The Differentiation of the Olfactory Placode in *Xenopus*

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Normal development of the olfactory placode was studied to describe the sequence of events involved in the development of the olfactory placode. It has been primarily concerned with the morphological differentiation of the sensory neurons, their initial growth, maturation patterns and the contacts of their axons with the primitive prosencephalic vesicle. The olfactory organ first appears at stage 23 as a paired thickening of the two ectodermal layers: the superficial non-nervous layer (NNL) and the inner nervous layer (NL). Receptor cells differentiate from the NL and the supporting cells develop from the NNL. After stage 26 the placodal cells begin to migrate toward the epithelial surface between the NNL cells and their apical processes reach the surface at stage 28. As the apical process reaches the epithelial surface, basal processes (presumptive axons) sprout from the base of the NL cells at stage 29/30. They penetrate the underlying telencephalon by stage 32. Sensory synaptic contacts first appear at stage 37/38. Some placodal cells remain at the olfactory epithelium as basal cells while other placodal cells differentiate into olfactory neurons.

The results confirmed that neurons originate exclusively from the nervous layer of the ectoderm while supporting cells originate from the NNL layer. The results also indicate that the development of olfactory neuron is independent of information from the target itssue.

KEY WORDS: Olfactory Placode, Olfactory Neuron

The development of the nervous system depends, to a large extent, on the sequential interaction between its parts. Peripheral sensory systems, for instance, play a very important role in the developement of the central nervous system (Shorey, 1909). Previous studies have shown that the olfactory system offers a good example of the occurrence of sequential events of specification and induction, as the peripheral embryonic placode, possibly induced by the underlying mesenchyme and prosencephalic vesicle, influences in its turn the development of the olfactory bulb and, possibly, of the entire telencephalon (Clairambault, 1976; Stout and Graziadei, 1980).

The ectodermal placodes in amphibians originate from the so called presumptive placodal thickening, PPT (Knouff, 1927, 1935). The PPT is recognizable in amphibians at the neural plate stage as a continuous thickening, immediately adjacent to the presumptive neural plate, of the nervous ectoderm (Fig. 1). The rostral and suprabranchial portions of the PPT continue to develop while the other parts disappear. By the time of neural tube closure, the rostral thickening of the PPT, called the sensory plate, differentiates into three structures- a hypophyseal placode and a pair of olfactory placodes (Knouff, 1935; Carpenter, 1937).

The development of the olfactory epithelium has been studied with the light and electron microscope in fish (Theisen, 1972), in chick
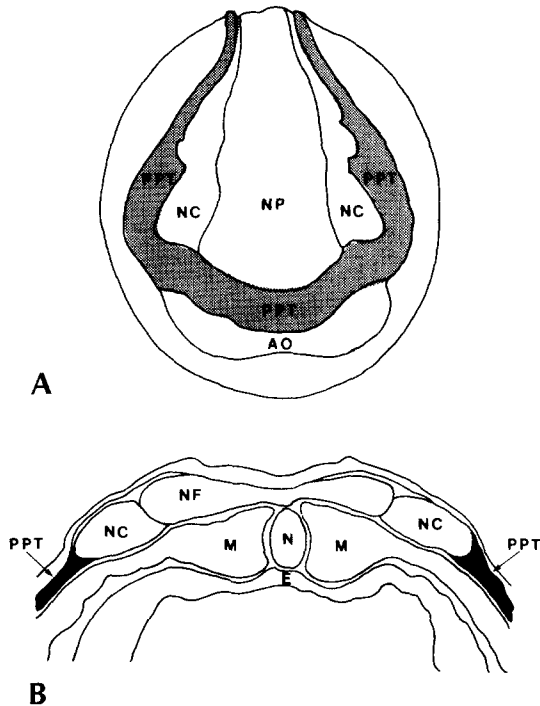
Fig. 1. (A) Diagram at the neural plate stage showing the relations of definitive neural plate, neural crest, placodal thickening. Diagram is projected on the anterodorsal surface (modified from Knouff, 1935). NC, neural crest; NP, neural plate; PPT, primitive placodal thickening; AO, adhesive organ. (B) Diagram of a cross section of mid neural fold stage amphibian showing the neural plate, neural crest and primitive placodal thickung. NC, neural crest; PPT, primitive placodal thickning; NF, neural fold; N, notochord; M, mesoderm; E, endoderm (modified from Sadachiani and Thiebaud, 1987).

