and sequence data were taken from Close et al. (2002): forward 5’-ATGGCTGAGCGCAAGACAGAG; reverse 5’–TCAGCAGATGTCGGCTGTCCG. We performed an in silico search for both c-jun and c-fos in the zebrafish Ensemble Database (V4) using sequence data from other animals. c-jun had not been identified by the database at that time although a putative fos sequence had been identified. We designed primers to amplify the predicted c-fos and c-jun transcripts we identified in this search. The following primers were used to amplify c-jun: forward 5’-
ATGTCTACCAAGATGGAAACTACT; reverse 5’-
TCAGAAGGTTTGCGAGCTGTTGTG. The c-fos primer sequences were:
forward 5’-ACAGGATGATGTTTACCAGCCTT; reverse 5’-
TTCATACTTGACGTCCGACCAT. Our c-jun and c-fos sequences have
been entered in GenBank under the following accession numbers: DQ003340
(c-jun) and DQ003339 (c-fos).

Cloning of olfactory receptors

OR11.1 and V1R were amplified from wild type genomic DNA (these
genes do not contain introns) using Platinum® Taq DNA Polymerase High
Fidelity (Invitrogen). OR11.1 (DQ442275) was cloned using the following
primers: OR11.1 Forward: 5’-ATGAACACCAGCGGCTCGGT and OR11.1
Reverse: 5’-TGCTCTCTTCATTGCATCTTG. V1R (AY279524; Pfister and
Rodriguez, 2005) was cloned using V1R Forward: 5’-
ATGGACCTGTGTGCACCAT and V1R Reverse: 5’-
TCTTGGCTCGACCTTGGCCA. Both PCR products were ligated into
pGEM®-T-Easy vector (Promega).

Generation of c-Jun and c-Fos protein alignments

Protein alignments comparing zebrafish c-jun and c-fos predicted
proteins with c-Jun and c-Fos from other animals were done using MegAlign
software (DNASTAR, Lasergene v6) software using the ClustalW method.
Zebrafish c-fos (DQ003339) was compared with goldfish (Carassius auratus;
AB111051), carp (Cyprinus carpio; U81505), cherry salmon (Oncorhynchus
masou; AB111054), chicken (Gallus gallus; NM_205508), mouse (Mus
musculus; NM_010234) and human (Homo sapiens; V01512). Zebrafish c-jun
(DQ003340) was compared with goldfish (Carassius auratus; AB111052), chicken (Gallus gallus; M57467), mouse (Mus musculus; NM_010591), rat (Rattus sp., X17163) and human (Homo sapiens; CR541752). Conserved domains (Jun-like and leucine zipper domains) were predicted using the CDD (conserved protein domain database) on the NCBI/Entrez website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=cdd).

**In Situ Hybridization**

Digoxigenin (DIG) labeled mRNA probes were made for egr1, c-jun, c-fos, OR11.1 and V1R using the SP6/T7 RNA labeling kit (Roche) using manufacturer’s instructions. Sense probes were made for all mRNA sequences and they did not show any staining. Zebrafish embryos were fixed in 4% paraformaldehyde and the processed for whole mount in situ hybridization according to Thisse *et al* (1993). Permeabilization was done by treatment with 10µg/ml proteinase K in PBST for 0 minutes for 24h, 2-3 minutes for 48h and 10-12 minutes for 72h. Embryos were hybridized with 100ng probe overnight at 65°C. To visualize the hybridized probe, embryos were incubated overnight at 4°C in alkaline phosphatase coupled anti-DIG antibody (1:5000; Roche). Coloration reaction was performed using a 1:50 dilution of NBT/BCIP (Roche) in staining solution (described by the manufacturer) until reaction product was sufficiently visible. Treated and untreated embryos that were paired for analysis were processed for in situ hybridization at the same time.
Odorant preparation and application

