

Cellular and molecular interactions in the development of the *Xenopus* olfactory system



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Development of the olfactory system in Xenopus laevis begins during gastrulation, with the induction of olfactory placodes at the rostral edge of the prospective neural plate. Initial placodal induction appears to involve cerberus, a molecule secreted from the involuting anterior endoderm. Possible downstream genes expressed in the anterior neural ridge and sense plate include the transcription factors Pax-6, X-dll2, X-dll3, and Xotx2. Forebrain development is dependent on the presence of the placode and subsequent innervation by olfactory axons, with the extent of this dependence declining as development advances. During metamorphosis thyroid hormones initiate extensive changes in the olfactory system, including the origins of new regions of the olfactory epithelium and olfactory bulb, and a change in olfactory projection patterns.

Key words: induction / metamorphosis / olfaction / olfactory receptor / *Xenopus*

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XENOPUS LAEVIS, the African clawed frog, has become a favorite model for studies of vertebrate development.¹ The frogs are easily kept and bred in the laboratory, develop relatively rapidly, and embryos are amenable to many types of experimental intervention that are difficult, if not impossible, in mammals. The ability to inject mRNA into embryos makes some genetic manipulations of early development possible,² and a technique for creating transgenic animals is now available.³ An excellent staging system also exists.⁴ In the following, we review the structure of the adult olfactory system of *Xenopus*, then examine early development and metamorphosis. We have two main goals: to introduce the unique features of the *Xenopus* system, and to examine the general implications of studies on *Xenopus* for understanding cellular and molecular interactions in vertebrate olfactory development.

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Structure of the adult olfactory system

Xenopus adults have three distinct peripheral olfactory epithelia, located in separate nasal chambers (Figure 1). These are the principal cavity (medial diverticulum) epithelium, the middle cavity (lateral diverticulum) epithelium, and the vomeronasal epithelium. The principal cavity appears to be used to sense airborne odors. It is filled with air even when the animal is submerged.⁵ The middle cavity is separated by a valve from the principal cavity, is always filled with water, and is used to sense water-borne odors.⁵ Its presence in *Xenopus* is correlated with the aquatic lifestyle of the adults; sensory epithelium is lacking in the middle cavity of most other frogs.⁶ The function of the vomeronasal organ is not clear, but it also appears to be filled with fluid.

The olfactory epithelia contain receptor, supporting, and basal cells,^{7,8} as in other vertebrates. Principal cavity receptor cells are ciliated (Figure 2A), as are

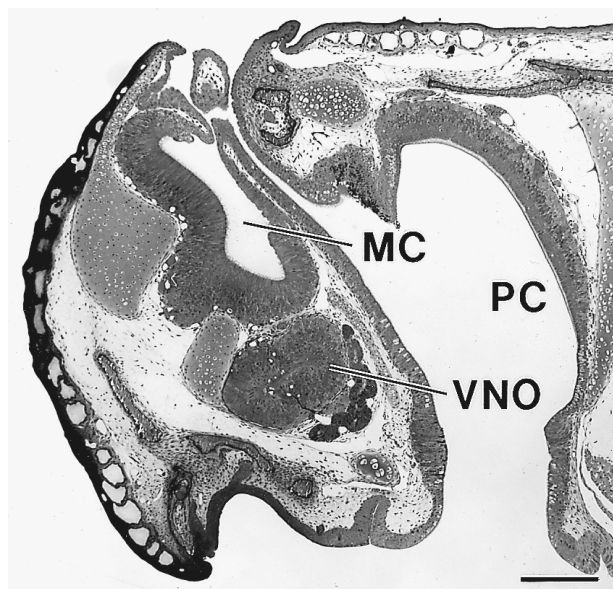


Figure 1. Sagittal section of a juvenile frog to show the three nasal cavities. Anterior to left. PC, principal cavity; MC, middle cavity; VNO, vomeronasal organ. Scale = 200 μ m.

the majority of mammalian olfactory receptor cells. The principal cavity epithelium projects to the dorso-medial part of the main olfactory bulb, with some fibers crossing to the contralateral side.^{8,9} Middle cavity receptor cells are of two types, one ciliated and one microvillar (Figure 2B), as in the olfactory

