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Interactions between the olfactory placode and the olfactory bulb during development in *Xenopus laevis*

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The University of Arizona, 1992
INTERACTIONS BETWEEN THE Olfactory Placode AND THE Olfactory Bulb DURING DEVELOPMENT IN XENOPUS LAEVIS

by

Christine Ann Byrd

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During Development in Xenopus laevis

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Christine A. Byrd
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LIST OF ABBREVIATIONS

CNS = central nervous system
OB  = olfactory bulb
OP  = olfactory placode
TTX = tetrodotoxin
ABSTRACT

The relationship between olfactory receptor-cell axons and the output cells of the olfactory bulb (the mitral/tufted cells) was analyzed during normal development and following experimental manipulation to investigate the role of olfactory afferent axons in the induction of olfactory-bulb formation. A light- and electron-microscopic study of *Xenopus* embryos, larvae, and adult frogs outlined the major events in olfactory-bulb formation. Axons were observed in the neural tube immediately before the future olfactory-bulb neurons began to differentiate. Quantitative analysis during normal development indicated that both olfactory-axon number and mitral/tufted-cell number increased and were positively correlated throughout larval development. By adulthood, however, the number of axons increased significantly more than the number of mitral/tufted cells. The correlation of axons to mitral/tufted cells in larvae and the time course of normal, larval development are consistent with the hypothesis that olfactory axons influence olfactory-bulb development.

To examine further the relationship between the axons and output cells, experimental manipulations to alter the number of incoming axons were performed. The effects of partial deafferentation of the olfactory bulb, by removal of one olfactory placode before differentiation of the neural tube, were analyzed. This manipulation had a major effect on the morphology of the olfactory bulb and resulted in a significant reduction in olfactory-axon number (to about one-half of
control values) and a corresponding decrease in mitral/tufted-cell number.

The effects of transplanting an extra olfactory placode on the development of the larval olfactory bulb were also examined. The transplanted tissue retained the normal appearance and cell types of olfactory epithelium. The volume of the olfactory bulbs in animals with a transplant was not significantly different from control values. While there was initially an increase in total axon number, by late-larval stages, the number of olfactory axons was not different from control values. This suggests that the olfactory bulb regulates the number of afferent fibers. The number of mitral/tufted cells at early-and late-larval stages was not affected by this manipulation. Therefore, it appears that there are reciprocal interactions between the olfactory epithelium and the olfactory bulb during development.
CHAPTER 1: INTRODUCTION

In several sensory systems, afferent axons have profound effects on the development of their target structures in the central nervous system (CNS). Experimental manipulations to alter the number of primary afferent axons have been performed in many of these systems in attempts to reveal the normal effects of axons on their target tissue. The following dissertation characterizes the interactions between olfactory afferent axons and their CNS target, the olfactory bulb (OB). Both deafferentation and transplantation methods were used to examine this relationship.

Deafferentation Studies

Deafferentation of a central brain structure during development has been used in several systems including the vertebrate visual system (Kollros, 1953; Sohal, 1976), invertebrate visual system (Power, 1943; Meyerowitz and Kankel, 1978; Macagno, 1979; Selleck and Steller, 1991), vertebrate auditory system (Levi-Montalcini, 1949; Parks, 1979; Born and Rubel, 1985; Moore, 1990), vertebrate somatosensory system (Belford and Killackey, 1979a, 1979b, 1980), antennal-lobe system of insects (Hildebrand et al., 1979; Oland and Tolbert, 1987; Tolbert and Sirianni, 1990), and vertebrate olfactory system (Burr, 1916a; Piatt, 1951; Clairambault, 1971, 1976; Graziadei and Monti-Graziadei, 1992). These are all
examples of experiments in which the sensory afferent axons were removed during development and the effects on their targets were analyzed. Other studies have analyzed the effects of deafferentation in adult animals and found that, even in adults, afferent axons are important in maintenance of their target (e.g. Cook et al., 1951; Matthews and Powell, 1962; Powell and Erulkar, 1962). These will not be examined further here as my main emphasis is on the effects of afferent axons on target-structure development. There are also numerous studies that analyze the effects on central structures when their peripheral target is removed (e.g. Hamburger, 1934; Cowan and Wenger, 1967; Prestige, 1967; Baptista et al., 1990).

In these studies, removal of a limb bud or peripheral organ produced severe alterations in the development of the area of the CNS that innervates those structures. These types of studies will also not be elaborated here; instead, I have concentrated on the effects of sensory afferent fibers on target CNS structures.

One example of a sensory system in which trans-synaptic alterations were created with experimental manipulations during development is the trigeminal pathway of the somatosensory system of rats and mice. In this pathway, the whiskers on the snout are innervated by sensory neurons that have synaptic relays through a series of central trigeminal structures. An anatomical map of the whiskers exists in each step of the relay from the medulla to the thalamus, ending in structures known as "barrels" in layer IV of the somatosensory cortex. Development of the map proceeds sequentially, beginning with the most peripheral
target (Belford and Killackey, 1979b). Damage to a row of whiskers during early postnatal development either prevents the formation or causes degeneration of the cortical barrels, three synapses away from the original sensory neuron (Van der Loos and Woolsey, 1973). It has been shown that injury at progressively older stages of development in the rat disrupts the normal formation of the map first in the medulla, followed by the thalamus, and finally the cortex (Belford and Killackey, 1979b). Thus, in this system, if the primary afferent fibers are damaged, defects occur in the primary target (medulla) as well as in the rest of the pathway.

The auditory system is another system in which alterations in the sensory periphery lead to profound changes in the central target. Here, ganglion cells of the VIII nerve receive synaptic input from the hair cells in the cochlea and send afferent axons to synapse in the cochlear nucleus (mammals) or the nucleus magnocellularis (birds) of the brainstem. After embryonic cochlear ablation, the number of brainstem neurons decreases (Levi-Montalcini, 1949; Parks, 1979; Born and Rubel, 1985; Moore, 1990), the neurons migrate abnormally (Levi-Montalcini, 1949; Parks, 1979), and protein synthesis is reduced (Born and Rubel, 1985; Steward and Rubel, 1985), suggesting that these target neurons depend upon primary auditory afferent input for their growth and maintenance.

Often, the effects of deafferentation appear to be due primarily to the cessation of activity, since just blocking activity can have deleterious effects on the development of target structures. In the auditory system, blocking activity in the
auditory nerve with the sodium-channel blocker tetrodotoxin (TTX) alters protein synthesis and cell size in the postsynaptic target in chicks and mammals (Born and Rubel, 1988; Sie and Rubel, 1992). In addition, cell number is decreased in the avian auditory system (Born and Rubel, 1988). Similarly, in the visual system of the kitten, visual deprivation by eyelid suture (which blocks patterned activity) results in marked histological changes in the primary target, the lateral geniculate nucleus (Wiesel and Hubel, 1963). These changes include shrunken nuclei and diminished cell areas. Furthermore, deprivation by eyelid suture in the monkey alters the formation of ocular dominance columns in layer 4 of the visual cortex (Hubel et al., 1977). These columns result from eye-specific segregation of axons from the lateral geniculate nucleus. Eyelid suture results in shrinkage of the cortical projections from the lateral geniculate nucleus that receives projections from the deprived eye and expansion of the cortical projections from the lateral geniculate nucleus of the remaining eye. This suggests that patterned vision is not necessary for the initiation of column formation but is important for completion of column formation. Injections of TTX, which silences all activity (both patterned and spontaneous), block the formation of ocular dominance columns altogether (Stryker and Harris, 1986), suggesting that spontaneous activity is the most important factor in the initiation of column formation. In addition, the formation of eye-specific layers in the lateral geniculate nucleus is also not dependent upon visual experience, since these layers develop before photoreceptor development is
complete (i.e. before patterned vision can occur) (Donovan, 1966). Also, in the development of the retinogeniculate pathway, spontaneous activity is responsible for eye-specific segregation of afferent fibers, as shown with TTX infusion (Shatz and Stryker, 1988). All of these studies involving disruption of the integrity or activity of afferent fibers show that normal sensory stimulation is crucial in the development of central brain structures.