embryo (Mendoza et al., 1982), in amphibians (McEwen, 1949), and in mammalian embryos (Cuschieri and Bannister, 1975a; Bossy, 1980; Farbman and Squinto, 1985). In most vertebrates the component cells of embryonic ectoderm are so similar that subpopulations are not easily identified. However, in amphibian embryos two discrete cellular layers are recognized in the olfactory placode area of the cranial ectoderm. The outer layer is called the non nervous layer (NNL) and the inner is called the nervous layer (NL). The nervous layer gives rise to nervous structures such as basal cells and neurons. Supporting cells which are nonnervous cells

develop from the NNL.

The olfactory epithelium of adult vertebrates contains three basic cell types: basal cells, neurons, and supporting cells (Graziadei, 1973a). The olfactory sensory neurons undergo continuous replacement throughout life. Basal cells differentiate into mature olfactory neurons (Moulton, 1974; Graziadei and Monti Graziadei, 1978, 1979; Constanzo and Graziadei, 1983).

Bells observed that the olfactory placodes, once determined, could develop independently of the brain (1907). Subsequently other workers also suggested that the maturation of olfactory neurons is independent of information obtained from the target tissue (Carpenter, 1937; Heckroth and et al., 1983). Not only do the neurons mature and persist in the absence of their target tissue but they can also project to foreign locations and modify them (Graziadei and Monti Graziadei, 1979; Stout and Graziadei, 1980; Graziadei and Kaplan, 1980; Magrassi and Graziadei, 1985, 1994; Dryer and Graziadei, 1994; Koo and Graziadei, 1995). Conversely removal of the olfactory placode in amphibian embryos inhibits the normal maturation of the olfactory bulb and other portions of the telencephalon (Burr, 1923a, b; Clairambault, 1976; Stout and Graziadei, 1980; Graziadei and Monti-Graziadei, 1992). This suggests that the normal maturation of the telencephalon depends, to a certain degree, on input from the peripheral olfactory organ. Taken above together, we can suggest that the olfactory system influence on the development of the olfactory bulb and, possibly, of the entire telencephalon. However, the study of the normal olfactory placode has not been systematically done in Xenopus. The study of the normal olfactory placode, of its differentiation and its connection with the primitive prosencephalic vesicle will serve as a background information for the interpretation of the experiments using the olfactory system.

Materials and Methods

African clawed frogs, Xenopus laevis were maintained in a room with 20-22°C and a 12/12 light cycle. Each frog was kept in a separate,

transparent 1 gallon aquarium with several holes on the cover for aeration. Adult frogs and juvenile frogs were fed a mixture of chopped beef liver and Nasco frog brittle (Nasco Co., Fort Arkinson, Wisconsin). From stage 43/44 to 50 tadpoles were fed nettle powder solution. Tadpoles from stage 51 through 66 (end of metamorphosis) were fed nettle powder solution and a frog brittle paste.

Embryos were obtained by injection of chorionic gonadotropin (Sigma Chemical Co.) into the dorsal lymph sac of adult frogs. 1050 I.U. of hormone diluted with double deionized water was injected into the female and 450 I.U. into the male at around 8-9 A.M. 8 hours after the injection, the pair was placed in the breeding tank. Fertilized embryos were separated from unfertilized ones and placed in a cold temperature incubator maintained at 18°C to slow down growth to acquire the right stages for prolonged time.

For light microscopy, embryos were collected in anesthetic solution containing MS-222 (ethyl m-amino benzonate methanesulfate, 1:4000). Embryo stages 15 through 66 were fixed by immersion in 1% glutaraldehyde, 0.4% paraformaldehyde and 0.1 M sodium dichromate/potassium chromate buffer at pH 7.2. Fixation was done at 4°C for 2 hours and animals were then stored overnight in 0.2 M sodium dichromate/potassium chromate buffer (pH 6.0) at 4°C (modification of Tranzer's method in 1976). The animals were postfixed in 1% osmium tetroxide, 0.1 M sodium dichromate/potassium chromate buffer (pH 7.2) and 1% potassium ferrocyanide (Karnowsky, 1971) at 0°C in the dark for 1 hour. The excess osmium was rinsed with 0.1 M sodium dichromate/potassium chromate and animals were then dehydrated through graded ethanol. Propylene oxide was used as a clearing agent prior to infiltration with araldite. The embedded embryos were sectioned at 1 micron on a Sorvall MT-2 Ultramichrotome with glass and diamond knives. The sections were cut in horizontal planes and stained with 1% toluidine blue. Light microscopic picture were photographed with a Zeiss Ultraphot II.