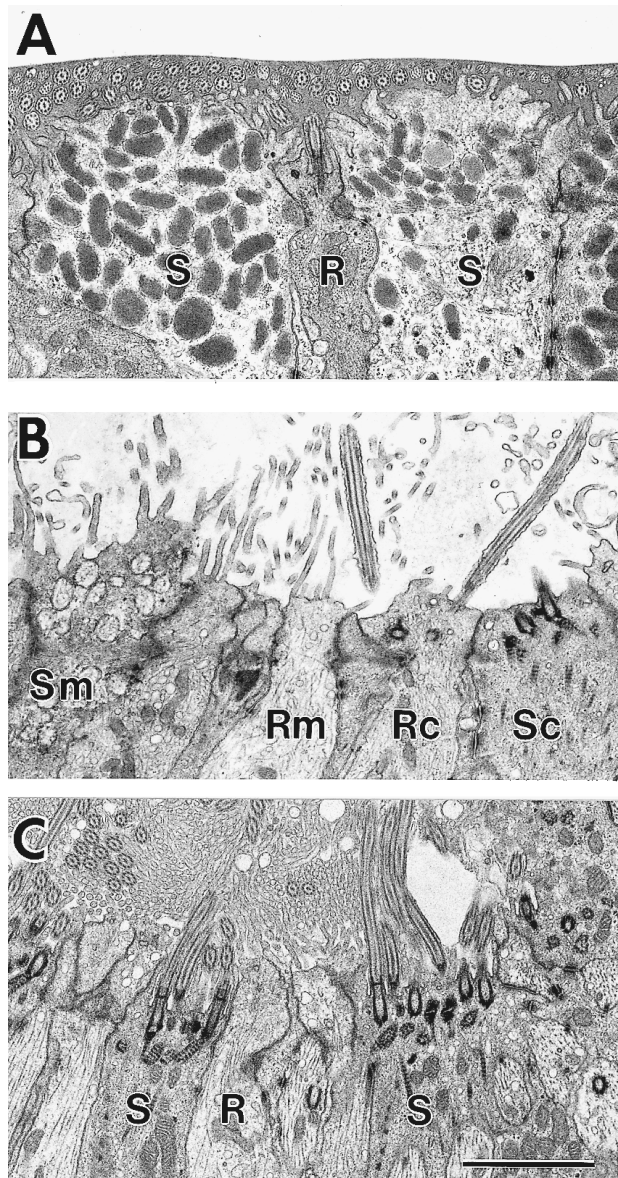


Figure 2. Electron micrographs to show differing surface specializations of receptor cells (R) and supporting cells (S) in the three nasal cavities. (A) Principal cavity. Ciliated receptor cells and microvillar supporting cells. (B) Middle cavity. Both ciliated (c) and microvillar (m) receptor and supporting cells. (C) Vomeronasal organ. Microvillar receptor cells and ciliated supporting cells. Scale = 2 μ m.

epithelium of many fishes.¹⁰ The middle cavity epithelium projects to the ventrolateral part of the main olfactory bulb.⁸ In addition, some olfactory receptor cells of the middle cavity have axons that project beyond the olfactory bulb, as far caudal as the diencephalon.¹¹ Vomeronasal receptor cells are exclusively microvillar (Figure 2C), as in most other tetrapods.¹⁰ The vomeronasal organ projects to the accessory olfactory bulb.⁸

Recently, 19 genes for *Xenopus* odorant receptor proteins have been cloned.¹² Interestingly, odorant receptor expression correlates with the functional and morphological differences between principal and middle cavity epithelia. Receptor proteins expressed in the principal cavity are members of mammalian receptor subfamilies, but receptor proteins of the middle cavity are more closely related to those of fishes. Several markers that distinguish olfactory neuron subclasses have also been described, including the lectin soybean agglutinin (SBA),^{7,13} the monoclonal antibody E7,¹⁴ and the monoclonal antibodies A5 and B2.¹⁵

The olfactory bulb is laminated; one can recognize olfactory nerve, glomerular, mitral cell/plexiform, and granule cell layers.¹⁶ Mitral/tufted cells are the primary output neurons of the olfactory bulb. Their dendrites receive synapses from olfactory axon terminals within knots of neuropil (glomeruli) located in the glomerular layer, their cell bodies are located in the mitral cell/plexiform layer, and their axons project to various telecephalic centers. As in all frogs,⁶ left and right olfactory bulbs are fused in the midline, allowing olfactory receptor axons and mitral/tufted cell dendrites to cross to the contralateral side.