Transplantation Studies

Converse experiments involving transplanting extra tissue have also been performed to examine the effects on the target. Examples of these include the vertebrate visual system (Twitty, 1932), the vertebrate auditory system (Richardson, 1932), and the vertebrate olfactory system (Burr, 1924, 1930; May, 1927; Stout and Graziadei, 1980). Several transplantation studies have been performed in amphibians, especially salamanders, in which a graft is taken from one species and transplanted into another species that is very different in size (heteroplastic transplantation). *Ambystoma tigrinum* and *Ambystoma punctatum* have been used in experiments in which an eye, ear, or nose was interchanged. Twitty (1932) interchanged one eye and found that the visual centers of the brain became enlarged or reduced depending on the size of the grafted eye. Richardson (1932) looked at the effects of heteroplastic transplantation with the ear vesicle in *Ambystoma* and also found marked hyperplasia or hypoplasia in the area acoustica.
of the medulla (center for endings of the VIIIth nerve fibers) depending on the size of the transplant.

**Olfactory System: Structure and Development**

The olfactory system is a model system in which to study afferent effects on development of a central structure because it is easily accessible, the structure is well-known, and the circuitry is well-understood (see for example, Pinching and Powell, 1971a, 1971b; Shepherd, 1974). The peripheral olfactory organ is the olfactory epithelium located in the nasal capsules. Odorants stimulate the neurons in the olfactory epithelium, the olfactory receptor cells. The axons of these cells (olfactory axons) form the first cranial nerve, the olfactory nerve; information is transmitted via this nerve to the brain for detection and perception of the odorant. The primary CNS target for the olfactory axons is the OB. Olfactory-axon terminals make synaptic connections in the glomerular layer with the dendritic processes of neurons of the OB. Some of the OB neurons that are postsynaptic to the olfactory axons are the output neurons, the mitral and tufted cells; these cells modify the information from the olfactory axons and send it to higher centers of the brain for identification. Many vertebrates possess a two-part olfactory system: the main olfactory system is primarily involved in general odorant detection, and the accessory system is most important in pheromone detection for reproductive behaviors. The sensory organ for the accessory olfactory system is the vomeronasal
organ. The axons of the receptor cells in this epithelium project to the accessory OB. The central projections of the main and accessory olfactory systems remain distinct (Winans and Scalia, 1970).

The olfactory epithelium develops from the olfactory placodes (OPs). These placodes arise from paired thickenings in the cranial ectoderm (Knouff, 1935; van Oostrom and Verwoerd, 1972). During development, olfactory receptor cells in the placode send their axons through the basal lamina to the underlying neural tube (Cuschieri and Bannister, 1975; Klein and Graziadei, 1983). Several lines of evidence suggest that these axons influence the development of the OB. For example, soon after the olfactory axons reach the neural tube in mouse embryos, the OB begins to differentiate (Hinds, 1972b). Also, the olfactory axons initially penetrate into the deep layers of the neural tube before retracting to their final positions in the superficial layers of the bulb (Hinds, 1972b). Since the axons are found in the deep layers of the OB where neurogenesis occurs, it is possible that they could influence the precursor cells of the bulb during very early stages of OB development. This idea is supported by the fact that the appearance of young neurons, including the major output neurons of the bulb (the mitral cells), coincides with the ingrowth of olfactory axons (Hinds, 1972b).
Olfactory System: Effects of Deafferentation

More direct evidence of the role of olfactory afferent axons on development of central olfactory structures has been provided by studies on early sensory deafferentation. For example, in the antennal (olfactory) lobe of the moth, *Manduca sexta*, removal of the antenna early in postembryonic development results in deafferentation of the lobe and subsequent failure in the formation of glomeruli (specialized neuropil structures) (Hildebrand et al., 1979; Oland and Tolbert, 1987). Even more dramatic changes have been observed in amphibians where removal of one OP early in embryonic life in *Ambystoma* (Piatt, 1951), *Rana* (Clairambault, 1971, 1976), and *Xenopus* (Stout and Graziadei, 1980; Graziadei and Monti-Graziadei, 1992) prevents the formation of the ipsilateral OB. Similar findings have been reported in chicks (Venneman et al., 1982) and mice (Giroud et al., 1965) where prevention of innervation by olfactory axons leads to disruption of the development of the OB. When no olfactory axons innervate the neural tube, no olfactory bulb forms; when only a few olfactory axons innervate the neural tube, a rudimentary, abnormal OB forms. These results suggest that olfactory afferent axons play a critical role in the development of target olfactory structures. The mechanisms by which olfactory axons exert their influence have not been elucidated.
Olfactory System: Effects of Transplantation

To define further the importance of olfactory axons on OB development, several studies have involved transplantation of an extra OP. Burr (1930) performed heteroplastic transplantation of olfactory organs in salamander species of different sizes and found that when the larger olfactory organ was transplanted into the smaller species, there was hyperplasia in the cerebral hemispheres. In the same study, Burr (1930) also transplanted an extra OP into Ambystoma and found that this resulted in hyperplasia of the cerebral hemisphere; however, it is unclear which cells were counted. Similarly, Stout and Graziadei (1980) reported that, in Xenopus, when the axons from an OP transplant grow into the OB, the bulb volume in young larvae is 40-60% larger than normal; however, no data was presented.

In addition, investigators have found that OPs transplanted to ectopic cranial regions innervate a variety of brain areas and appear to induce significant changes at these sites. Stout and Graziadei (1980) transplanted OPs to a variety of head regions in Xenopus. When the transplanted OPs penetrated the diencephalon, the olfactory axons innervated the dorsal thalamus. Stout and Graziadei (1980) reported that this results in hyperplasia and formation of "glomeruli-like structures". No cell counts were performed, so it is possible that a rearrangement of neurons in the innervated structure was misinterpreted as an increase in cell number. Because the determination of glomeruli was from light-level analysis, however, the
presence of synaptic connections was not established. These structures could simply be bundles of olfactory axons similar to neuromas found in injured peripheral nerves. When the nerve entered the myelencephalon, no hyperplasia or glomerular formation was observed. In a similar study, Magrassi and Graziadei (1985) removed the optic vesicle in *Xenopus* and transplanted two OPs in its place. In all cases, they observed that the transplants fused with the host OP, but the fused structure had additional nerves. The extra nerves from the this fused structure penetrated a protrusion from the diencephalon and formed a "glomerular layer" with presumptive synaptic connections between olfactory axons and dendrites from the surrounding neurons. Some possible explanations for their data are suggested by the authors including the hypothesis that a piece of the telencephalon was transplanted with the placode or that cells migrated from the placode to form the new cell population.

These results, which suggest that olfactory axons can profoundly influence areas of the CNS that are not their normal targets, are consistent with earlier findings by Burr (1924, 1930) and May (1927). Burr transplanted an extra olfactory organ to the head region in *Ambystoma* and found that often the nerve entered the diencephalon and established connections with the dorsal thalamus (Burr, 1924); this resulted in hyperplasia of that area (Burr, 1930). May (1927) excised the otic placode in two other amphibians, *Rana* and *Bufo* (in separate experiments), and transplanted an olfactory organ in its place. He found that innervation of the
medulla by the graft resulted in hyperplasia (4.26%) and a cellular migration toward the nerve. The amount of hyperplasia was related to the number of penetrating axons.

One of the problems with these early studies of amphibians (May, 1927; Burr, 1930) is the lack of consistency in staging the animals. These studies were performed before staging tables were widely accepted, so that it is difficult to be certain when in the development of the system the surgeries were performed. Regardless, the above studies provide evidence that molecular factors released from olfactory receptor-cell axons in the OP or cellular interactions between olfactory axons and the embryonic neural-tube neurons are responsible for inducing OB development and are also capable of organizing forebrain tissue.