Same procedures for fixation and embedding, as described in above section of light micrpscopy,

were also used for electron microscopy. Ultra thin sections (about 500 A) were mounted on naked copper grids, stained with 7.5% uranyl acetate on the 40°C plate for 45 minutes and Reynold's lead citrate for 30 min in room temperature. Stained sections were examined and photographed with a TEOL 1200 transmission electron microscope.

Results

The ectoderm of *Xenopus laevis* embryo consists of two morphologically discrete layers. The inner nervous layer (NL) consists of squamous cells in horizontal section that stain darkly with toluidine blue. The outer non-nervous layer (NNL) is columnar and its cells stain lightly. The morphology of the olfactory placode in the embryo is best seen in horizontal sections of the head, at the level of the optic vesicles. The placode develops as a region where the NL becomes thick and is located on the ventrolateral side of the head, rostral and lateral to the telencephalon. A small cleft divides the placodal structure from the forebrain. At around stage 24 this cleft begins to get filled with mesenchyme. The olfactory placode is first histologically recognizable at stage 22/23 as a pair of areas situated in front of the eyes (Fig. 2). At this stage the placode does not show an obvious thickening of the two layers as it is represented only by the two single rows of NL and NNL cells. The cells of the placodal area are larger than NL cells of the other regions and this makes it possible to distinguish them. The thickeness of the nervous layer selectively increases at stage 23-25 while the non nervous layer is still represented by a single cell line (Fig. 3). The placodal cells of the NL are arranged in two or three layers at these stages. At stage 26 the NL cells begin to invade and comingle with the NNL (Fig. 4). However, most of the NL cells still have morphological characteristics of the basal cells. A layer of mesenchyme becomes obvious between the placodal area and the wall of the forebrain at the level of optic vesicle. From stage 26 the boundary between the cells of the NL and NNL cells becomes difficult to distinguish because more placodal cells invade the NNL layer.
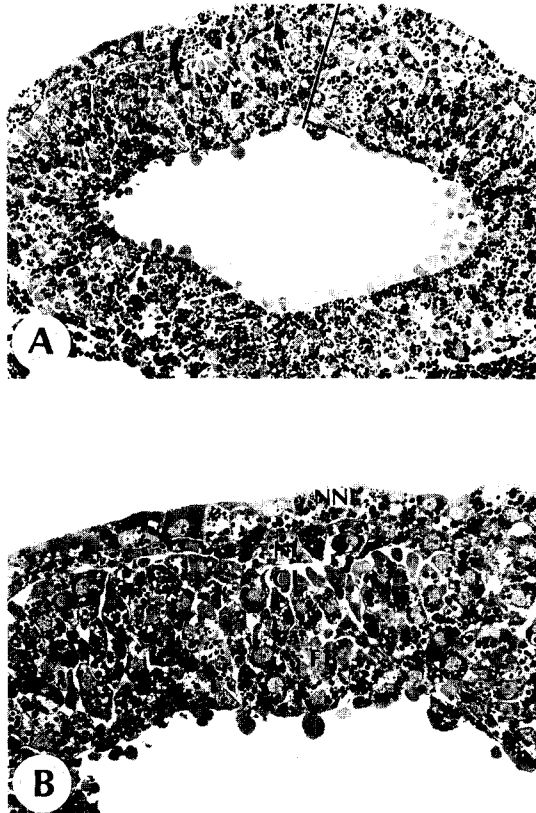
Fig. 2. Light micrographs of 1 micron thick resin section of stage 22/23 *Xenopus laevis* embryo. Sections are cut in the horizontal plane and stained with toluidin blue. (A) Midline is indicated by a bar. The presumptive placodal area is indicated between two arrows. B, brain. × 250, (B) Higher magnification of the same section shown in Fig. (A). At this stage the placode consists of two cell layers: the more superficial nonnervous layer (NNL) and the deeper nervous layer (NL). A small cleft (CL) divides the placodal structure from the forebrain (FB). × 400.