Early olfactory development: morphology and gene expression

Figure 3 summarizes the timing of important events in the development of the *Xenopus* olfactory system. The olfactory epithelia develop from paired olfactory placodes, which arise within a thickened region of the anterior ectoderm called the sense plate.¹⁷ Fate mapping studies¹⁸⁻²⁰ have shown that the placodes derive from the ectoderm of the anterior neural ridge (Figure 4). They are composed of a superficial layer, which gives rise to the olfactory supporting cells, and a deep (sensory or nervous) layer, which gives rise to the olfactory receptor cells.¹⁷ The placodes also give rise to some cells that migrate into the brain, including the precursors of the GnRH neurons.^{21,22}

The olfactory bulbs, like the placodes, derive from the lateral parts of the anterior neural ridge.^{18,19} Prospective placodal and bulbar cell populations are separated when the neural folds fuse at the end of the neurulation. The neurons of the larval olfactory bulb are born in a definite sequence. Mitral cells of stage 62 tadpoles are labeled by ³H-thymidine injections as early as stages 11–12, but periglomerular and granule cells are only labeled by injections after stage 38.²³

A large number of genes are known to be expressed in the olfactory placode, olfactory bulb, or their precursor tissues in *Xenopus*. The time and location of expression for a variety of transcription factors, cell

surface receptors, and signaling molecules that show localized expression in the developing olfactory system are summarized in Table 1. Expression of a number of cell adhesion molecules in the olfactory placodes and/or olfactory bulb has also been described (e.g. N-CAM,³⁹ EP-cadherin,⁴⁰ and N-cadherin⁴¹). Of particular interest is the fact that most genes are expressed both in the olfactory placodes and the olfactory bulbs. This pattern may reflect the common origin of the placodes and bulbs from the anterior neural ridge.¹⁹

Induction and specification of the olfactory placodes

The appearance of the olfactory placodes is the first sign of differentiation in the developing olfactory system. What cellular and molecular interactions lead up to this event? To answer this question, we must examine a number of basic developmental questions: (1) at what stage is the ectoderm *specified* to form placodes? (2) what factors *induce* the ectoderm to form placodes? (3) which ectoderm is *competent* to respond to the inductive signals? and (4) when is the








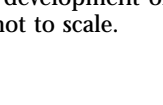


STAGE	DEVELOPMENTAL EVENTS	
14	neural plate formed	
20	neural tube closed	
23	placodes distinct	
30	olfactory axons enter brain	
32	bulb begins to differentiate	
36	first synapses form in bulb	
37/38	vomerinal organ and principal cavity distinct	
44	laminar organization of bulb clear	
48	accessory olfactory bulb distinct	
50	left and right bulbs begin to fuse	
51	middle cavity appears	
52	metamorphosis begins	
54	dorsal bulb begins to form	
59	principal cavity projection gone from ventral bulb	
66	metamorphosis complete	

Figure 3. Timeline of important events in development of the *Xenopus* olfactory system. Illustrations not to scale.