Another study that illustrates the powerful influence olfactory axons have on their target was performed on the antennal-lobe system of Manduca. Male moths possess a specialized region of neuropil that is absent in females. Transplantation of an antennal imaginal disk from a male into a female produces a gynandromorph. In these animals, the female antennal lobe receives projections from the male antenna; this results in the development of male-specific neurons in the antennal lobe of the female (Schneiderman et al., 1982). Clearly, the olfactory receptor-cell axons in the moth antennae, like the receptor cells in other animals, are capable of restructuring CNS targets.
Goals of This Dissertation

Many of the studies outlined above have been performed in insects and amphibians because of the accessibility of the olfactory system even at very early stages of development. I have examined the influences of afferent axons on the development of the OB in the clawed frog *Xenopus laevis* for a number of reasons. First, these frogs can be bred year round and are oviparous, so that even early embryonic stages are available. Second, the embryos have tremendous regenerative capabilities and can withstand extensive experimental manipulations. Third, because the olfactory structures are not encased in bone, as they are in mammals, they are easily accessible, especially in embryonic and larval stages. Finally, the larvae are transparent, so that the olfactory structures are quite obvious and the results of experimental manipulations can be seen without dissection.

The goal of this research was to make a step toward understanding the cellular interactions and potential molecular factors that control development of the OB. I hope that this work eventually leads to future experiments to determine whether there is an inductive influence of olfactory axons on the birth, differentiation, or migration of cells of the OB. My analysis of this question began with a study of the normal development of the OB in *Xenopus laevis* since there was no published work outlining OB development in this frog. Once this was accomplished, experiments to analyze the interactions between the OP and OB were designed. To test the hypothesis that olfactory axons are crucial in OB
development, I experimentally altered the number of sensory afferent axons innervating the developing OB prior to OB differentiation.

These studies were designed to examine the effect of olfactory axons on the induction of OB development. Partial deafferentation of the OBs was accomplished by removing one OP at embryonic stages; conversely, transplantation of an extra OP was performed in an attempt to provide supernumerary innervation to the OB. These studies are different from previously published work in these areas in that my studies were performed at well-defined stages, when no OB differentiation had occurred, and they involve comprehensive quantitative analyses, unlike previous qualitative studies. My results suggest that there are cellular interactions between olfactory axons and cells of the neural tube or molecular factors released from the olfactory axons that are responsible for influencing OB development, and in addition that there appear to be cellular or molecular signals from the OB that influence the development of the olfactory epithelium.
CHAPTER 2: DEVELOPMENT OF THE OLFATORY BULB IN *XENOPUS*

The first step in the analysis of the interactions between olfactory axons and the OB during development was to investigate the normal development of the OB in *Xenopus*. Thus, I performed a morphological and quantitative analysis of OB development. The goal of the morphological study was to determine the time course of development of the OB. The quantitative study was performed to analyze the relationship between the number of olfactory afferent axons and the number of OB output neurons. The output neurons of the OB are the mitral and tufted cells; in these studies they are grouped together as mitral/tufted cells because the two cell types are mixed together in *Xenopus*. These cells were counted because they receive direct synaptic input from olfactory axon terminals. Thus, it is expected that these cells will be involved in important developmental interactions with the olfactory axons. The ratio between olfactory axons and mitral/tufted cells is the convergence ratio; this number may be functionally related to the efficiency of olfactory acuity, as will be discussed later. In this chapter, I report that the time course of OB development and the numerical relationship between olfactory axons and mitral/tufted cells throughout larval development are consistent with the hypothesis that the olfactory afferent axons influence OB development. Reports of the data presented here appeared in Byrd and Burd (1990, 1991a, 1991b).
MATERIALS AND METHODS

Tissue preparation

Embryos were obtained from gonadotropin-induced matings of *Xenopus laevis* breeders (obtained from either Nasco or a natural breeding population). The offspring were reared in 6-liter tanks with rearing solution (see Burd, 1991) and fed boiled nettle (Wunderlich-Diez Corp., Hasbrouck Heights, NJ) daily. They were staged according to criteria established by Nieuwkoop and Faber (1956).

Forty-eight animals from several breedings and at various stages of development were anesthetized with 0.02% (embryos and larvae) or 2% (adults) MS222 (tricaine methanesulfonate; Sigma) and either immersed in (up to stage 48) or perfused with (beyond stage 48) 4% glutaraldehyde (Ted Pella), 0.6% paraformaldehyde (Fisher) in 0.035M cacodylate buffer (pH 7.4) (Ted Pella). They were left in the fixative solution overnight; then either the entire embryo (for young stages) or only the nasal capsules and brains (for older stages) were postfixed in 2% osmium tetroxide (Electron Microscopy Sciences), dehydrated in a series of graded alcohols, and embedded in Epon (Ted Pella) (for light and electron microscopy) or Durcupan (Electron Microscopy Sciences) (for light microscopy). Serial, one- or two-micron sections were made of the tissue in either the horizontal or coronal plane. The sections were stained with toluidine blue (1% toluidine blue, 1% sodium borate). At selected areas, thin-sections were made, collected on
formvar-coated slot grids, stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 electron microscope.

**Quantitative analysis**

The axons in the olfactory nerve, which includes olfactory and vomeronasal receptor-cell axons, and the cells in the mitral-cell/plexiform layer of the main and accessory OBs were counted in larvae at stages 50 (n=3), 54 (n=3), 56 (n=3), 57 (n=3), and 58 (n=6) and in adult, female frogs (n=2). It was not possible to differentiate between axons originating from the sensory epithelium of the vomeronasal, principal, or middle cavities. Therefore, for quantitative analysis, I had to group together axons from these areas as well as mitral/tufted cells from all areas of the main and accessory OBs. Even though this grouping might mask some interesting differences between the main and accessory olfactory systems, they were analyzed together because differential data analysis was not possible. Counts of axons in the olfactory nerve were made from electron-micrographic montages of one olfactory nerve in each animal as described by Burd (1991). Because there is no significant difference in the number of olfactory axons in each olfactory nerve, the axons in one nerve were counted and multiplied by two to get the total number of olfactory axons in the animal (Burd, 1991). The total area of axons in the nerve, excluding glial cells and extracellular space, was measured in electron-micrographic montages at 4000x, the number of axons in 10-20% of each measured area was
counted, and then the total number of axons in the olfactory nerve was calculated (see Burd, 1991). Because the olfactory nerve in adults is significantly larger than that in larvae, the method of quantifying the number of olfactory axons was modified. A montage through the entire olfactory nerve was made at 1000x to permit calculation of the area of the nerve (after extracellular space and glial cells were eliminated). Photomicrographs for counting axons were taken at 4000x at representative areas, the area occupied by axons in each 4000x photomicrograph was measured, and the axons in 10-20% of that area were counted. This number, which represented about 1% of the total nerve, was then extrapolated to the total area to get the total number of axons in adult frog olfactory nerves. Because the olfactory receptor-cell axons do not branch before entering the OB (Scalia, 1976), the number of olfactory axons estimated with these techniques is actually an estimate of the number of mature or nearly mature olfactory receptor cells in the olfactory epithelium.