Fig. 3. Stage 25 *Xenopus laevis* embryo. The placodal area (indicated by two arrows) shows a selective increase in the thickness of the nervous layer (NL) while the nonnervous layer (NNL) is still represented by a single layers of cells. B, brain. OV, optic vesicle. × 400.
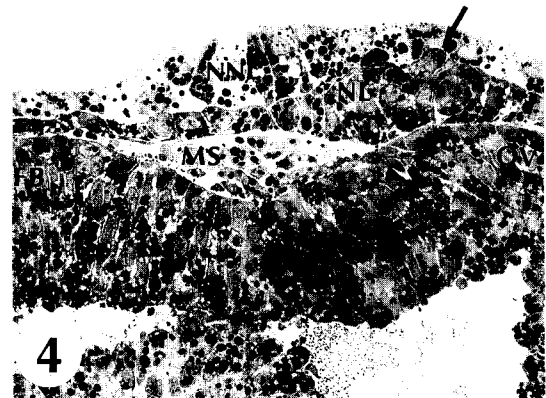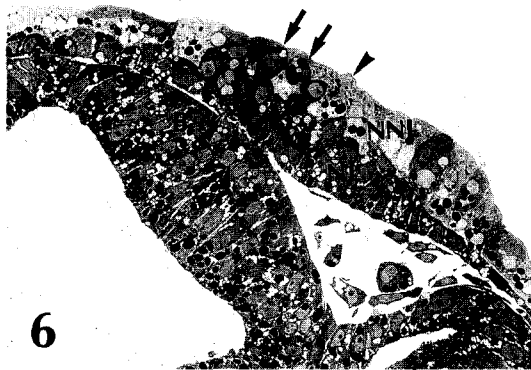

Fig. 4. Horizontal section through the haed of a stage 26 embryo. The cells of the nervous layer (NL) begin to invade the nonnervous layer (NNL) at arrow. A layer of mesenchyme (MS) appears between the placodal area and the forebrain (FB). OV, optic vesicle. × 400.

Many of the darkly staining distal processes of the NL cells penetrate between the NNL cells. Some of the superficial dark cells reach the free epithelial surface with their apical processes at stage 28 (Fig. 5). From stage 28 the ectoderm of the olfactory region aquires the characteristics of the olfactory epithelium because the two ectodermal layers are no longer discretely separated. Several of the NNL cells show apical protuberances at stage 29. These apical mounds are filled with large vesicles. NL cells and NNL
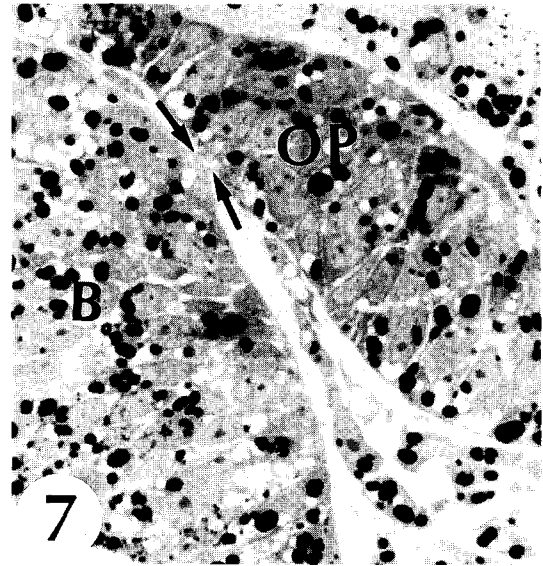
cells continue to comingle. At stage 29/30 (Fig. 6) cell processes, presumptive olfactory growth cones, begin to project from the placodal base to the surface of the forebrain. At stage 32 (Fig. 7) these processes develop more obviously at the base of the placode toward the forebrain wall and few sensory axons begin to reach the brain. From

Fig. 5. Horizontal section of stage 28 embryo at the level of the optic vesicle (OV). Midline is indicated by a bar. The boundary between the olfactory placode (OP) and the NNL is much less distinct than it was at stage 26. At this stage apical process of some cells of the nervous layer reach the epithelial surface (arrow) and the placodal area of this layer becomes stratified. The nonnervous layer (NNL) is still formed by a simple layer of cells. B, brain. × 400.

Fig. 7. Stage 32 embryo. At the base of the placode cellular bridge with the forebrain wall become more evident. Few sensory axons begin to invaginate the brain (arrow). B, brain; OP, olfactory placode. × 400.