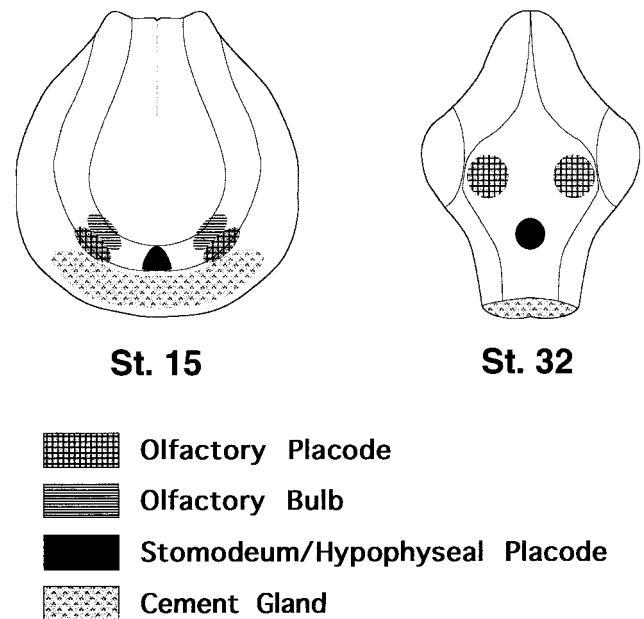


Figure 4. Diagram showing the location of the prospective olfactory placodes, olfactory bulbs, cement gland, and stomodeum/hypophyseal placode at stage 15, when placodes are specified, and of the differentiating structures at stage 32, when olfactory axons have just entered the brain. Anterior views, not to scale.

Table 1. Gene expression in the *Xenopus* olfactory system

Gene	Tissue and stage of expression							Refs
	Ant Ect (<13)	Ant NR* (13–18)	Sense Pl* (19–22)	Olf plac (23–36)	Olf ep (37+)	Pros† (19–36)	Olf Bulb (37+)	
Transcription factors								
<i>NeuroD</i>	–	–	–?	+		+		24
<i>Pax-6</i>	–	+	+	+	+	+	+	25
<i>X-dll2</i>	–	+	+	+	+	–	–	26
<i>X-dll3</i>	–	+	+	+	+	+	+	26, 27
<i>X-dll4</i>		–	–	–	–	+	+	27, 28
<i>XANF-1, 2</i>	+	+	–	–	–	–	–	29
<i>XFKH4</i>	–	+	+	+		+		30
<i>Xlim-1</i>	–	–	–		+	–?	–?	31, 32
<i>Xotx1, 4</i>	–	–	–	+	+	–	–	33
<i>Xotx2</i>	+	+	–	+	+	+	+	33, 34
<i>Xsna</i>	+	+	–	–	–	+		35
Receptors								
<i>X-Notch-1</i>	+	+	+	+	+	+	+	2, 26
Signaling molecules								
<i>X-Delta-1</i>	+	+	+	+	+	+	+	2, 26
<i>BMP-2</i>	–?	+	+	+		–		36, 37
<i>X-bhh, X-chh</i>	–	+						38

*Scored only for expression in prospective olfactory epithelium.

†Scored only for expression in prospective olfactory bulb.

Abbreviations: Ant Ect, anterior ectoderm; Ant NR, anterior neural ridge; Sense Pl, sense plate; Olf Plac, olfactory placode; Olf Ep, olfactory epithelium; Pros, prosencephalon; Olf Bulb, olfactory bulb. Symbols: +, some expression reported; –, lack of expression reported, ?, interpretation unclear. A blank indicates no available information.

ability to form olfactory epithelium *restricted* to the placodes?

Specification of the placodes in *Xenopus* appears to occur by neural fold stages (approximately stage 15).⁴² This is well before any overt signs of placodal differentiation (stage 23), although somewhat later than the neural plate stages found for placodal determination in heteroplastic transplantation experiments on other amphibians.⁴³

Induction of the placodes has not been explicitly analysed in *Xenopus*, but a large body of work on other amphibians has shown that the underlying endoderm of the archenteron roof and the adjacent neural plate are each capable of inducing olfactory placodes under certain conditions (reviewed in refs 44, 45). This suggests that influences from both the archenteron roof and the prospective neurectoderm cooperate in the normal induction of the olfactory placodes (as has been shown for the lens placodes⁴⁶).