The number of mitral/tufted cells in the OB was determined from serial, one-micron, horizontal sections through the entire OB in larvae and from two-micron sections in adults. In every twentieth section, cells in the mitral cell/plexiform layer were counted by using a grid reticle in a light microscope. All of the cells in this layer of both the main and accessory OBs were counted, and so the number of mitral/tufted cells actually includes mitral cells, tufted cells, and glial cells. Glial cells were included because it is difficult to distinguish them at the
light-microscope level, but they do not appear to be present in high numbers. A preliminary analysis of the percentage of cells in the mitral-cell/plexiform layer that are neuroglia was performed. The glial cell nuclei were counted in one, 1-μm section (at the level where both accessory OBs are present) of three animals at stage 58. The criteria for identification as a glial cell were the following: a darkly-stained nucleus that was smaller (and often more irregularly-shaped) than the large, round, lightly-stained nuclei of the mitral/tufted cells. These cells made up an average of 3% of the total number of cells. Therefore, it was concluded that the inclusion of glia in the estimate of mitral/tufted-cell number did not severely affect the analysis. The accessory OB was included in these studies because the nerve to the accessory bulb (the vomeronasal nerve) runs with the olfactory nerve and is included in the number of olfactory axons. The total number of mitral/tufted cells was calculated using the following formula (adapted from Abercrombie, 1946; Konigsmark, 1970): total number of cells = total nuclei counted x (total number of sections/number of sections counted) x (section thickness/nuclear diameter). The average mitral/tufted-cell nuclear diameter was obtained by measuring the diameters of 20 nuclei in the mitral cell/plexiform layer of animals at each stage. The average mitral/tufted-cell nuclear diameter for all stages was 7.49±0.07 (n=13). The nuclear diameter increased only slightly with stage (7.36±0.17 (n=3) at stage 50; 7.42±0.12 (n=3) at stage 54; 7.54±0.13 (n=3) at stage 56; 7.63±0.17 (n=3) at stage 58; and 7.55±0.05 (n=2) in adults).
RESULTS

Morphology of the developing olfactory bulb

The structure of the developing OB was examined at both the light- and electron-microscopic levels. The OB developed from the area of the neural tube directly underneath the OPs. An overview of the development of the OB at the light-microscopic level is shown in Figures 2.1 and 2.2. The area underneath the OP had the characteristics of undifferentiated neural tube (see below) when it was contacted by the axons of the olfactory receptor cells at stage 29/30 (Figure 2.1A). The first sign of differentiation was evident at stage 32 (Figure 2.1B). Olfactory axons continued to grow into the expanding neural tube, and both the olfactory nerve and the olfactory nerve layer were present by stage 36 (Figure 2.1C). The neural tube continued to enlarge in the region of the OB, and the olfactory nerve layer was larger and more obvious by stage 40 (Figure 2.1D). By stage 44, all of the layers present in the mature OB were apparent, but the elements comprising them were loosely organized (Figure 2.1E); by stage 48/49, the organization of layers was indistinguishable from the mature pattern (Figure 2.1F). The basic structure of the OB remained constant throughout late larval life and into adulthood (Figures 2.2A and B).

The olfactory axons have been reported to reach the prosencephalon at stage 32 (Nieuwkoop and Faber, 1956; Klein and Graziadei, 1983). In our study, olfactory receptor-cell axons were first seen below the basal lamina of the neural
FIGURE 2.1. Early development of the olfactory bulb. Light-microscopic photomicrographs show, in horizontal sections, the transition from undifferentiated neural tube to olfactory bulb. The rostral pole is at the top of the figures. A: Stage 29/30: the neural tube shows no signs of differentiation. op, olfactory placode; ob, future site of the olfactory bulb; V, ventricle. B: Stage 32: differentiating cells (arrow) are present in the neural tube. C: Stage 36: the olfactory nerve (arrow) is distinct, the olfactory nerve layer is evident (arrowheads), and the intermediate zone (iz) can be differentiated from the ventricular zone (vz) (separated here by a dashed line). D: Stage 40: many more olfactory axons are present at the surface of the developing olfactory bulb (arrowheads), and the olfactory nerve is thicker (arrow). E: Stage 44: all the layers of the olfactory bulb are present. onl, olfactory nerve layer; gl, glomerular layer; mc/pl, the mixed mitral-cell/plexiform layer; gel, granule cell layer. F: Stage 48/49: the accessory olfactory bulb (aob) is separate from the main olfactory bulb and has the same layers as the main bulb. Scale bar for A-E and for F = 50 μm.
FIGURE 2.2. Further development of the olfactory bulb. Light-microscopic photomicrographs show the bulbs from a stage-58 larva (A) and an adult frog (B). Abbreviations are the same as in Figure 2.1. Scale bars = 100 μm.
tube at stage 30, but they were much more prevalent by stage 32 (Figure 2.3A). The axons in cross-section were small, round profiles with numerous microtubules.

Between stages 26 and 30, when the OP begins to develop (Klein and Graziadei, 1983), the neural tube remains very thin, with only a slight thickening apparent on the lateral sides (Nieuwkoop and Faber, 1956). At these stages, the cells of the neural tube appeared to be undifferentiated cells of the ventricular zone (Figure 2.1A). These cells had irregularly-shaped nuclei with large amounts of dense heterochromatin, processes that appeared to extend to both the apical surface and the ventricle, and scant cytoplasm packed with ribosomes and containing few other organelles (Figure 2.3B).

The first signs of differentiation of the OB were apparent at stage 32, just after the olfactory axons were seen in the neural tube. Some of the cells of the neural tube had lightly-stained, large, round nuclei with centrally-located nucleoli and contained more numerous and elaborate Golgi complexes, more mitochondria, and slightly more rough endoplasmic reticulum than the undifferentiated cells in the ventricular zone. (Figures 2.1B and 2.3C). These criteria have been used in other studies to distinguish between undifferentiated and differentiated cells (Tennyson, 1970; Hinds, 1972b)

At the next stage, stage 33/34, some of the differentiating cells began to extend processes (Figure 2.4). These processes were similar to the tangential processes of developing mitral cells described by Hinds (1972b). Golgi complexes
FIGURE 2.3. Initial events in differentiation of the olfactory bulb occur at stage 32. A: Olfactory axons (arrows) are first seen below the basal lamina (arrowheads) of the neural tube at stage 32. B: Most of the cells of the neural tube are still undifferentiated cells of the ventricular zone, but some differentiating cells with large, round nuclei (C) are seen. Scale bar = 1 μm (A) or 2 μm (B,C).
FIGURE 2.4. Differentiating cell with process. At stage 34, cells begin to extend processes towards the incoming olfactory axons. These processes have numerous Golgi stacks (arrowheads), many mitochondria (examples at *), and occasionally contain yolk granules (Y). Scale bar = 1 μm.
and rough endoplasmic reticulum were present in the proximal portion of these processes. These processes were easily distinguished from the processes of the undifferentiated cells of the ventricular zone because the processes from those cells lacked a mature Golgi complex at their base, had many more ribosomes, and had fewer organelles (compare Figure 2.3B with 2.4).

At subsequent stages, typical elements of the OB began to emerge. By stage 36, the olfactory axons had formed a thick bundle identifiable as the olfactory nerve (Nieuwkoop and Faber, 1956), and many more olfactory axons had entered the neural tube to form a definite olfactory-nerve layer (Figure 2.1C). Also at stage 36, the neural tube was thicker, and an intermediate zone was apparent above the ventricular zone. This intermediate zone contained the large, round, light nuclei of differentiating neurons. Many of these cells extended processes toward the incoming axons. A few synapses were found as early as stages 36 and 38, usually on the bases of growing processes of young neurons (Figure 2.5A). By stage 40, the glomerular layer was present and had the marbled appearance that is typical of older stages, with dark olfactory axons and pale dendrites (Figure 2.5B). Before this stage, it was difficult to distinguish dendritic processes from glial processes.

At stage 44, all the OB layers appeared to be present (Figure 2.1E). There was a definite olfactory nerve layer, a well-developed glomerular layer, the first sign of a mitral cell/plexiform layer, and a thin granule cell layer. The olfactory nerve layer was a distinct layer at this stage (Figure 2.6A), but the axons were neither as tightly
FIGURE 2.5. Development of the glomerular layer. An early synapse (arrow) between an olfactory axon and a proximal process of an olfactory-bulb neuron is present at stage 38 (A). The glomerular layer is well-developed by stage 40 (B) with dark olfactory axons (a), light dendrites (d), and synapses (arrow). Scale bars = 1 \mu m.
packed nor as uniform in size as at later stages. The glomerular layer was formed, but the olfactory axon terminals had fewer synaptic vesicles than those seen at later stages (Figure 2.6B). There were numerous mitral/tufted cells, and there were a few synapses on the mitral/tufted-cell bodies and on processes running through the area between the mitral/tufted cells (Figure 2.6C). The granule cells were first distinguishable at this time as small cells with a thin rim of cytoplasm around a dark nucleus. Many undifferentiated cells still lined the ventricle.