Odors used were prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) (Cayman Chemical), 4-pregnen-17,20β-diol-3-one,20-sulphate (17,20P), sodium salt (Steraloids) and Taurocholic acid (Sigma-aldrich). Stocks of PGF$_{2\alpha}$ and 17,20P were prepared in 95% or 100% ethanol (according to package instructions), and stored at 4°C. Stock solutions were diluted in distilled water to $10^{-6}$M and $10^{-8}$M for application ($10^{-8}$M and $10^{-10}$M final concentrations, respectively, for treatment groups after application). Taurocholic acid stock was prepared by dilution into distilled water for application. Ethanol diluent control was made to reflect highest concentration of ethanol present in any odorant application $10^{-5}$M. Distilled water control was added at same concentration as odorant in dH$_2$O, 2mls into 200mls. New odorants were made from stock on the day of fertilization for each experiment. To control for the possibility of effects caused by temperature differences between odorants and embryo medium, all odorants were stored at room temperature for the duration of experiment. Embryos from multiple crosses were collected shortly (within 1 hour) after fertilization, mixed, then randomly assigned to a treatment group. Each treatment group was placed in 200 ml of embryo medium (Westerfield, 1993). Odorant was applied no more than 2.5 hours after fertilization, at 24 hours post fertilization, and 47 hours post fertilization (1 hour before collection time).

Cell Counts

Embryos processed for in situ hybridization were mounted in glycerol and viewed ventrally at 40x magnification. Cells with clearly identifiable gene expression were counted (Fig.6 A-C for examples). The pattern of gene
expression was not the same when comparing the two OE in a single fish thus each OE was counted as an individual sample.

**Statistical Analysis**

The data collected on the number of cells expressing *c-fos, OR11.1* and *V1R* were analyzed using the Mann-Whitney test, 2 tailed for a non-normal distribution. Statistical analysis was done using the Vassar Stats program available at the Vassar Stats website ([http://faculty.vassar.edu/lowry/VassarStats.html](http://faculty.vassar.edu/lowry/VassarStats.html)).

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CHAPTER 5
DISCUSSION AND FUTURE DIRECTIONS

There are three main conclusions that can be drawn from the data presented in this dissertation: there is little cell mixing between the cells of the OP and cranial neural crest during placode formation, zebrafish form and retain olfactory memories of odorants they experience during development and the olfactory environment experienced during development can alter gene expression in the olfactory epithelium.

**There is little cell mixing between placode and crest cells**

*Summary*

The experiments in chapter two explore the formation of the OP with respect to its interaction with neural crest cells. These two fields of cells simultaneously migrate anteriorly, dorsal to the eye. While previous studies have examined the other migratory routes of neural crest, this is one of the few studies to examine the route CNC takes to populate the frontal mass. In addition, the experiments presented in chapter two simultaneously visualized CNC migration and OP convergence during OP formation.

**OP and CNC cell movements**

The data in chapter two demonstrate that during formation of the vertebrate head, there appears to be little mixing between the CNC and OP cellular fields. OP convergence occurs simultaneously with CNC migration and while these groups of cells associate with one another during anterior migration they do not extensively mix during this process. As the CNC cells
encounter the forming OP they move around rather than between the OP cells. Initially, the CNC cells move ventral to the OP cells and eventually surround the OP once it is formed. However, it was difficult to resolve the extent of mixing at the borders between the CNC and OP fields as they moved anteriorly. We followed CNC cell movements using a transgenic zebrafish line that expresses GFP under the control of the sox10 promoter. sox10 is known to be involved in the differentiation of glial, neuronal and pigment cell types and not bone or cartilage (Kelsh, 2006). sox10's role in the differentiation of a subset of CNC-derived cell types suggests that the CNC cells that we observe surrounding the OP are likely to become neurons, glia or pigment cells and not the structural part of the nose (i.e. the nasal capsule). It is possible that some of the CNC cells surrounding the OP will become the glia of the olfactory nerve. We did not observe cells that express both CNC and OP markers. The lack of co-expression of CNC and OP markers indicates that while CNC cells are a pluripotent cell type, they are not likely to change fate to become a "placode" cell despite their close association with OP cells before, during and after anterior migration. This is not surprising when one considers that there are many differences between the molecular signaling and transcription factor cascades that are involved in the establishment of the placodal and neural crest cellular fields before anterior migration (Schlosser, 2006).

