

The olfactory placodes of the zebrafish form by convergence of cellular fields at the edge of the neural plate

Kathleen E. Whitlock^{1,*} and Monte Westerfield²

¹Field of Genetics and Development, Department of Molecular Biology and Genetics, 445/449 Biotechnology Building, Cornell University, Ithaca, NY 14853, USA

²Institute of Neuroscience, University of Oregon, Eugene, OR 97403-1254, USA

*Author for correspondence (e-mail: kew13@cornell.edu)

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SUMMARY

The primary olfactory sensory system is part of the PNS that develops from ectodermal placodes. Several cell types, including sensory neurons and support cells, differentiate within the olfactory placode to form the mature olfactory organ. The olfactory placodes are thought to arise from lateral regions of the anterior neural plate, which separate from the plate through differential cell movements. We determined the origins of the olfactory placodes in zebrafish by labeling cells along the anterior-lateral edge of the neural plate at times preceding the formation of the olfactory placodes and examining the later fates of the labeled cells. Surprisingly, we found that the olfactory placode arises from a field of cells, not from a discrete

region of the anterior neural plate. This field extends posteriorly to the anterior limits of cranial neural crest and is bordered medially by telencephalic precursors. Cells giving rise to progeny in both the olfactory organ and telencephalon express the *distal-less 3* gene. Furthermore, we found no localized pockets of cell division in the anterior-lateral neural plate cells preceding the appearance of the olfactory placode. We suggest that the olfactory placodes arise by anterior convergence of a field of lateral neural plate cells, rather than by localized separation and proliferation of a discrete group of cells.

Key words: *distal-less 3*, fate map, lineage tracing, telencephalon

INTRODUCTION

In vertebrates, some derivatives of the peripheral nervous system (PNS), including primary sensory neurons of the inner ear and the olfactory epithelium, develop from specialized tissues called placodes. Placodes are thought to arise from cells along the lateral edge of the anterior neural plate lying between neural and non-neural ectoderm (Landacre, 1910; Verwoerd and van Oostrom, 1979; Northcutt and Gans, 1983; Northcutt, 1996). Placodes of the PNS appear as distinct structures once the neural tube has formed, yet the morphogenetic movements leading to their formation are still unclear.

Although many studies have described the development of the olfactory sensory system after the appearance of the placodes (Farbman, 1988, 1992; Hansen and Zeiske, 1993), few studies have examined the processes leading to the formation of the olfactory placodes. The currently accepted hypothesis is that the olfactory placodes arise from groups of cells that separate from the lateral edge of the anterior neural plate through differential cell movements (Verwoerd and Van Oostrom, 1979; Farbman, 1992). Later, these isolated groups of cells proliferate to give rise to the olfactory placodes. Although fate-map studies in the chick support this hypothesis that the olfactory placodes arise from the neural ridge (Couly and Le Douarin, 1985), isolated groups of cells lying next to the anterior neural plate have not been described. Thus, it is

still unclear whether a group of cells separates from the edge of the anterior neural plate to form each olfactory placode or whether the olfactory placodes develop within the anterior neural plate.

To understand the cellular events leading to placode formation, we have investigated the development of the olfactory placodes in the zebrafish, *Danio rerio*, where we can follow the fates of individual cells in the neural plate. In the zebrafish, the olfactory placodes appear as thickenings of the ectoderm by 17-18 hours after fertilization (h) and these thickenings later invaginate to form the naris, or opening of the nose, by 32 h (Hansen and Zeiske, 1993). Previously we have shown that the first axons projecting from the olfactory placodes reach the central nervous system (CNS) by 22 h. These early axons extend from pioneer neurons and establish the initial pathway later followed by the axons of the olfactory sensory neurons (Whitlock and Westerfield, 1998). The pioneer neurons arise in the anterior end of the neural plate from a distinct lineage that does not include olfactory sensory neurons (Whitlock and Westerfield, 1998). Thus, by the 4-somite stage (12 h), olfactory fates are already regionalized within the developing neural plate.

To understand how the placodes form, we injected single cells with lineage tracer dyes in the anterior end of the neural plate prior to the morphological appearance of the olfactory placode and followed the fates of these cells until after the

olfactory placode formed. Surprisingly, we found that a large field of cells widely distributed along the edge of the anterior neural plate contributed to the olfactory placode. This field of cells is regionalized such that cells in specific anteroposterior locations give rise to specific parts of the developing olfactory organ. We found that cells adjacent and medial to this olfactory placode field give rise to the telencephalon. This telencephalic field is also regionalized along the anteroposterior axis, such that anterior olfactory bulb arises from cells adjacent to the region from which the first sensory neurons derive. Thus, in contrast to the hypothesis that the olfactory placodes arise from groups of cells that separate from the neural plate, we show here that the placodes arise from large fields of cells along the anterior-lateral neural plate. We suggest that they form by anterior convergence of these cellular fields.

MATERIALS AND METHODS

Animals

Zebrafish embryos were obtained from natural spawnings of laboratory strains of wild-type fish (AB; Walker, 1998). Fish were staged by morphology (Kimmel et al., 1995) and developmental times are expressed as hours postfertilization at 28.5°C (h).

Cell labeling and lineage tracing

Embryos at the 4- to 5-somite stage (approx. 12 h) were embedded in agar with a window cut to expose the lateral edge of the neural plate. These blocks were mounted on a microscope slide and covered with physiological saline. Microelectrodes (approximately 300 M Ω resistance) were pulled (Sutter Instruments) and backfilled with a mixture of Rhodamine dextran dye (2%; M_r 3000, Molecular Probes) and Fluorescein dextran dye (2%; M_r 3000, Molecular Probes) in 0.2 M KCl. Cells were visualized with a 40 \times water immersion lens using a fixed-stage microscope (Zeiss). After penetration of a single cell, dye was passed into the cell by 'ringing' the electrode using capacitance compensation and an oscilloscope to measure the amplitude and duration of the ringing. Successful filling of the cell was judged by a rapid check of the Rhodamine fluorescence. Preparations for lineage tracing were allowed to develop to the desired age and then fluorescent images were collected on a confocal microscope (Zeiss) and reconstructed using the Voxel View and Voxel Math programs (Vital Images). Non-fluorescent images were obtained by using the anti-Fluorescein antibody (Boehringer Mannheim, 1:1000) coupled with the alkaline phosphatase Fast Red (Boehringer Mannheim, red) or NBT/BCIP (Boehringer Mannheim, blue). In general, only one side of the embryo was labeled in the lineage tracing experiment, except when labeling the pioneer neurons. To label cells in the region of the pioneer neurons, animals were mounted dorsal side up and approached from the anterior end. This made it possible to label cells on both sides of the neural plate so these preparations had two placodes to score per animal. The fate map was constructed from the results of 175 successful single cell labels out of 468 cells attempted representing an overall success rate of 37%, although on different days the success rate varied greatly. The average clone size was 2.8 (s.d. \pm 1.0) cells with a range of 2-5 cells.

