INTRINSIC AND EXTRINSIC CONTRIBUTIONS TO THE DEVELOPMENT OF THE ZEBRAFISH OLFACTORY EPITHELIUM

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Maegan Veronica Rivard Harden

January 2007

INTRINSIC AND EXTRINSIC CONTRIBUTIONS TO THE DEVELOPMENT OF THE ZEBRAFISH OLFACTORY EPITHELIUM

Maegan Veronica Rivard Harden, Ph. D.
Cornell University 2007

I have used the zebrafish, *Danio rerio* to study the following stages of olfactory sensory system development: the cell movements underlying olfactory placode (OP) formation, environmentally induced gene expression changes in the differentiating OP and modulation of gene expression changes in sensory neuron development.

Cranial neural crest (CNC) and placodes both contribute to vertebrate sensory structures. Little is understood about the extent of cellular mixing between the CNC and OP fields during OP formation. I used live imaging and molecular markers in fixed tissue to follow the CNC and OP fields during OP formation. I found that while the CNC cells associate with and eventually surround the OP, little cell mixing occurs between the fields during this process.

The OP develops into the olfactory epithelium (OE). I explored the effects of the environment on gene expression in the developing OE. My lab previously showed that zebrafish form and retain olfactory memories of the odorant phenylethyl alcohol (PEA). I performed microarray analysis using the OEs of PEA imprinted fish to identify upregulated genes. One of the genes I identified was the transcription factor *otx2. Otx2* is expressed in the developing and adult OE. The number of *otx2* expressing cells is significantly increased in

juvenile and adult OE of PEA exposed fish. I showed that *otx2* cells also express neuronal markers suggesting that PEA exposure leads to an expansion of a neuronal precursor population that is maintained throughout life.

The environmentally induced gene expression changes I observed could be a consequence of activating the immediate early genes (IEGs), which are transcription factors that are rapidly upregulated in response to sensory stimuli. To test if IEG expression was affected by odorant exposure my lab and I exposed juvenile zebrafish to a number of odorants and examined the expression of three IEGs in the developing OE. We found a significant change in the expression of the IEG, *c-fos* when fish were exposed to odorants of behavioral relevance. My studies provide a better understanding of how the OP forms and how the environment affects the differentiation of cells within the OE.

BIOGRAPHICAL SKETCH

Maegan Veronica Rivard Harden was born on May 10, 1979 to Nancy and Keith Rivard. Maegan was also raised by her stepfather Bill Karhan. She grew up in Ashburnham, MA and graduated from Oakmont Regional High School in Ashburnham. Maegan became interested in a career in science after her high school chemistry teacher recommended her for a biotechnology summer camp at the local community college. At that summer camp, Maegan learned how mutations in genes could cause human disease. This intrigued her and she thought that it would be great if she could figure out how to fix these genetic errors in order to eradicate these diseases. Maegan's father passed away the fall after that summer camp of a heritable form of heart disease. This event in her life reinforced Maegan's desire to understand how gene mutation results in disease, which led her to study biotechnology in college. Maegan received her Bachelor of Science degree in Biotechnology (high distinction) from Worcester Polytechnic Institute (WPI) in Worcester, MA in 2001. Maegan did her undergraduate research in Dr. Elizabeth Ryder's laboratory working on neuronal migration in *C. elegans*. This work became her senior thesis project (a.k.a Major Qualifying Project, MQP) and Maegan was given the MQP award for having the best undergraduate Biology/ Biotechnology thesis in her graduating class. The summer after her sophomore year, Maegan was awarded an Office of Research undergraduate research fellowship at the University of Massachusetts Medical School in Worcester. Maegan worked in the lab of Dr. Allan Jacobson and completed a synthetic lethal screen identifying genes involved in mRNA decay in yeast. After her junior year, she was awarded a Pfizer summer undergraduate

research fellowship, which allowed her begin her senior thesis work over the summer. Before coming to Cornell to begin her Ph.D. Maegan completed a summer internship at Abbott Laboratories in Worcester, MA. At Abbott, she worked with the molecular biology group on characterizing several kinases that were potential drug targets for a new therapy to relieve the symptoms of rheumatoid arthritis. Maegan began her Ph.D. work at Cornell University in the fall of 2001 and completed her dissertation work on the development of the zebrafish olfactory system with Dr. Kathleen Whitlock in the fall of 2006.

Maegan also enjoys cooking, music, theater, traveling, yoga and spending time with her family. She also loves animals, especially her two cats. Maegan was lucky enough to marry her best friend, Matthew Harden on May 7, 2005. Maegan hopes to move back to New England where she wants to start a career in the biotechnology/ pharmaceutical industry.

For my parents and my husband.
Your love and support made this possible.

ACKNOWLEDGMENTS

I am grateful to many people who have been an instrumental part in helping me complete this work. First and foremost, I thank my advisor, Dr. Kathleen Whitlock for her constant encouragement and support throughout my time in her lab. Kate was a wonderful advisor in every way. She is a creative, enthusiastic and talented scientist and it was a privilege to be her graduate student. One of the main reasons I chose to come to Cornell for my graduate studies was the opportunity to work with Kate. I was interested in her research when I first read the about it in the booklet about the Genetics and Development faculty members. After meeting her during the interview process, I knew that I would definitely rotate with her and most likely work in her lab. Throughout my dissertation work Kate has taken the time to work with me in the laboratory, nurture my ideas and has continuously guided me with compassion and concern for what is best for my dissertation project and my career. Kate also challenged me to be better everyday: a better speaker, a better writer, a better teacher, a better scientist and a better person. This is what an advisor does and I hope that other graduate students have the same good fortune that I had. Kate is going to begin a new life in Chile and I wish her all the best. The graduate students in Chile are lucky.

I also want to thank my committee members Dr. Ken Kemphues and Dr. David Lin. Both Ken and Dave were always willing to help in whatever way they could. They provided helpful advice and suggestions on my research projects throughout my studies. I also appreciated their comments on the published manuscript presented as chapter three of this dissertation.

There are a few other faculty members that I would like to

acknowledge. I would like to thank Dr. John Ewer for his general support, endless reading of the imprinting manuscript and for technical advice. I thank Dr. David Dietcher and Dr. Kelly Liu for being additional faculty members on my A exam committee. I also thank Dr. Eric Alani (former Director of Graduate Studies) for trying to help me during a very difficult time.

I want to thank all of the wonderful people that I have had the opportunity to work with in the Whitlock lab. All of the Whitlock lab members made it a fun and enjoyable place to work. In particular, I want to mention Chad Westmiller and Cheri Jackmin who were the fish facility managers during my time in the lab. Their excellent fish care made these experiments possible. I also want to acknowledge a fantastic undergraduate, Melissa McKenzie, who worked with me on the experiments presented in chapter four of this dissertation. It was a pleasure to work with Melissa and her hard work was instrumental in completing that project.

While I have made many great friends while at Cornell I would particularly like to thank two of my best friends Karen Hoffman and Bonnie Bolkan. Karen was my roommate at WPI and by some strange coincidence we both ended up coming to Cornell for graduate school (Karen was in the Chemistry Department). Karen is a great friend, study-buddy and wonderful person. We have always supported each other and I hope we will be able to do so for many years to come. As many people know, Bonnie has been my best friend in the department. I don't know exactly when Bonnie and I got to know one another but I feel truly blessed to have her as a friend. I thank her for discussions about my research, a lot of fun times (including great food) and for many afternoon coffee breaks. I expect that we will be friends for a long time.

I also want to acknowledge my family. I thank my brother, Bryan and sister-in-law, Anne Marie for their love, support and showing me around Ithaca and Cornell when I first got here. I thank my parents-in-law, Sue and Joe, for their love and encouragement. I am lucky to have parents that have always encouraged me to follow my dreams and I cannot thank my Mom and Stepfather enough for their constant love and support throughout this endeavor. Finally, I thank my husband Matt, whose unwavering belief in me leaves me speechless. I hope I make him feel as loved and supported as he makes me feel every day.

Lastly, I would like to thank the following institutions for financial support: Field of Genetics and Development NIH Training Grant (GM07617), GAANN (Graduate Assistants in Areas of National Need) Fellowship P200A000118, Center for Vertebrate Genomics Graduate Research Fellowship and NIH Grant R01 DC04218 (awarded K. Whitlock).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

17, 20P- 4-pregnen-17,20-diol-3-one,20-sulphate

CNC-cranial neural crest

CNS- central nervous system

GPCRs- G-protein coupled receptors

h- hours post fertilization

IEG- immediate early gene

INP- immediate neuronal precursor

nt- neural tube

OB- olfactory bulb

OE- olfactory epithelium

OMP- olfactory marker protein

OP- olfactory placode

OR- olfactory receptor

OSNs - olfactory sensory neurons

PEA- phenylethyl alcohol

$PGF_{2\alpha}$ - Prostaglandin $F_{2\alpha}$

PNS- peripheral nervous system

QRT-PCR- quantitative reverse transcriptase polymerase chain reaction

s- somites

VNO- vomeronasal organ

VR- vomeronasal receptor

CHAPTER 1

INTRODUCTION

Organization of the olfactory sensory system

Olfaction serves as the primary window into the sensory world for most species of animals (Ache and Young, 2005). The ability to detect odorant molecules is ubiquitous in the animal kingdom and is essential for reproduction, predation and social interaction (Dryer and Berghard, 1999). The olfactory sensory system senses olfactory stimuli in the environment through sensory neurons located in the periphery that project axons directly to the olfactory bulb in the central nervous system (CNS). The olfactory sensory neurons (OSNs) are located within an epithelium lining in the nasal cavity and are able to regenerate throughout life (Farbman, 1992). The epithelium lines the valleys and sides of the folds of a nasal membrane. The part of the membrane lacking sensory epithelium is covered with a non-chemosensory epithelium. In addition to the OSNs, the epithelium houses support cells (a.k.a sustentacular cells) and basal cells, which give rise to the OSNs. Generally, mature sensory neurons are found at the apical surface of the epithelium while immature neurons are found more basally (Farbman, 1992). Bundles of the unmyelinated sensory axons form the olfactory nerve, which enters the CNS and terminates synapses on secondary neurons in the olfactory bulb (Farbman, 1992). The olfactory bulb is located in the most anterior or rostral region of the CNS and is the location where the first synapses are formed. The OSNs synapse with mitral/tufted and periglomerular cells located within the glomerular layer of the bulb. The synapses between the OSNs and the second order neurons in the bulb occur at characteristic structures called glomeruli,

which are ovoid to spherical regions containing a dense network of nerve endings (Farbman, 1992). Axons of the second order neurons send olfactory information to stereotypical positions of higher olfactory centers of the brain, namely the olfactory cortex. The olfactory cortex also sends reciprocal projections back to the olfactory bulb (Farbman, 1992; Komiyama and Luo, 2006). This basic plan of relaying olfactory information is remarkably conserved across the animal species (Farbman, 1992; Hildebrand and Shepherd, 1997).

The organization of the zebrafish olfactory system is similar to that described above in that they have an olfactory epithelium (OE) (Fig. 1.1, arrows) where the OSNs are located (Whitlock, 2004). The OSNs project axons to the olfactory bulb (Fig. 1.1, arrowheads), which transmits signals to the brain. Like all teleost (boney) fish, the paired olfactory organs of the adult zebrafish lie on the dorsal side of the head and directly interact with the environment. Water enters in through an inlet and out through an outlet (Hansen and Zeiske, 1998). The gross morphology of the zebrafish epithelium is similar to that of the other Cypriniformes (carps and minnows) in that the epithelium is found on a multi-folded structure located in an olfactory chamber (Hansen and Zielinski, 2005). The structure of the adult organ is a bilaterally symmetrical rosette, which grows as the fish grows. The folds of the nasal membrane, or lamellae, project outward from the center of the rosette, which is called the midline raphe. The oldest and largest lamellae are located at the caudal end of the organ and new lamellae develop at the rostral end of the midline raphe (Hansen and Zeiske, 1998).

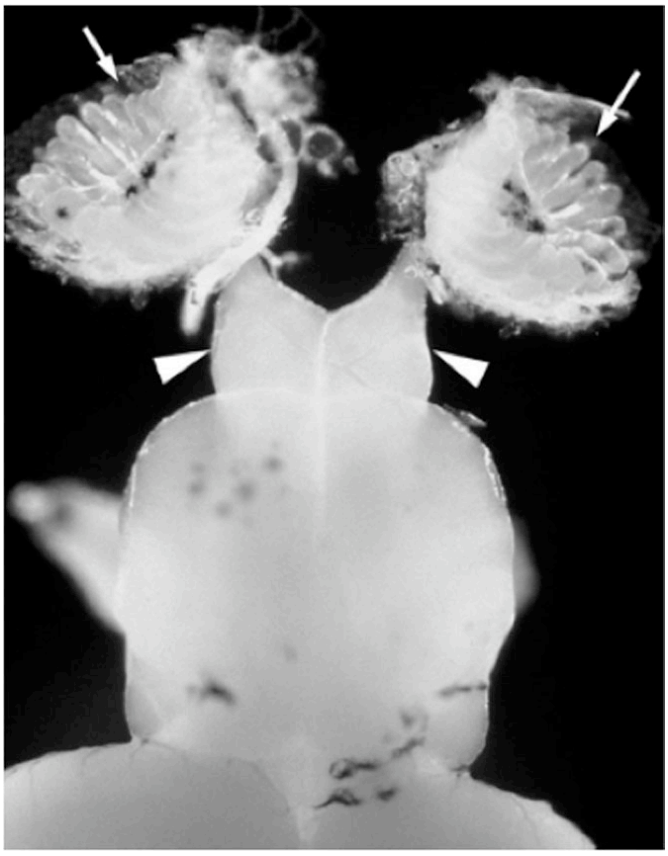


Figure 1.1 The olfactory system of the adult zebrafish. Photo of a brain of the adult zebrafish connected to the olfactory bulbs (arrowheads) and olfactory rosettes (arrows) via the olfactory nerve. Dorsal view, anterior to the top of the page. Reproduced with permission from Whitlock, KE (2004).

Development of the olfactory epithelium in the zebrafish

Like other vertebrate paired sense organs (e.g. ears, lenses of the eyes, and lateral line), the olfactory epithelia arise from placodes. Sensory placodes are defined as neuroectodermal thickenings located in the region of the developing head. In the zebrafish, the olfactory placode gives rise to the OSNs as well as the non-neural support cells (Hansen and Zeiske, 1993; Baker and Bronner-Fraser, 2001; Whitlock, 2004). This is not true for all teleosts, in some cases (e.g. sturgeons) only the sensory neurons are derived from the placode while the support cells arise from epithelial cells (Zeiske et al., 2003; Hansen and Zielinski, 2005). In zebrafish, the olfactory placode is evident by 17-18h (hours post fertilization), ORs are expressed in some cells by 24h, and sensory neuron axons can be observed by 40h (Barth et al., 1996; Whitlock and Westerfield, 1998; Whitlock and Westerfield, 2000).

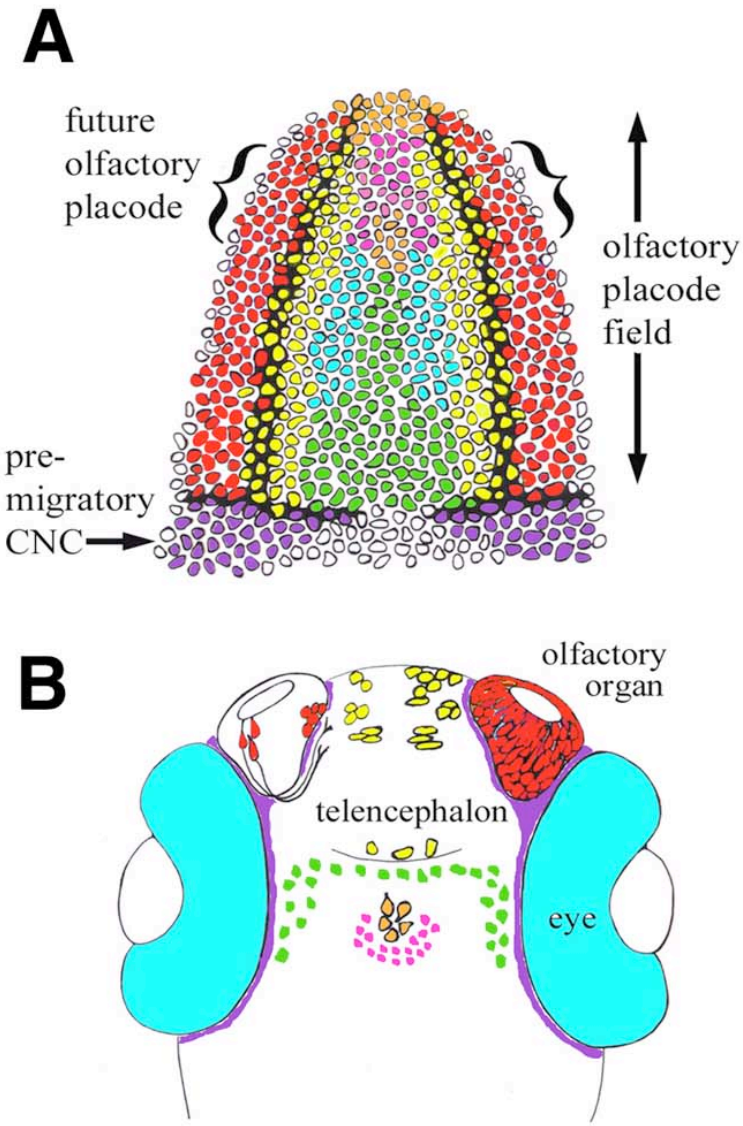
Studies using electron microscopy have described the formation of the OE in zebrafish in detail (Hansen and Zeiske, 1993). Briefly, Hansen and Zeiske (1993) showed that at the 6 somite stage, the field of cells that will converge to become the olfactory placode is observed as a continuous line of subepidermal cells that have a distinct morphology compared to cells of the epidermis and developing forebrain. The placodal cells are loosely packed and extend processes to interact with one another. Between 6 somites (12h) and 24h, the placode cells fill the space between the developing forebrain and eyes and the placode is formed at 24h. The placode is oval shaped in the rostro-caudal direction and semicircular in the dorsoventral direction. The olfactory pits can be observed as small slits at 34-36h. Thick bundles of axons can also be observed leaving the placode, toward the brain at this stage. At

48-50h the olfactory pit widens and becomes oval shaped and sensory neurons bearing cilia and microvilli are also apparent. The axons of the sensory neurons aggregate to form the olfactory nerve, which spans between the olfactory organ and the forebrain. Opening of the olfactory pit continues for several days. At day 14 the first fold of the epithelium is evident in the rostrocaudal direction and it develops into the midline raphe. The second fold, the first lamella, is observed at 33 days. At 40-42 days the olfactory pit is divided into the incurrent and excurrent nostrils. Lamellae are constantly added during this time and throughout the life of the zebrafish (Hansen and Zeiske, 1993; Hansen and Zeiske, 1998).