The olfactory placodes — like the lens and ear placodes — arise at the borders of the neural plate, and it is not surprising that a wide variety of studies support a connection between placodal and neural induction. Neural induction and patterning has been an extremely active area of research in recent years, stimulated by the discovery of a number of endogenous neural inducing molecules (reviewed in refs 47,

48). Classic work by Nieuwkoop and colleagues⁴⁹ supported a ‘two-signal’ model of neural induction. In this model, an initial signal from involuting dorsal mesoderm during gastrulation induces neural tissue of an anterior character (‘activation’), and a second (‘transforming’) signal then posteriorizes the more caudal parts of the activated ectoderm. Thus in this model, neural induction and anteroposterior patterning go hand-in-hand. Recent work has supported this general model, although suggesting that neural induction (activation) and some anteroposterior patterning may occur prior to gastrulation, via planar signaling through the ectoderm from the preinvolution dorsal lip tissue (reviewed ref 50; see ref 51 for an alternative interpretation).

In the initial model proposed by Nieuwkoop and colleagues,⁴⁹ activation spreads laterally and anteriorly through the ectoderm, but with decreasing strength, and the boundaries of the neural plate are determined by a drop in the level of activation below the threshold required for neural induction. The induction of neural crest (and by implication, placodes) is considered the response of ectoderm to levels of activation below the threshold for neuralization. A modified version of this model was later developed in which the boundary of the neural plate is determined by the interaction between the spread

of activation and a stage-dependent loss of neural competence in the responding ectoderm.⁵² This model predicts that placodal competence of the ectoderm should be retained after neural competence is lost, and is supported by studies of lens induction in *Xenopus*.⁵³ However, there is some evidence that the boundary of the neural plate is at least provisionally established much earlier, in late blastula stages.^{54,55}

The second element of the two-signal model, transformation, is concerned with the establishment of anteroposterior identity. The olfactory organs are some of the most anterior ectodermal structures of *Xenopus*. As shown in Figure 4, they lie immediately behind the cement gland (the most anterior structure^{56,57}) and lateral to the stomodeal-hypophyseal anlage. Agents such as retinoic acid that suppress the development of anterior structures suppress the olfactory organs as well as the cement gland, eyes and forebrain.^{58,59} Moreover, as predicted by the two-signal model, the recently identified neural inducers noggin,⁶⁰ follistatin,⁶¹ chordin,⁶² and cerberus⁶³ all induce both cement gland and anterior neural markers (e.g. *Xotx2*) — but not posterior neural markers — in cultured animal caps.

Interestingly, injection of *cerberus* mRNA into a single blastomere of an eight-cell embryo ventralized by UV treatment results in formation of a head-like structure that has multiple olfactory organs connected to a mass of brain tissue. Moreover, of the identified neural inducers, only cerberus has been shown to induce histologically recognizable olfactory placodes in animal caps.⁶³ This result is consistent with the location of *cerberus* message — but not *noggin*, *follistatin*, or *chordin* — in the endoderm that immediately underlies the prospective placodes at the end of gastrulation. The strongest interpretation of these results would be that cerberus is a signaling molecule necessary for olfactory placode induction.

Little is known about the path that leads from induction of prospective placodes to their precise localization, although patterns of gene expression in the anterior neural ridge (Table 1) give some hints about processes that may be involved. The transcription factors *X-dll3* and *XFKH4* are expressed in the anterior neural ridge, continue to be expressed in the sense plate, and then become restricted to the olfactory placodes.^{26,27,30} In contrast, *XANF-2* is first expressed throughout the anterior neural ridge, but expression is retained only in the medial part of the ridge, the hypophyseal anlage.²⁹ The complementarity of these expression patterns could be interpreted as reflecting a progressive restriction of cell fate within

these regions. However, cell labeling experiments²⁰ suggest that the complementarity may instead result from migration of cells within the anterior neural ridge and sense plate, leading to a sorting out of the prospective olfactory and hypophyseal cell populations.