Double RNA in situ hybridization

Cells at the edge of the neural plate and subsequently in the developing olfactory placode (Ekker et al., 1992; Akimenko et al., 1994) and in the developing forebrain (Papalopulu and Kintner, 1993) express the gene *distal-less 3* (*dlx3*). Premigratory cranial neural crest cells express the *forkhead 6* gene (*fkh6*; Odenthal and Nüsslein-Volhard, 1998). RNA probes generated with template for the zebrafish

dlx3 and *fkh6* genes were used for in situ hybridization. RNA in situ hybridization was performed using digoxigenin (DIG)-labeled RNA probes (Thisse et al., 1993) for the *dlx3* gene and fluoresceinated probes for the *fkh6* gene. The probe colors were also reversed to compare results. We found that it is better to label the weaker probe with DIG because it gives a stronger signal. The NBT/BCIP blue coloration was performed first to prevent 'blueing' in the subsequent red coloration. The reaction was stopped, the preparations rinsed once in dH₂O, rinsed in high pH buffer (pH 8.2), rinsed once in dH₂O, the probe stripped (0.1 M glycine, pH 1.8), rinsed 3 \times in PBS, and then followed by the Fast Red reaction. After rinsing in PBS, the preparations were postfixed in 4% paraformaldehyde. Again the signal from the NBT/BCIP blue coloration was generally stronger than that from the Fast Red coloration.

Gene expression and single-cell labeling

We correlated the *dlx3* expression domain with our lineage tracing

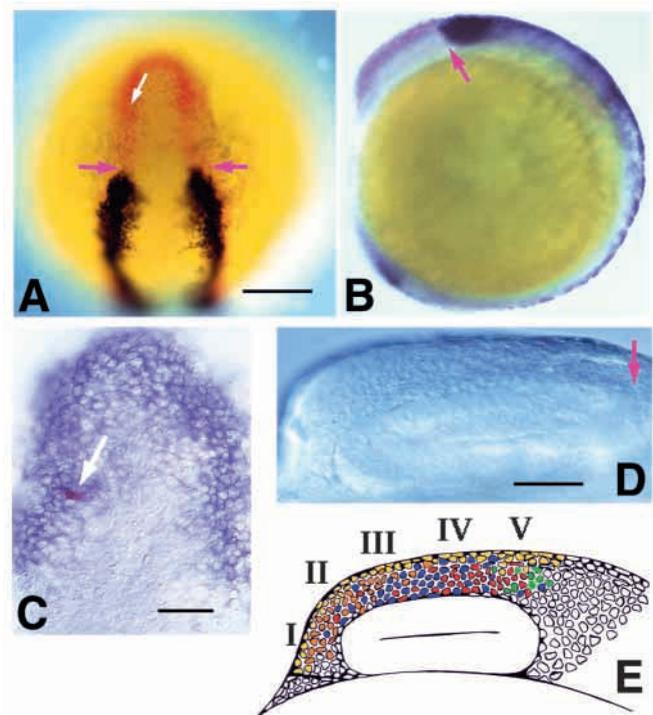


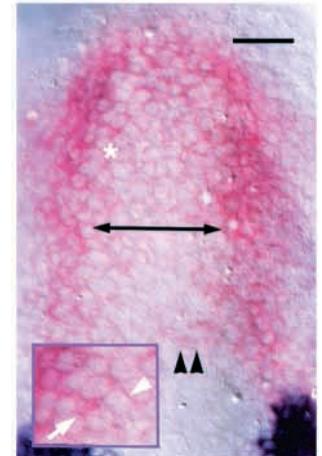
Fig. 1. The *dlx3* and *fkh6* gene expression domains subdivide the anterior neural plate. Whole-mount RNA in situ hybridizations and single-cell labeling at the 4- to 5-somite stage (12 h). (A,B) Embryo double labeled for *dlx3* (red) and *fkh6* (blue) gene expression. The field giving rise to the olfactory placode expresses high levels of *dlx3*. Pink arrows indicate posterior edge of developing eye at 12 h. (A) White arrow indicates posterior region shown in higher magnification in C. Dorsal view with anterior toward top of page. (B) Lateral view with anterior toward the left margin of same embryo shown in A. (C) A preparation double labeled for lineage tracer dye in red (white arrow) and then immediately processed for in situ hybridization to show *dlx3* expression (blue). Dorsal view of 12 h embryo, anterior is toward the top of the page. (D) Lateral view of live embryo at 12 h, anterior is to the left. Embryos were mounted in this orientation for single-cell labeling. Arrow indicates posterior edge of developing eye. (E) Cells color coded in neural plate with respect to future fates in the olfactory organ: Red, orange, green and blue cells contributed to the olfactory organ, yellow cells gave rise to clones in the telencephalon. Roman numerals I-V indicate regions along the anteroposterior axis within the neural plate. Scale bars: A,B, 250 μ m; C, 50 μ m; D,E, 80 μ m.

fate map by combining lineage tracing labeling and in situ hybridization using the *dlx3* mRNA probe. Embryos were fixed immediately after injecting a single cell, rinsed, heat blocked for 1 hour at 70°C, incubated in blocking solution (PBDT: 0.1 M phosphate buffer, 2% bovine serum albumin, 1% dimethylsulfoxide [DMSO] and 0.5% Triton X-100 with 2% normal goat serum) and processed with alkaline phosphatase anti-Fluorescein antibody and reacted with Fast Red. After the reaction was complete, the embryos were rinsed, postfixed and processed for *dlx3* gene expression (see preceding section). In this way, we could align our single cell labeling with the expression domain of the *dlx3* gene.

Cell division immunocytochemistry

Embryos were collected and maintained at 28.5°C in embryo medium (13.7 mM NaCl, 0.54 mM KCl, 1.3 mM CaCl₂, 1.0 MgSO₄, 0.044 mM KH₂PO₄, 0.025 mM NaH₂PO₄, and 0.42 mM NaHCO₃ at pH 7.2). They were allowed to develop until 6- (approx. 12 h), 10- (approx. 14 h), 14- (approx. 16 h), and 18- (approx. 18 h) somite stages and 20 embryos from each stage were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer with 0.15 mM CaCl₂ and 4% sucrose (pH 7.3). After rinsing 2× in PBST, embryos were preabsorbed in 2% normal goat serum (NGS) in PBDT (0.1 M phosphate buffer, 2% bovine serum albumin, 1% dimethylsulfoxide [DMSO] and 0.5% Triton X-100). Animals were incubated with anti-phosphorylated Histone H3, (Upstate Biotechnology, NY) 1:800 in PBDT+2%NGS overnight at 4°C, which labels cells in M phase (Henzel et al., 1997), then rinsed 3× over 2 hours and placed in goat anti-rabbit secondary antibody, (Sternberger Monoclonals Inc.) at 1:200. After a series of washes, embryos were incubated in peroxidase-rabbit anti-peroxidase complex (Sternberger Monoclonals Inc.) overnight at 4°C. Embryos were then rinsed in 0.1 M phosphate buffer and 1% DMSO 3× for 1 hour. The coloration reaction was done using 0.02% DAB (diaminobenzidine) in 1:1 dH₂O/PBS with 1% DMSO and 0.003% hydrogen peroxide. The preparations were processed through a glycerol series (30%, 50%, 70%, 90%, glycerol: 0.1 M phosphate buffer), mounted in 100% glycerol and viewed with Nomarski optics.