By stage 48/49, all of the features of the mature frog OB were in place (Figure 2.1F). The cellular elements were indistinguishable from those in the adult, all of the layers of the main and accessory OBs were in their proper positions, and the OB had the correct orientation (i.e. the nerve entered the bulb at its most rostral region). Features typical of the *Xenopus* OB are shown in Figures 2.7 and 2.8. Neural elements of the OB at this stage appeared more mature than those at stage 44. In the glomerular layer, the axon terminals were packed with vesicles, and their cytoplasm was darker than axon terminals at stage 44 (compare Figure 2.6B with Figure 2.7C). The mitral/tufted cells at stage 48/49 (Figure 2.8A) were lighter and less packed with ribosomes than the mitral cells at earlier stages. Also at this stage, the rostral part of the right and left OBs began to fuse to form a single OB (Nieuwkoop and Faber, 1956). After stage 48/49, the only changes in the structure of the OB appeared to be an increase in size and in number of components (compare Figure 2.1F to Figure 2.2). No structures of later stage
FIGURE 2.6. Further development of the layers. The olfactory nerve layer, glomerular layer, and plexiform layer are present by stage 44. A: The olfactory nerve layer contains axons that are not as uniform in diameter as they are at later stages. B: The glomerular layer contains more synapses (arrows) than at stage 40, and some dense-core vesicles (arrowheads) are seen in both axons and dendrites. C: The plexiform layer contains few processes with some dense-core vesicles (arrowheads) and synapses (arrows). Scale bar = 2 μm (A) or 0.5 μm (B,C).
FIGURE 2.7. Ultrastructure of the bulb at stage 48/49. **A:** Low-power electron micrograph showing the olfactory nerve layer (onl), and glomerular layer (gl). Note that the axons in the olfactory nerve layer are more tightly packed and are more uniform than at stage 44. **B:** A periglomerular cell (pg) in the glomerular layer and a glial cell (g) in the nerve layer are shown. This is the first stage in which periglomerular cells are identified. **C:** At stage 49, the olfactory axon terminals (a) in the glomerular layer are packed with vesicles, and the dendrites contain either mostly flattened vesicles (*) or predominantly spherical vesicles (.). Scale bar = 2 μm (A,B) or 1 μm (C).
FIGURE 2.8. More ultrastructural features of the bulb at stage 48/49. A: A typical mitral cell with a process and the surrounding plexiform layer are shown. Note the large Golgi stacks (arrow) at the base of the process, mitochondria (m), rough endoplasmic reticulum (arrowheads), and lysosome (*). B: The plexiform layer at stage 49 contains numerous processes and synapses (arrows). Many processes contain dense-core vesicles (arrowheads) and flattened vesicles (*). C: Granule cells have a mature appearance at this stage. They are usually packed close together and have a thin rim of cytoplasm (arrowheads). Scale bar = 1 μm.
larvae or adult OBs are pictured here as they are unchanged from those of stage-48/49 OBs. Table 2.1 summarizes the major events in OB development in *Xenopus*.

**Relationship of the number of olfactory axons to the number of mitral/tufted cells**

A quantitative analysis of two elements of the olfactory system was performed to determine the convergence ratio at various stages of development. The number of olfactory axons innervating the OB and the number of cells in the mitral cell/plexiform layer of the main and accessory OBs were ascertained in larvae between stages 50 and 58 and in adult frogs. The latter number included the output cells of the OB, mitral and tufted cells, as well as any glial cells located in this layer. From this analysis, we show that there was an increase in axon number with each stage (Figure 2.9A and Burd, 1991), with a 16-fold increase in axon number between stage 58 and adulthood. The number of mitral/tufted cells also increased with stage, but the increase between stage 58 and adulthood was only 2.3 fold (Figure 2.9B). Glomeruli were not counted because they could not be clearly resolved in one-micron, plastic sections.

The quantitative relationship between the olfactory receptor-cell axons and the mitral/tufted cells was also examined in larvae and adults. The convergence ratio of olfactory axons onto mitral/tufted cells was an average of 5:1 for larval stages; values for all larval stages ranged from 3.6 to 7.0. The number of mitral/tufted cells was correlated with the number of olfactory axons at all stages
<table>
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<td>ST 40</td>
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¹Times are approximate for conditions in our facility.
²NT=neural tube; ON=olfactory nerve; ONL=olfactory nerve layer; GL=glomerular layer; OB=olfactory bulb; AOB=accessory olfactory bulb.
FIGURE 2.9. Quantitative analysis of olfactory axons and mitral/tufted cells during normal development. **A:** The number of olfactory axons increases with stage. Larval stages are indicated by blank bars; adults by solid bars. Note the different scale for adults. **B:** The number of mitral/tufted cells also increases with stage.
FIGURE 2.10. Quantitative relationship between number of olfactory axons and number of mitral/tufted cells across developing stages. **A:** The number of olfactory axons is correlated with the number of mitral/tufted cells during larval development (n=18, r=0.952). The ratio between these numbers is maintained at about 5:1. **B:** In adults, the increase in the number of olfactory axons is significantly greater than the increase in the number of mitral/tufted cells, so the convergence ratio increases to about 34:1.
Relationship of Axons to Mitral/Tufted Cells in larval development

![Graph showing the relationship between axon number and mitral/tufted cell number for different stages of larval development.]

Relationship of Axons to Mitral/Tufted Cells in larvae and adults

![Graph showing the relationship between axon number and mitral/tufted cell number for larvae and adults.]

Legend:
- △ stage 50
- ○ stage 54
- □ stage 56
- ◇ stage 57
- ○ stage 58

Legend:
- ○ larvae
- ○ adults
of larval development examined (Figure 2.10A). There was a very strong correlation between these two elements in larvae, since the correlation coefficient was very near 1.0 (n=18, r=0.952). The convergence ratio changed significantly sometime after metamorphosis (Figure 2.10B); for adults, the convergence ratio was 34:1. This change was due to the dramatic increase in number of olfactory axons with a less significant increase in the number of mitral/tufted cells.

DISCUSSION

The sequence of events that lead to the formation of the OB has been well-studied in rodents using a variety of techniques including autoradiography (Hinds, 1968; Bayer, 1983), Golgi analysis (Hinds and Ruffett, 1971; Hinds, 1972a), and electron microscopy (Hinds, 1972b). Studies have also been performed in non-mammalian species such as ducks (Rebiere and Dainat, 1981), lungfish (Derivot, 1984b), and snakes (Holtzman and Halpern, 1990); but, to my knowledge, this is the first report to follow the complete development through adulthood in a non-mammalian species. The general developmental scheme of OB formation has been reviewed elsewhere (see Brunjes and Frazier, 1986), but will be summarized here. At the time at which the olfactory axons contact the neural tube, there is an inner, ventricular zone and an outer, intermediate layer (Hinds, 1972a). Soon after this, the major output neurons, the mitral cells, are born, followed by the other output
neurons, the tufted cells (Hinds, 1968). The interneurons, both granule cells and periglomerular cells, are the last bulb neurons to form. The birthdates of OB cells in *Xenopus* have not yet been reported. The glomerular neuropil contains the first synapses of the bulb, and the external and internal plexiform layer synapses form shortly after those in the glomerular layer (Hinds and Hinds, 1976).

The development of the OB in *Xenopus* proceeds very much like the development of the mammalian OB. The first signs of differentiation do not occur until after the olfactory axons enter the neural tube. Interestingly, in the mouse, the incoming olfactory axons penetrate deep into the intermediate zone of the neural tube during the early stages of development before retracting to their final location in the superficial layers (Hinds, 1972b). Penetration of olfactory nerve fibers coincides with the appearance of numerous young neurons, including the output neurons of the OB, the mitral cells (Hinds, 1972b). Since the incoming olfactory axons are found in the deep layers of the neural tube where neurons are generated and begin their outward migration, it is possible that the axons stimulate neurogenesis or induce differentiation in their CNS target neurons in the OB. Penetration of olfactory axons has not yet been reported in the frog.