The competence of ectoderm to respond to placodal induction has not been systematically examined in *Xenopus*, but prospective ventral epidermis loses its competence to respond to lens induction at early neural plate stages,⁵³ similar to the timing for loss of olfactory competence in other amphibians.⁴³ In contrast, the complete restriction of competence to the olfactory epithelium is a very late event in *Xenopus*. Regeneration occurs in 80–90% of placodal extirpations through stage 40, but does not occur in extirpations performed from stage 42 on.^{64,65} When extirpations are performed at stages 33–34, cell labeling has shown that the cells comprising regenerated olfactory placodes are derived from the surrounding sense plate.²⁰

Interrelations of the developing nose and brain

Extirpation of the developing sense plate or olfactory placodes has shown that normal brain development depends on the presence of placodes and subsequent innervation by olfactory axons.^{64–67} The extent of this requirement appears to be stage-dependent. If the sense plate is extirpated at stages 22–23, the entire telencephalon fails to form.⁶⁶ Regeneration of a single olfactory organ results in formation of an unpaired telencephalon. These results are quite striking, and are difficult to reconcile with the almost normal formation of the non-olfactory telencephalon in *Pax-6* mutant mice, in which olfactory placodes and bulbs never form.^{68,69} The situation clearly needs further study.

If one olfactory placode is removed somewhat later, at stages 26–31, either the ipsilateral olfactory bulb doesn't form, or a single midline olfactory bulb forms.⁶⁵ The remainder of the telencephalon appears relatively normal. Interestingly, partial regeneration of the placode leads to formation of a partial olfactory bulb, with a direct relationship between the number of olfactory axons and the number of mitral/tufted cells (their target population in the bulb) when examined at stage 58. This suggests that receptor cell number may regulate mitral/tufted cell number. Supporting this hypothesis, transplantation of an additional olfactory placode to a normal host appears

to cause an increase in size of the olfactory nerve and bulb, as well as receptor cell and mitral/tufted cell number, at young larval stages.^{64,70} However, by stage 58 there is no significant increase in receptor cell number or size of the olfactory bulb in animals with an additional transplanted placode.⁷⁰ This suggests that the olfactory bulb may also be able to regulate receptor cell number.

Finally, bilateral removal of the olfactory organ at stages 45–46, after the normal laminated structure of the bulb has formed, results in loss of lamination and a reduction in bulbar volume.⁶⁷ The normal fusion of the bulbs is also prevented. It is not clear whether the mitral/tufted cells of the denervated side die or dedifferentiate.

Heterotopic transplantation of the olfactory placode has shown that receptor cell axons can penetrate areas of the brain other than the prospective olfactory bulbs.^{21,64,71} Transplantation of an olfactory placode to the side of the head of a host embryo, with or without removal of the optic vesicle, results in the formation of glomerular-like structures in the wall of the diencephalon and apparent local hyperplasia.^{64,71} In contrast, nerves originating from placodes transplanted further caudally, in the region of the extirpated otic vesicle, penetrate the myelencephalon but do not cause hyperplasia or glomerular-like structures.⁶⁴ The apparent local hyperplasia in the more anterior transplantations may be due to migration of cells from the transplanted placode into the brain,²¹ but if this is true it is difficult to understand why a similar effect would not be seen in more caudal transplantations.

Metamorphosis

Metamorphosis in *Xenopus* involves fundamental restructuring of the olfactory system (Figure 5). In young larvae there are only two sensory epithelia, the vomeronasal and the principal cavity.^{7,8,72} The principal cavity receptor cells resemble those of the adult middle cavity,⁸ and appear to be used to sense water-borne odors. The middle cavity arises as a small bud at the anterior end of the principal cavity around stages 50–51, and middle cavity receptor cell axons reach the olfactory bulb by stage 52,⁷³ overlapping with principal cavity axons in the ventrolateral olfactory bulb. This overlap in principal and middle cavity projections is retained through later larval stages, but between stages 57 and 59 the principal cavity projection to the ventrolateral bulb is lost, thus resulting in

the adult projection pattern.⁸ Principal cavity receptor cell morphology changes at this time, and it appears likely that the larval receptor cells are completely replaced during metamorphosis. In contrast, no significant metamorphic changes appear to occur in the vomeronasal epithelium or projection.

Control of the metamorphic change in the principal cavity olfactory projection has been examined by unilateral extirpation of the developing middle cavity epithelium.⁷³ Even in the absence of middle cavity afferents, principal cavity axons still withdraw from the ventrolateral olfactory bulb during metamorphosis. This suggests that rather than some sort of competitive interaction with middle cavity axons, intrinsic cues may direct the outgrowth of principal cavity axons to the dorsomedial bulb during metamorphosis.