One model for the formation of the olfactory placode is that it arises from a patch of cells at the edge of the neural plate that becomes detached by growth of non-neural tissue around it (Farbman, 1992). In contrast, fate mapping of the anterior neural plate of the zebrafish at 12h demonstrated that the olfactory placode arises from fields of cells on either side of the developing telencephalon (Fig. 1.2, red cells) (Whitlock and Westerfield, 2000). These fields of cells converge anteriorly, in the absence of cell division, to form the olfactory placode (Whitlock and Westerfield, 2000). Therefore, the olfactory placode develops by the convergence of a field of cells rather than localized cell division. Recent studies in chick demonstrated that the otic placode also arises via the convergence of a large cellular field (Streit, 2002). These data suggest that cellular convergence is a common mechanism for placode formation (Whitlock, 2004).

The olfactory bulbs and the olfactory placode develop in concert, with the placode providing an inductive signal for the development of the bulb

Figure 1.2 Fate map of the anterior neural plate of the zebrafish. (A) Schematic of a dorsal view of the anterior neural plate at 4-5 somites (12h), anterior at the top of the page. The olfactory placode is formed by a convergence of the two fields of cells (red) on either side of the developing telencephalon (yellow). Pre-migratory neural crest cells are shown in purple. Cells of the anterior pituitary placode are shown in orange. Other colors show the location of other cell types that are not related to this dissertation. The future location of the olfactory placodes are indicated by brackets. (B) Schematic of a zebrafish head at 50h after anterior migration has occurred. Ventral view, anterior at the top of the page. The formed olfactory organ is shown in red. The migratory route of the neural crest cells is shown in purple. Reproduced with permission from Whitlock KE (2004).


A
future
olfactory
placode
olfactory
placode
field
pre-
migratory
CNC
B
olfactory
organ
telencephalon
eye

through the axonal projections of the OSNs (Gong and Shipley, 1995). In zebrafish, the first connections between the olfactory placode and olfactory bulb are made by a special set of neurons called pioneer neurons. The pioneer neurons establish a pathway to the olfactory bulb for the axons of the OSNs to follow. The pioneers extend processes into the region of the developing telencephalon by 20h and undergo apoptosis once connections are made between axons of the mature OSNs and the bulb (Whitlock and Westerfield, 1998).

Olfactory behavior in fish: focus on olfactory imprinting and zebrafish as a behavioral model

Odor signals serve to communicate information in a diverse array of demanding behavioral contexts (Ache and Young, 2005). The information content of the chemical signal is rarely, if ever, a single compound. Rather, they are complex mixtures of compounds that are combined at very specific ratios. While individual compounds do illicit physiological responses, it is usually these multi-component mixtures that result in complete biological activity (Ache and Young, 2005). Fish smell odorants dissolved in the surrounding water. They sense four main types of odorants: amino acids, gonadal steroids, bile acids and prostaglandins. Like other animals, olfaction plays a role in fish behaviors related to feeding, reproduction, and predation/avoidance (Laberge and Hara, 2001). Amino acids are generally regarded as being a trigger for promoting feeding behavior. Gonadal steroids and prostaglandins appear to be involved in reproductive behaviors. The role of bile acids largely remains unclear but some data indicate that taurocholic acid is important for recognition of kin (Laberge and Hara, 2001; Zhang et al.,

2001). Importantly, bile acids elicit a behavioral response in many fish, including zebrafish (Vitebsky et al., 2005).

Olfactory imprinting

Behavioral imprinting is the formation of a long-term memory of a sensory stimulus in the absence of re-exposure. Olfactory imprinting is life-long memory of an odor stimulus experienced during development (Hudson, 1993). Imprinting usually occurs during a critical period, or specific window of developmental time which is particularly favorable for the generation of this long-term memory (Hudson, 1993). The formation of long-term olfactory memories is conserved across animals and has been shown to occur in *C. elegans* (Remy and Hobert, 2005), rabbits (Hudson and Distel, 1998), pigeons (Gagliardo et al., 2001) and humans (Porter and Winberg, 1999).

One of the most spectacular and well-known examples of olfactory imprinting is that exhibited by salmon. Salmon imprint on their natal stream as juveniles and then migrate to the ocean to mature. As adults, salmon return to their natal stream to spawn and eventually die (Hasler and Scholz, 1983; Dittman and Quinn, 1996). Hasler and Scholz (1983) demonstrated that salmon use olfaction to return home. They exposed hatchery-raised salmon to one of two artificial odorants, morpholine or phenylethyl alcohol (PEA), during smolt stage. The fish were tagged and released into Lake Michigan. Two years later, when the salmon were ready to return to their natal stream to spawn, Hasler and Scholz spiked one stream near the release site with morpholine and another stream with PEA. Of the fish that were recovered, more than 90% were found in the stream spiked with the chemical to which they were imprinted. Though the fish probably imprint on a mixture of

chemicals in the natural environment, Hasler and Scholz showed that one artificial chemical was enough to guide them (Hasler and Scholz, 1983).

While it is clear that olfaction is essential for homing, the molecular mechanisms that lead to olfactory imprinting remain unclear (Dittman and Quinn, 1996). The basis of olfactory imprinting lies partially within the central nervous system (CNS) (Wilson et al., 2006), however some intriguing evidence has been presented to suggest that the peripheral nervous system (PNS) is also involved in imprinting. Nevitt *et al.* (1994) used patch clamping to study the sensitivity of OSNs from PEA-imprinted and non-imprinted coho salmon. They found that OSNs isolated from PEA-exposed fish showed more robust responses to PEA than the cells isolated from PEA-naïve fish. This was not a general increase in odorant responsiveness in these cells since both PEA-exposed and PEA-naïve cells responded similarly to L-serine (Nevitt et al., 1994). Dittman et al., (1997) showed that a signaling protein found in OSNs is sensitized in PEA-imprinted coho salmon when compared to controls (Dittman et al., 1997). There is also preliminary evidence that olfactory receptor (OR) expression changes during the parr-smolt transition in atlantic salmon (Dukes et al., 2004). The parr-smolt transition may be a critical period for imprinting in salmon (Ebbesson et al., 1996) and altered OR expression during this time suggests that the olfactory receptors may play a role in imprinting (Dukes et al., 2004).

Zebrafish as model for olfactory behavior

Zebrafish are an ideal system to use for studies of olfactory behavior. They have a well-developed sense of smell, which is used for food localization, detection of predators and other types of communication

(Korsching et al., 1997). Odorant stimuli for zebrafish are water-borne, which makes it easy to administer odorants. While the structure of the zebrafish olfactory system is similar to the stereotyped structure observed in mammals, it is smaller, which makes it more ideal for anatomical and functional studies (Korsching et al., 1997). Olfactory behavior assays have been developed to test zebrafish response to general odorants such as amino acids (Lindsay and Vogt, 2004; Vitebsky et al., 2005) as well as social cues like kin odors (Mann et al., 2003). Zebrafish can also be used to study olfactory behavior from very early in development. For example, zebrafish can detect and exert an aversive response to the amino acid L-cysteine at three days of development (Vitebsky et al., 2005). In addition, olfactory behavior mutants have been isolated and characterized demonstrating that it is a useful system for dissecting the molecular mechanisms underlying olfactory driven behaviors (Vitebsky et al., 2005). Zebrafish have also been used for physiological studies. Electro-olfactograms have shown that like other fish, zebrafish have strong responses to amino acids and bile acids (Michel and Lubomudrov, 1995). Optical recording of neuronal activity by anterograde labeling of OSN axons with a voltage-sensitive dye showed that the olfactory bulbs of zebrafish are activated by amino acids, nucleotides, bile acids (including taurocholic acid), the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 4-pregnen-17,20-diol-3-one,20-sulphate (17-20P). Furthermore, each class of odorants activated specific regions of the olfactory bulb (Friedrich and Korsching, 1998). Interestingly, $PGF_{2\alpha}$ and 17,20P are important reproductive pheromones in goldfish and may play a similar role in zebrafish though this has not been directly shown (Sorensen et al., 1988; Sorensen et al., 1998).

The olfactory sensory neurons and the olfactory receptors

The olfactory sensory neurons

OSNs are bipolar cells that have a relatively short dendrite and a longer axon (Farbman, 1992). The dendrite terminals have several slender appendages; either microvilli or cilia that amplify the cell surface and contain receptor proteins. The OSNs are unique because they are the only primary sensory neurons in vertebrates that have axons located so close to the periphery (Farbman, 1992). The OSNs are also special because they continually regenerate in vertebrates. This is thought to occur as a protective mechanism against the elements of the external environment that can damage the sensory neurons (Farbman, 1992). Both ciliated and microvillous sensory neurons are found in the epithelium of the zebrafish (Hansen and Zeiske, 1998). The zebrafish sensory epithelium also contains crypt cells, which bear both cilia and microvilli and have an axon that aggregates with the axons of the other sensory neurons of the epithelium. The crypt cell is a new type of OSN that has been found in fish (Hansen and Zielinski, 2005).

Studies in mouse have shown that the OSN lineage differentiates through a sequence of distinct intermediate cell types. Presumably, there is a self-renewing stem cell located in the basal layer of the OE that gives rise to the OSN lineage. However, isolating this stem cell population has proven to be difficult (Beites et al., 2005). The stem cell gives rise to a mitotic progenitor cell that is characterized by expression of the mouse *achaete scute* homologue, *Mash1*. *Mash1* positive cells give rise to a second population of mitotic progenitors called the immediate neuronal precursors (INPs), which are characterized by expression of the proneural gene *Neurogenin 1 (Ngn1)*. INP division results in daughter cells that differentiate into the OSNs. Stem cells,

Mash1+ progenitor cells and INPs are all located in the basal layer (Fig. 1.3, light gray) of the mouse OE while post-mitotic, maturing OSNs lie atop these progenitor cells with the least mature (Fig. 1.3, dark gray, black) nearest the basal cells and the most mature sensory neurons (Fig. 1.3, checkered) located in the apical part of the epithelium (Calof et al., 1996; Calof et al., 2002; Beites et al., 2005). The basal cells in the zebrafish epithelium lie at the edge of the basal lamina between the support cells (Hansen and Zeiske, 1998). It is currently unclear whether or not the OSNs in zebrafish go through a similar differentiation program as the one that has been described for mouse but it does appear that the developing zebrafish OE is striated. Analysis of gene expression in the developing zebrafish OE has shown that markers for cell division (e.g. PCNA) and newly differentiated neurons (e.g. HuC) are expressed in cells found basally in the OE (Mueller and Wullimann, 2003) while markers for differentiated OSNs (e.g. OMP) are found apically (Celik et al., 2002).

The olfactory receptors

The binding of odorants to olfactory receptors (ORs) triggers a biochemical cascade within the sensory neurons. This cascade ultimately results in depolarization and initiation of action potentials that propagate along sensory axons to the olfactory bulb and higher processing centers in the brain (Dryer and Berghard, 1999). Therefore, the OR is the interface between the environment and the nervous system and represents the first crucial step in the processing of olfactory information. Buck and Axel first described the ORs of the main olfactory epithelium of the rat in 1991 and were awarded the Nobel Prize in Physiology and Medicine in 2004 for this work (Buck and Axel, 1991).

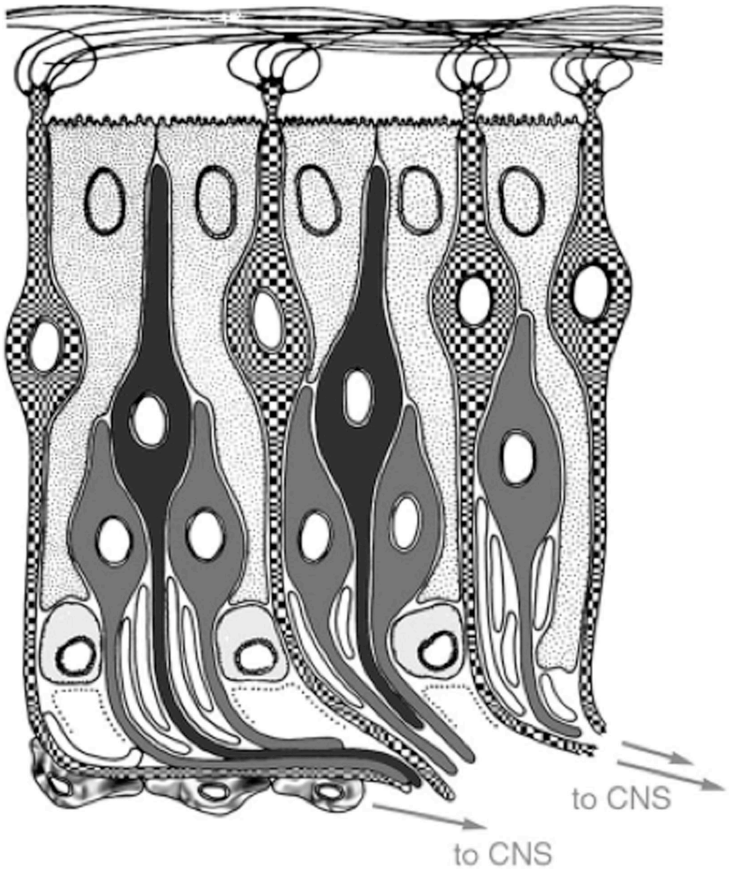


Figure 1.3 Schematic depicting the olfactory epithelium in cross-section. The olfactory sensory neurons (checkered) detect odorants in the environment, project axons directly to the CNS and regenerate throughout life. The basal cells (light gray) give rise to the OSNs, which are located in the more basal layers of the epithelium when they are immature (dark gray) and move apically as they mature (black). Reproduced with permission from Whitlock KE (2004).

ORs belong to the superfamily of G-protein coupled receptors (GPCRs). ORs comprise the largest family of GPCRs in all species studied and encode a multigene family of unparalleled size and diversity. Vertebrate ORs belong to the rhodopsin-like class of GPCRs. This class also includes receptors for dopamine, histamine, norepinephrine, opioids, adenosine and other signaling molecules (Dryer, 2000). Olfactory receptors contain seven transmembrane α-helices separated by three extracellular and three intracellular loops. They have an extracellular amino-termimus and an intracellular carboxy-terminus. It is thought that the region between the second and sixth transmembrane domains of ORs form a pocket for ligand binding and that it is the sequence variability in this region that results in the ability of ORs to bind many different odorants without changing the overall structure of the receptor (Dryer and Berghard, 1999; Kratz et al., 2002). In general, OR genes that are at least 80% identical to each other at the amino-acid level are considered members of the same subfamily. However, ORs in some species cannot be divided into families based on sequence similarity (Dryer and Berghard, 1999). In addition to the ORs of the main OE, another group of ORs have been described that are expressed in the vomeronasal organ (VNO), which is an additional olfactory organ. VNO receptor genes (VRs) differ from those of the main OE. There are two VR gene families (V1Rs and V2Rs), both of which appear to encode GPCRs (Dryer and Berghard, 1999).

In the nematode, multiple receptor genes are expressed in each OSN, whereas flies and vertebrates express only one or a few receptor genes per OSN (Strausfeld and Hildebrand, 1999; Troemel, 1999). In addition, vertebrates express the chosen OR gene from a single allele, either the maternal or paternal allele (Mombaerts, 2001). While the exact mechanism

regulating this one-receptor-one-OSN relationship is not understood, two models have emerged. The deterministic model proposes that an individual OR gene is chosen by a unique combination of transactivators that activate only a single receptor. An alternative model, termed the stochastic model, suggests that expression of all OR genes are activated by a single group of proteins that randomly select one allele of one OR to activate. Maintenance of expression of the selected OR is also complex. The deterministic model suggests that the OR of choice is dictated before expression occurs. In the case of the stochastic model, there may be a feedback mechanism involved, which leads to the commitment of the cell to maintain expression of the randomly selected OR (Shykind, 2005; Komiyama and Luo, 2006). OSNs expressing the same OR converge upon the same few glomeruli in the olfactory bulb (Mombaerts, 2001).

ORs in zebrafish

ORs were first isolated in the zebrafish by Barth *et al.* (1996) using a PCR strategy, which designed primers to amplify sequences that were similar to ORs in rats and catfish. These sequences were expressed in the developing and adult zebrafish OE. Since the first identification of the ORs in zebrafish, additional ORs have been identified and their expression patterns analyzed in the developing and adult epithelium (Barth et al., 1996; Weth et al., 1996; Barth et al., 1997; Dugas and Ngai, 2001). Genomic analysis has shown that like the mammalian ORs, the zebrafish ORs can be grouped into families based on amino acid similarity and that the members of each family are clustered in the genome in the same transcriptional orientation, which is likely to be important for transcriptional regulation (Barth et al., 1997; Dugas

and Ngai, 2001). Analysis of OR expression patterns has shown that the location and timing of expression appears to be random between the two epithelia in a single animal and between different individuals early in development (Barth et al., 1997; Vogt et al., 1997) but individual receptors may be regionalized in the adult epithelium (Weth et al., 1996). Until recently, the OR repertoire of the zebrafish was still incomplete. Recent advances in the zebrafish genome allowed Alioto and Ngai (2005) to present the most complete description of the zebrafish ORs to date. They identified 143 intact ORs in the zebrafish genome and a number of partial OR sequences. These sequences fall into 8 families and are located on seven out of the 25 haploid chromosomes, which is different than the widespread distribution of ORs in the murine genome. Some sequences are still yet to be assigned to a chromosomal location and it is likely that there are more OR genes to be identified (Alioto and Ngai, 2005). Receptors similar to the vomeronasal receptors in mouse have also been identified in the zebrafish even though zebrafish do not have a VNO. A single receptor similar to the V1R family and a V2R-like receptor are expressed in the main OE of the adult zebrafish (Pfister and Rodriguez, 2005). Analysis of the zebrafish genome has led to the identification of a current total of 88 V2R genes and pseudogenes that can be subdivided into 12 subfamilies however, no additional V1R genes were identified (Hashiguchi and Nishida, 2005). The recent characterization of the OR repertoire of zebrafish will allow for a complete analysis of their expression patterns and regulation.

Dissertation Organization

The experiments presented in this dissertation provide insight into the development of the vertebrate olfactory epithelium (OE) by exploring two stages of its maturation: formation of the olfactory placode and differentiation of the cells within the olfactory placode once it is formed. The zebrafish, *Danio rerio*, has been used for all of the studies in this dissertation. The zebrafish has emerged as a leading model organism for the study of vertebrate development because of its rapid development, the ability to obtain large numbers of embryos from a single female, and the optical clarity of the embryos, which allows for the visualization of developmental processes from the moment of fertilization. Many molecular tools have also been developed for the zebrafish including the ability to label cell populations during development in either live animals or fixed tissue. With the advent of a complete genome sequence, zebrafish is also emerging as a wonderful organism for genetic and genomic studies. As stated above (see section on olfactory behavior), the zebrafish is a good model for the study of olfactory development and behavior. The studies presented in this dissertation take advantage of these attributes of the zebrafish in order to advance the current understanding of how the vertebrate olfactory system develops.

Chapter two describes studies that further characterize the intrinsic program of cellular movements involved in placode formation. As the olfactory placode is being formed, the cranial neural crest (CNC) cells are also migrating anteriorly, dorsal to the eye to contribute to the sensory and structural parts of the vertebrate head. Studies in this chapter explore how the simultaneous, anterior movement of the CNC cells affects the formation of the

OP. A similar analysis was done in relation to the anterior pituitary placode, whose cells are closely associated with the cells of the olfactory placode early in development (Fig. 1.2, orange cells). This chapter also presents experiments that investigate the role of three placodally expressed genes (*dlx3b, six4.1 and eya1)* in olfactory placode formation. The data in this chapter show that while the OP and CNC cells associate during OP formation, there is not extensive cell mixing between these two cell populations during this process.