Fig. 6. Stage 29/30 embryo. Comingling of the nervous layer (NL) and nonnervous layer (NNL) cells is evident at arrows. Many of the placodal cells have migrated from NL, obscuring the separation between NL and NNL. At this stage the basal processes begin to project from the placodal base to the surface of the forebrain as indicated by large arrowheads. Some of the NNL cells show apical protuberances (small arrowheads). B, brain; OV, optic vesicle. × 400.

stage 32 the frontal part of the telencephalon where olfactory axons arrive becomes very complex because of the intermingling of dendritic branches of the brain cells with olfactory axons (Fig. 8). With increasing age, more cells in the olfactory neuroepithelium have both apical and basal processes (presumptive dendrites and axons). The dark cells have the characteristics of neurons in various stages of differentiation and the ones that are near the surface show most mature features. The cells nearest to the surface aquire the characteristics of mature neurons and contain more rER, mitochondria and microtubules, and Golgi complexes. The olfactory nerve fascicles develop connections with the brain, while many cells are still in differentiation into mature olfactory neurons. Round dark cells still remain at the base of the neuroepithelium and many of these cells show mitotic figures. At stage 35/36 the placodes show the beginning of the surface invagination of the olfactory pit (Fig. 9). The NNL cells elongate and project processes toward the base of the neuroepithelium. These cells become more similar to the supporting cells of the mature neuroepithelium. All of the components of the
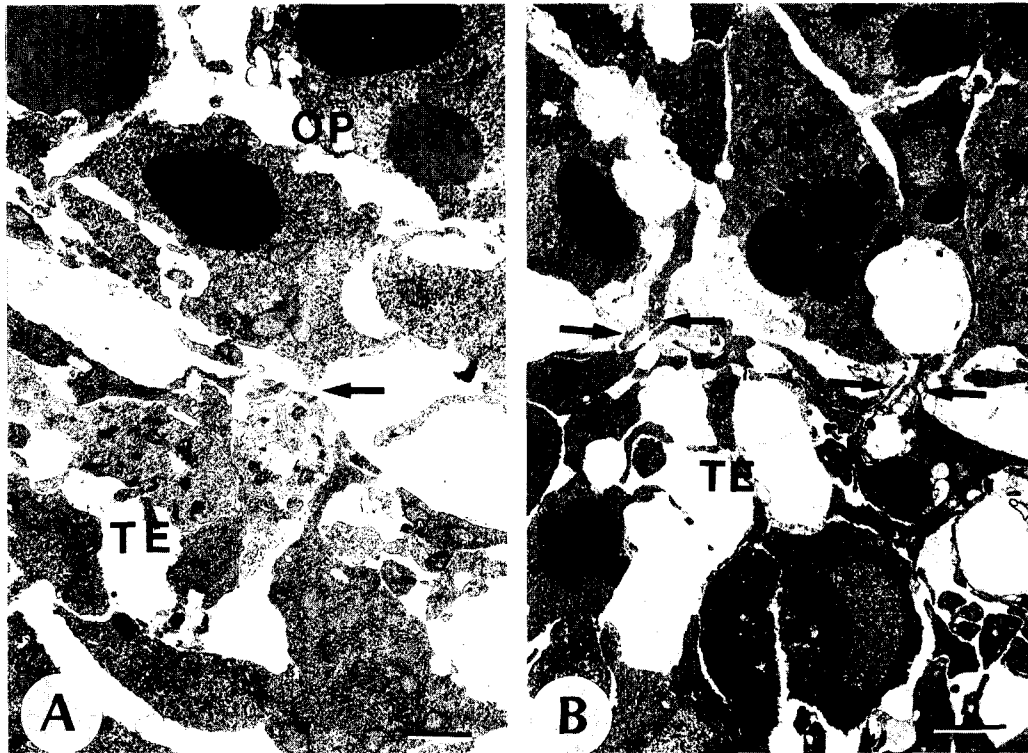
Fig. 8. Electron micrograph of stage 32 embryo. (A) One growth cone-like structure reaches the brain (arrow). The frontal part of the telencephalon where olfactory axons arrive becomes complex. TE, telencephalon; OP, olfactory placode. × 10,000. (B) A few axons reach the telencephalon at the arrows. TE, telencephalon; OP, olfactory placode. The black blobs are pigment granules. × 4,000. bar = 3 microns.

olfactory epithelium, basal cells, neurons, and supporting cells can be first seen in their developing forms at stage 29/30. By stage 37/38 (Fig. 10) the neuroepithelium displays the organization of the mature organ. The neurons closest to the free surface of the epithelium have the characteristics of olfactory receptor cells (Fig. 11). They are bipolar cells with thick apical processes and thin basal processes. Apical processes of many cells terminate at the body surface and often extended as a knob.