\textit{six4.1 and dlx3b}

The data presented in chapter two also provides some insight into the possible roles that the pan-placodally expressed genes, \textit{dlx3b}, \textit{six4.1} and \textit{eya1}, might be playing in the formation of the OP. \textit{dlx3b} and \textit{six4.1} were found to be localized to different regions of the OP. At 4-8s, \textit{dlx3b} is expressed more
broadly in the OP field than six4.1. This difference is evident when comparing dlx3b and six4.1 fields of expression in relation to the CNC field. The dlx3b field lies adjacent to the CNC field while there is a gap between the six4.1 field and CNC field. Both six4.1 and dlx3b are important for specifying the pre-placodal region (Bailey and Streit, 2006). The expression of dlx3b immediately adjacent to the pre-migratory CNC field suggests that it may play an additional role in establishing the OP/CNC border. dlx genes have been proposed to be important for defining the neural borders of the forming neural plate (Beanan and Sargent, 2000; Bailey and Streit, 2006; Phillips et al., 2006) but establishment of the OP/CNC border has not been closely examined.

By 24h, the OP is formed and surrounded by CNC cells. At 24h and 48h dlx3b expression becomes localized to the ventral region of the OP while six4.1 remains expressed throughout the OP. By 48h, dlx3b expression is localized to the antero-ventral and postero-ventral regions of the OE, which is reminiscent of the expression of the pro-neural genes notch1a, ngn1 and HuC (see Fig. 3.7) (Mueller and Wullimann, 2003). This expression pattern may suggest a role for dlx3b in the differentiation of OSNs. In zebrafish, knock-down of dlx3b results in smaller OPs at 18s and 24h (Solomon and Fritz, 2002) however, a detailed analysis of the OPs in dlx3b morphants has not been performed at later stages of OE development to determine if loss of dlx3b affects the differentiation of OSNs.

eya1

Chapter two also describes the OP phenotype in a mutant for the eya1 gene. There were no discernible defects in the OP field (as judged by six4.1 and dlx3b expression) at 5 somites. This does not signify that eya1 is not
important for OP formation because its role may be masked due to functional redundancy of the Eya proteins. At 48h, eya1 mutant fish exhibit a range of OSN defects. This suggests a role for eya1 in the differentiation of OSNs and is consistent with what has been shown for placodally derived cell types in other tissues (Kozłowski et al., 2005; Nica et al., 2006).

CNC cells and the anterior pituitary

We examined the association of the CNC cells with the cells of the anterior pituitary placode. We found that the anterior pituitary cells are closely associated with ventral neural crest cells and not the dorsal CNC cells we describe associating with the OP. The association of neural crest cells with the anterior pituitary is consistent with the fact that the connective tissues of the anterior pituitary are neural crest derived (Hall, 1999).

Future directions

CNC and OP cell movements

While the CNC and OP cell movement studies indicate that there is little cell mixing between the CNC and OP cell populations during OP formation, there is still the possibility that some cellular interactions were missed because our analyses were done in fixed, staged embryos. This study may have also missed rare events where a single cell expresses both markers. If this analysis were repeated using fluorescence, time-lapse imaging, where rare events such as those described above have been observed (Kulesa et al., 2005), then both of these issues would be avoided. Efforts were made to generate a zebrafish line that expressed six4.1:dsRed (a red fluorescent protein) and sox10:GFP (Green fluorescent protein) in an attempt to perform live imaging
studies. Unfortunately, we did not obtain transgenic line that recapitulated the six4.1 expression pattern despite multiple attempts.

**dlx3b**

If dlx3b plays a role in establishing the border between the OP and CNC fields then loss of dlx3b in the OP field may result in an expansion of the CNC field. An expansion of the CNC field would shift the OP/CNC border anteriorly in a dlx3b mutant fish compared to wild type animals. Because there is not a dlx3b mutant zebrafish line it would be necessary to knock-down Dlx3b protein function using morpholinos. dlx4b is also expressed in the OP field and there is probably functional redundancy between these two proteins (Solomon and Fritz, 2002). Therefore, it would be necessary to knock-down the function of dlx3b alone, dlx4b alone and dlx3b and dlx4b together to fully examine the role of the dlx genes at the OP/CNC border.