Chapters three and four present experiments that demonstrate that the environment, an extrinsic influence, can play a role in the differentiation of cells within the formed olfactory placode by altering gene expression. Experiments are presented in chapter three that demonstrate that like salmonids, zebrafish can form and retain olfactory memories of PEA. A combination of microarray analysis and *in situ* hybridization were used to explore the molecular changes that take place in the OE (the PNS) during olfactory imprinting. The data presented in this chapter indicate that olfactory imprinting is correlated with changes in gene expression in the OE. One of these gene expression changes is evident during development of the OE and is maintained through adulthood. These results suggest a role for the environment in controlling gene expression in the developing PNS.

Chapter four explores the effect of the olfactory environment on expression of the immediate early genes. The immediate early genes (IEGs) are a class of transcription factors that are rapidly up-regulated in response to environmental stimuli and could be a link between the environment and downstream gene expression changes (Herdegen and Leah, 1998; Tischmeyer and Grimm, 1999). IEGs have been shown to be up-regulated in

the olfactory bulb in response to odor stimuli (Guthrie et al., 1993; Montag-Sallaz and Buonviso, 2002; Kawamoto et al., 2003; Baraban et al., 2005) but to date, there is only one description of the effects of odor stimuli on IEG expression in the OE (Norlin et al., 2005). Chapter four describes the expression patterns of three IEGs (*egr1, c-fos* and *c-jun)* in the developing zebrafish olfactory system and the expression changes observed in response to odor exposure during development. There was no change in expression in response to PEA. A change in the frequency of *c-fos* expressing cells was observed in response to taurocholic acid and $PGF_{2\alpha}$ but no change was seen when fish were exposed to 17,20P. The experiments presented in this chapter demonstrate that expression of the IEG *c-fos* is modulated by exposure to behaviorally relevant odorants. This data support the idea first proposed in chapter three of this dissertation, that the olfactory environment can alter gene expression in the developing OE.

Chapter five summarizes the major results presented in chapters two through four of this dissertation and presents some future research directions that would build upon this work. Additional experiments related to chapter three are provided as an appendix. The data presented in this dissertation furthers the understanding of the formation and differentiation of the zebrafish olfactory epithelium.

REFERENCES

Ache BW, Young JM. 2005. Olfaction: Diverse Species, Conserved Principles. Neuron 48:417-430.

Alioto TS, Ngai J. 2005. The odorant receptor repertoire of teleost fish. BMC Genomics 6.

Baker CV, Bronner-Fraser M. 2001. Vertebrate cranial placodes I. Embryonic induction. Developmental Biology 232:1-61.

Baraban SC, Taylor MR, Castro PA, Baier H. 2005. Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. Neuroscience 131:759-768.

Barth AL, Dugas JC, Ngai J. 1997. Noncoordinate expression of odorant receptor genes tightly linked in the zebrafish genome. Neuron 19:359-369.

Barth AL, Justice NJ, Ngai J. 1996. Asynchronous onset of odorant receptor expression in the developing zebrafish olfactory system. Neuron 16:23-34.

Beites CL, Kawauchi S, Crocker CE, Calof AL. 2005. Identification and molecular regulation of neural stem cells in the olfactory epithelium. Experimental Cell Research 306(2):309-316.

Buck L, Axel R. 1991. A Novel Multigene Family May Encode Odorant Receptors: A Molecular Basis for Odor Recognition. Cell 65:175-187.

Calof AL, Bonnin A, Crocker C, Kawauchi S, Murray RC, Shou J, Wu HH. 2002. Progenitor cells of the olfactory receptor neuron lineage. Microsc Res Tech 58:176-188.

Calof AL, Hagiwara N, Holcomb JD, Mumm JS, Shou J. 1996. Neurogenesis and cell death in olfactory epithelium. Journal of Neurobiology 30:67-81.

Celik A, Fuss SH, Korsching SI. 2002. Selective targeting of zebrafish olfactory receptor neurons by the endogenous OMP promoter. European Journal of Neuroscience 15:798-806.

Dittman A, Quinn T. 1996. Homing in Pacific salmon: mechanisms and ecological basis. J Exp Biol 199:83-91.

Dittman AH, Quinn TP, Nevitt GA, Hacker B, Storm DR. 1997. Sensitization of olfactory guanylyl cyclase to a specific imprinted odorant in coho salmon. Neuron 19:381-389.

Dryer L. 2000. Evolution of odorant receptors. BioEssays 22:803-810.

Dryer L, Berghard A. 1999. Odorant receptors: a plethora of G-protein-coupled receptors. Trends Pharmacol Sci 20:413-417.

Dugas JC, Ngai J. 2001. Analysis and Characterization of an Odorant Receptor Gene Cluster in the Zebrafish Genome. Genomics. Genomics 71:53-65.

Dukes JP, Deaville R, Bruford MW, Youngson AF, Jordan WC. 2004. Odorant receptor gene expression changes during the parr-smolt transformation in Atlantic salmon. Mol Ecol 13:2851-2857.

Ebbesson SO, Smith J, Co C, Ebbesson LO. 1996. Transient alterations in neurotransmitter levels during a critical period of neural development in coho salmon (Oncorhyncus kisutch). Brain Res 742:339-342.

Farbman AI. 1992. Cell Biology of Olfaction. Cambridge: Cambridge University Press.

Friedrich RW, Korsching SI. 1998. Chemotopic, combinatorial, and noncombinatorial odorant representations in the olfactory bulb revealed using a voltage-sensitive axon tracer. Journal of Neuroscience 18:9977-9988.

Gagliardo A, Ioale P, Odetti F, Bingman VP. 2001. The ontogeny of the homing pigeon navigational map: evidence for a sensitive learning period. Proc Biol Sci 268:197-202.

Gong Q, Shipley MT. 1995. Evidence that pioneer olfactory axons regulate telencephalon cell cycle kinetics to induce the formation of the olfactory bulb. Neuron 14:91-101.

Guthrie KM, Anderson AJ, Leon M, Gall C. 1993. Odor-induced increases in c-fos mRNA expression reveal an anatomical "unit" for odor processing in olfactory bulb. Proc Natl Acad Sci U S A 90:3329-3333.

Hansen A, Zeiske E. 1993. Development of the olfactory organ in the zebrafish, Brachydanio rerio. Journal of Comparative Neurology 333:289-300.

Hansen A, Zeiske E. 1998. The peripheral olfactory organ of the zebrafish, Danio rerio: an ultrastructural study. Chemical Senses 23:39-48.

Hansen A, Zielinski BS. 2005. Diversity in the olfactory epithelium of boney fishes: Development, lamellar arrangement, sensory neuron cell types and transduction components. Journal of Neurocytology 34:183-208.

Hashiguchi Y, Nishida M. 2005. Evolution of vomeronasal-type odorant receptor genes in the zebrafish genome. Gene 362:19-28.

Hasler AD, Scholz AT. 1983. Olfactory Imprinting and Homing in Salmon. Berlin Heidelberg: Springer-Verlag. 134 p.

Herdegen T, Leah JD. 1998. Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res Brain Res Rev 28:370-490.

Hildebrand JG, Shepherd GM. 1997. Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. Annual Review of Neuroscience 20:595-631.

Hudson R. 1993. Olfactory Imprinting. Current Opinion in Neurobiology 3.

Hudson R, Distel H. 1998. Induced peripheral sensitivity in the developing vertebrate olfactory system. Annals of the New York Academy of Sciences:109-115.

Kawamoto M, Tamura M, Ohno K, Kawasaki Y, Mori K, Shikina T, Noda K, Kubo T. 2003. The alteration of odor-induced c-Fos immunoreactivity in the rat olfactory bulb after olfactory nerve transection. J Neurol 250:51-54.

Komiyama T, Luo L. 2006. Development of wiring specificity in the olfactory system. Current Opinion in Neurobiology 16:67-73.

Korsching SI, Argo S, Campenhausen H, Friedrich RW, Rummrich A, Weth F. 1997. Olfaction in zebrafish: what does a tiny teleost tell us? Seminars in Cell and Developmental Biology 8:181-187.

Kratz E, Dugas JC, Ngai J. 2002. Odorant receptor gene regulation: implications from genomic organization. Trends in Genetics 18:29-34.

Laberge F, Hara TJ. 2001. Neurobiology of fish olfaction: a review. Brain Research Reviews 36:46-59.

Lindsay SM, Vogt RG. 2004. Behavioral responses of newly hatched zebrafish (Danio rerio) to amino acid chemostimulants. Chemical Senses 29:93-100.

Mann KD, Turnell ER, Atema J, Gerlach G. 2003. Kin recognition in juvenile zebrafish (Danio rerio) based on olfactory cues. Biological Bulletin 205:224-225.

Michel WC, Lubomudrov LM. 1995. Specificity and sensitivity of the olfactory organ of the zebrafish, danio rerio. Journal of Comparative Physiology A 177:191-199.

Mombaerts P. 2001. How smell develops. Nature Neuroscience 4 Supplement:1192-1198.

Montag-Sallaz M, Buonviso N. 2002. Altered odor-induced expression of c-fos and arg 3.1 immediate early genes in the olfactory system after familiarization with an odor. J Neurobiol 52:61-72.

Mueller T, Wullimann MF. 2003. Anatomy of neurogenesis in the early zebrafish brain. Brain Research Developmental Brain Research 140:137-155.

Nevitt GA, Dittman AH, Quinn TP, Moody WJJ. 1994. Evidence for a peripheral olfactory memory in imprinted salmon. Proceedings of the National Academy of Sciences U S A 91:4288-4292.

Norlin EM, Vedin V, Bohm S, Berghard A. 2005. Odorant-dependent, spatially restricted induction of c-fos in the olfactory epithelium of the mouse. J Neurochem 93:1594-1602.

Pfister P, Rodriguez I. 2005. Olfactory expression of a single and highly variable V1r pheromone receptor-like gene in fish species. Proc Natl Acad Sci U S A 102:5489-5494.

Porter RH, Winberg J. 1999. Unique salience of maternal breast odors for newborn infants. Neurosci Biobehav Rev 23:439-449.

Remy JJ, Hobert O. 2005. An interneuronal chemoreceptor required for olfactory imprinting in *C. elegans*. Science 309:787-790.

Shykind BM. 2005. Regulation of odorant receptors: one allele at a time. Human Molecular Genetics 14:R33-R39.

Sorensen PW, Christensen TA, Stacey NE. 1998. Discrimination of pheromonal cues in fish: emerging parallels with insects. Current Opinion in Neurobiology 8:458-467.

Sorensen PW, Hara TJ, Stacey NE, Goetz FWM. 1988. F Prostaglandins Function as Potent Olfactory Stimulants That Comprise the Postovulatory Female Sex Pheromone in Goldfish. Biology of Reproduction 39:1039-1050.

Strausfeld NJ, Hildebrand JG. 1999. Olfactory systems: common design, uncommon origin. Current Opinion in Neurobiology 9:634-639.

Streit A. 2002. Extensive cell movements accompany formation of the otic placode. Developmental Biology 249:237-254.

Tischmeyer W, Grimm R. 1999. Activation of immediate early genes and memory formation. Cell Mol Life Sci 55:564-574.

Troemel ER. 1999. Chemosensory signaling in C. elegans. BioEssays 21:1011-1020.

Vitebsky A, Reyes R, Sanderson MJ, Michel WC, Whitlock KE. 2005. Isolation and characterization of the laure olfactory behavioral mutant in the zebrafish Danio rerio. Developmental Dynamics 234:229-242.

Vogt RG, Lindsay SM, Byrd CA, Sun M. 1997. Spatial Patterns of Olfactory Neurons Expressing Specific Odor Receptor Genes in 48-Hour-Old Embryos of Zebrafish Danio Rerio. The Journal of Experimental Biology 200:433-443.

Weth F, Nadler W, Korsching S. 1996. Nested expression domains for odorant receptors in zebrafish olfactory epithelium. Proceedings National Academy of Sciences USA 93:13321-13326.

Whitlock KE. 2004. A new model for olfactory placode development. Brain, Behavior and Evolution 64:1-11.

Whitlock KE, Westerfield M. 1998. A transient population of neurons pioneers the olfactory pathway in the zebrafish. Journal of Neuroscience 18:8919-8927.

Whitlock KE, Westerfield M. 2000. The olfactory placode of the zebrafish form by convergence of cellular fields at the edge of the neural plate. Development 127:3645-3653.

Wilson DA, Kadohisa M, Fletcher ML. 2006. Cortical contributions to olfaction: Plasticity and perception. Semin Cell Dev Biol 17:462-470.

Zeiske E, Kasumyan A, Bartsch P, Hansen A. 2003. Early development of the olfactory organ in sturgeons of the genus Acipenser: a comparative and electron microscopic study. Anat Embryol (Berl) 206:357-372.

Zhang C, Brown SB, Hara TJ. 2001. Biochemical and physiological evidence that bile acids produced and released by lake char (Salvelinus namaycush) function as chemical signals. J Comp Physiol [B] 171:161-171.

CHAPTER 2

SIMULTANEOUS VISUALIZATION OF CRANIAL NEURAL CREST CELL MIGRATION AND OLFACTORY PLACODE FORMATION IN THE ZEBRAFISH[1]

ABSTRACT

Vertebrate sensory organs originate from both cranial neural crest (CNC) and placodes. CNC migration into the branchial arches is well studied. However, the migratory route of CNC cells dorsal to the eye is not well understood. In addition, little is known about the interactions between CNC cells and the placodes forming in the frontal mass. We followed the development of the forming olfactory placode (OP) and determined its association with the dorsally migrating CNC cells in the zebrafish embryo. Using live imaging and molecular markers in fixed tissue we show that during migration, the CNC cells associate with and eventually surround the forming OP. In spite of the close association between the CNC and OP fields, little cell mixing occurs during this process. Furthermore, we find that the OP markers *dlx3b* and *six4.1* are localized to different domains of the developing OP.

INTRODUCTION

The origin of the highly specialized structures of the vertebrate head, including the sensory organs, was concurrent with the appearance of neural crest and neurogenic placodes in craniates (Northcutt and Gans, 1983; Northcutt, 1996). The neural crest cells are multipotent cells that contribute to

[1] This chapter will be submitted to the journal *Developmental Dynamics.* I performed all of the experiments presented in this chapter. Dr. Kathleen E. Whitlock is corresponding author on this manuscript.

a wide variety of cell types including neurons, glia, endocrine cells, and melanocytes (Le Douarin and Kalcheim, 1999). The cranial or cephalic neural crest (CNC) cells migrate into the developing head and differentiate into the cartilage, bone, cranial neurons, glia and connective tissues of the face (Le Douarin and Kalcheim, 1999). In order to contribute to a vast array of tissues in the frontal mass, CNC cells follow definite migratory routes at precise developmental times from their origin in the mesencephalic regions of the pre-migratory neural crest (Osumi-Yamashita et al., 1994). CNC cells migrate into the developing head via two different routes: ventrally, caudal to the eye where they contribute to formation of the jaw and craniofacial muscles and anteriorly, dorsal to the prosencephalon where they populate the frontal mass (Le Douarin and Kalcheim, 1999). Great strides have been made in the understanding of the patterning, differentiation and molecular signals involved in the ventral CNC migration (Creuzet et al., 2005; Brugmann et al., 2006; Noden and Francis-West, 2006) but little is understood about the CNCs that migrate dorsally. One of the genes expressed in pre-migratory neural crest that is important for CNC differentiation is the transcription factor *sox-10* (Dutton et al., 2001). *sox-10* is thought to play a role in the specification of glial, neuronal and pigment cell types. *sox10* is expressed transiently in migrating cells and expression is lost as differentiation occurs. Glial cells, which express *sox-10* throughout development provide an exception to this rule (Kelsh, 2006).

Neurogenic placodes are neuroectodermal thickenings in the region of the developing head that contribute to the paired sense organs (i.e. nose, lens, ear and lateral line) and the cranial sensory ganglia (Graham and Begbie, 2000; Baker and Bronner-Fraser, 2001; Schlosser, 2006). Placodes

arise from a unique territory in the head ectoderm termed the pre-placodal region (Torres and Giraldez, 1998; Bailey and Streit, 2006). The pre-placodal region is induced by activation of FGF and antagonists of the BMP and Wnt signaling cascades (Litsiou et al., 2005) and is characterized by the expression of genes in a horseshoe shaped pattern at the edge of the anterior neural plate (Torres and Giraldez, 1998; Streit, 2001; Streit, 2004; Bailey and Streit, 2006; Schlosser, 2006). The Six, Eya and Dlx gene families are among the genes that are expressed in the pre-placodal region (Bailey and Streit, 2006). In zebrafish, *six4.1* (Kawakami et al., 2000; Kobayashi et al., 2000), *eya1* (Sahly et al., 1999) and *dlx3b* (Akimenko et al., 1994) are expressed in the pre-placodal region and their expression persists throughout the formation multiple placodes including the olfactory placodes (OPs). Previously, we fate mapped the anterior neural plate of the zebrafish starting at 12h and demonstrated that the OPs arise from fields of cells on either side of the developing telencephalon (Whitlock and Westerfield, 2000). These fields of cells converge anteriorly, in the absence of cell division, to form the OPs (Whitlock and Westerfield, 2000). Once formed, the OPs give rise to non-neural support cells as well as the olfactory sensory neurons of the peripheral nervous system. (Farbman, 1992; Baker and Bronner-Fraser, 2001; Whitlock, 2004b). In chick, the otic placode also arises through directed cellular movements of a large field of otic precursor cells scattered throughout the embryonic ectoderm (Streit, 2002).

As placodes are being formed, CNC cells are migrating anteriorly to populate the developing head and both placode and CNC cells are known to contribute to mature sensory structures. For example, the neurons of the cranial sensory ganglia arise from both neural crest and placodes (Northcutt,

1993). The neurons of cranial nerves V, VII, IX and X that are derived from placodes are larger and located distally to the smaller, proximal neurons, which are of crest origin (D'Amico-Martel and Noden, 1983; Le Douarin and Kalcheim, 1999). The neuroglia of all of the cranial ganglia are exclusively neural crest derived (Le Douarin and Kalcheim, 1999). Furthermore, the gonadotropin releasing hormone cells (GnRH) of the terminal nerve (cranial nerve 0) are closely associated with the OP (Whitlock, 2004a) and we have recently demonstrated that they are of neural crest origin (Whitlock et al., 2005). The neural crest origin of the terminal nerve GnRH cells and their close association with the OP raises the question of cellular mixing between these two fields during OP formation.