A primitive form of synaptic formation appears as a few presynaptic vesicles and synaptic thickenings at stage 37/38 (Fig. 12). By stage 41 obvious axodendritic synapses are formed in the brain (Fig. 13).

Discussion

The olfactory neuroepithelium is derived from the olfactory placode which consists of two morphologically discrete layers: the ectodermal nervous layer (NL) and the ectodermal nonnervous layer (NNL) which covers the NL. In the development of the olfactory epithelium from the olfactory placode the following sequential embryological processes appear to occur. (1) stem cell proliferation (2) the initial differentiation of receptor cells by formation of dendrites and dendritic knobs (3) the differentiation of non nervous elements (supporting cells) (4) outgrowth of receptor axons (5) the maturation of receptor cells (6) the formation of synapses between olfactory receptor cells and brain cells.

During the early stages of embryonic development, I have observed that, the NL cells
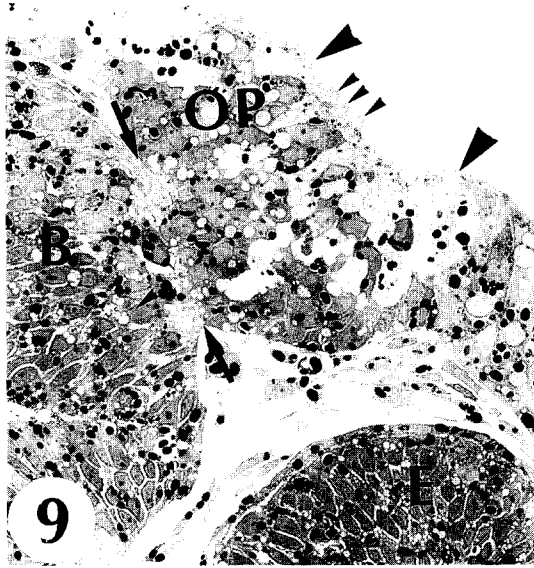
Fig. 9. Stage 35/36 embryo. The placode demonstrates comminging of the cells of the two layers (NL and NNL) and many nerve fascicles (arrow) link them with the forebrain. Round and dark cells remain at the base of the neuroepithelium. The surface invagination of the olfactory pit (large arrowheads) begins. Protuberances of NNL (nonnervous layer) cells are more prominent (small arrowheads). B, brain; E, eye; OP, olfactory placode. × 400.

are morphologically similar to the cells of the neural tube while the NNL cells maintain their epithelial characteristics. The olfactory receptor cells derive from a region of the nervous layer of the olfactory placode located at the boundary of the neural folds. These observations are consistent with the findings of previous authors (Jacobson, 1959; Van Oostrom and Verwoerd, 1972).

The NL cells show a high degree of morphological maturation by stage 28 when some of their dendrites have already reached the free surface. Their axons exit through the basal lamina of the olfactory epithelium at stage 29/30 indicating that the olfactory neurons are morphologically mature before their axons reach their target, namely the telencephalon. At this time the cells of the telencephalon are still in the early stages of differentiation and they only begin to differentiate after the olfactory axons reach them. This indicates that the maturation of the olfactory neurons is not influenced by the target
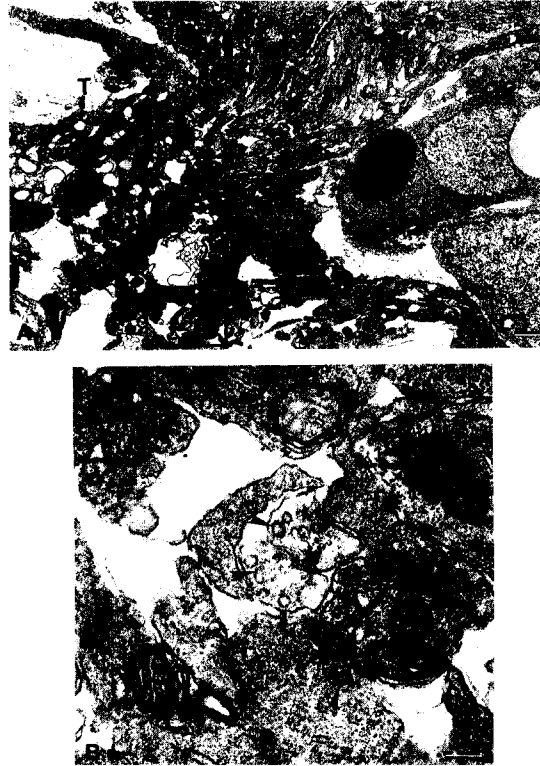