To determine whether or not the msx genes are antagonizing the dlx genes at this border (see discussion, chapter two), the location of the border could be examined in animals that are mutant for the dlx genes and morphant for the msx genes. If the dlx and msx genes antagonize each other at the OP/CNC border then the shift observed in dlx mutant animals might be rescued in the dlx/msx mutants because the shift observed in dlx3b mutants might be due to an overactivation of msx genes at the border. A similar experiment was performed by Phillips et al. (2006) to examine the role of dlx3b and dlx4b in establishing the neural/ non-neural border of the anterior neural plate. They knocked down the function of multiple Msx proteins in a deficiency mutant that covers the dlx3b and dlx4b genes and were able to restore the neural plate widening observed in the deficiency mutant. The caveat to this
experiment is that the deficiency mutant, b380, covers more genes than just dlx3b and dlx4b. The alternative to this approach would be to use morpholinos to knock down protein function of Dlx3b, Dlx4b, MsxB and MsxC however, this could result in gross morphological defects as a result of injecting so many morpholinos into the embryos. Repeating the Phillips et al. (2006) experiment to examine the OP/CNC border would be an initial way of determining whether or not the dlx and msx genes play a role in establishing OP/CNC border.

eya1

The loss of OSNs observed in eya1 mutants suggests that Eya1 is necessary for the differentiation of the OSNs but it is unclear if the OSNs are undergoing apoptosis as is the case for the ear hair cells (Kozlowski et al., 2005) or remaining in an undifferentiated state like the cells of the anterior pituitary (Nica et al., 2006). One way to eliminate the possibility that OSN loss is due to cell death is to determine whether or not there is an increase in the number of apoptotic cells in the olfactory epithelia of eya1 mutant juveniles.

The environment alters gene expression in the developing olfactory epithelium

Summary

In chapter three we have demonstrated that the environment alters gene expression in the PNS. We have shown that zebrafish are able to form and retain olfactory memories of the artificial odorant PEA. Microarray and in situ hybridization experiments demonstrated that the olfactory environment experienced during development leads to an upregulation of the transcription factor otx2. Upregulation of otx2 manifests itself as an increase in the number
of cells expressing otx2 during development. The increase in otx2 cell number is maintained in the adult epithelium. In addition, the cells that express otx2 also express pro-neural genes but not differentiated OSN markers, suggesting that PEA exposure results in an increase of the number of cells in a particular neuronal precursor pool.

The data presented in chapter four support the finding that the olfactory environment is able to alter gene expression in the developing OE. The expression patterns of three immediate early genes (IEGs) were characterized in the developing zebrafish olfactory system. The expression of one of the IEGs, c-fos, is modulated by exposing juvenile zebrafish to two different behaviorally relevant odorants during development. Together, the data presented in chapters three and four suggest that the olfactory environment plays a role in controlling gene expression in the developing OE.

**Future directions**

**Olfactory imprinting**

Zebrafish are able to imprint on the odorant PEA but further study is necessary to narrow down the critical period for PEA imprinting in zebrafish. In our behavioral imprinting studies, juvenile zebrafish were exposed to PEA for three weeks but an increase in otx2 expression was observed after just one day of PEA exposure. This suggests that the molecular mechanisms involved in PEA imprinting may be established prior to three weeks of age. Narrowing down the critical period for PEA would involve exposing juveniles for defined periods of time (e.g. 1 week, 2 weeks, 3 weeks) to determine the shortest period of exposure necessary to form odor preference.
Additional behavioral experiments could also characterize the gene expression changes in the PNS of fish exposed to odorants other than PEA. Comparison of the genes involved in imprinting of many different odorants would allow for a distinction to be made between genes that respond ubiquitously to novel odorant exposure versus genes that respond to specific odorants.