In an effort to understand how the CNC and OP fields interact during craniofacial development we used molecular markers and imaging techniques to follow their cellular movements as they migrate anteriorly. We were particularly interested in determining the extent of cell mixing between the cells of the OP field and CNC cells during placode formation. Our observations suggest a model where there is little cell mixing between the CNC and OP cellular fields as the OPs are formed. We used *dlx3b* and *six4.1* to visualize the OP field and found that *dlx3b* is initially expressed more broadly in the placode field than *six4.1.* Later in development, *dlx3b* expression becomes localized to the ventral part of the OE compared to *six4.1*. Because *eya1* is expressed in the OP field throughout placode development (Sahly et al., 1999) we examined OP formation in the zebrafish *eya1* mutant (Kozlowski et al., 2005). We found that while the OP fields are normal in *eya1* mutant zebrafish they exhibit a range of olfactory sensory neuron defects at later stages. Lastly, because the OP field is closely associated with the anterior pituitary placode

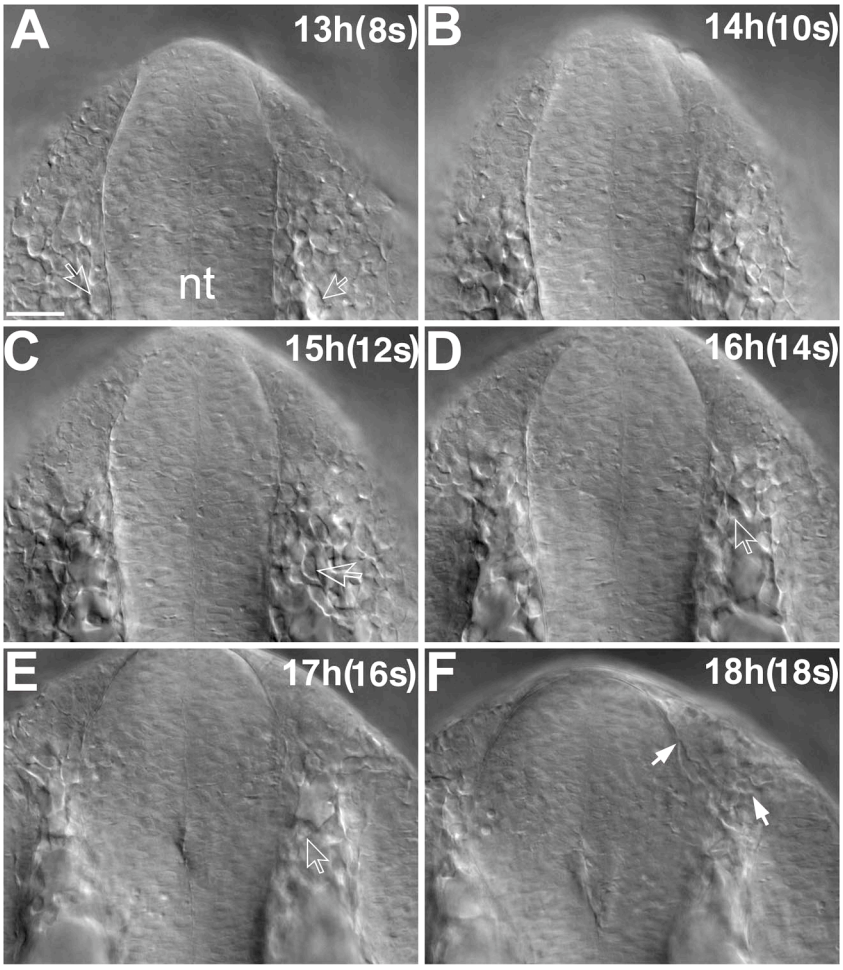
early in development (Whitlock and Westerfield, 2000; Chapman et al., 2005) and because the connective tissues of the anterior pituitary are neural crest derived (Hall, 1999), we examined the extent of cell mixing between the CNC cells and the forming anterior pituitary placode. We found that the anterior pituitary cells are not associated with CNC cells that migrate anteriorly but are associated with ventral neural crest cells.

RESULTS

Imaging of olfactory placode formation

Previously, we used single cell lineage tracing to define the fields of cells that form the OP. We found that the placode is formed by a convergence of two large fields of cells on either side of the neural plate (Whitlock and Westerfield, 1998; Whitlock and Westerfield, 2000; Whitlock, 2004b). To better understand the cellular movements involved in the formation of the OP we visualized this process using time lapse Nomarski imaging (Fig. 2.1). We observed the cell movements at the anterior edge of the neural tube during the time period when the OP is formed. We imaged the anterior neural plate from 8 s (somites) until 18s when the placode is clearly visible (Whitlock and Westerfield, 2000). At 8s the cells that migrate anteriorly (Fig. 2.1A, open arrows) lie immediately adjacent to the forming neural tube (nt). By 10s (Fig. 2.1B) these cells begin to move anteriorly and by 12-14s (Fig. 2.1C,D) there are streams of cells moving forward as neural tube is being formed. The cells actively extend and retract filopodia to interact with one another and their environment (Fig. 2.1C, open arrow). At 16s there are very few cells (Fig. 2.1E, open arrow) still moving anteriorly compared to 14s (Fig. 2.1D, open arrow) and the placode is visible by 18s (Fig. 2.1F, arrows).

Figure 2.1. Time-lapse imaging of OP formation from 8-18 somites. A-F: Still images taken from time-lapse imaging of the anterior convergence of the OPs. Developmental age is shown in the upper right-hand corner of each image. All images are dorsal views, anterior to the top of the page. The cells of the OP (open arrows) move anteriorly alongside the forming neural tube (nt). The edge of the olfactory placode can be observed by 18h (18s) (F, white arrows). h=hours post fertilization; s= somite stage. Scale bar (A)= 30μm.


A
13h(8s)
nt
B
14h(10s)
C
15h(12s)
D
16h(14s)
E
17h(16s)
F
18h(18s)

We found that as cells migrate anteriorly they move as a loose aggregate of cells extending filopodia.

Imaging of anterior migration of cranial neural crest cells using *sox10:egfp* zebrafish

During the time when OP precursors are converging anteriorly the CNC cells are also migrating in the same region. We visualized CNC cells *in vivo* using a transgenic zebrafish line that expresses GFP in pre-migratory CNC under control of the *sox10* promoter (Dutton et al., 2001; Wada et al., 2005). At 4-5s the *sox10:egfp* fish express GFP in a similar pattern to that observed for *sox10* mRNA (Fig. 2.2A,B). By 14-15 somites *sox10* mRNA is being down-regulated (Fig. 2.2C) but GFP expression persists in the *sox10:egfp* fish due to the perdurance of the GFP protein (Fig. 2.2D) in cells that expressed *sox10* mRNA.

The perdurance of GFP allowed us to use time-lapse imaging to visualize CNC cells as they migrated anteriorly, dorsal to the eye. We began imaging CNC cell movements at 5s (Fig. 2.3A). The most anterior *sox10-GFP* cells in the pre-migratory CNC field moved ventrally toward the branchial arches (data not shown). The CNC cells that move dorsal to the eye began migrating at around +1h (hour) and were beginning to move over the eye at +1h40m (minutes) (Fig 2.3A). These cells moved as a group along the developing neural tube and were dorsal to the developing eye at +2h (Fig. 2.3A). By +2h40m the CNC cells have moved into the most anterior limits of the forming neural tube.

We recorded time-lapse images at a more oblique angle through the dorsal third of the eye (2.3B). Once the most anterior CNC cells moved into

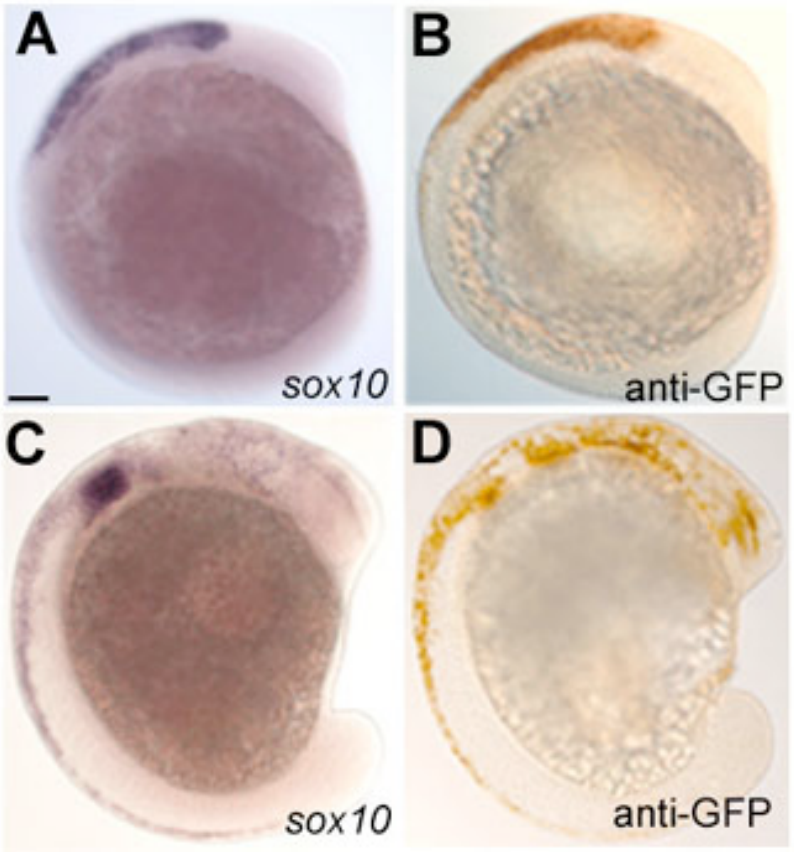
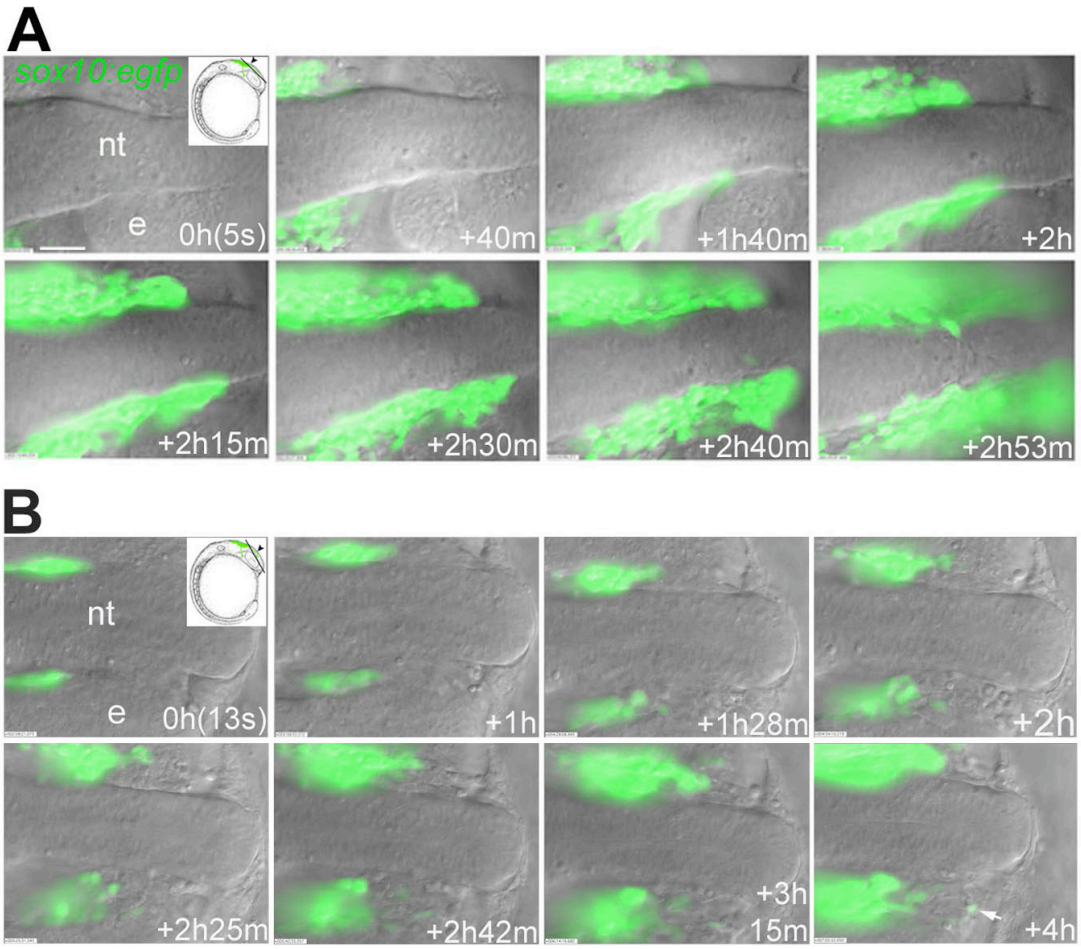


Figure 2.2 . Perdurance of the GFP protein in *sox10:egfp* transgenic zebrafish. *sox10 in situ* hybridization (A,C) and anti-GFP immunocytochemistry (B,D) in *sox10:egfp* embryos. At 5 somites (A,B) *sox10* mRNA expression (A) is similar to GFP expression (B) but at 14-15 somites (C,D) GFP is expressed (D) in more cells than *sox10* mRNA (C). All images are lateral views, anterior to the right, dorsal to the top of the page. Scale bar (A)= 100μm.

Figure 2.3. Time-lapse imaging of anterior migration of CNC cells in *sox10:egfp* zebrafish. A: Still images documenting the beginning of neural crest cell migration as the cells move along the neural tube (nt) dorsal to the developing eye (e). These images were taken starting at 5s (0h time point). Each still is labeled with the time it was captured in relation to the 0h time point. B: Still images documenting neural crest cell movements into the anterior part of the embryo. Images were taken starting at 13s (the 0h time point). White arrow (+4h) indicates a cell that has migrated into the region of the developing OP. A,B: Embryos were imaged from dorsal (inset, arrowhead) at two different focal planes (inset, line). All images are looking down on dorsal, anterior to the right. h=hours after the beginning of imaging, m=minutes, nt=neural tube, e=eye, s=somite stage. Scale bar=30μm.


A
sox10.egfp
nt
e
0h(5s)
+40m
+1h40m
+2h
+2h15m
+2h30m
+2h40m
+2h53m
B
nt
e
0h(13s)
+1h
+1h28m
+2h
+2h25m
+2h42m
+3h
15m
+4h

the anterior region of the forming neural tube they did not remain as a tight group of cells; rather they begin to spread out at 2h (Fig. 2.3B). Two to four hours into imaging, individual CNC cells were observed moving in the region of the forming OP (Fig. 2.3B, arrow). Our analysis shows that the CNC migration involves two distinct steps: CNC cells migrate dorsal to the eye as a group and then individual CNC cells separate as they move into the region of the forming OP.

CNC cells surround the forming olfactory placode

At 4-6 s, the CNC cells flank the posterior border of the olfactory field (Whitlock and Westerfield, 2000; Whitlock, 2004b). Our time-lapse studies suggested that the CNC cells and the cells of the OP may mix during anterior migration. To determine the extent of cell mixing between the OP and CNC cells we labeled both fields using markers for the CNC and OP cells in fixed, staged embryos. We used an anti-GFP antibody to visualize the CNC cells in the *sox10:egfp* zebrafish line. The OP field was labeled using *in situ* hybridization with digoxigenin-labeled mRNA probes for two different genes that were previously described to be expressed in the OP field: *six4.1* (Fig. 2.4A, purple) (Kobayashi et al., 2000)and *dlx3b* (Fig. 2.4B, blue) (Akimenko et al., 1994).

Initial expression of OP and CNC markers

dlx3b and *six4.1* are expressed beginning at 2s in a horseshoe-shaped domain around the anterior neural plate, which includes the OP fields (Fig. 2.4). *dlx3b* (Fig. 2.4B, blue) is more broadly expressed in the posterior region of the horseshoe than *six4.1* (Fig. 2.4A, purple). At 2s *sox10-GFP* is in pre-

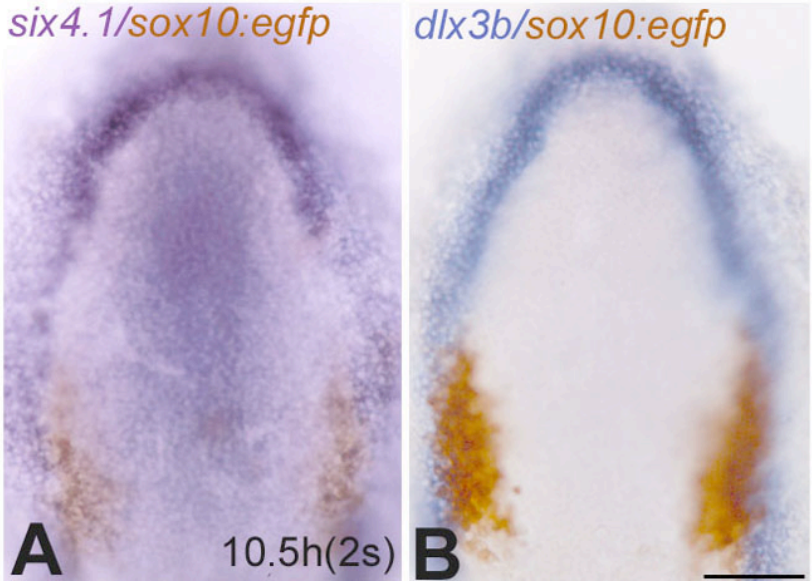


Figure 2.4. Double labeling of the OP field and CNC cells in *sox10:egfp* embryos at 10.5h (2s). *six4.1* (A, purple) and *dlx3b* (B, blue) are expressed in the OP field at 2s. Both genes are expressed in a horseshoe shape around anterior neural plate. *six4.1* is more restricted than *dlx3b*. At this stage the pre-migratory CNC cells (A,B, brown) flank the posterior domains of the *six4.1* (A, purple) and *dlx3b* (B, blue) fields. A,B: Dorsal views, anterior to the top of the page. h=hours post fertilization, s=somite stage. Scale bar=100μm.

migratory CNC (Fig. 2.4, brown), which is adjacent to the posterior *six4.1* and *dlx3b* domains.

six4.1 *and* sox10 *during early somitogenesis*

We double labeled with *six4.1* (Fig. 2.5) or *dlx3b* (Fig. 2.6) and *sox10-GFP* to follow the development of the OP and CNC cellular fields. Using *six4.1* (Fig. 2.5, blue) with *sox10-GFP* (Fig. 2.5, brown) we showed that there is initially a large gap separating the OP and CNC fields (Fig. 2.5A-C). During development, the gap narrows so that at 10s the fields lie adjacent to one another (Fig. 2.5D). From 12-14s (Fig. 2.5 E, E1 and F, F1) the CNC cells began to move ventrally to the OP cells. More than one focal plane is shown at 12s (Fig. 2.5 E and E1) and 14s (Fig. 2.5 F and F1) because the curve of the embryos at these stages made it difficult to visualize all labeled cells in one plane of focus. The OP becomes evident at 16-18s (Fig. 2.5 G, H) and the CNC cells surround it as the OP border becomes more defined from 18-20s (Fig. 2.5 H-K).

dlx3b *and* sox10 *during early somitogenesis*

Because the pattern of *dlx3b* expression differs from that of *six4.1* we also examined this OP marker in the *sox10-GFP* fish. At 4s the expression domain of *dlx3b* (Fig. 2.6A, blue) appears to extend more posterior than the *six4.1* (Fig. 2.5A) field. In contrast to the gap observed between *six4.1* and the CNC field (Fig. 2.5A), the cells that express *dlx3b* lie adjacent to the CNC field (Fig. 2.6A). This difference in expression between the two genes is maintained as the CNC cells move anteriorly at 6s and 8s (Fig. 2.5B,C and Fig. 2.6B,C).