Fig. 2. The intensity of *dlx3* expression differs in medial and lateral domains in the anterior neural plate. Flattened whole-mount preparation of anterior neural plate from a 12 h embryo hybridized with a probe to *dlx3*. Expression at the edge of the neural plate is higher than that in the center (double-headed arrow). This difference in levels of expression is evident at higher magnification (inset from region with asterisk; white arrow, lateral; white arrowhead, medial). Double arrowheads mark posterior border of medial expression domain of *dlx3*. Scale bar, 50 μm.



RESULTS

The *dlx3* and *fhk6* gene expression domains subdivide the anterior neural plate

Initially, we used morphological landmarks visible in live embryos to define cellular fields and then we correlated the positions of these fields with the gene expression domains of *dlx3* and *fhk6*. In the zebrafish, cells thought to form the olfactory and auditory placodes express *dlx3* at early segmentation stages (Akimenko et al., 1994; Ekker et al., 1992). At the 4- to 5-somite stage (12 h), a time prior to the morphological differentiation of the olfactory placode, cells in a broad, lateral stripe extending around the anterior end of the neural plate to the posterior edge of the eyes express *dlx3* (Fig. 1A,B, red). In addition, cells in the more medial part of the

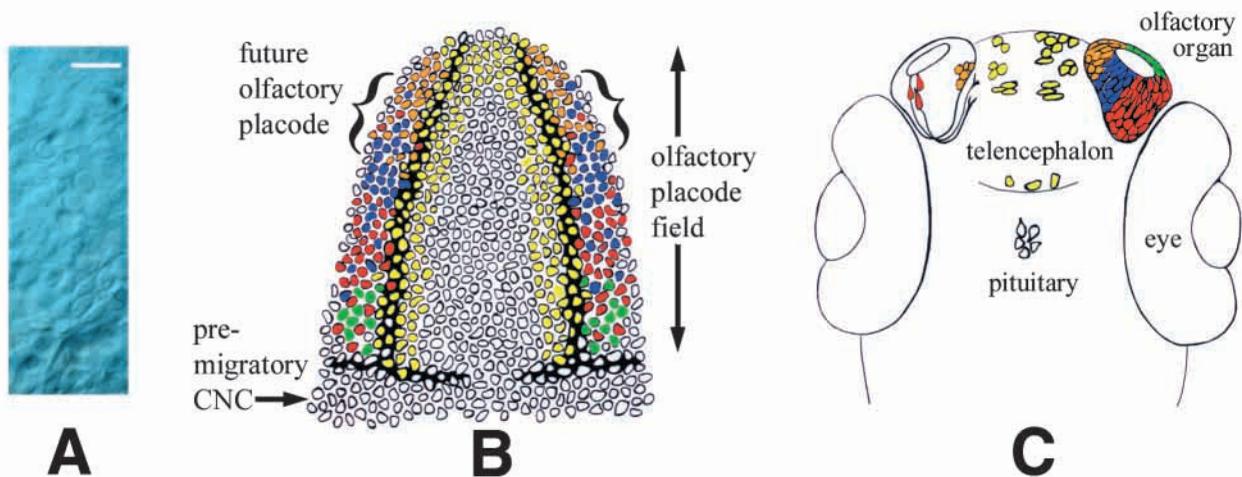


Fig. 3. Lineage tracing in the neural plate at 4-5 somites (12 h) shows fates of cells at 50 h in the olfactory organs and telencephalon. (A) Olfactory placode field domain viewed under Nomarski optics. Dorsal view with anterior towards the top of the page. Scale bar, 20 μm. (B) Schematized dorsal view of the anterior end of 12 h embryo with anterior towards the top of the page. The blackened border extending along the anteroposterior axis approximately represents the border between the fields of cells giving rise to olfactory organ and those giving rise to telencephalon. The transverse blackened border represents the anterior limits of premigratory cranial neural crest. The cells are color coded with respect to their future fates in the developing olfactory organ and bulb at 50 h. (C) Schematized ventral view of a 50 h zebrafish head with anterior towards the top of the page. Right-hand olfactory organ: the olfactory organ has been subdivided into four colored areas. Clones in these areas were color coded in diagram B. Left hand olfactory organ: sensory neurons with axons extending into the CNS at 50 h come predominantly from red cells, pioneer neurons come from orange cells but only in the region abutting the telencephalic field anteriorly. The pituitary clones come from the non-colored cells most anterior in the neural plate between the two blackened borders.

anterior neural plate express low levels of *dlx3* (Figs 1A, 2). This difference in the intensity of *dlx3* expression at the edge versus the center of the developing neural plate was observed in 1 μ m confocal optical sections through the anterior neural plate (data not shown) suggesting that it is due to differences in the level of expression, not to the thickness of the layer of cells expressing *dlx3* in these regions. As the olfactory placode becomes morphologically distinct (by 17 h) expression of *dlx3* is localized within the region of the placode with weaker expression in the developing telencephalon.

Cells of the premigratory neural crest express the gene, *fhk6* (Odenthal and Nüsslein-Volhard, 1998), and by the 4- to 5-somite stage, the anterior limit of *fhk6* expression (Fig. 1A,B, blue) aligns with the posterior border of the broad *dlx3* expression domain (Fig. 1A,B, red). The interface of these two expression domains also corresponds to the posterior border of the developing eye (Fig. 1A,B,D, pink arrow). Thus the locations of cells expressing *dlx3* and *fhk6* at the 4- to 5-somite stage can be predicted based on their positions relative to the developing eyes. Posterior to the developing eyes and lateral to the *fhk6* domain, a strip of cells approximately one cell diameter wide expresses *dlx3*. This expression domain extends posteriorly and broadens in the area where the ear will form.

Olfactory organ arises from a large field of cells at the anterior lateral neural plate

To define the region of the neural plate giving rise to the olfactory placode, we constructed a fate map of cells in the anterior neural plate (Fig. 3A) at the 4- to 5-somite stage (12 h: Fig. 1E, lateral view; Fig. 3B, dorsal view). In a lateral view at the 4- to 5-somite stage (12 h), the outline of the developing eye can be seen in the live embryo (Fig. 1D). Using this border as a landmark, we subdivided the field of cells extending from the anterior end of the neural plate into five regions where region I is the area at the anterior end of the neural plate and regions II-V lie medial to the developing eye field at

progressively more posterior positions (Fig. 1E). Each region was approximately eight cell diameters along the anteroposterior axis. At 12 h (4-5 somites), we labeled single cells in the region extending from the anterior end of the neural plate to the posterior edge of the developing eye (Fig. 1D, arrow). We allowed the labeled embryos to develop until 50 h and examined the positions and morphologies of the clones resulting from the injected cells.