The mature structure of the *Xenopus* OB is present long before metamorphosis, but the size increases over subsequent larval stages and into adulthood. The OB of *Xenopus* has the same basic features that have been described in the OBs of other animals. Ultrastructurally, the major neural elements
are similar to those described for mammals and fish. The glomerular layer contains electron-dense olfactory axon terminals and pale dendritic processes that contain either round or flattened vesicles (Pinching and Powell, 1971b). There are numerous axo-dendritic synaptic contacts as well as dendro-dendritic contacts like those described in the rat OB (Pinching and Powell, 1971b). The mitral cells, granule cells, and periglomerular cells are similar to those described for other animals (rat: Price and Powell, 1970; cat: Willey, 1973; goldfish: Ichikawa, 1976). Short-axon cells and other interneurons described in rodents (Price and Powell, 1970; Pinching and Powell, 1971a; Schneider and Macrides, 1978) may be present in the *Xenopus* OB, but they were not identified in this study.

While the mature structure of the OB in *Xenopus* was found generally to be similar to that described for mammals, subtle differences did appear. In particular, the mitral cells do not form a distinct layer; instead, they are scattered throughout the region equivalent to the external and internal plexiform layers in other animals. In this respect, the OB in *Xenopus* follows the pattern in many non-mammalian species including *Ambystoma* (Herrick, 1924), goldfish (Ichikawa, 1976), and bullfrog (Burton et al., 1990). The OB in *Xenopus*, like that of the African lungfish (Derivot, 1984a), also differs from that of mammals in that the neuropil of the glomerular layer does not form glomeruli that are as distinct as those found in mammals. Our impression is that the glial elements and periglomerular cells that surround the glomeruli in mammals are fewer in number in *Xenopus*, rendering the
borders of the glomeruli less distinct (C.A.B. and G.D. Burd, unpublished observations). A third difference is that the right and left OBs in *Xenopus* fuse together at their rostral end. This fusion begins at stage 50 and is complete by stage 58. Consequently, axons from the olfactory epithelium on both sides have some projections to the contralateral OB (Chapter 4; Ebbesson et al., 1986; Byrd and Burd, submitted).

A quantitative study was performed to determine the convergence ratio at various stages of development. The ratio of olfactory axons to mitral/tufted cells is 5:1 in larvae and increases to 34:1 in adult frogs. The number of axons in animals just post-metamorphosis could not be quantified because the distance between the olfactory epithelium and the OB decreased at this time, and the olfactory nerve was too short to section (see Burd, 1991). Therefore, I analyzed mature, breeding, adult frogs. The increase in the convergence ratio in adults is a result of the fact that the number of olfactory axons increases much more than the number of mitral/tufted cells after metamorphosis. The number of mitral/tufted cells increases only slightly in comparison to the number of olfactory axons, but no evidence for cell death was found. This increase in convergence ratio should result in increased sensitivity to stimuli (as shown in a theoretical and mathematical model by van Drongelen et al., 1978). But the ramifications of this increased convergence for odor coding are not clear since it is not known whether the newly added receptors maintain the larval pattern of sensitivity to particular odorants or
represents a new pattern. Interestingly, in *Xenopus*, beginning around stage 54, a new region of olfactory epithelium is added with the formation of the middle cavity (Key, 1986). If these new receptor cells are sensitive to different stimuli and converge onto the same mitral/tufted cells as the receptor cells from the other cavities, then these mitral/tufted cells may become responsive to more complex inputs from both air and water sources. Until more is known about olfactory coding, the types of receptor cells, and the projection patterns of these cells, I can only speculate about the significance of increased afferent axon convergence in adulthood.

Comparison of the number of olfactory receptor cells or olfactory axons in the olfactory nerve of different species has revealed considerable diversity (human: 6 million axons, Moran et al., 1982; rabbit: 100 million receptor cells, Allison and Warwick, 1949; pig: 12 million axons, Gasser, 1956; rat: 32 million, Meisami, 1991; garfish: 20 million axons, Easton, 1971; burbot fish: 10 million axons, Gemne and Døving, 1969; young pike: 10 million axons, Kreutzberg and Gross, 1977; bullfrog larvae: 2 million axons and adult bullfrog: 40 million axons, Burton et al., 1990; hawkmoth: 300,000 antennal-nerve axons, Oland and Tolbert, 1988). For estimates of axonal number in *Xenopus*, the accuracy of the estimate was increased by counting the axons in a larger percentage of the area of the nerve than usual. My data reveal many fewer (4.6 million) olfactory axons in adult *Xenopus* than in
another species of frog (adult bullfrog: 40 million, Burton et al., 1990), but this is not surprising since *Xenopus* is smaller than the bullfrog, *Rana catesbeiana*.

The number of mitral/tufted cells in the OB also varies among species, but less widely. The total number of mitral/tufted cells in both sides of the OB in the frog was similar to the number of these cells in the two OBs of mammals (compare 135,000 mitral/tufted cells for adult *Xenopus* to human [25 years old]: 102,000 mitral cells, Bhatnagar et al., 1987; rabbit: 90,000 mitral cells, 350,000 mitral/tufted cells, Allison and Warwick, 1949; rat: 96,000-142,000 mitral cells, Panhuber et al., 1985). My counts overestimate the number of mitral/tufted cells because I was unable to distinguish mitral/tufted cell classes, and also because I included glial cells. Glial cells appear to make up only a small percentage of the total number of cells in the mitral-cell/plexiform layer (see Materials and Methods), and they do not appear to be a significant factor in my analysis.

These numbers permit the calculation of convergence ratios, presumably an important factor in olfactory sensitivity. In these calculations, I assume that each output neuron receives the same number of afferent fibers, and that each afferent fiber does indeed synapse directly onto the output neurons. Thus in rabbits, the ratio of olfactory axons to mitral cells is about 1000:1 and axons to mitral and tufted cells is about 290:1 (from Allison and Warwick, 1949); in bats, the ratio is comparable at 900:1 (Bhatnagar and Kallen, 1975); in the hawkmoth, an insect with exquisite odor detection capabilities, about 450:1 (from Oland and Tolbert, 1988;
Homberg et al., 1988); compare these to adult *Xenopus* which is 34:1. In the burbot fish, the convergence ratio is about 1000:1 (Gemne and Døving, 1969), but this may be an overestimate of the number of mitral/tufted cells because the number of axons in the olfactory tract was counted and this includes many centrifugal axons projecting to the bulb. In the lungfish, a continually growing animal, the ratio increases with the size of the animal, from 35:1 to 185:1 (Derivot, 1984b).

My study follows the convergence ratio throughout development and into adulthood. A similar developmental study was performed in the rat (Meisami, 1989) and found that the convergence ratio increased in development from 25:1 at birth to 250:1 in weanlings to 800:1 in adults (Meisami, 1989, 1991). This increase in ratio is accompanied by an increase in olfactory sensitivity (i.e. higher sensitivity at low odor concentrations). Thus, the increase in convergence ratio in *Xenopus*, although not as dramatic as in the rat, may result in increased olfactory sensitivity for adult frogs.

I found a positive correlation between olfactory axons and mitral/tufted cells throughout larval development. Because this relationship lasts throughout the larval period of OB development, the olfactory axons could play an instructive role in OB development. The fact that the convergence ratio changes after larval stages might suggest that the influence of olfactory axons could become less effective with age.
This hypothesis could be addressed with experiments to examine the importance of olfactory axons in maintenance of the cells in the OB.

In conclusion, the numerical relationship during development and the time course of ingrowth of olfactory axons and OB development in *Xenopus* are consistent with the hypothesis that the olfactory axons stimulate or induce OB formation. My quantitative results, however, are also consistent with an alternative hypothesis, that the OB influences olfactory receptor-cell number. Differentiating between these two hypotheses is the subject of the following chapters.
CHAPTER 3: EFFECTS OF PARTIAL DEAFFERENTATION

The previous chapter on morphological and quantitative analysis of the normal development of the OB in *Xenopus* supports the hypothesis that there is an important relationship between olfactory axons and the output neurons of the OB. I showed that the number of olfactory axons and the number of mitral/tufted cells in the OB are positively correlated throughout larval stages of development. The increase in axon number parallels the increase in mitral/tufted-cell number at least until the stages of metamorphic climax.