Extensive cell genesis occurs in the olfactory bulb during metamorphosis. In particular, the entire dorsal bulb (that part receiving principal cavity projections) arises at this time, and many new cells are also generated in the ventral bulb.²³ Unlike the situation in embryonic and early larval development, all cell types appear to arise simultaneously. There also appears to be extensive cell genesis in the olfactory epithelium — the number of receptor cell axons increases four-fold between stages 54 and 60.⁷⁴ More-

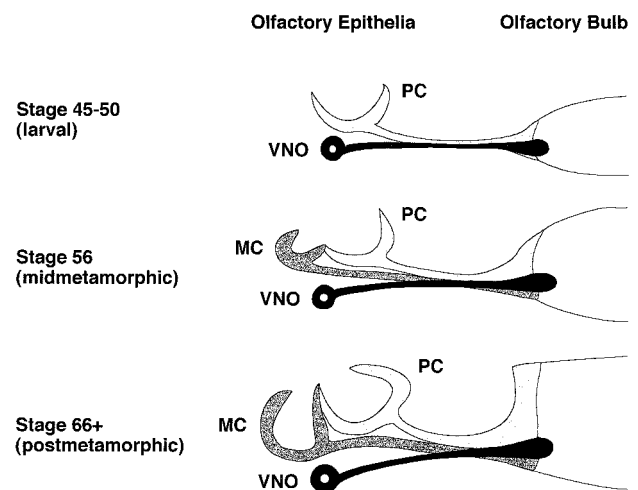


Figure 5. Diagrammatic lateral view of metamorphic changes in the olfactory system. The middle cavity is absent prior to metamorphosis. In midmetamorphic animals the middle cavity and principal cavity axons both project to the ventral part of the main olfactory bulb, but in postmetamorphic animals principal cavity axons project only to the dorsal part of the main olfactory bulb. PC, principal cavity; MC, middle cavity; VNO, vomeronasal organ.

over, the ratio of olfactory axon number to mitral/tufted cell number is dramatically different in larvae and adults.¹⁶ The 5:1 ratio observed at stage 58 changes to 34:1 in the adult. This increase in the convergence of axons to bulb output neurons may reflect an increase in olfactory sensitivity in adult frogs.¹⁶

Most of the metamorphic changes occurring in frogs are due to the direct or indirect actions of thyroid hormones, which are detectable in *Xenopus* plasma by stage 54 and rise rapidly through metamorphic climax at stages 58–64.⁷⁵ Treatment of tadpoles with propylthiouracil (PTU), a blocker of thyroid hormone synthesis, results in developmental arrest of tadpoles at stage 52, although growth continues. PTU treatment significantly reduces the normal increase in axon number and olfactory epithelium thickness at metamorphosis.^{76,77} Conversely, hyperthyroidism induced by exogenous thyroid hormone increases both cell genesis and volume in the sensory epithelium of premetamorphic tadpoles.⁷⁸ This increase appears to be a direct effect of thyroid hormone on the epithelium, as shown by the differential effect on the two sides in tadpoles with unilateral implantation of a cholesterol pellet containing thyroxine.⁷⁹

Conclusion

Our knowledge of olfactory development in *Xenopus* is clearly extensive, but there are a number of areas in which significant gaps remain. Important problems for future research include the following: (1) What factors are responsible for the initial formation of the olfactory placodes? (2) Through what mechanisms do the olfactory placodes and nerves influence development of the brain? (3) Are all metamorphic changes the direct effect of thyroid hormones, or are tissue interactions important as well? The integration of classical experimental embryological techniques and studies of gene expression offers great potential for answering these questions. Moreover, perturbation of gene expression either through over- or under-expression may help elucidate the role of particular genes in the development of the olfactory system. Because of its experimental tractability, we expect that *Xenopus* will remain an important animal for examining the causal mechanisms involved in vertebrate olfactory development for many years to come.

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