We scored the fates of the progeny of the injected cells and color-coded them according to fate on a diagram of the anterior neural plate (Figs 1E, 3B,C). The lateral edge of the neural plate (Fig. 3B, border between medial and lateral is depicted by the heavy black shading extending along the anteroposterior axis) gave rise to cells in the olfactory organ. This domain of cells always giving rise to fates in the olfactory organ extends an average of four cell diameters (cell diameter approx. 12 μ m) medially from the edge of the developing eye (Fig. 1D), and strongly expresses *dlx3* (Fig. 1C). This 'olfactory organ domain' (Fig. 3B, multicolor) extended posteriorly to the premigratory cranial neural crest domain, indicated by the anterior border of *fhk6* expression (horizontal heavy black shading, Fig. 3B).

Cells in different regions of the olfactory domain of the neural plate contributed to characteristic regions of the olfactory organ. The anterior neural plate gave rise to the anterior, medial part of the olfactory organ (Fig. 3B,C, orange) whereas the lateral region, lying next to the eye, arose from more posterior cells in the neural plate (Fig. 3B,C, red). The most posterior region of the olfactory organ domain gave rise to the most dorsal region of the olfactory organ (Fig. 3B,C, green). This general spatial organization was consistent from embryo to embryo. There were no sharp borders and cells were somewhat intermixed with respect to their future fates (Fig. 3B), although this may result from slight differences when trying to match positions in different embryos.

Cells lying medially more than four cell diameters from the

Table 1. Cells labeled at the 4- to 5-somite stage produce clones of cells of distinct cell types

	Olf. Neuron¶	Structure	Support	Telencephalon	Pituitary	Total
REGION I						
medial	0	0	0	11 (vent. telen)	0	11
lateral	3	12	0	5	4	24
REGION II						
medial	5*	2	1	6 (vent. telen.)	0	14
lateral	22*	0	3	0	0	25
REGION III						
medial	0	0	0	5 (vent. telen.)	0	5
lateral	21	0	2	1	0	24
REGION IV						
medial	0	0	0	11§ (ant. tel.)	0	11
lateral	24§	0	0	1	0	25
REGION V						
medial	0	5	0	12 (dor. tel.)	0	17
lateral	13	5	1	0	0	19
						n _t =175

NEURONAL CLONE SIZES:

Olfactory sensory and telencephalon=2.8±1.0

Pioneer neurons 1.0±0

*Regions containing pioneer neuron precursors.

§Regions giving rise to first neurons extending axons.

¶The 88 clones in this column had the following phenotypes: 64 with no axons, 13 with axons, 9 pioneers, 2 ventral cells with axons.

eye, (Fig. 2, line with double-head arrow) gave rise to progeny in the telencephalon and showed lower *dlx3* expression. This telencephalic domain (Fig. 3B,C, yellow) was organized so that the most anterior region gave rise to the most ventral posterior region of the telencephalon near the postoptic commissure whereas the most posterior medial neural plate contributed to dorsal telencephalon (Table 1). Like the olfactory domain, the telencephalic domain in the anterior neural plate extended posteriorly to the premigratory cranial neural crest domain (Fig. 3B). This fate map demonstrates that the olfactory placode and the telencephalon develop from distinct cellular fields at and adjacent to the edge of the neural plate.

Different olfactory cell types arise from distinct regions of the anterior neural plate

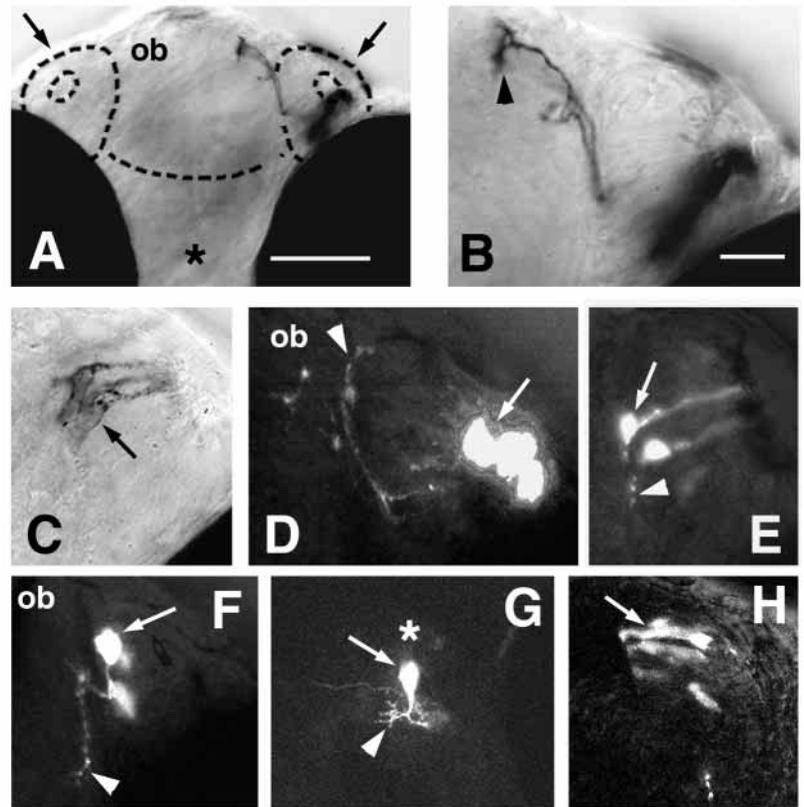
The injected cells in the lateral domain of the neural plate gave rise to clones of progeny primarily in the olfactory organ (Table 1). Whole-mount preparations were viewed from the ventral side (Fig. 4A) because this was the best orientation from which to see the entire olfactory organ at 50 h. Clones containing sensory neurons with axons that were the first to extend into the developing olfactory bulb, arose from progenitors in the area posterior to where the nose placode later arose (Table 1, region IV; Figs 1E, 3B,C, red cells). The cell bodies of these neurons lay laterally within the olfactory organ and their axons extended into the region of the developing olfactory bulb (Fig. 4B, arrowhead). The neurons in the anterior region of the olfactory organ at 50 h (Fig. 4C) arose primarily from region II (Figs 1E, 3B,C, orange; Table 1). Clones of cells in the dorsal olfactory organ came primarily from cells in region V (Figs 1E, 3B,C, green; Table 1). In general, the majority of labeled neurons (excluding pioneer neurons) in the olfactory organ had

cell bodies and dendrites, but no axons by 50 h (51/64; Table 1). The few neurons with axons (Fig. 4A,B,D) had projections that turned dorsally in the developing olfactory bulb (Fig. 4D, arrowhead). With the exception of pioneer neurons, cells with axons always had dendrites. Thus, the common pattern was to extend dendrites first, then axons later (Fig. 4E). Occasionally we observed a cell type in the extreme ventral part of the nose, which we termed 'ventral neuron' ($n=2$; Table 1; Fig. 4F). The axons of these ventral neurons grew dorsally to meet the olfactory nerve and then abruptly turned posteriorly and extended in the anterior commissure (Fig. 4F, arrowhead). The most anterior part of region I of the *dlx3* domain gave rise to structural elements of the nose and ethmoid plate, and neurons in the pituitary (Fig. 4G), but rarely ($n=3/24$) to olfactory neurons. Also, cells scattered throughout the lateral *dlx3* expression domain produced support cells of the olfactory epithelium. These cells generally extended processes from the apical to basal borders of the olfactory epithelium and were fairly uniform in width throughout their length (Fig. 4H, arrow).