The present study was designed to determine whether manipulation of the number of sensory afferent axons innervating the developing OB could influence the number of output neurons that form in the OB. To do this, I removed one OP from *Xenopus* embryos and analyzed the effects on the number of mitral/tufted cells. This is the only study to date that has examined the quantitative relationship between experimentally-induced changes in the sensory afferent innervation and corresponding changes in neuron number in the developing OB. I report here that removal of one OP results in a decreased number of olfactory axons and a parallel reduction in the number of mitral/tufted cells. Some of the data presented here have been previously reported (Byrd and Burd, 1991c, in preparation).
MATERIALS AND METHODS

Deafferentation Procedure

*Xenopus laevis* embryos were collected from eight gonadotropin-induced matings of adult breeders in our colony (obtained from either Nasco or a natural breeding population). Embryos between stages 26 and 31 (staging series by Nieuwkoop and Faber [1956]) were deafferented. During these stages, the OP is pigmented and the olfactory axons are just beginning to contact the neural tube (Chapter 2; Klein and Graziadei, 1983; Byrd and Burd, 1991a) but have not made synaptic connections in the neural tube (Chapter 2; Byrd and Burd, 1991a). For surgical deafferentation, embryos were anesthetized with 0.02% tricaine methanesulfonate (MS-222, Sigma, St. Louis, MO) in Steinberg’s solution (58 mM sodium chloride, 0.67 mM potassium chloride, 0.34 mM calcium nitrate, 0.83 mM magnesium sulfate, 5 mM Tris; pH 7.4) and positioned in holes in clay dishes. The right OP was removed from 294 embryos using a glass needle and a loop made with baby hair. In some experiments, the entire sensory plate (which includes the OPs and surrounding tissue) was removed from embryos at these same stages, and an OP from another embryo was transplanted into the wound left by the excised sensory plate; two animals that developed with a single nasal capsule as a result of this surgery are included in this study. As a control for possible neural-tube damage during deafferentation, the part of the neural tube directly beneath the right OP was removed, without damage to the placode, in 20 embryos. For all
animals, after the wound healed for 15 minutes, the embryo was transferred to a 100 ml plastic beaker with 50% Steinberg's solution and 50% rearing solution (Burd, 1991). The following day, the embryos were put into individual plastic trays in 400 ml of 100% rearing solution. When animals reached approximately stage 40, they were fed boiled nettle (Wunderlich-Diez Corp., Hasbrouk Heights, NJ) daily, and the solution was changed weekly. Control animals from each population were reared under the same conditions.

**Tissue Preparation**

Animals used for quantitative analysis (including those with partial deafferentation or neural-tube removal and their age- and stage-matched controls) were sacrificed when they reached stage 58 (onset of metamorphic climax). They were anesthetized with 0.02% tricaine methanesulfonate, and their weights and lengths were recorded. They were then perfused transcardially with either 2% glutaraldehyde and 1.6% paraformaldehyde in 0.035 M sodium cacodylate buffer or 4% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer and left in fixative solution overnight. The next day, the nasal capsules (with olfactory nerves attached) and telencephalon of each animal were post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a graded series of alcohols, and embedded in Epon (nasal capsules) or Durcupan (brains). Thick, one-micron sections and thin, 70-nm sections were taken through the olfactory nerves at the
region just caudal to where the trigeminal nerve enters the nasal capsule. Thin sections were collected on Formvar-coated slot grids (Formvar resin, Ted Pella), stained with uranyl acetate and lead citrate, and viewed with a JEOL 1200 EX electron microscope. Serial, one-micron, horizontal sections were made of the OBs and stained with toluidine blue (1% toluidine blue in 1% sodium borate).

Some embryos with partial deafferentation or neural-tube removal (not included in the 294 surgeries) were immersed in one of the above-mentioned fixative solutions immediately after surgery or one day later and processed as whole embryos for light and electron microscopy as above. These were sectioned and analyzed to determine the amount of tissue removed.

Quantitative Analysis

The olfactory receptor-cell axons and the cells in the mitral-cell/plexiform layer of the OB were counted in stage-58 animals with partially-deafferented OBs, with partial removal of the neural tube, and with no surgery (stage-matched, sibling control animals). Quantitative data on the number of olfactory axons were obtained as previously described (Burd, 1991). Briefly, this procedure involves measuring the total area of axons in the olfactory nerve (excluding glial cells and extracellular space) in electron-micrographic montages. The axons in 10-20% of the measured area are counted, and the total number of axons in the olfactory nerve is calculated. For control animals, the axons in one olfactory nerve were counted,
and this number was multiplied by two to get the total number of olfactory axons in the animal. This procedure was followed because there is no significant difference between the number of olfactory axons in each olfactory nerve (Burd, 1991). For animals with partial deafferentation, the axons in the solitary olfactory nerve were counted. For all animals, this number includes axons from the olfactory and vomeronasal epithelia because the two nerves run together (see Byrd and Burd, 1991a).

The number of mitral/tufted cells in the OB was estimated by counting the cells in the mitral-cell/plexiform layer in every twentieth section of serial, one-micron, horizontal sections through the entire OB as previously described (Chapter 2; Byrd and Burd, 1991a). This number includes mitral cells, tufted cells, and glial cells, as these are very difficult to distinguish at the light-microscope level. An estimate of the percentage of cells in the mitral-cell/plexiform layer that are glial cells was made (see Chapter 2, Materials and Methods); these results suggested that glial cells make up a small percentage of the total number of cells. The percentage of cells that were glia was not different between controls (3%; n=3) and animals with partial deafferentation (3%; n=3). Thus, the inclusion of glial cells should not significantly affect the estimate of mitral/tufted-cell number.

Statistical calculations were performed using the SAS System for personal computers (SAS Institute, Inc.). To determine whether parametric analyses could be used to compare groups, the Shapiro-Wilks test for normality was performed on
stage-58, control animals. The results of this test allowed me to assume that the number of olfactory axons and the number of mitral/tufted cells were distributed normally (p > 0.05 in both cases). For all comparisons Student's t-tests were performed on the ranked data. When performed on ranked data, the t-test becomes a more powerful test for small sample sizes (Conover and Iman, 1981). For all statistical analyses, a significance level of 0.05 was used.

These experiments were designed with each experimental animal paired to a sibling, control animal that was matched in both age and stage. The statistical analysis that was originally planned was the Wilcoxon signed-rank test; this is a paired test that compares each experimental animal with its matched control animal. This was not done for several reasons. First, the Wilcoxon T distribution is conservative and not very powerful for the small numbers of data to which I was limited (Dr. Jean Weber, personal communication). Second, the results of a previous study from our laboratory showed that same-stage animals reared under similar conditions but from different populations do not differ significantly in weight, length, head width, or olfactory-nerve length (Burd, 1992). Because animals from different populations do not appear to be significantly different in size, control animals from different populations were grouped together, and experimental animals from different populations were grouped together; the unpaired t-test on ranked data was then used instead of paired analyses.
Analysis of Projections

In two animals with a partially-regenerated nasal capsule but no apparent nerve to the OB, the projections of the regenerated nasal capsule were analyzed with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes, Eugene, OR), using a method similar to that described by Godement and colleagues (1987). The animals were anesthetized when they reached stage 58 as described above, immersed in a fixative solution of 4% paraformaldehyde in 0.1M phosphate buffer, and left in the fixative solution overnight. The next day, a crystal of DiI was inserted into the regenerated nasal capsule, and the animals were left in vials of the fixative solution for 3 weeks. They were then analyzed as whole mounts with a Zeiss fluorescence microscope.