Fig. 10. Horizontal section of the embryo head. (A) Low power view of the stage 37/38 embryo shows the invasion of the forebrain by the sensory axons (arrow). Arrowhead indicates the olfactory neuron that its dendrite reaches the epithelial surface. Midline is indicated by bar. B, brain; OP, olfactory placode; E, eye. × 250. (B) Stage 41 embryo. Nerve fascicles of olfactory axons are more obvious (arrow) at this time. OP, olfactory placode; FB, forebrain. × 375.

tissue, instead, the subsequent differentiation of the target temporally follows the input from the olfactory neurons. The same observations were made in adult mammals (Monti Graziadei, 1989). This is unlike other nervous system segments where the target tissue influences the development of the receptor neurons (Lavelle and Lavelle, 1970; Jacobson, 1993). Further evidence in support of the independent development of the olfactory neurons comes from transplantation experiments. In *Amblystoma* several authors have shown that the nasal ectoderm cells are committed even before the appearance of the neural folds
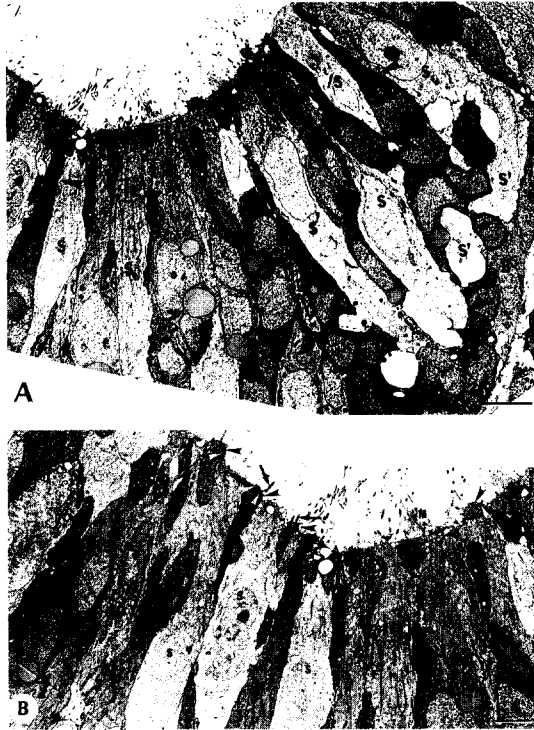
Fig. 11. Electron micrograph of the olfactory epithelium at stage 41. (A) Olfactory neuroepithelium contains both dark (neuron)(N) and light (supporting)(S) cells. Many elongated dark cells show the characterisrics of olfactory neurons (arrowhead). They are bipolar cells with thick dendrite (D) and thin axons. Small dark cells (B) are located close to the basal lamina. The light cells at the surface of the epithelium project their processes (S') toward the base of the epithelium. × 2,000. bar= 7.5 microns. (B) Higher magnification of stage 41 olfactory epithelium shown in (A). Dendrites of olfactory neurons often form dendritic knobs (large arrowheads) with basal bodies(white arrows) and cilia (arrows). Apical mounds of the supporting cells contain several secretory granules (small arrowheads). The small dark areas are pigment granules. S, supporting cells; N, olfactory neurons; M, microvilli; F, neurofilament bundles. × 4,000. bar = 4 microns.

Fig. 12. Electronmicrograph of stage 37/38. (A) Nerve fascicles (NF) of olfactory receptor cells connect with the telencephalon (T). The boxed area will be shown in (B). OP, olfactory placode. × 10,000. bar = 1 micron. (B) Higher magnification of the boxed area in (A). Primitive forms of synaptic formation appear. Synaptic membrane thickenings appear between neighbering axons and dendrites. A number of synaptic vesicles also appear (large arrowheads). Small arrowheads indicate dense cored vesicle. × 60,000. bar = 200 nm.