Figure 2.5. Development of the OP and CNC fields in *six4.1/*anti-GFP double labeled embryos. Visualization of OP convergence using *six4.1 in situ* hybridization (blue) and the CNC field using anti-GFP immunocytochemistry (brown) in *sox10:egfp* embryos. A-I: Dorsal views, anterior to the top of the page, of fixed, staged, double-labeled embryos. Embryos were examined every 2 somites. E, E1: Two different focal planes of same embryo at 15h (12s). F, F1: Two different focal planes at 16h (14s). E and F focus on the dorsal (dor) olfactory placode, E1 and F1 focus on the ventral (ven) edge of the olfactory placode. The neural crest cells are ventral to the olfactory fields at these stages. J, K : Ventral views, anterior to top, of the formed OP surrounded by CNC cells at 18h (18s) (J) and 19h (20s) (K). h=hours post fertilization, s= somites. Scale bars A(for A-K); K (for J, K)= 30μm. 20 embryos were examined per time point.

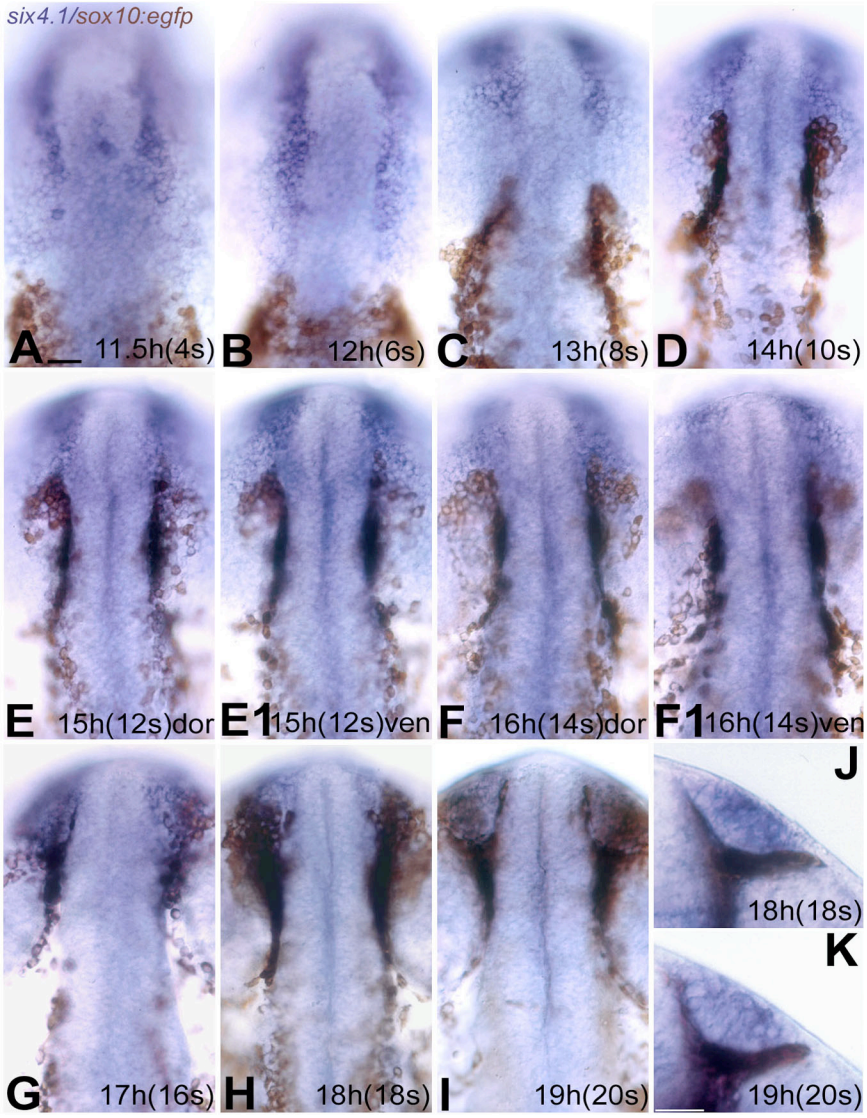
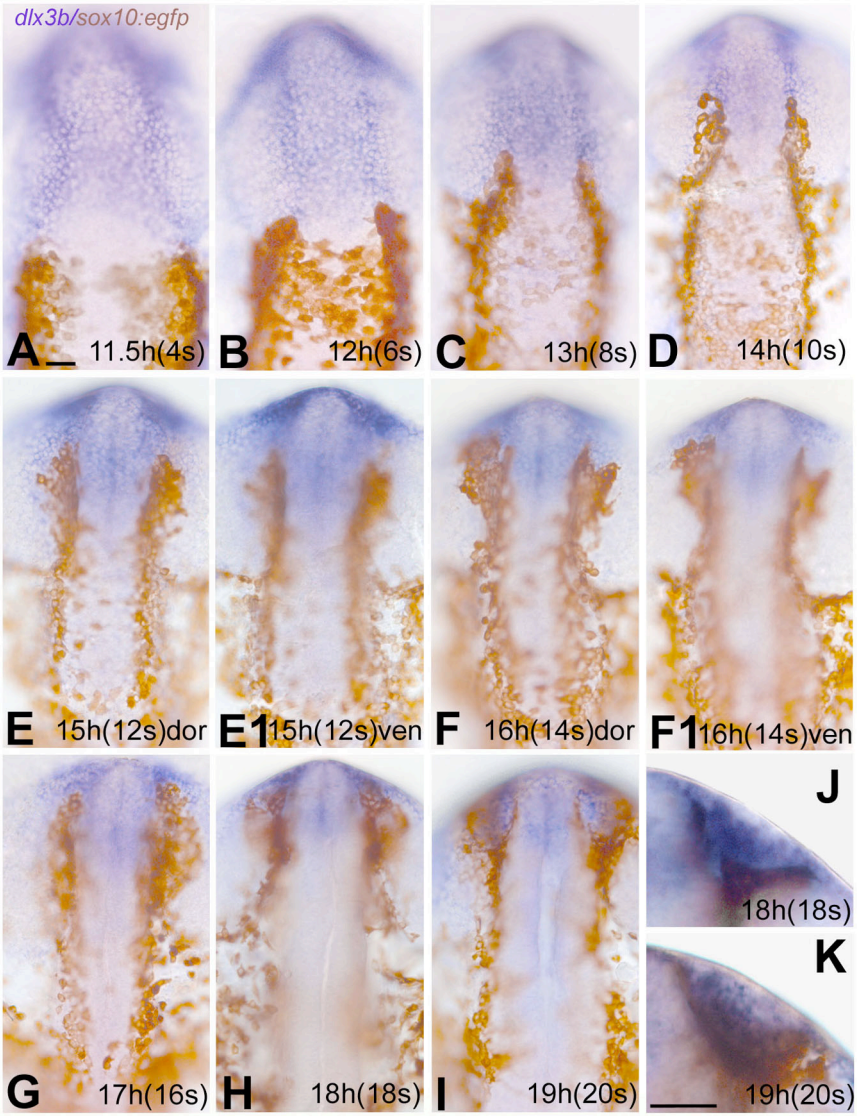

six4.1/sox10:egfp
A 11.5h(4s)
B 12h(6s)
C 13h(8s)
D 14h(10s)
E 15h(12s)dor
E1 15h(12s)ven
F 16h(14s)dor
F1 16h(14s)ven
J
18h(18s)
K
19h(20s)
G 17h(16s)
H 18h(18s)
I 19h(20s)

Figure 2.6. Development of the OP and CNC fields in *dlx3b/* anti-GFP double-labeled embryos. Visualization of OP convergence using *dlx3b in situ* hybridization (blue) and the CNC field using anti-GFP immunocytochemistry (brown) in *sox10:egfp* embryos. A-I: Dorsal views, anterior to the top of the page, of fixed, staged, double-labeled embryos. Embryos were examined every 2 somites. E, E1: Two different focal planes of same embryo at 15h (12s). F, F1: Two different focal planes at 16h (14s). E and F focus on the dorsal (dor) olfactory placode, E1 and F1 focus on the ventral (ven) edge of the olfactory placode. The CNC cells are ventral to the olfactory fields at these stages. J, K : Ventral views, anterior to top, of the formed olfactory placode surrounded by CNC cells at 18h (18s) (J) and 19h (20s) (K). h=hours post fertilization, s= somites. Scale bars A(for A-K); K (for J, K)= 30μm. 20 embryos were examined per time point.

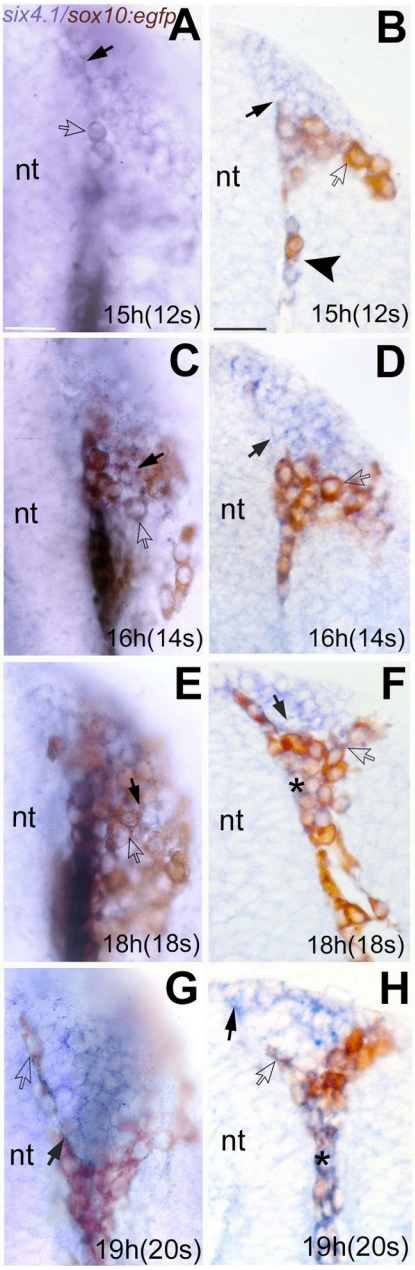

dlx3b/sox10:egfp
A 11.5h(4s)
B 12h(6s)
C 13h(8s)
D 14h(10s)
E 15h(12s)dor
E1 15h(12s)ven
F 16h(14s)dor
F1 16h(14s)ven
G 17h(16s)
H 18h(18s)
I 19h(20s)
J
18h(18s)
K
19h(20s)

At 10s the CNC cells were found dorsal to the eye, along the neural tube. The CNC cells are associated with the olfactory field at 10s as judged by both *six4.1* (Fig. 2.5D) and *dlx3b* (Fig. 2.6D) expression. This coincided with the time point just before the CNC cells move ventrally around the OP field. At 12s (Fig. 2.5 and 2.6 E, E1) and14s (Fig. 2.5 and 2.6 F, F1) the OP cells began to coalesce in the front of the head and the CNC cells began to dive ventrally, around the OP cells. By 16s (Fig. 2.5 and 2.6 G) the OP was discernable and the CNC cells are found ventral to the forming OP. By 18s the OP is clearly visible and is encompassed by CNC cells (Fig. 2.5H and J; Fig. 2.6 H and J). The OP became more defined from 18s to 20s and a clear border between the OP and CNC cells was observed (Fig. 2.5 I and K; Fig. 2.6 I and K).

The CNC cells do not extensively mix with cells in the OP field during placode formation

Up until 12s the CNC cells remain posterior to the OP field (see Fig. 2.5 and 2.6). At 12s the CNC cells began to move ventral to the OP cells. We closely examined the later stages of OP formation in whole mount (Fig. 2.7A, C, E and G) and sectioned (Fig. 2.7B, D, F and H) *six4.1/* anti-GFP preparations to determine the extent of mixing between the two groups of cells as the CNC cells surround the OP. At 12-14s, *six4.1* expressing cells are evident within the OP, thus defining the OP border (Fig. 2.7, A-D, black arrows). As CNC cells (Fig. 2.7, brown) moved anteriorly along the forming neural tube (nt) individual cells expressing GFP were observed outside the border of the OP (Fig. 2.7, open arrows). At 12s we observed rare cases where CNC cells were observed next to OP cells (Fig. 2.7B, arrowhead)

Figure 2.7. CNC cells move ventral to and surround the forming OP. Whole mount preparations (A,C,E,G) and cryostat sections (B,D,F,H) of *six4.1* (blue)*/* anti-GFP (brown) double-labeled embryos. All images are dorsal views, anterior to top of the page. At 15h (12s) (A,B) the neural crest cells were first seen meeting the edge of the olfactory placode. At 12s we observed rare cases where CNC and OP cells were found next to eachother (B, arrowhead). At 16h (14s) (C,D) the CNC cells began to aggregate at the posterior border of the OP. At 18h (18s) (E,F) the neural crest cells surrounded the OP. The border of the placode was refined at 19h (20s) (G,H). *six4.1* expressing cells (black arrows) were observed at the edge of the forming OP. anti-GFP labeled CNC cells (open arrows) are found at the edge of the placode move ventral to the placode as it forms. Some cells (F,H, asterisks) appeared to be double labeled. Upon close inspection they are not. nt= neural tube, h= hours post fertilization, s= somites; Scale bars A (for A-G) and B (for B-H)= 30μm. 5 whole mount and sectioned embryos were examined at high magnifcation per time point.


six4.1/sox10:egfp
A
nt
15h(12s)
B
nt
15h(12s)
C
nt
16h(14s)
D
nt
16h(14s)
E
nt
18h(18s)
F
nt
*
18h(18s)
G
nt
19h(20s)
H
nt
*
19h(20s)

however, this was not a frequent occurrence in our preparations. Starting at 18s GFP expressing cells (Fig. 2.7, E-H brown) were observed surrounding the OP in whole mount (Fig. 2.7, A, C, E and G) and sectioned (Fig. 2.7 B, D, F, H) preparations. During our analysis we did not observe any cells that expressed both GFP and *six4.1* or *dlx3b*. In some of our sectioned preparations some cells appeared as if they could be expressing both markers (Fig. 2.7 F, H, asterisk) however, upon close examination it was clear that these cells were not double labeled. While we rarely observed GFP positive cells amongst the *six4.1*/*dlx3b* positive cells or vice versa it was difficult to resolve the border between the CNC and OP cells in some cases and we may have inadvertently overlooked some cell mixing at the border. Overall these two fields do not appear to extensively mix as they populate the frontal mass. Therefore, our findings suggest that CNC and OP cells largely remain as two distinct groups of cells as they move anteriorly.

dlx3b **and** ***six4.1*** **are expressed in different regions of the olfactory placode**

In order to define the *six4.1* and *dlx3b* expression domains in the developing OP field we double labeled for *six4.1* and *dlx3b.* The difference in expression patterns was first evident at 4s (compare Fig. 2.5 and 2.6, A-C) and it is clearly seen at 8s (Fig. 2.8A and A1). At 8s, the CNC cells are found adjacent to the developing eye (e) (Fig. 2.8A and A1, brown). There is a gap between the field of *six4.1* (Fig. 2.8A) expression and the CNC cells (bracket). In contrast, cells expressing *dlx3b* (Fig. 2.8A1) are found adjacent to the CNC cells (Fig. 2.8A and A1,asterisk). Therefore, *dlx3b* expression extends throughout the OP field and *six4.1* is restricted to the anterior OP field. By 18s-

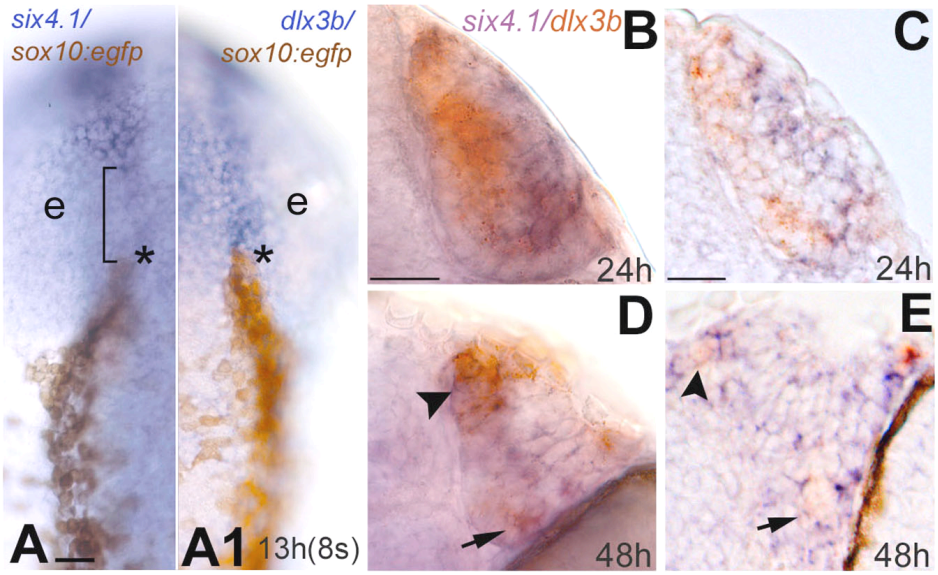


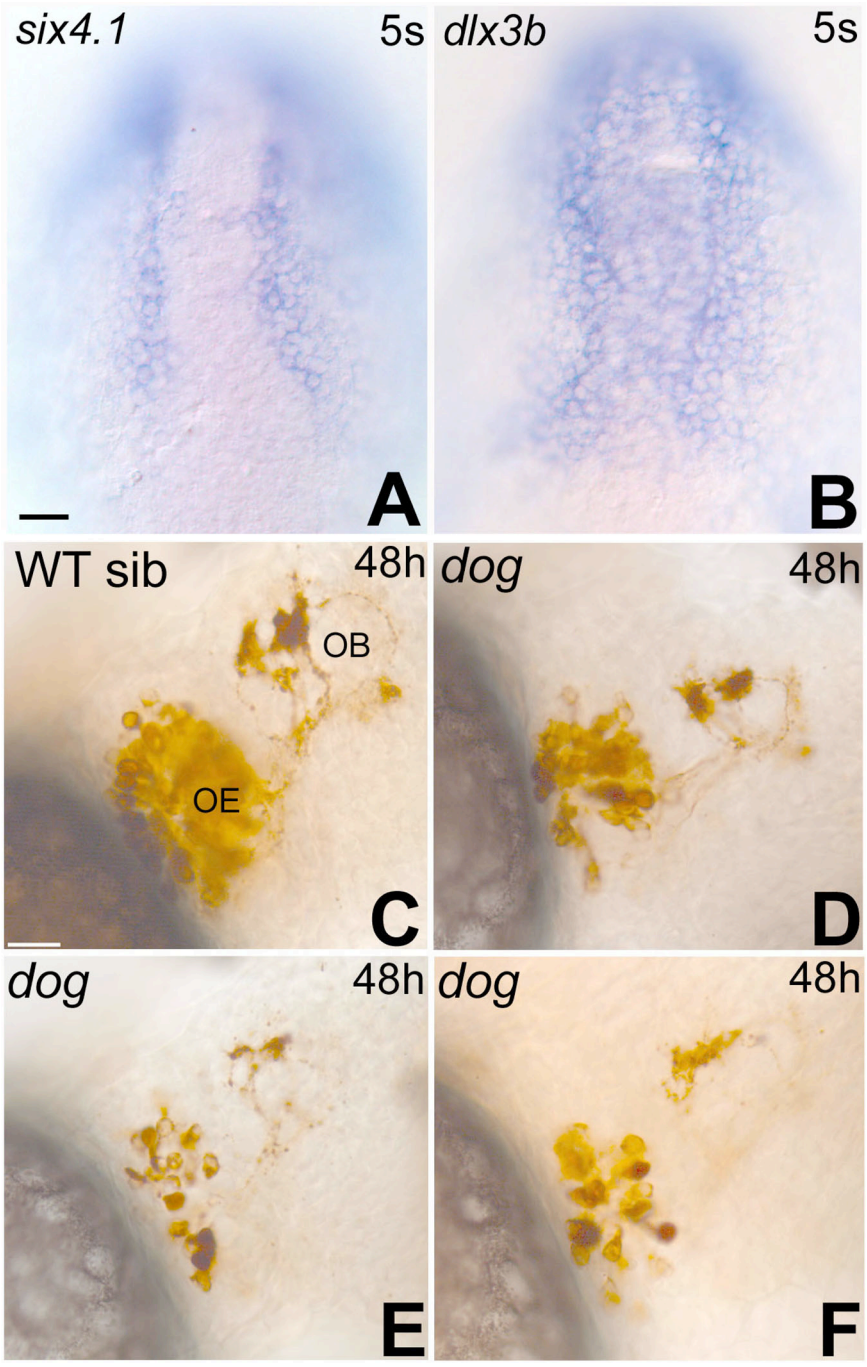
Figure 2.8. *dlx3b* and *six4.1* are localized to different regions of the forming olfactory placode. A, A1: At 13h(8s) there is a gap (bracket) between the placode cells expressing *six4.1* (A, blue) and the neural crest cell field (brown, asterisk) while the *dlx3b* expressing cells (A1, blue) abut the neural crest cells (brown, asterisk). Dorsal view, anterior to the top of the page. B-E: Double *in situ* hybridization of *dlx3b* (red) and *six4.1* (blue) at 24h (B,C) and 48h (D,E). Ventral views, anterior to the top of the page. B, C: *dlx3b* is localized to the ventral edge of the placode while *six4.1* is found throughout the placode. D, E: At 48h *dlx3b* expression is localized to the antero-ventral region (arrowhead) and posterior-ventral region (arrow). e=eye, h= hours post fertilization, s= somites. Scale bars A, B (for B and D), C (for C and E)= 30μm.