The role of *otx2* in the developing OE

We observed that the olfactory environment is able to alter the gene expression of two transcription factors, *otx2* and *c-fos*. It is important to note that while *otx2* and *c-fos* are both transcription factors that can activate downstream target genes, they are expressed in very different locations in the zebrafish OE and they have very different functions in the literature.

*otx2* is expressed in a very defined region of the OE. It is only found in ventral anterior part of the epithelium, and spans the basal to apical axis. The *otx2* expressing cells form a cluster and the increase of *otx2* cells occurs by an expansion of this cluster. The majority of the *otx2* cells are also clustered in the caudal lamellae and midline raphe regions of the adult OE. Importantly, the caudal lamellae are the first lamellae to form during development suggesting that the increase in *otx2* cell number observed in juveniles is maintained through adulthood. The clustering of *otx2* expressing cells in the developing OE suggests that *otx2* is activating the transcription of genes that will lead to the specification of a yet-to-be determined property that this OSN precursor population has in common. This commonality could be expression of the same OR or same family of ORs. Alternatively, these cells could have a functional commonality, meaning they may express different ORs but all of the ORs
expressed by these OSNs might bind and therefore be activated by a similar class of odorant (e.g. alcohols or amino acids). A model where the otx2 cells express ORs that bind similar odorants is attractive because the increase in otx2 cells was only observed when fish were exposed to PEA (an alcohol) and not L-cysteine (an amino acid) or vanilla (a mixture of compounds but the majority is vanillin, which is an aldehyde). A "common odorant class" model is also attractive because expression of individual ORs is not regionalized (like otx2 expression is) in the developing OE rather, each OR is thought to be randomly dispersed at 48h (Barth et al., 1996; Vogt et al., 1997). Furthermore, this type of model is consistent with what is known in C. elegans where the otx genes specify chemosensory neuron type (i.e. what type of environmental stimulus a neuron detects) (Lanjuin et al., 2003; Lanjuin and Sengupta, 2004). In C. elegans, one otx homologue specifies neurons that sense volatile odorants while a different otx homologue specifies neurons that detect temperature (Lanjuin et al., 2003; Lanjuin and Sengupta, 2004). Examining otx2 expression after exposing juveniles to a different alcohol would determine if the increase in the number of otx2 expressing cells is related to exposure to alcohols.

The role of c-fos in the developing OE

otx2 expression is found in a cluster of cells in a very specific region of the developing OE. In contrast, c-fos is expressed in cells found sporadically throughout the developing OE and unlike otx2, its expression pattern is not reproducible from OE to OE. We find that c-fos expression is apparent at 24h and that its expression is modulated at 48h by odorant exposure. Upregulation of c-fos expression in the olfactory bulb is used as a measure of neuronal
activity in response to odorant exposure (Guthrie et al., 1993). It is unlikely that c-fos is acting as a measure of neuronal activity in the developing OE at the early stages we describe here because very few OSNs have made functional connections with the olfactory bulb at 48h (Whitlock and Westerfield, 1998). However, olfactory receptor expression is evident in the OE at 24h (Barth et al., 1996) suggesting that c-fos could be upregulated after odorant binding and activating transcription of downstream factors. Upregulation of c-fos may affect many different processes, possibly even OR choice and/or maintenance.

Importantly, we show that c-jun is expressed ubiquitously in the developing OE. c-fos must heterodimerize with c-jun in order to bind DNA (Herdegen and Leah, 1998). The sporadic expression of c-fos compared to c-jun suggests that c-fos expression conveys selectivity for transcriptional activation in the developing OE. Examination of c-fos expression in response to additional odorants may reveal that its expression is only modulated in response to particular odorant classes. In addition, we have only examined the expression of three IEGs in the olfactory sensory system of zebrafish. There are many more IEGs (Herdegen and Leah, 1998) whose expression may be modulated in the developing OE after odorant exposure.

Are ORs involved?