Cells lying adjacent to the olfactory placode field contribute to the telencephalon

To determine how far medially the field of cells giving rise to the olfactory organ extended, we labeled single cells (Fig. 5A, red) more medially, in an area where the intensity of *dlx3* gene expression is lower (Fig. 2, double-headed arrow; Fig. 5A). Cells in this medial domain gave rise to progeny that populated the telencephalon, including the developing olfactory bulb (Table 1). In region II (Fig. 1E), they gave rise to progeny in the ventral telencephalon near the anterior commissure, whereas cells labeled in region III (Fig. 1E) contributed to the

Fig. 4. Single cells in the lateral domain labeled at 12 h give rise to clones in the olfactory organ and anterior pituitary. All preparations viewed from the ventral side (anterior toward the top of the page). (A) Olfactory organs are paired structures (arrows) with the developing olfactory bulbs (ob) lying in the CNS between the two olfactory organs at 50 h. The anterior commissure (dashed line) lies posterior to developing bulbs and the postoptic commissure (asterisk) further posterior between the eyes. (B) Higher magnification (from A) of clone of cells in the olfactory organ with axons (arrowhead) terminating in the developing olfactory bulb. (C) Clone in anterior region of the olfactory organ (arrow) with dendrites extending toward the naris. (D) Clone of cells (arrow) in dorsal part of the nose with axons extending into the developing olfactory bulb (arrowhead). (E) Clone of cells in ventral medial part of the olfactory organ (arrow) with dendrites extending toward the naris and axons (arrowhead) initiating extension towards the CNS. (F) Ventral cell with cell body lying in the extreme ventral part of the olfactory organ (arrow) and an axon exiting the olfactory organ then turning into the anterior commissure (arrowhead). (G) Clone of anterior pituitary cells lying posterior to the level of the postoptic commissure (asterisk). The most ventral cell (arrow) with extensive processes (arrowhead), a second cell lies behind. (H) A clone of support cells (arrow) in the olfactory organ. Scale bars: A, 75 μm ; B-H, 25 μm .



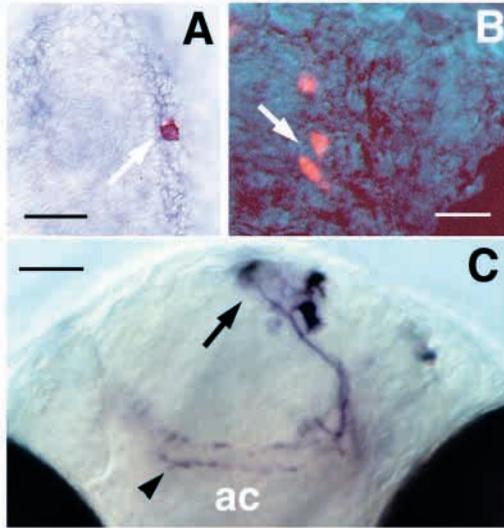


Fig. 5. Single cells labeled in the medial domain give rise to clones in the telencephalon. (A) Dorsal view of a 12 h embryo (anterior towards the top of the page). A single cell (red) labeled (arrow) in the medial domain expressing *dlx3* (blue). The cell is slightly swollen, a phenotype often observed immediately after dye injection. (B,C) Ventral views of 50 h embryo with anterior toward top of the page. (B) A clone of cells (red) in the ventral telencephalon of a living embryo at 50 h. The cells arose from a single cell labeled more anterior to that shown in A. (C) A clone of interneurons whose cell bodies (black arrow) lie in the anterior olfactory bulb and axons (arrowhead) extend into the anterior commissure (ac). This clone arose from a single cell labeled at the position shown in A. Scale bars: A, 50 μm ; B, 25 μm ; C, 75 μm .

ventral half of the developing olfactory bulb. Cells labeled medially in regions I, II, III and V of the neural plate lacked axons at 50 h (Fig. 5B, arrow). In contrast, cells labeled in region IV (Table 1, medial; Fig. 5C, arrow) produced clones with axons, allowing us to identify them positively as interneurons. These interneurons had cell bodies in the anterior developing olfactory bulb (Fig. 5C, arrow) and axons that extended in the anterior commissure to the contralateral side (Fig. 5C, arrowhead). With Nomarski optics, there was no apparent morphological border within the neural plate between cells giving rise to olfactory organ and cells giving rise to telencephalon. Rather, there was an apparent mixing of cells in a transition zone where the probability of labeling a cell giving rise to telencephalon increased as cells were labeled more than four cell diameters from the edge of the developing eye. However, a single labeled precursor never gave rise to a clone of mixed fates i.e. olfactory organ and telencephalic cells.

Pioneer neurons are postmitotic by 12 h

Previously we described pioneer neurons in the zebrafish olfactory system that arise from a specific region of the neural plate and establish the initial axon pathway to the CNS (Whitlock and Westerfield, 1998). We present here additional data showing the initial outgrowth of the axons of the pioneer neurons as they exit the placode and the sizes of clones generated from their precursors in the neural plate. Clones derived from single cells labeled in the region of the neural plate that gives rise to pioneer cells (Whitlock and Westerfield,

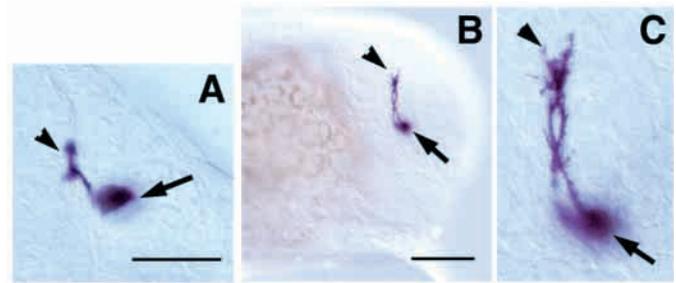


Fig. 6. Pioneer neurons extend axons without forming dendrites. Single cells labeled in the pioneer domain at 12 h and scored at 22–38 h. (A) Ventral view with anterior towards the top of the page. (B,C) Lateral view with anterior to the right. (A) Single pioneer scored at 22 h in the olfactory placode (arrow) with an axon exiting the olfactory placode and starting to enter the telencephalon (arrowhead). (B) Clones of pioneer cells (two cells were labeled) at 28 hours. Two cell bodies lie in the ventral anterior placode (arrow) and extend into the developing CNS (arrowhead). (C) Higher magnification view of A showing the very bushy growth-cone-like appearance of the axons in the CNS (arrowhead). Scale bars: A,C, 40 μm ; B, 25 μm .