20s *dlx3b* is localized to the ventral part of the forming OP, and *six4.1* remains expressed throughout the placode (data not shown). In order to determine if *six4.1* and *dlx3b* were localized to different regions of the OP once it was formed we performed double *in situ* hybridization in at 24h and 48h. At 24h, in both whole mount (Fig. 2.8B) and sectioned (Fig. 2.8C) preparations, *six4.1* (Fig. 2.8 B, C, blue) is expressed throughout the OP while *dlx3b* (Fig. 2.8 B, C, red) is localized to the ventral, basal region of the placode. At 48h, *six4.1* remains expressed throughout the OP, only at a lower level of expression (Fig. 2.8 D, E, blue). In contrast, *dlx3b* becomes localized to the antero-ventral part of the OE (Fig. 2.8D and E, arrowhead) and the posterior-ventral OE (Fig. 2.8D and E, arrow). Thus, prior to OP formation (8s) *dlx3b* had a broad expression domain extending to the CNC. In contrast, *six4.1* was localized more anteriorly in the OP field. Yet as development proceeded *dlx3b* became localized to restricted domains within the OP at 48h while *six4.1* remained broadly expressed.

Formation of the olfactory placode in the *dog* mutant

The *dog* mutant (Whitfield et al., 1996) was recently shown to result from a mutation in the *eya1* gene (Kozlowski et al., 2005). Because *eya1* is expressed in the OP field early during development and throughout OP formation (Sahly et al., 1999), we examined whether OP development is disrupted in dog^{tm90b} (*eya1*) mutants. We examined the expression of both *six4.1* and *dlx3b* in the placode field in *dog* mutant embryos at 5s (Fig. 2.9A and B) and found that the expression patterns of both of these genes appeared normal in all of the embryos from *dog* heterozygous adults (n=100 embryos per gene).

Figure 2.9. OP formation in *dog* mutant zebrafish. A,B: At 5 somites, *six4.1* (A) and *dlx3b* (B) expression is normal in the OP fields of all embryos in clutches from *dog* heterozygous parents. C-F: anti-caleretinin immunocytochemistry of wild type sibling (C) and *dog* mutant zebrafish (D-F) at 48h. All are frontal views, dorsal to the top of the page. *dog* mutants exhibit a range of olfactory sensory neuron defects. OE= olfactory epithelium, OB= olfactory bulb, h= hours post fertilization, s=somites. Scale bars A (for A and B), C (for C-F), = 30μm.


six4.1
5s
A
dlx3b
5s
B
WT sib
48h
OB
OE
C
dog
48h
D
dog
48h
E
dog
48h
F

Visual inspection of *dog* mutants at 48h showed the presence of an OP. To visualize the olfactory sensory neurons in the mutants we used the anti-calretinin antibody (Winsky et al., 1989) in 48h *dog* embryos. We previously described normal anti-calretinin staining in the developing olfactory system of 48h zebrafish (Vitebsky et al., 2005). As we reported previously, axonal projections in the developing olfactory bulb (OB) form three main axonal bundles termed lateral (closest to the OE), medial (furthest from the OE) and central (in between lateral and medial bundles). Compared to wild type siblings (Fig. 2.9C) *dog* mutant embryos (n=12/52, 23%) showed varying degrees of olfactory sensory neuron loss and a concomitant reduction of axonal branching in the CNS (Fig. 2.9 D-F). These defects are similar to those seen for the sensory neurons of the otic vesicle in *eya1* mutants (Kozlowski et al., 2005). Therefore, *dog* mutant embryos exhibit a range of sensory neuron defects in multiple sensory systems. However, *eya1* does not appear to be necessary for the initial patterning of the OP fields.

CNC cells do not mix with cells of the anterior pituitary

In order to determine if the CNC cells moving dorsal to the eye become associated with the anterior pituitary we examined the anterior pituitary cells and the CNC cells in *sox10:egfp* embryos. We used the *lhx3* marker (Glasgow et al., 1997; Herzog et al., 2003) to follow the anterior pituitary cells and an anti-GFP antibody to visualize the CNC cells in the *sox10:egfp* fish line. At 22h the CNC cells surrounded the OPs (Fig. 2.10, brown) and populated the area between them. At 22h the anterior pituitary *lhx3* expressing cells are present as a string of cells extending along the ventral, anterior neural tube (Fig. 2.10, arrows). CNC cells located between the OPs (Fig. 2.10, open arrow) were not

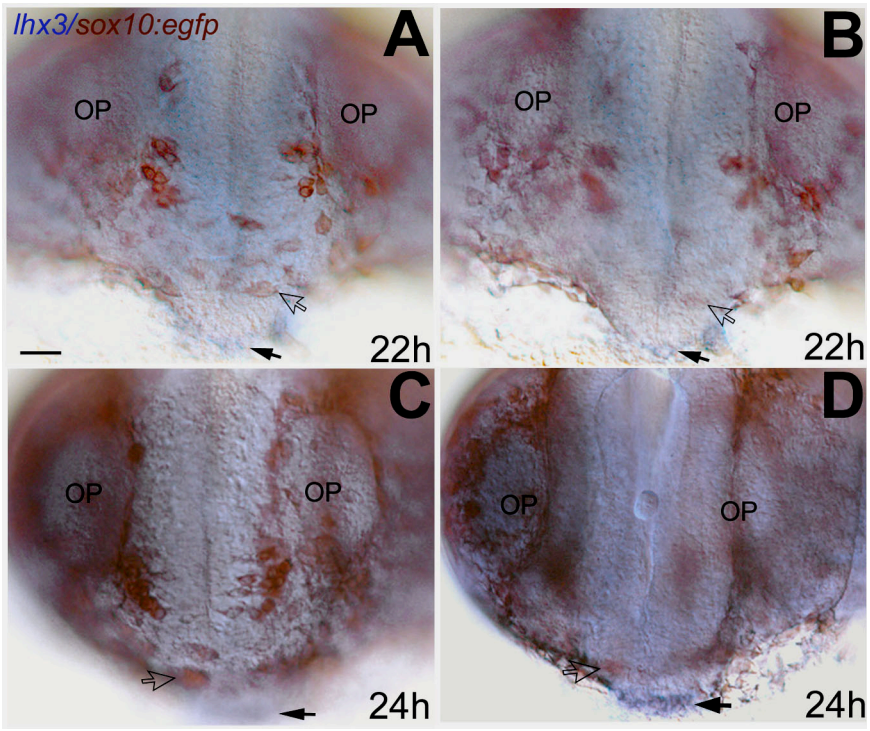


Figure 2.10. CNC cells are not associated with the developing anterior pituitary placode. A-D: Frontal views (dorsal to the top of the page) of 22h (A,B) and 24h (C,D) *sox10:egfp* embryos double labeled for *lhx3 in situ* hybridization (blue) to visualize the anterior pituitary placode and anti-GFP immunostaining (brown) to view neural crest cells. The CNC cells that have migrated to the front of the developing head (open arrows) are more anterior (A,C) than the *lhx3* expressing cells (black arrows), which are visualized in a more posterior focal plane (B,D). OP= olfactory placodes. Scale bar (A)= 30μm.

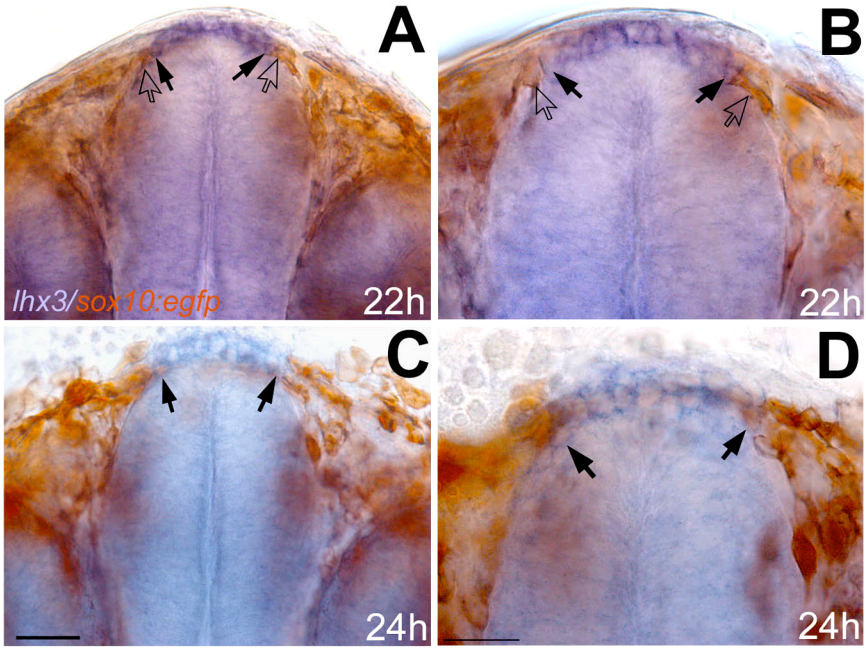


Figure 2.11. Ventral CNC cells lie adjacent to but do not mix with cells of the anterior pituitary placode. A-D: Ventral views (anterior to the top of the page) of 22h (A,B) and 24h (C,D) *sox10:egfp* embryos double labeled for *lhx3 in situ* hybridization (blue) to visualize the anterior pituitary placode and anti-GFP immunostaining (brown) to view neural crest cells. B and D are higher magnification images of A and C. The CNC cells (open arrows) found on the ventral side of the head lie adjacent to the anterior pituitary placode (black arrows) at 22h (A,B). At 24h (C,D) more *lhx3* positive cells (black arrows) are present and they have moved ventral to the CNC cells. Scale bars C (A and C) and D (B and D)= 30μm.

associated with the forming anterior pituitary cells (Fig. 2.10, arrows) at 22h (Fig. 2.10 A,B) or 24h (Fig. 2.10 C,D). Clear visualization of both groups of cells was not possible in the same focal plane.

Surprisingly, when we examined these preparations from the ventral side (Fig. 2.11) we found that the string of *lhx3* positive anterior pituitary cells were flanked on either side by GFP positive cells at 22h (Fig. 2.11 A,B). The GFP cells (Fig. 2.11 A and B, open arrows) lie adjacent to but are not mixed with the anterior pituitary cells (Fig. 2.11A and B, arrows). Due to their position these GFP positive cells are most likely not part of the population we have described here that associate with the OP. At 24h the ventral neural crest cells were no longer associated with the anterior pituitary cells (Fig. 2.11C and D). Thus, these ventral neural crest cells are transiently associated, but do not mix with the cells that form the anterior pituitary.

DISCUSSION

OP Formation in the zebrafish

Based on our previous studies, we proposed a model for the formation of the OP in vertebrates in which the OPs are formed through the convergence of cellular fields found on either side of the developing telencephalon (Whitlock, 2004b). Our model for placode formation is supported by fate mapping studies in the chick that demonstrated that the otic, olfactory and lens placodes also form through the rearrangement of cellular fields via directed cell movements (Streit, 2002; Bhattacharyya et al., 2004; Streit, 2004). Together, these studies suggest that cellular convergence may be a common mechanism for placode formation. Upon examining placode formation using time-lapse microscopy and molecular markers in fixed, staged embryos we

find that the cells that form the OP stream anteriorly along the forming neural tube starting at approximately 6-8s. The placode cells move over the developing eye and have migrated into the anterior part of the developing head by 14s. The placode is initially observed at 16s and it is refined and organized through 20s. As the cells move anteriorly, it is evident in our time-lapse imaging that the cells are extending and retracting filopodia as they interact with one another. This is consistent with previous electron microscopy studies that describe the cells forming the zebrafish OP as having small "pseudopodia-like" extensions to make connections with other cells (Hansen and Zeiske, 1993).

Cellular interactions of the CNC and OP cells during OP formation

In this study we have visualized the convergence of the OP field and determined the extent of cell mixing between the OP field and CNC cells. Formation of the OP is accompanied by the anterior migration of the CNC cells. We did not observe any mixing between the CNC and OP fields as they moved anteriorly. Furthermore, we did not observe the co-expression of *sox10-GFP* with either *six4.1* or *dlx3b* in any of the cells. This is consistent with fate mapping experiments that demonstrated that the OP field lies adjacent to the pre-migratory CNC field and that cells within the CNC domain do not contribute to the OP (Whitlock and Westerfield, 2000; Whitlock et al., 2003). Our findings agree with a model proposed for the chick embryo where there is an orderly positioning of the pre-placodal and CNC fields at the neural plate stage before craniofacial morphogenesis begins (Meulemans and Bronner-Fraser, 2004; Bailey and Streit, 2006). The positioning of the pre-placodal and CNC fields is governed by a complex network of BMP, FGF and

Wnt signaling that results in expression of placodal and CNC specifying transcription factors (e.g. Six and Sox genes respectively) that lead to the activation of different signaling cascades (Brugmann and Moody, 2005; Litsiou et al., 2005; Bailey and Streit, 2006; Schlosser, 2006). The early activation of placodal and CNC signaling results in the specification of placodal and CNC cells before they migrate into the frontal mass. While our data are in agreement with a separation between placode and CNC cells, there is not always a clear separation between these fields in the early embryo. For example, the otic precursors in chick are initially intermingled with CNC cells and then parse out as development proceeds (Streit, 2002). In rare cases the OP and CNC cells mix at the border between the fields but we did not observe extensive mixing between the CNC and OP fields. Our findings for the OP in the zebrafish suggest that overall, the OP and CNC cells begin and remain as separate cellular populations as they migrate anteriorly to populate the frontal mass.

While the CNC cells do not move between the OP cells, they are closely associated with the OP cells during anterior migration in that the CNC cells move around the OP cells and surround the OP once it is formed. CNC cells form the skeletal part of the nose (Langille and Hall, 1988; Le Douarin and Smith, 1988; Le Douarin and Kalcheim, 1999) but it is unlikely that the *sox10:egfp* cells we visualize surrounding the OP at 18-20h will differentiate into the structural part of the nose. This is because *sox10* is thought to play a role in the differentiation of neuronal, glial and pigment CNC derivatives but not structural CNC derivatives (Kelsh, 2006). Previously, we have shown that some of the CNC cells differentiate into the terminal nerve GnRH cells (Whitlock et al., 2003; Whitlock et al., 2005). The CNC cells we observe

surrounding the OP may also differentiate into glia of the olfactory nerve, which would be consistent with what has been shown for other placodes.

The role of *dlx3b, six4.1*and *eya1* in the OP field

We found that *dlx3b* is expressed more broadly in the OP field compared to *six4.1* at 4-8s. The *dlx3b* expression domain lies adjacent to the CNC domain and there is a gap between the CNC cells and the *six4.1* domain. *dlx3b* and *six4.1* are both likely to play a role in specifying the OP field but *dlx3b*'s expression adjacent to pre-migratory CNC field suggests that it may also be involved in specifying the posterior border between the OP and CNC cells. A role for *dlx3b* at the OP, pre-migratory CNC border is consistent with a proposed role for the Dlx genes as "border specifying" genes in the neural plate (McLarren et al., 2003; Meulemans and Bronner-Fraser, 2004; Litsiou et al., 2005; Bailey and Streit, 2006). In the chick embryo, mis-expression of *Dlx5* leads to the upregulation of neural plate border genes (including the pre-placodal specifying gene *Six4)* but not neural crest specifying genes (McLarren et al., 2003).

A role for *dlx3b* in establishment of the posterior OP border could be mediated through the opposing activities of the *dlx* and *msx* genes. The *msx* genes are a family of vertebrate genes that are homologous to the *Drosophila* muscle segment homeobox (*msh*) gene (Ekker et al., 1997). The Msx and Dlx proteins are thought to antagonize each other by competing for regulatory elements *in vivo* (Bendall and Abate-Shen, 2000). In zebrafish, *msxB* and *msxC* are expressed in the CNC cells and their expression domains abut the *dlx3b* expression domain at 4-6s (Ekker et al., 1997; Phillips et al., 2006). Zebrafish that contain a deficiency that covers the *dlx3b* gene have a wider

neural plate than controls (Fritz et al., 1996; Solomon and Fritz, 2002; Phillips et al., 2006). Phillips et al. (2006) showed that knock-down of multiple Msx proteins using morpholinos is able to rescue the neural plate widening defect observed in the deficiency line. This suggests that the antagonizing activities of Msx and Dlx proteins refine the neural plate border in the zebrafish. It is yet to be determined if the Dlx and Msx proteins play a similar role in establishing the border between the posterior OP field and pre-migratory CNC cells but the expression patterns of these genes is consistent with this possibility.

Because *eya1* is expressed in the OP field before convergence we examined the OP field in *eya1* mutants to determine if *eya1* plays a role in the OP field early in development. We found that the OP fields are normal in *dog (eya1)* mutant zebrafish, which is consistent with what has been described previously for the otic, lateral line and anterior pituitary placodes in *dog* mutants (Kozlowski et al., 2005; Nica et al., 2006). Induction of the otic vesicle and lateral line placodes appears to occur normally in *dog* mutant embryos (Kozlowski et al., 2005). Similarly, anterior pituitary placode development (as judged by *lhx3* expression) appears normal for the first two days of development (Nica et al., 2006). It is unclear why there are no defects observed in these placodes early during placode formation when *eya1* is expressed throughout the pre-placodal region (Sahly et al., 1999). It is likely that there is functional redundancy between *eya1* and the other three *eya* genes (*eya2, eya3* and *eya4)* that have recently been identified in the zebrafish (Schonberger et al., 2005; Nica et al., 2006). In mouse, *Eya1* and *Eya2* are both expressed throughout the cranial placodes, including the OP (Xu et al., 1997).To date, there has not been a detailed analysis of the expression of all of the zebrafish *eya* genes during OP development.