(Carpenter, 1937). When an extra olfactory placode is transplanted in different locations, the olfactory neurons mature in the abnormal environment even though their axons do not reach their target (Bell, 1907; Stout and Graziadei, 1980; Heckroth and et al., 1983; Monti Graziadei, 1992; Valverde and et al., 1992; Koo and Graziadei, 1995)

Autoradiographic and other experimental observations support the idea that supporting cells do not develop from the same ectodermal layers as the receptor neurons (Graziadei, 1973b; Graziadei and Monti Graziadei, 1979). Results also clearly show that the supporting cells derive from the non nervous ectodermal layer and not from the remaining stem cells situated in the superficial layer of the olfactory epithelium as reported in mammals by Cushieri and Bannister (1975a, b)

As the NL cells migrate to the epithelial surface the NNL cells elongate and extend their processes toward the basal lamina while remaining in contact with the epithelial surface. These NNL cells

Fig. 13. Electron micrograph of synaptic formation in the presumptive olfactory bulb at stage 41. Synaptic formations between axons of olfactory neurons and presumptive dendritic profiles of telencephalic cells are indicated by arrows. M, mitochondria. × 30,000. bar = 750 nm.

acquire a secretory capability and other characteristics of supporting cells (Theisen, 1972; Graziadei, 1973a, b; Yamamoto et al., 1985). My observations show that these cells begin to differentiate at stage 27 which is approximately 4 hours before olfactory axons begin to exit from the basal lamina. However, different results were observed in rats where differentiation of supporting cells occurs right after the olfactory axons reach the olfactory bulb (Cushieri and Bannister, 1975b)

At stage 32 (1 day and 16 hours) the axons of the olfactory neurons reach the presumptive olfactory bulb. However, the development of synapses between receptor axons and their target cells in the telencephalon is not seen until stage 37/38 (2 days and 5 hours). Only after stage 41 can distinctive synapses with several presynaptic vesicles and synaptic thickenings be observed. This delay in synaptogenesis has also been observed in the olfactory bulb of mouse (Hinds

and Hinds, 1976b) and in the tectum of chick (Crossland et al., 1974). As opposed to the cortex, where synaptogenesis starts before neurogenesis is completed, these authors have observed that the presynaptic endings and the cells on which they will synapse stay close for days before they form synaptic connections.

The present study establishs two aspects of differentiation of the olfactory organ in Xenopus. First, the study demonstrates the origin of the olfactory neuroepithelium from two distinct populations of cells. It has emphasized that the olfactory organ anlagen is part of the nervous layer of the ectoderm, located at the boundaries of the neural plate. Second, the study demonstrates that the olfactory axons develop from the base of the olfactory epithelium and mature before reaching their target. Synaptogenesis starts by innervation of the olfactory axon when brain is still undergoing primitive differentiation.

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Xenopus 후각원판의 분화
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*Xenopus*를 사용하여 후각원판이 정상적인 상태에서 어떠한 과정을 거쳐 발달하는지 연구하였다. 특히 뉴런의 형태적 분화, 초기 발달과 성숙 양상, 축색과 원시전뇌의 접촉 등에 초점을 맞추었다. 후각원판은 stage 23에 외배엽이 두터워진 형태로 처음 나타나는데, 쌍을 이루며 각각은 표피쪽의 비신경층(NNL)과 안쪽의 신경층(NL)의 두 층으로 되어 있다. Stage 26 후에 원판 세포는 NNL세포 틈을 비집고 상피쪽으로 이동하기 시작하며, stage 28이 되면 선단 돌기가 표피 끝에 도달한다. Stage 29/30에는 NL의 기부에서 기부 돌기(미래의 축색)가 나타나 stage 32무렵에 종뇌에 도달한다. 시냅스는 stage 37/38에 처음 나타난다. 일부 원판 세포들이 후각뉴런으로 분화하는 동안 많은 원판 세포들은 기저세포로서 후각상피에 그대로 남아 있다. 연구 결과는 뉴런은 외배엽의 NL에서 기원하고 지지세포는 NNL층에서 기원함을 보여주었다. 또한 시냅스 형성 전에 뉴런의 분화가 완성됨으로써 후각뉴런의 분화는 뇌의 발달과 독립적으로 일어나며 뇌의 영향을 받지 않는다는 사실을 알 수 있었다.