1998; Table 1, region II; Fig. 1E) included neurons with large cell bodies (Fig. 6, arrows) and axons extending into the developing olfactory bulb by 28 h (Fig. 6, arrowheads). In 22 h preparations, we observed initial axon outgrowth from the pioneer neurons (Fig. 6A). As a pioneer axon grew out to meet the telencephalon, it had a large simple growth cone (Fig. 6A, arrowhead) that lacked the fine processes evident later, after it entered the region of the developing olfactory bulb (see Fig. 6B,C, arrowheads). Upon closer inspection, the axons of these older cells were very ‘bushy’ in appearance (Fig. 6B,C, arrowheads). In all cases, the cell bodies of pioneer neurons were large, round and devoid of dendrites (Fig. 6, arrows) and extended a single axon out of the olfactory placode.

Pioneer neurons developed from neural plate precursors without further cell division. The cell depicted in Fig. 6A was labeled at 12 h and scored at 22 h after it had sprouted an axon. The two cells depicted in Fig. 6B,C resulted from labeling two adjacent cells at the 4- to 5-somite stage. When scored at 28 h there were still only two cells and the cells had extended axons. This was true for all pioneer precursors ($n=9$) labeled in this study (see Table 1). This indicates that the cells labeled at 12 h were postmitotic and differentiated directly into pioneer neurons. In contrast, neural plate cells that gave rise to telencephalic or olfactory sensory neurons produced clones with an average size of 2.8 cells (s.d. \pm 0.99) indicating continued proliferation before differentiation.

The olfactory placode forms in the absence of regionalized pockets of cell division

Localized cell division is another mechanism that could potentially contribute to the formation of the olfactory placode. We examined patterns of cell division in the 6 hours (12–18 h) prior to the morphological appearance of the olfactory placodes. To do so, we collected and fixed animals at 12 h (4–6 somites), 14 h (10 somites), 16 h (14 somites) and 18 h (18 somites) stages, and then assayed them for cell division using an antibody that recognizes dividing cells. At 12 h, there is very little cell division in the anterior neural plate (Fig. 7A). At 14

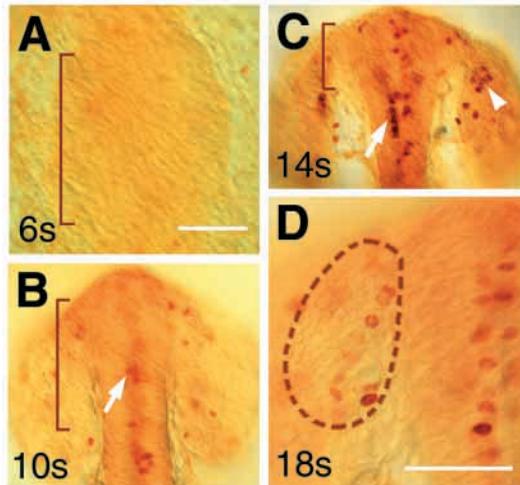


Fig. 7. Cells undergoing mitosis are rare in the 6 hours preceding the appearance of the olfactory placode (17–18 h) as assayed with anti-phosphorylated Histone H3 antibody. All views are dorsal with anterior towards the top of the page. Brown bracket marks developing olfactory field region. (A) Anterior neural plate of 12 h (6 somite) embryo showing no cell division in the region of the olfactory field. (B) At 14 h (10 somites), cell division is evident in the ventricular region (white arrow). (C) Cell division has increased at 16 h in both the ventricular region (white arrow) and the developing eye region (white arrowhead). (D) The olfactory placode is evident (dashed outline) at 18 h (18 somites) and dividing cells are seen within the olfactory placode. Scale bars: A–C, 75 μ m; D, 40 μ m.

h (10 somites), there is cell division in the forming ventricle (Fig. 7B, arrow), but few cells label in the developing olfactory placode field (Fig. 7B, brown bracket). At 16 h (14 somites), dividing cells continue to be localized in the developing ventricles (Fig. 7C, arrow) and, in addition, the developing eyes (Fig. 7C, arrowhead). Still no localized cell division is observed in the region of the developing olfactory placode (Fig. 7C, brown bracket). With Nomarski optics, the olfactory placode is apparent at 17–18 h in the living embryo. At this time, cell division is observed within the formed olfactory placode (Fig. 7D, dashed outline). Therefore, from 12 h through 18 h, the time period preceding the formation of the olfactory placodes, we found no evidence of extensive cell division in the regions where the olfactory placodes will form.

DISCUSSION

The olfactory placode develops within the neural plate, not in the overlying ectoderm

Early studies of the developing olfactory placode suggested that the placode develops from ectoderm adjacent to the neural plate (Zwilling, 1940; Emerson, 1944, 1945). Transplantation experiments in amphibians further suggested that the olfactory placodes arise as a result of an inductive interaction between the neural plate and the overlying ectoderm (Haggis, 1956) with the placodes arising from the ectoderm rather than the neural plate. In contrast, more recent studies using serial reconstruction of staged mouse embryos (Verwoerd and Van Oostrum, 1979) suggested that the olfactory placodes arise from the neural plate rather than from overlying ectoderm.

In support of this hypothesis, chick-quail chimeras and transplantation experiments (Couly and Le Douarin, 1985, 1987) showed that the anterior-lateral region of the neural plate contributes to the olfactory placodes. Our data in zebrafish (Whitlock and Westerfield, 1998; present study) further support the hypothesis that the olfactory placodes arise from the neural plate and are not induced from overlying ectoderm as the neural tube forms. Moreover, our results show no evidence for distinct developmental events where neural plate gives rise to the telencephalon and adjacent non-neural ectoderm gives rise to olfactory placode. Rather, these two regions of the developing nervous system, one central and the other peripheral, arise from adjacent fields of cells within the neural plate.

The nose arises from convergence of a large field of cells with regionalized cell fates