The roles of *dlx3b* and *eya1* within the formed OP

At 24 and 48h, *dlx3b* becomes localized to the ventral part of the formed OP whereas *six4.1* remains expressed throughout the OP. The pattern of *dlx3b* expression in the OP at 48h is reminiscent of the expression of genes that promote neuronal differentiation (Mueller and Wullimann, 2003) suggesting that *dlx3b* may also play a role in promoting neuronal differentiation during later stages of OP development. The localization of *dlx3b* to the basal part of the OP suggests that it is expressed in cells that have entered into but not completed differentiation because the majority of terminally differentiated olfactory sensory neurons are located in the apical part of the OE at 48h (Celik et al., 2002) (Harden and Whitlock, unpublished observations). Similarly, it has been shown that in the mouse epidermis, *dlx3* is expressed in cells that have recently stopped dividing and initiated a terminal differentiation program (Morasso et al., 1996; Beanan and Sargent, 2000). Further investigation is necessary to fully understand the role of *dlx3b* in the OP but it is clear that *dlx3b* is essential for OP development in zebrafish because knock-down of the Dlx3b protein using morpholinos results in a reduction of the OP at 18s and 24h (Solomon and Fritz, 2002).

Investigation of the formed OPs in *dog (eya1)* mutants demonstrated that they show a range of olfactory sensory neuron defects indicative of a role for *eya1* in sensory neuron differentiation. Again, this is in agreement with what has been observed for the otic, lateral line and anterior pituitary placodes. Characterization of the otic placode phenotype showed that *dog* mutants have either a reduced number or completely lack cristae hair cells at 48h. This indicates that *eya1* is not required to make hair cells but may be

required for their maintenance (Whitfield et al., 1996; Kozlowski et al., 2005). Consistent with this model, there is an increase in apoptosis in the otic vesicles of *dog* mutants from 24-48h suggesting that hair cells differentiate but are lost because they undergo cell death (Kozlowski et al., 2005). In the lateral line, trunk neuromasts are completely absent at 120h in *dog* embryos and the number of anterior neuromasts is reduced at 60h (Whitfield et al., 1996; Kozlowski et al., 2005). *dog* mutants also exhibit defects in the differentiation of specific lineages of cells that arise from the anterior pituitary placode beginning at 48h (Nica et al., 2006). In this case, there is no indication of increased apoptosis or trans-differentiation into cell types that arise from other placodes, which suggests that the anterior pituitary cells remain in an undifferentiated state (Nica et al., 2006). Additional studies are necessary to determine the cause of the defects observed in the olfactory sensory neurons.

CNC cells and the anterior pituitary placode

Simultaneous visualization of the CNC and anterior pituitary cells demonstrated that the anterior pituitary cells are not associated with CNC cells that migrate dorsally but they are found adjacent to ventral GFP positive cells at 22h. This finding is in agreement with recent fate mapping data from Wada et al. (2005) who also used the *sox10:egfp* transgenic zebrafish line to examine the CNC migratory routes between and ventral to the developing eye. They show that the CNC cells that populate the region of the developing anterior pituitary at 22-24h originate at the very anterior region of the pre-migratory CNC field and that they do not take a dorsal migratory path. Instead they take a more ventral path that goes between the dorsal part of the developing eyes (Wada et al., 2005). This is likely to be a more ventral CNC

route than the one we describe here that interacts with OP fields. It is possible that the CNC cells they observe in the anterior pituitary region are the same cells we observed moving ventrally at the beginning of our time-lapse imaging analysis (Harden and Whitlock, data not shown). Together, these data indicate that there is a transient association of CNC and anterior pituitary cells during development. The close association of these two cell populations has also been demonstrated in mouse (Mackenzie et al., 1991). These studies found that a marker of CNC-derived tissues, *Hox 7.1*, is expressed in the developing anterior pituitary at 11 days of gestation, which suggests that neural crest cells interact with the anterior pituitary earlier during development (Mackenzie et al., 1991).

In this study we have characterized the cellular movements of the OP and CNC cells during normal development of the frontal mass. Disruption of this process results in birth defects in humans. Recent findings have linked exposure to antifungal agents (such as those found in agrochemicals) during development with craniofacial malformations that result from abnormal neural crest migration (Groppelli et al., 2005; Menegola et al., 2005). In the future, we will be able to utilize our understanding of the dorsal movements of the CNC and OP fields to determine how teratogens in the environment, such as antifungal agents, can disrupt normal craniofacial development.

METHODS

Animals

Zebrafish were crossed as described previously (Westerfield, 1993) and embryos were collected the morning of fertilization. Wild type embryos were the New Wild Type (NWT) strain, which originated in the Whitlock laboratory.

The *sox10:egfp* transgenic zebrafish line (Wada et al., 2005) was provided by the Kelsh laboratory and maintained in our fish facility. *dog*^{tm90b} embryos (Kozlowski et al., 2005) were collected from pairwise matings of heterozygous *dog* adults provided by the Kozlowski laboratory. All animal procedures were approved by the Cornell University Institutional Animal Use and Care Committee.

Time lapse Imaging

Imaging of either wild type or *sox10:egfp* embryos was performed using a Leica DMRA2 microscope using OpenLab 4.0.4 software. For imaging of *sox10:egfp* embryos, sequential Nomarski and fluorescence images were taken every 2 minutes and then merged. Embryos were embedded in agar in an Attofluor cell chamber (Molecular Probes) and covered with embryo medium (Westerfield, 1993), a window of agar was removed to allow for visualization of the dorsal side of the developing head. The Attofluor chamber allowed imaging for about six hours at approximately 26^{o}C without replenishing embryo medium.

***In situ* hybridization**

After collection, embryos were staged as described by (Kimmel et al., 1995). Embryos collected during somite stages were placed at 31^{o}C to accelerate development and the number of somites were counted to ensure accurate staging. Embryos were fixed in phosphate buffered- 4% paraformaldehyde. Digoxigenin and fluorescein labeled mRNA probes were made using the SP6/T7 DIG RNA labeling kit (Roche) following the manufacturers instructions. Probes were made to *dlx3b* (Ekker et al., 1992),

six4.1 (Kobayashi et al., 2000), *lhx3* (Glasgow et al., 1997) and *sox10* (Dutton et al., 2001). Embryos 20 somites and younger were dechorionated after fixation. *In situ* hybridization was performed as described by Thisse et al. (1993). The duration of the proteinase-K (Sigma) permeabilization step was as follows: 24h and earlier: no permeabilization was performed, 36h: 1 minute; 48h: 3 minutes. Double *in situ* hybridizations were performed as described in (Schulte-Merker, 2002) with the exception that the anti-fluorescein antibody (Roche) was used at 1:10,000. Blue coloration reaction was done using NBT/BCIP (Roche) and red coloration reaction using INT/BCIP (Roche) following the manufacturer's instructions.

Immunocytochemistry

Fixed, Staged embryos (described above) were processed for immunocytochemistry. Fixed embryos were rinsed in phosphate buffered saline (PBS) and blocked in PBST (PBS and 0.1% Tween 20) with 6% normal donkey serum (Jackson Immuno Research). Embryos 24h and younger did not undergo any permeabilization treatment. Embryos were incubated overnight at 4^{o}C in anti-GFP antibody (rabbit, Invitrogen/Molecular Probes, 1:500) to detect cells expressing GFP in *sox10:egfp* embryos. Embryos were rinsed several times with PBST and incubated with goat anti-rabbit secondary antibody (Sternberger Monoclonals Inc., 1:200) for 5 hours at room temperature, rinsed several times with PBST and incubated with peroxidase-rabbit anti-peroxidase complex (Sternberger Monoclonals Inc., 1:500). Coloration reaction was performed using DAB (diaminobenzidine, Sigma) as described in Whitlock and Westerfield (2000). Anti-calretinin antibody labeling was performed as described in Vitebsky et al. (2005).

Double Immunocytochemistry and *In situ* hybridization

sox10:egfp embryos were double labeled using anti-GFP antibodies and *in situ* hybridization by sequentially performing the procedures described above. Immunocytochemistry was performed first with care to use RNase free solutions. Embryos were not placed in methanol because this resulted in a lack of anti-GFP staining. During all blocking/ antibody binding steps 0.5μl/ml of RNase Out (Invitrogen) was added. After DAB reaction, embryos were rinsed several times in PBST and then *in situ* hybridization was performed as described above.

Cryostat sections of zebrafish embryos

Embryos already processed for immunocytochemistry and *in situ* hybridization were used for cryostat sections. Embryos were embedded in agar as described previously (Westerfield, 1993) except that agar embedded embryos were placed in Shandon M-1 Embedding Matrix for frozen sections (Thermo Electron Corporation). Sections were 10-12μm thick.

ACKNOWLEGEMENTS

The authors thank the Kobayashi laboratory for the *six4.1* cDNA, M. Rebbert for the *lhx3* cDNA, the Kelsh laboratory for the *sox10* cDNA and the Westerfield laboratory for *dlx3b* . We thank Dr. Robert Kelsh and Dr. David Kozlowski for providing the *sox10:egfp* and *dog* fish, respectively. We also thank L.Sanders for imaging assistance and S. Twomey for cryostat sectioning. This work was supported by Cornell Center for Vertebrate Genomics Graduate research fellowship (MVH) and NIHR01DC0421802 (KEW).

REFERENCES

Akimenko MA, Ekker M, Wegner J, Lin W, Westerfield M. 1994. Combinatorial expression of three zebrafish genes related to *distal-less*: Part of a homeobox gene code for the head. The Journal of Neuroscience 14:3475-3486.

Bailey AP, Streit A. 2006. Sensory organs: making and breaking the pre-placodal region. Curr Top Dev Biol 72:167-204.

Baker CV, Bronner-Fraser M. 2001. Vertebrate cranial placodes I. Embryonic induction. Developmental Biology 232:1-61.

Beanan MJ, Sargent TD. 2000. Regulation and Function of Dlx3 in Vertebrate Development. Developmental Dynamics 218:545-553.

Bendall AJ, Abate-Shen C. 2000. Roles for Msx and Dlx homeoprotein in vertebrate development. Gene 247:17-31.

Bhattacharyya S, Bailey AP, Bronner-Fraser M, Streit A. 2004. Segregation of lens and olfactory precursors from a common territory: cell sorting and reciprocity of Dlx5 and Pax6 expression. Dev Biol 271:403-414.

Brugmann SA, Moody SA. 2005. Induction and specification of the vertebrate ectodermal placodes: precursors of the cranial sensory organs. Biology of the Cell 97:303-319.

Brugmann SA, Tapadia MD, Helms JA. 2006. The Molecular Origins of Species-Specific Facial Pattern. Current Topics in Developmental Biology 73:1-42.

Celik A, Fuss SH, Korsching SI. 2002. Selective targeting of zebrafish olfactory receptor neurons by the endogenous OMP promoter. European Journal of Neuroscience 15:798-806.

Chapman SC, Sawitzke AL, Campbell DS, Schoenwolf GC. 2005. A three-dimensional atlas of pituitary gland development in the zebrafish. Journal of Comparative Neurology 487:428-440.

Creuzet S, Couly G, Le Douarin NM. 2005. Patterning the neural crest derivitives during development of the vertebrate head: insights from avian studies. Journal of Anatomy 207:447-459.

D'Amico-Martel A, Noden DM. 1983. Contributions of placodal and neural crest cells to avian cranial peripheral ganglia. American Journal of Anatomy 166:445-468.

Dutton KA, Pauliny A, Lopes SS, Elworthy S, Carney TJ, Rauch J, Geisler R, Haffter P, Kelsh RN. 2001. Zebrafish colourless encodes *sox10* and specifies non-ectomesenchymal neural crest fates. Development 128:4113-4125.

Ekker M, Akimenko MA, Allende ML, Smith R, Drouin G, Langille RM, Weinberg ES, Westerfield M. 1997. Relationships among msx gene structure and function in zebrafish and other vertebrates. Mol Biol Evol 14:1008-1022.

Ekker M, Akimenko MA, Bremiller R, Westerfield M. 1992. Regional Expression of Three Homeobox Transcripts in the Inner Ear of Zebrafish Embryos. Journal of Neuron 9:27-35.

Farbman AI. 1992. Cell Biology of Olfaction. Cambridge: Cambridge University Press.

Fritz A, Rozowski M, Walker C, Westerfield M. 1996. Identification of selected gamma-ray induced deficiencies in zebrafish using multiplex polymerase chain reaction. Genetics 144:1735-1745.

Glasgow E, Karavanov AA, Dawid IB. 1997. Neuronal and neuroendocrine expression of lim3, a LIM class homeobox gene, is altered in mutant zebrafish with axial signaling defects. Developmental Biology 192:405-419.

Graham A, Begbie J. 2000. Neurogenic placodes: a common front. Trends Neurosci 23:313-316.

Groppelli S, Pennati R, De Bernardi F, Menegola E, Giavini E, Sotgia C. 2005. Teratogenic effects of two antifungal triazoles, triadimefon and triadimenol, on Xenopus laevis development: craniofacial defects. Aquatic Toxicology 73:370-381.

Hall BK. 1999. Neural Crest in Development and Evolution. New York. 313 p.

Hansen A, Zeiske E. 1993. Development of the olfactory organ in the zebrafish, Brachydanio rerio. Journal of Comparative Neurology 333:289-300.

Herzog W, Zeng X, Lele Z, Sonntag C, Ting JW, Chang CY, Hammerschmidt M. 2003. Adenohypophysis formation in the zebrafish and its dependence on sonic hedgehog. Developmental Biology 254:36-49.

Kawakami K, Sato S, Ozaki H, Ikeda K. 2000. Six family genes- structure and function as transcription factors and their roles in development. BioEssays 22:616-626.

Kelsh RN. 2006. Sorting out Sox10 functions in neural crest development. Bioessays 28:788-798.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995. Stages of Embryonic Development of the Zebrafish. Developmental Dynamics 203:253-310.

Kobayashi M, Osanai H, Kawakami K, Yamamoto M. 2000. Expression of three zebrafish Six4 genes in the cranial sensory placodes and the developing somites. Mechanisms of Development 98:151-155.

Kozlowski DJ, Whitfield TT, Hukriede NA, Lam WK, Weinberg ES. 2005. The zebrafish dog-eared mutation disrupts eya1, a gene required for cell survival and differentiation in the inner ear and lateral line. Developmental Biology 277:27-41.

Langille RM, Hall BK. 1988. Role of the neural crest in development of the cartilaginous cranial and visceral skeleton of the medaka, Oryzias latipes (Teleostei). Journal of Anatomy and Embryology 177:297-305.

Le Douarin NM, Kalcheim C. 1999. The Neural Crest. Cambridge: Cambridge University Press.

Le Douarin NM, Smith J. 1988. Development of the peripheral nervous system from the neural crest. Annual Reviews in Cell Biology 4:375-404.

Litsiou A, Hanson S, Streit A. 2005. A balance of FGF, BMP and WNT signalling positions the future placode territory in the head. Development 132:4051-4062.

Mackenzie A, Leeming GL, Jowett AK, Ferguson MW, Sharpe PT. 1991. The homeobox gene Hox 7.1 has specific regional and temporal expression patterns during early murine craniofacial embryogenesis, especially tooth development in vivo and in vitro. Development 111:269-285.

McLarren KW, Litsiou A, Streit A. 2003. DLX5 positions the neural crest and preplacode region at the border of the neural plate. Dev Biol 259:34-47.

Menegola E, Broccia ML, Di Renzo F, Massa V, Giavini E. 2005. Craniofacial and axial skeletal defects induced by the fungicide triadimefon in the mouse. Birth defects research. Part B, Developmental and reproductive toxicology 74:185-195.

Meulemans D, Bronner-Fraser M. 2004. Gene-regulatory interactions in neural crest evolution and development. Dev Cell 7:291-299.

Morasso MI, Markova NG, Sargent TD. 1996. Regulation of Epidermal Differentiation by a Distal-less Homeodomain Gene. Journal of Cell Biology 135:1879-1887.

Mueller T, Wullimann MF. 2003. Anatomy of neurogenesis in the early zebrafish brain. Brain Research Developmental Brain Research 140:137-155.

Nica G, Herzog W, Sonntag C, Nowak M, Schwarz H, Zapata AG, Hammerschmidt M. 2006. Eya1 is required for lineage-specific differentiation, but not for cell survival in the zebrafish adenohypophysis. Dev Biol 292:189-204.

Noden DM, Francis-West P. 2006. The differentiation and morphogenesis of craniofacial muscles. Developmental Dynamics 235:1194-1218.

Northcutt RG. 1993. A reassessment of Goodrich's model of cranial nerve phylogeny. Acta Anatomica 148:71-80.

Northcutt RG. 1996. The origin of craniates: Neural crest, neurogenic placodes, and homeobox genes. Israel Journal of Zoology 42:S-273-S-313.

Northcutt RG, Gans C. 1983. The genesis of neural crest and epidermal placodes: A reinterpretation of vertebrate origins. The Quarterly Review of Biology 58:1-28.

Osumi-Yamashita N, Ninomiya Y, Doi H, Eto K. 1994. The Contribution of both Forebrain and Midbrain Crest Cells to the Mesenchyme in the Frontonasal Mass of Mouse Embryos. Journal of Developmental Biology 164:409-419.

Phillips BT, Kwon HJ, Melton C, Houghtaling P, Fritz A, Riley BB. 2006. Zebrafish msxB, msxC and msxE function together to refine the neural-nonneural border and regulate cranial placodes and neural crest development. Dev Biol 294:376-390.

Sahly I, Andermann P, Petit C. 1999. The zebrafish eya1 gene and its expression pattern during embryogenesis. Development, Genes and Evolution 209:399-410.

Schlosser G. 2006. Induction and specification of cranial placodes. Dev Biol 294:303-351.

Schonberger J, Wang L, Shin JT, Kim SD, Depreux FFS, Zhu H, Zon L, Pizard A, Kim JB, MacRae CA, Mungall AJ, Seidman JG, Seidman CE. 2005. Mutation in the transcriptional coactivator EYA4 causes dilated

cardiomyopathy and sensorineural heariing loss. Nature Genetics 37:418-422.

Schulte-Merker S. 2002. Looking at embryos. In: Nusslein-Volhard C, Dahm R, editors. Zebrafish. Oxford: Oxford University Press. pp 41-50.

Solomon KS, Fritz A. 2002. Concerted action of two dlx paralogs in sensory placode formation. Development 129:3127-3136.

Streit A. 2001. Origin of the vertebrate inner ear: evolution and induction of the otic placode. J Anat 199:99-103.

Streit A. 2002. Extensive cell movements accompany formation of the otic placode. Developmental Biology 249:237-254.

Streit A. 2004. Early development of the cranial sensory nervous system: from a common field to individual placodes. Dev Biol 276:1-15.

Thisse C, Thisse B, Schilling TF, Postlethwait JH. 1993. Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. Development 119:1203-1215.

Torres M, Giraldez F. 1998. The development of the vertebrate inner ear. Mechanisms of Development 71:5-21.

Vitebsky A, Reyes R, Sanderson MJ, Michel WC, Whitlock KE. 2005. Isolation and characterization of the laure olfactory behavioral mutant in the zebrafish Danio rerio. Developmental Dynamics 234:229-242.