The studies presented in this dissertation do not link expression of particular ORs to exposure of any of the odorants used. In the case of PEA exposure, OR expression was examined using both quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) and microarray analysis but no positive correlation was found (see Appendix). A caveat of these studies was that only a small percentage of the complete OR repertoire was
examined because they were carried out before the complete zebrafish OR family was described by Alioto and Ngai (2005).

Now that the entire OR repertoire is known, microarray analysis could be used to examine changes in OR expression upon odorant exposure during development. In the zebrafish, OR expression is observed at 24h, which makes the involvement of the odorant environment in OR choice and/or maintenance possible in this species (see olfactory receptor expression, chapter four) (Barth et al., 1996; Whitlock and Westerfield, 1998). The environment may be playing a role in the feedback mechanism that is a key part of stochastic model for OR choice and maintenance (Shykind, 2005).

**Conclusion**

Elucidation of the development of the olfactory system results in a better understanding of how animals use olfaction to interact with the world around them. This dissertation advances our knowledge of the development of the olfactory epithelium. The results from these studies further our understanding of how the olfactory epithelium forms and how individual cells within the epithelium differentiate.
REFERENCES


In order to determine whether olfactory imprinting is accompanied by changes in OR expression, quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) was used to examine the expression of a subset of ORs in imprinted and control fish. At the time this analysis was carried out, sequence was only available for approximately 25% of the ~100 zebrafish olfactory receptor genes. Only recently has the entire OR repertoire of zebrafish been described (Alioto and Ngai, 2005). The known genes at that time fell into six families: 2, 4, 5, 7, 9 and 13. Primers for QRT-PCR were designed to conserved regions of these OR receptor families. Families, as opposed to individual ORs, were examined as a preliminary way to determine whether or not there may be expression differences. This was a faster and more economical way to narrow down which receptors may be of interest. If statistically significant differences were observed with any of the families then the next step would have been to design primer pairs to individual receptors within a family to see if we could determine the specific OR(s) with an expression difference. To design primers to conserved OR family regions cDNA sequences of individual family members were aligned and then primers were designed using the guidelines specified by Applied Biosystems (SYBR®-Green PCR and RT-PCR Reagents Protocol, 2001, www.appliedbiosystems.com). An example of one of these alignments for family 9 is shown in Fig. A1; locations of the selected primers are indicated (Fig. A1, arrows). The primer sequences for the families used for QRT-
Figure A1. Primer design for QRT-PCR reactions. Primers for QRT-PCR were made to each olfactory receptor family as opposed to individual receptors in order to initially explore OR expression at a broad level. cDNA sequences of the members of each OR family were obtained from GenBank and aligned. A portion of the OR family 9 alignment is shown. Primers (arrows) were designed to regions of high conservation within the families.
PCR are provided in Table A1. PCR products from reactions using these primers were cloned and sequenced to confirm that the primers amplified multiple family members and to determine if the primers showed any preference for individual members. The results of this analysis are also indicated in Table A1. Primers were tested in QRT-PCR using RNA isolated from individual OEs of wild type fish. These experiments determined that the primers for families 2 and 13 did not give reliable results in QRT-PCR despite working well in conventional PCR. To determine if there were gene expression changes in OR families 4, 5, 7 and 9 between PEA-imprinted and control fish, RNA was isolated from OEs of two clutches of fish (1111 and 1106) that showed a high level of preference for PEA in the behavioral assay (see chapter three for details of behavioral analysis). The RNA from the individuals within these clutches was pooled and used in QRT-PCR. The results from these analyses are shown in Fig. A2. No statistically significant differences were observed in OR family expression between PEA-imprinted and control fish.

Methods for QRT-PCR analysis

RNA isolation and cDNA synthesis

Olfactory epithelia were isolated from adult zebrafish, flash-frozen in liquid nitrogen and stored at -80°C until use. Total RNA was isolated from epithelia using the RNeasy® kit (Quiagen). An On-column DNase digestion was performed using the RNase-Free DNase Set (Qiagen) to ensure that there was no genomic DNA contamination in RNA samples. SuperScript™II