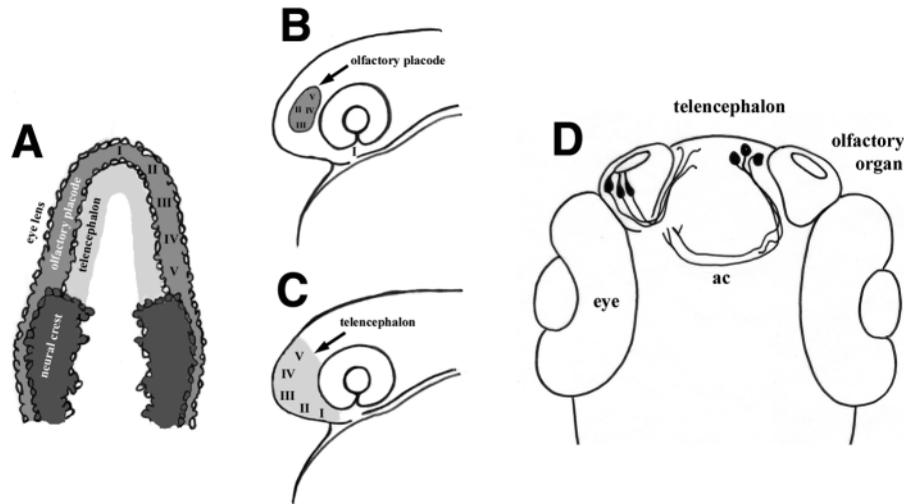
Currently, the favored model for the origin of the olfactory placode (Farbman, 1992) proposes that a piece of tissue separates from the neural plate through differential cell movements and gives rise to the olfactory placode. In contrast, our data suggest that the olfactory placode develops through convergence of a field of cells within the neural plate rather than from a small piece of detached neural plate. By lineage tracing, we found that olfactory placode precursor cells occupy a long narrow band, about four cells wide, at the 4- to 5-somite stage (12 h) and, by 17–18 h, these cells have converged to form an obvious placodal structure. The field of olfactory placode precursor cells in the neural plate is continuous with, and adjacent to, the field of cells that later form the telencephalon. As the olfactory placode field converges anteriorly, it maintains regionalized cell fates such that cells in the anterior olfactory placode field contribute to the anterior medial part of the olfactory organ and posterior cells form the lateral part of the nose (Fig. 8A,B).

Previous time-lapse video analysis of zebrafish development (Karlstrom and Kane, 1996) revealed a significant anterior movement of cells during early segmentation stages in the developing head. Part of this movement is due to the CNS moving forward, and part to the cranial neural crest cells migrating forward to populate the frontal mass. We propose that the anterior convergence of the olfactory placode field is part of this movement, and that this morphogenesis brings about the formation of the olfactory placodes.

The telencephalon arises from neural plate cells lying medial to the olfactory placode field

Our fate map also shows that cells medial to the olfactory placode field along the anterior edge of the neural plate contribute to the telencephalon. Many cells that we labeled in this region produced clones of labeled cells within the developing olfactory bulb. The positions of cells in the medial anterior neural plate at 12 h are indicative of their future fates within the developing telencephalon. Thus, cells lying most posteriorly populate the dorsal telencephalon, whereas those lying very anteriorly populate the most ventral, posterior telencephalon (Fig. 8A,C). The final positions of these clones result from both movements of the posterior cells anteriorly (Karlstrom and Kane, 1996) and flexure of the brain (Kimmel et al., 1995) that brings cells lying ventral-anterior to a ventral-posterior position. These results suggest that the telencephalon

Fig. 8. Summary diagram of regionalized fates of olfactory placode and telencephalic fields. (A) Schematized anterior neural plate where dark gray is the premigratory neural crest domain, medium gray is the olfactory placode field and the light gray the telencephalic field. The roman numbers depict the regions I-V described in the text. (B) The olfactory placode at 24 h, depicted in medium gray, arises from the edge of the developing neural plate with positions II-V marked within the placode. Cells from region I gave rise to clones in the region of the pituitary (ventral to the eye). (C) The telencephalon at 24 h, depicted in light gray, arises from the telencephalic field of the neural plate. Regions I-V are depicted relative to their origins in the anterior neural plate. (D) Neurons having axons when scored at 50 h are depicted in a ventral view with anterior to the top of the page. Interneurons (right) in olfactory bulb have cell bodies in area where the sensory neurons (left) have axons extending into the olfactory bulb. Both types of neurons arise from the level of region IV (see A) in the developing neural plate. ac, anterior commissure.



(thought of as an anterior structure like the olfactory placode) originates from a field of cells medial to the olfactory field, which extends from the anterior part of the neural plate posteriorly to the cranial neural crest domain at the 4-somite stage. Although there is no morphologically apparent border separating the olfactory placode field from the telencephalic field, the fates that we observe suggest that, at 12 h, the cells in these fields are committed to a single fate. Indeed, we never observed clones containing mixed olfactory organ and telencephalic fates arising from a single cell, indicating strict cell fate restrictions even in the absence of a clear morphological border. Within both fields, cells at the same anteroposterior position (region IV, Fig. 8A) were the first to differentiate. In the lateral field, these cells gave rise to the first olfactory sensory neurons with axons and, in the medial field, they gave rise to the first interneurons in the telencephalon with axons. The axons of these first sensory neuron extended into the area of the developing olfactory bulb where the cell bodies of the interneurons lie (Fig. 8D). It is striking that the first olfactory sensory neurons and interneurons differentiate in the same position (region IV) in adjacent fields indicating the possibility of developmentally coordinated signals controlling differentiation of olfactory sensory neurons and their neuronal partners within the future olfactory bulb. These results show, for the first time, that in zebrafish the olfactory placode and olfactory bulb develop in parallel from adjacent fields of cells in the neural plate.

Cellular fields form the olfactory placode in the absence of cell division

In principle, localized cell proliferation could contribute to the thickening of the developing olfactory placode. Our results, however, suggest that the olfactory placodes, which are apparent by 17-18 h, form with very little cell division. This implies that cell movement, rather than proliferation, is the major contributor to the initial formation of the olfactory placodes. This finding is consistent with previous work by Harris and Hartenstein (1991) who showed that when cell division is blocked in *Xenopus* at the beginning of gastrulation,

development proceeds rather normally through neural induction and CNS differentiation, indicating that these events can take place in the absence of cell division. In zebrafish, cell cycles lengthen at the end of gastrulation (10 h) and become more variable (Kimmel et al., 1994) making it less likely that we would have found large numbers of dividing cells during early segmentation stages (12-18 h).

Our lineage tracing results also show that a specific cell type, the pioneer neurons, differentiates in the absence of cell division. Previous work in zebrafish has defined neurons generated by the end of gastrulation (10 h; cycle 16) as 'primary neurons' (Kimmel and Westerfield, 1990; Kimmel et al., 1994). The pioneer neurons, whose precursors were labeled at 12 h and had differentiated into neurons by 24 h in the absence of cell division, fit the criteria as being early differentiating primary neurons. In contrast, we found that individual olfactory sensory neuron precursors in the neural plate produced two to five cells in the placode when scored at 50 h, indicating that their differentiation is accompanied by cell division. This cell division occurs after the initial formation of the placode, 12-18 h, which was the time period when we observed very little cell division. Thus, at the 4- to 5-somite stage, the precursors of pioneer neurons are already postmitotic whereas olfactory sensory neuron precursors divide twice, on average, before differentiating. The olfactory placode fields converge to form the placodes and in doing so gather cells of different mitotic futures: the postmitotic pioneer neurons that will undergo axonogenesis and then die, and the sensory neuron precursors that will divide and differentiate.

Placodal development in the anterior neural plate

We have shown that the olfactory placode arises from a continuous field of cells in the anterior neural plate extending posteriorly to the anterior limits of premigratory cranial neural crest. This is very surprising because previous work predicted that the lens placode should lie between the olfactory placode region and neural crest (Rudnick, 1944; Jacobson, 1963). In our studies, the lens appears to arise from non-neural ectoderm lying lateral to the field giving rise to the olfactory placode (K.