Wada N, Javidan Y, Nelson S, Carney TJ, Kelsh RN, Schilling TF. 2005. Hedgehog signaling is required for cranial neural crest morphogenesis and chondrogenesis at the midline in the zebrafish skull. Development 132:3977-3988.

Westerfield M, editor. 1993. The Zebrafish Book: A guide for the laboratory use of zebrafish (*Brachydanio rerio*). Eugene: University of Oregon Press.

Whitfield TT, Granato M, van Eeden FJM, Schach U, Brand M, Furutani-Seiki M, Haffter P, Hammerschimdt M, Heisenberg CP, Jiang YJ, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Nusslein-Volhard-C. 1996. Mutations affecting development of the zebrafish inner ear and lateral line. Development 123:241-254.

Whitlock KE. 2004a. Development of the nervus terminalis: Origin and migration. Microscopy Research and Techniques 65(1-2):2-12.

Whitlock KE. 2004b. A new model for olfactory placode development. Brain, Behavior and Evolution 64:1-11.

Whitlock KE, Smith KM, Kim H, Harden MR. 2005. A role for *foxd3* and *sox10* in the differentiation of gonadotropin-releasing hormone (GnRH) cells in the zebrafish, *Danio rerio*. Development 132:5491-5502.

Whitlock KE, Westerfield M. 1998. A transient population of neurons pioneers the olfactory pathway in the zebrafish. Journal of Neuroscience 18:8919-8927.

Whitlock KE, Westerfield M. 2000. The olfactory placode of the zebrafish form by convergence of cellular fields at the edge of the neural plate. Development 127:3645-3653.

Whitlock KE, Wolf CD, Boyce ML. 2003. Gonadotropin-releasing hormone (GnRH) cells arise from cranial neural crest and adenohypophyseal regions of the neural plate in the zebrafish, *danio rerio*. Developmental Biology 257:140-152.

Winsky L, Nakata H, Martin BM, Jacobowitz DM. 1989. Isolation, partial amino acid sequence, and immunohistochemical localization of a brain-specific calcium-binding protein. Proceedings of the National Academy of Sciences U S A 86:10139-10143.

Xu PX, Woo I, Her H, Beier DR, Maas RL. 1997. Mouse Eya homologues of the Drosophila eyes absent gene require Pax6 for expression in lens and nasal placode. Development 124:219-231.

CHAPTER 3

OLFACTORY IMPRINTING IS CORRELATED WITH CHANGES IN GENE EXPRESSION IN THE OLFACTORY EPITHELIA OF THE ZEBRAFISH[2]

ABSTRACT

Odors experienced as juveniles can have significant effects on the behavior of mature organisms. A dramatic example of this occurs in salmon where the odors experienced by developing fish determine the river to which they return as adults. Further examples of olfactory memories are found in many animals including vertebrates and invertebrates. Yet, the cellular and molecular bases underlying the formation of olfactory memory are poorly understood. We have devised a series of experiments to determine whether zebrafish can form olfactory memories much like those observed in salmonids. Here we show for the first time that zebrafish form and retain olfactory memories of an artificial odorant, phenylethyl alcohol (PEA), experienced as juveniles. Furthermore, we demonstrate that exposure to PEA results in changes in gene expression within the olfactory sensory system. These changes are evident by *in situ* hybridization in the olfactory epithelium of the developing zebrafish. Strikingly, our analysis by *in situ* hybridization demonstrates that the transcription factor, *otx2*, is up regulated in the olfactory

[2] This chapter is has been accepted for publication and is reproduced with permission from the *Journal of Neurobiology.* Harden MV, Newton LA, Lloyd RC and Whitlock KE. Olfactory imprinting is correlated with changes in gene expression in the olfactory epithelia of the zebrafish. *Journal of Neurobiology.* 2006 Sep 29; [Epub ahead of print]. LA Newton assisted in behavioral experiments. RC Lloyd performed statistical analysis of behavioral experiments. I performed microarray experiments and all experiments related to analysis of *otx2*. Dr. Kathleen E. Whitlock is corresponding author on this publication.

sensory epithelia in response to PEA. This increase is evident at two and three days post-fertilization and is maintained in the adult animals. We propose that the changes in *otx2* gene expression are manifest as an increase in the number of neuronal precursors cells olfactory epithelium of the odor-exposed fish. Thus, our results reveal a role for the environment in controlling gene expression in the developing peripheral nervous system.

INTRODUCTION

Memory is the ability to recover information of past events or knowledge. The assessment of whether a memory is formed and retained is generally assayed through behavioral tests. Behavioral imprinting is a type of memory that involves exposure to a stimulus during early development and a memory of the stimulus is retained long-term (e.g. throughout life or until major life changes such as puberty) in the absence of priming by the stimulus. Olfactory imprinting is the long-term retention of an odor memory experienced as a juvenile. In vertebrates, mammals show distinct behaviors driven by olfactory memories: pups of the European rabbit (*Oryctolagus cuniculus*) imprint *in utero* on chemical cues associated with the mother's diet and retain this preference until adulthood (Bilko et al., 1994; Hudson and Distel, 1998); mouse pups alter their genetic identification of self, as judged by olfactory choice, by imprinting through cross fostering of pups (Yamazaki et al., 1991). Thus the formation of long lasting olfactory memories is a behavior conserved across animals and for which the mechanisms are poorly understood. Perhaps the most widely known olfactory imprinting behavior is that observed in salmon. As juveniles, salmon imprint on the odors of their natal stream and then migrate to sea where they feed as adults. The adult salmon then return to

their natal stream to reproduce by navigating through the environment using a variety of olfactory cues (Hasler and Scholz, 1983; Dittman and Quinn, 1996). The previous work of Hasler and Scholz (1983) demonstrated that salmon can imprint on artificial odorants, phenylethyl alcohol (PEA) and morpholine.

The cellular and molecular basis of olfactory memory lies, at least in part, within the central nervous system (CNS) in both vertebrates (Wilson and Stevenson, 2003) and invertebrates (Siwicki and Ladewski, 2003). What is less well known is the role of the peripheral nervous system (PNS) in the process of sensory imprinting. Experiments examining changes in the olfactory sensory neurons from imprinted animals have shown that the physiological response to the imprinting odor is increased in rabbits as assayed by electro-olfactograms (Semke et al., 1995) and in salmon as assayed by single cell recordings (Nevitt et al., 1994; Dittman et al., 1997). These experiments suggest that olfactory experience during early development results in long lasting physiological changes.

We have used zebrafish to explore the role of the peripheral nervous system in olfactory imprinting in fishes. In order to do so we first show that zebrafish can make and retain olfactory memories of phenylethyl alcohol, a chemical odorant used previously in salmon studies. Furthermore we demonstrate that the olfactory environment causes changes in gene expression within the olfactory epithelium as assayed by *in situ* hybridization on tissues from developing and adult zebrafish. Specifically, exposure of the developing zebrafish to PEA results in an olfactory imprinting response in the adult. This behavioral response is correlated with an increase in the number of cells in the olfactory epithelium expressing the transcription factor *otx2*.

RESULTS

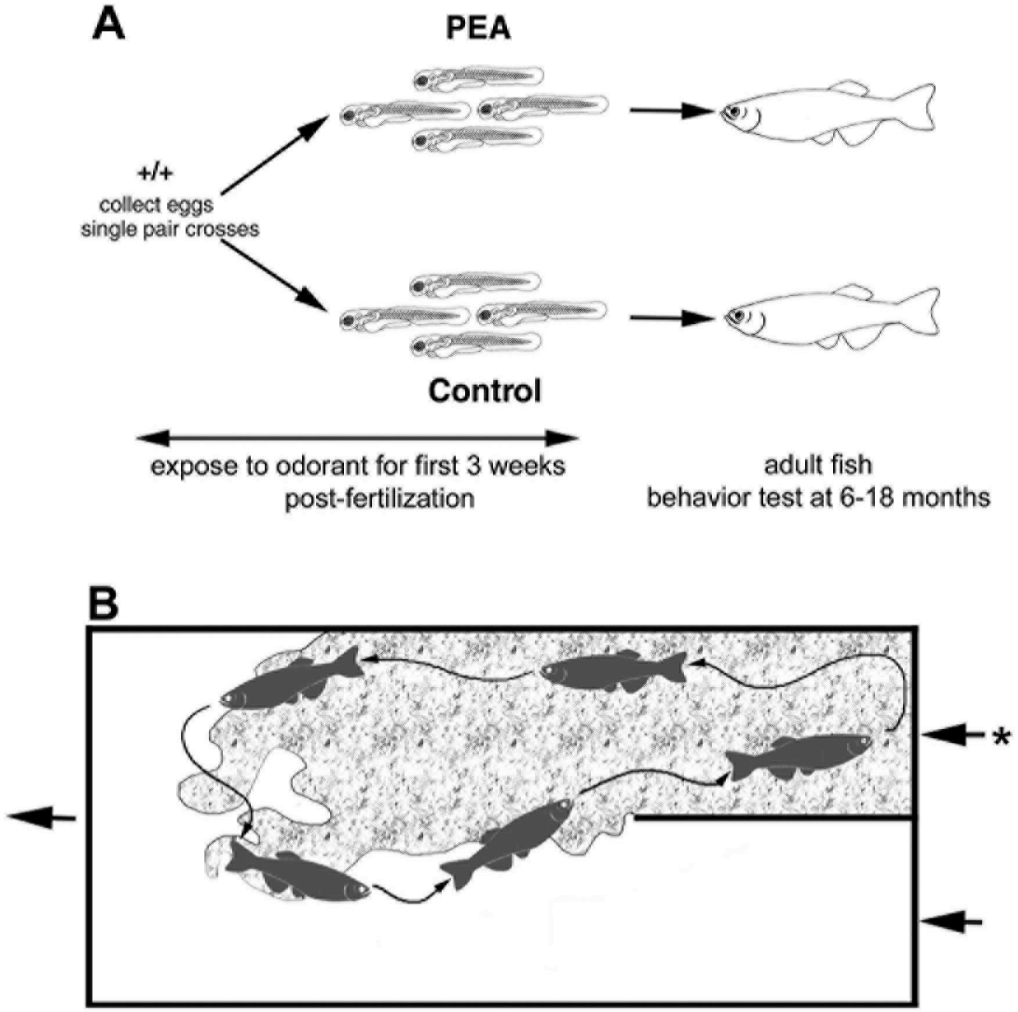
Behavioral analysis

Previous investigations using salmon have shown that the sensitive period for imprinting varies according to the species and ranges from the juvenile stage (while still in the chorion) through the metamorphosis-like parr-smolt transition (Quinn, 2005). Because the relevant developmental stage for imprinting in zebrafish is unknown, we exposed fish to PEA or distilled water from fertilization through three weeks of age, when they appear to undergo a metamorphosis-like change as judged by changes in pigment patterns (McClure, 1999). This time period encompasses the developmental time when the axons of the first olfactory sensory neurons arrive in the CNS (Hansen and Zeiske, 1993; Whitlock and Westerfield, 1998; Whitlock and Westerfield, 2000). After three weeks of odor exposure the fish were moved to larger tanks and allowed to grow to adulthood without further odorant exposure and tested in a Y-maze at ages ranging from 6-18 months (Figs. 3.1B, 3.2A).

Movement of fish in Y-maze

In observing the behavior of the fish in the Y-maze, they did not cluster in the PEA baited arm of the Y-maze, rather the fish moved in and out of the odor plume (Figs. 3.1B, 3.2 A). This is a behavior common to animals tracking odors much like a dog tracking a pheasant (Gibbons, 1986), or a moth tracking a pheromone (Willis and Arbas, 1991). In testing our fish, groups of either PEA treated or control fish (5-11 fish per group) were allowed to swim in the presence of a stream of system water plus PEA in one arm, and system water plus control water in the other. We then recorded their behavior over the course of four minutes (see Methods).

Figure 3.1. Description of behavioral imprinting paradigm in zebrafish. (A) Single clutches of wild type (+/+) embryos were collected, half of the embryos in each clutch were exposed to PEA and half to distilled water (control) for the first three weeks of development. Fish were then reared to adult in the absence of odorant and tested in a Y-Maze. (B) Fish track the odor plume within the Y-maze. In observing the movements of the zebrafish within the Y-maze they did not cluster at the site of odor entry (asterisk) rather they moved in and out of the odor plume (solid arrows).

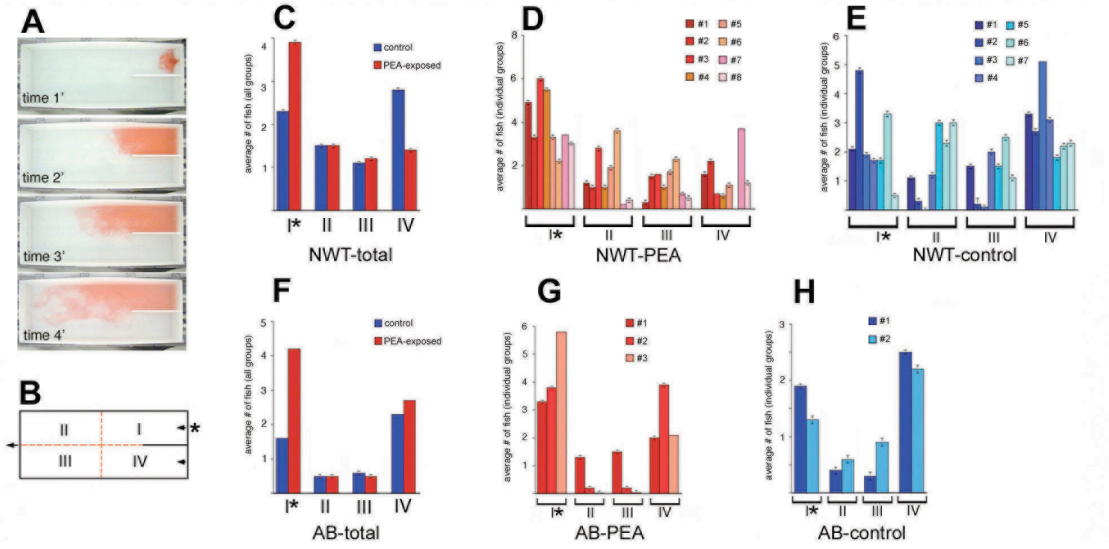

A
PEA
+/+
collect eggs
single pair crosses
Control
expose to odorant for first 3 weeks
post-fertilization
adult fish
behavior test at 6-18 months
B
*

Zebrafish exposed to PEA as juveniles prefer this odor as adults

We represented movements of the fish groups in the Y-maze graphically by recording the distribution of the individual fish within the group during the behavior trial and then plotting the average number of fish per quadrant summed over time (Fig. 3.2C-H). A summary of all NWT fish groups (Fig, 3.2C, n=15) and AB fish groups (Fig. 3.2F, n=5) shows that PEA-exposed fish (Fig. 3.2C, F, red) prefer the PEA-baited arm of the Y-maze (quadrant I, asterisk) when compared with control groups (Fig. 3.2C, F, blue). Each group of fish tested differed slightly in their distribution in the Y-maze (Fig. D, E, G, H). All fish show a natural preference for moving water which is why the average number of fish in quadrants I and IV (water inputs; Fig. 3.1B, 3.2B, arrows) is generally higher than quadrants II and III in controls.

In our statistical analysis of the data we found that the PEA-exposed fish were more likely to be found downstream of the odor ($p < 0.0001$). This effect was profound: a 95% confidence interval showed the proportion of PEA-exposed fish in the odor plume was greater than that for the non-PEA-exposed fish. We further analyzed the different strains: NWT strain (tested at 6-8 months of age, Fig. 3.2 C-E), and the AB strain, (tested at 18 months, Fig. 3.2 F-H). We found that the older fish (AB) were less likely than the younger fish (NWT) to be downstream of the odor ($p = 0.0067$). This effect was also pronounced: a 95% confidence interval showed that the proportion of younger fish (NWT) downstream of the odor plume was greater than that for the older fish (AB). The interaction between strain type and PEA-exposed/control fish was not statistically significant ($p = 0.5771$). Thus, zebrafish clearly remember the PEA odor to which they were exposed as juveniles and this memory may fade with age, although differences between inbred lines cannot be ruled out.

Figure 3.2. Zebrafish retain an olfactory memory of odorant experienced as juveniles. (A) Temporal dynamics of plume within Y-maze taken at 1 minute intervals visualized using food color. Food coloring was only added for visualizing the odor plume and was not added during behavioral trials. Two strains of wild type fish, AB and NWT, were used. (B) Video data were analyzed by dividing the Y-maze into quadrants, and counting the number of fish /quadrant every second [asterisk = quadrant with baited arm (I)]. (C-H), Average number of PEA-exposed (red) and control (blue) fish in each quadrant summed over 4 minutes ($\pm$S.E.M.). (C-E) NWT fish where (C) is all fish groups tested (n=15 groups) and (D, E), are the individual groups. (F-H) AB fish where (F) is all fish groups tested (n=5 groups) and (G, H) are the individual groups. Fish exposed to PEA as juveniles preferred the baited arm (I, with asterisk) over the control arm (IV). A modified analysis of covariance (see Methods) was performed on these data demonstrating that PEA-exposed fish were more likely to be found downstream (quadrants I and II combined) of the odor ($p < 0.0001$; see text).


A
time 1'
time 2'
time 3'
time 4'
B
II
I
*
III
IV
C
control
PEA-exposed
average # of fish (all groups)
I*
II
III
IV
NWT-total
D
#1
#2
#3
#4
#5
#6
#7
#8
average # of fish (individual groups)
NWT-PEA
E
NWT-control
F
AB-total
G
AB-PEA
H
AB-control

Gene expression in the developing olfactory epithelia

After the testing the adult fish in the Y-maze we collected OE from the control and PEA-exposed fish and performed an initial microarray analysis to look for changes in gene expression between the two groups. We found that a number of genes were up regulated in the PEA-exposed fish (Table 3.1). Because of the known role of the *otx* class of genes in olfactory sensory neuron development (Sagasti et al., 1999) we focused on the transcription factor *otx2* which was up-regulated 3.26 fold in the OE of the PEA-exposed versus control adult animals (Table 3.1). In order to localize the expression of *otx2* in the developing and adult zebrafish, we performed *in situ* hybridization using digoxigenin labeled mRNA probe (Whitlock and Westerfield, 2000) made from *otx2* cDNA. We found that in addition to the well-documented expression pattern in the midbrain (Li et al., 1994) this gene was expressed in the OE of the developing zebrafish starting at 24 hours post fertilization (h). To determine whether PEA odorant exposure affected *otx2* expression we repeated our imprinting paradigm with experimental (PEA-exposed) and control fish through three days of development (72h). Strikingly, the PEA-exposed fish showed a significant increase in the number of cells expressing *otx2* in the OE (Fig. 3.3, A-E). This increase was seen as early as 24h with 2-3 cells per OE expressing *otx2* in the PEA-exposed fish vs. <1 cell/OE in control fish (data not shown). The greater number of cells expressing *otx2* was maintained through 48h with an average of 12.6 cells expressing *otx2* in odor-exposed fish vs. 8.5 cells in controls (Fig. 3.3A, B, E). At 72h the overall number of cells expressing *otx2* decreased but the difference between PEA vs. control fish was maintained (5.9 cells vs. 3.6 cells in PEA vs. control,