W., unpublished data). Because the olfactory placodes arise from cellular fields extending along the anteroposterior neural plate, this makes the olfactory field continuous with the anterior limits of the premigratory cranial neural crest domain (Fig. 8A). A characteristic of vertebrate evolution is the 'invention' of neural crest, a cell population proposed to arise after the appearance of cephalochordates. Our findings of adjacent olfactory placode and neural crest domains suggest that they may have arisen from the same single ancestral domain. Recent studies using *Branchiostoma floridae* (amphioxus; a cephalochordate) have shown that cells at the edge of the neural plate express *distal-less* during development (Holland et al., 1996). This observation led to the proposal that *Branchiostoma* may have a preneural crest like population. An alternative is that, in amphioxus, *distal-less* gene function is involved in setting aside peripheral sensory structures, and that the subsequent (in evolutionary time) expression of genes such as *fkh6* may have subdivided this domain at the edge of the neural plate to allow for the specialization of neural crest and its derivatives. Future work coupling gene expression and cell fates in both zebrafish and amphioxus will help us to better understand the developmental and evolutionary relationship between neural crest and sensory placodes.

Our results suggest a new mechanism, different from the previously proposed model, for olfactory placode development. Rather than arising from a patch of cells that detaches from the edge of the anterior neural plate, a field of cells converges anteriorly to form the olfactory placode in the absence of localized pockets of cell proliferation. In addition, our findings show that similar morphogenesis of more medial cells produces the telencephalon. Future work following cell movements in living embryos will help us unravel the complex cellular behaviors that produce a distinct peripheral sensory structure, the olfactory placode, and its central target, the olfactory bulb.

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REFERENCES

- Akimenko, M. A., Ekker, M., Wegner, J., Lin, W. and Westerfield, M. (1994). Combinatorial expression of three zebrafish genes related to *distal-less*: part of a homeobox gene code for the head. *J. Neurosci.* **14**, 3475-86.
- Couly, G. F. and Le Douarin, N. M. (1985) Mapping of the early neural primordium in quail-chick chimeras. I. Developmental relationships between placodes, facial ectoderm, and prosencephalon. *Dev. Biol.* **110**, 422-39.
- Couly, G. F. and Le Douarin, N. M. (1987) Mapping of the early neural primordium in quail-chick chimeras. II. The prosencephalic neural plate and neural folds: Implications for the genesis of cephalic human congenital abnormalities. *Dev. Biol.* **120**, 198-214.
- Ekker, M., Akimenko, M. A., Bremiller, R. and Westerfield, M. (1992). Regional expression of three homeobox transcripts in the inner ear of zebrafish embryos. *Neuron* **9**, 27-35.
- Emerson, H. S. (1944). Embryonic grafts in regenerating tissue. III The development of dorsal and ventral ectoderm of *Rana pipiens* gastrulae. *J. Exp. Zool.* **97**, 1-19.
- Emerson, H. S. (1945). The development of late gastrula explants of *Rana pipiens* in salt solution. *J. Exp. Zool.* **100**, 497-521.
- Farbman, A. I. (1992). *Cell Biology of Olfaction*. Cambridge University Press, Canada.
- Farbman, A. I. (1988). Cellular interactions in the development of the vertebrate olfactory system. In *Molecular Neurobiology of the Olfactory System*. (ed. F. L. Margolis and T. V. Getchell). New York and London: Plenum Press.
- Haggis, A. J. (1956). Analysis of the determination of the olfactory placode in *Amblystoma punctatum*. *J. Embryol. Exp. Morph.* **4**, 120-139.
- Hansen, A. and Zeiske, E. (1993). Development of the olfactory organ in the zebrafish, *Brachydanio rerio*. *J. Comp. Neurol.* **333**, 289-300.
- Harris, W. A. and Hartenstein, V. (1991). Neuronal determination without cell division in *Xenopus* embryos. *Neuron* **6**, 499-515.
- Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P., Allis, C. D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348-360.
- Holland, N. D., Panganiban, G., Henyey, E. L. and Holland L. Z. (1996). Sequence and developmental expression of *AmphiDll*, an amphioxus *Distal-less* gene transcribed in the ectoderm, epidermis and nervous system: insights into evolution of craniate forebrain and neural crest. *Development* **122**, 2911-2920.
- Jacobson, A. G. (1963). The determination and positioning of the nose, lens and ear. I. Interactions within the ectoderm, and between the ectoderm and underlying tissues. *J. Exp. Zool.* **154**, 273-283.
- Karlstrom, R. O. and Kane, D. A. (1996). A flipbook of zebrafish embryogenesis. *Development* **123**, 461.
- Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.
- Kimmel, C. B., Warga, R. M. and Kane, D. A. (1994). Cell cycles and clonal strings during formation of the zebrafish central nervous system. *Development* **120**, 265-276.
- Kimmel, C. B. and Westerfield, M. (1990). Primary Neurons of the Zebrafish. In *Signal and Sense*. (ed. G. M. Edelman, W. E. Gall and M. W. Cowan), pp. 561-588. New York Wiley-Liss.
- Landacre, F. L. (1910). The origin of cranial ganglia in *Ameiurus*. *J. Comp. Neurol.* **20**, 309-411.
- Northcutt, R. G. (1996). The origin of craniates: neural crest, neurogenic placodes, and homeobox genes. *Israel J. Zool.* **42**, S-273-S-313.
- Northcutt, R. G. and Gans, C. (1983). The genesis of neural crest and epidermal placodes: a reinterpretation of vertebrate origins. *Q. Rev. Biol.* **58**, 1-28.
- Odenthal, J. and Nusslein-Volhard, C. (1998). *fork head* domain genes in zebrafish. *Dev. Genes. Evol.* **208**, 245-258.
- Papalopulu, N. and Kintner, C. (1993). *Xenopus* *Distal-less* related homeobox genes are expressed in the developing forebrain and are induced by planar signals. *Development* **117**, 961-975.
- Rudnick, D. (1944). Early history and mechanics of the chick blastoderm. *Quart. Rev. Biol.* **19**, 187-212.
- Thisse, C., Thisse, B., Schilling, T. T. & Postlethwait, J. H. (1993). Structure of the zebrafish *snail 1* gene and its expression in wild-type, *spadetail* and *no tail*. *Development* **119**, 1203-1215.
- Verwoerd, C. D. and van Oostrom, C. G. (1979). Cephalic neural crest and placodes. *Adv. Anat. Embryol. Cell Biol.* **58**, 1-75.
- Walker, C. (1998). Haploid screens and gamma-ray mutagenesis. In *The Zebrafish; Genetics and Genomics*. (ed. H. W. Dietrich, M. Westerfield and L. I. Zon). New York: Academic Press.
- Whitlock, K. E. and Westerfield, M. (1998). A transient population of neurons pioneers the olfactory pathway in the zebrafish. *J. Neurosci.* **18**, 8919-8927.
- Zwilling, E. (1940). An experimental analysis of the development of the anuran olfactory organ. *J. Exp. Zool.* **84**, 291-318.