RESULTS

Morphological Analysis

Controls. For my experimental animals, an OP was removed from Xenopus embryos (Figure 3.1a) at stages before any differentiation of the neural tube had occurred (Chapter 2; Byrd and Burd, 1991a). While care was taken to minimize the damage to the underlying neural tube during this procedure, the neural tube, which lies directly underneath the OP, could have been damaged by the surgery. To check the amount of injury to the neural tube, some embryos with partial
FIGURE 3.1. Tailbud-stage embryos showing the tissue removed in the partial-deafferentation procedure. A: Two stage-30 embryos are shown. One is lying on its side, and the otic vesicle (*) and cement gland (C) are labeled. The other is embedded in clay such that only the top of its head is visible. Arrowhead points to one of the pigmented OPs. B: Coronal section through an embryo 15 minutes after the OP on the left side of this figure was excised showing the amount of tissue removed in the surgery. The entire placode was removed, and the neural tube was only slightly damaged. OP=olfactory placode, NT=neural tube, C=cement gland. Scale bar = 1 mm (A) or 100 μm (B).
deafferentation were processed for light microscopy 15 minutes after surgery. Sections through these embryos revealed that the entire OP was removed with some damage to the neural tube in a few animals (Figure 3.1b) and with no apparent damage in others. To control for the possibility that some neural tube was removed with the placode and that this damage may have affected the development of the OB, I removed a portion of the underlying neural tube, leaving the placode undisturbed, and processed the animals for light microscopy 15 minutes later. In these animals, the entire portion of the neural tube directly beneath the OP was missing and the placode appeared nearly normal (Figure 3.2a). Analysis of other animals revealed that this region of the neural tube had already regenerated one day after surgery and was developing at a rate similar to that of the undisturbed side (Figure 3.2b). Animals with partial neural-tube removal that were examined at stage 58 revealed that the neural tube completely regenerated and that the animal developed two normal nasal capsules, two olfactory nerves, and a normal OB (Figures 3.2c and d).

Unilateral Olfactory-Placode Removal. At the stages used for analysis in this study, the excised OP usually regenerated into a nasal capsule that had an olfactory nerve projecting to the OB. In some of these cases, the regenerated nasal capsule was slightly reduced from normal, but these were not analyzed in the present study. I analyzed only animals with a single olfactory nerve to the OB, because there was
FIGURE 3.2. Injury to the neural tube does not affect development of the OB. A: Coronal section through an embryo 15 minutes after a portion of the neural tube was removed. Neural tube (NT) directly under one of the olfactory placodes (OP) was removed (arrow) without damage to the placode. B: One day later, coronal sections through an animal with the same procedure show that the neural tube has almost completely regenerated (arrow), is innervated by the olfactory nerve (arrowhead), and is differentiating at a rate similar to the unoperated side. OP=olfactory placode, C=cement gland. C: Dorsal view of the head of a stage-58 animal that had part of the neural tube removed early in development shows that the animal appears normal and has two nasal capsules (N) and an olfactory bulb (OB). D: Horizontal section through the OB of one of these animals shows that the bulb developed normally and the operated side (arrow) is indistinguishable from the unoperated side. Scale bar = 100 $\mu$m (A and B), 2 mm (C), or 250 $\mu$m (D).
a large amount of variability in the number of olfactory axons in the olfactory nerves of animals of the same stage, and I was interested in examining significant decreases in axon number. The following is an analysis of 21 cases that resulted in innervation of the OB by only one olfactory nerve.

Whole-animal examination of stage-58 larvae from which one OP had been excised at stages 26-31 showed that there were three categories of results. In most animals with an excised OP, the placode regenerated (93%). In the remaining animals, the OP either partially regenerated but did not innervate the OB (6 animals) or did not regenerate (15 animals) (Figure 3.3). Animals with a single nasal capsule (or a partially-regenerated nasal capsule) had one olfactory nerve that projected either to the ipsilateral, rostral telencephalon, leaving the other side uninnervated (Figure 3.3b; 16 animals), or to the midline of the rostral telencephalon, innervating both sides of the OB (Figure 3.3d; 5 animals). Some animals with a partially-regenerated nasal capsule that did not appear to have an olfactory nerve that innervated the OB (Figure 3.3c) were studied with tract-tracing procedures. Analysis with DiI of the nerves projecting from the nasal capsule in two of these animals confirmed that there were no projections to the OB (data not shown). Therefore, either there were no olfactory axons projecting from the nasal capsule, or the olfactory axons followed the normal projection path of the trigeminal nerve into the mesencephalic nucleus of the Vth nerve. I have not distinguished between these two possibilities, but for this study, the important fact
FIGURE 3.3. Dorsal view of the heads of a control animal and animals with partial deafferentation. **A:** Control animal at stage 58. **B:** Animal (D1 in Table 3.1) with only one olfactory nerve that innervates one side of the rostral telencephalon. **C:** In this animal (D2 in Table 3.1), the excised placode regenerated, but does not have an olfactory nerve innervating the telencephalon. The olfactory axons probably follow the trigeminal nerve (arrowhead) to the mesencephalon. The single olfactory nerve innervates one side of the telencephalon. **D:** This animal (D7 in Table 3.1) had the entire sensory plate removed and an olfactory placode transplanted in its place. A single, centrally-located nasal capsule developed in this animal, but whether the tissue is from a regenerated olfactory placode or the transplanted placode is unknown. There is a single olfactory nerve innervating the midline of the rostral telencephalon. Scale bar = 2 mm.
is that there are no olfactory projections to the OB from these regenerated nasal capsules.

Histological examination of the OBs of animals with partial deafferentation revealed that there were two different morphologies of the OBs, depending on where the single remaining olfactory nerve entered the telencephalon. Because animals with innervation to only one side of the telencephalon were very different morphologically from those with a nerve to the midline of the telencephalon, I present the data from these two groups separately in the following sections.

Animals with a Single Olfactory Bulb; Nerve to One Side. The normal *Xenopus* OB at stage 58 consisted of two main OBs fused at the center with two accessory OBs located at the lateral edges of the main OB (Figure 3.4a). Experimental animals with a single nerve innervating one side of the telencephalon had one-half of the normal main OB structure, that is, a single OB (Figure 3.4b). Note that when I refer to a single OB I mean one-half of a fused OB structure, in view of the fact that two separate OBs develop and then fuse beginning around stage 50 (Chapter 2; Nieuwkoop and Faber, 1956; Byrd and Burd, 1991a). This OB developed in the area innervated by the single nerve, possessed the normal features of the stage-58 OB, and had a single accessory OB. The other side of the telencephalon did not have any evidence of an OB; it appeared to consist only of
FIGURE 3.4. Horizontal sections through the olfactory bulb of a control animal and animals with partial deafferentation.  

A: The stage-58, control OB is a fused structure with two ventricles (one labeled at v), bilateral accessory bulbs (one labeled at a), and a laminated main bulb. For this and all olfactory-bulb figures: short arrow=olfactory nerve layer; long arrow=glomerular layer; m=mitral-cell/plexiform layer; ge=granule cell layer. 

B: Olfactory bulb from a partially-deafferented animal (D1 in Table 3.1) that had a single olfactory nerve entering one side of the telencephalon. The side innervated by the olfactory nerve has the typical laminated structure and single accessory OB (arrowhead) characteristic of one-half of the normal bulb. The other side did not develop into an olfactory bulb and appears to contain only ventricular cells and neurons of more dorso-caudal areas of the telencephalon. 

C: Olfactory bulb from an animal (D5 in Table 3.1) in which the single olfactory nerve innervated the midline of the telencephalon. In this case, a fused olfactory bulb developed with two ventricles and a single accessory bulb (arrowhead). 

D: This case (D7 in Table 3.1), where a single olfactory nerve innervates the midline of the telencephalon resulted from removing the sensory plate and transplanting an olfactory placode in its place. In this animal, a fused bulb developed with two accessory bulbs (arrowheads) and a single ventricle (v). Scale bar = 250 μm.
the cells normally surrounding the ventricle in the telencephalon dorso-caudal to the location of the OB.

**Animals with a Fused OB; Nerve to Midline.** Experimental animals with a single nerve innervating the rostral midline of the telencephalon had two different types of morphology. All of these animals had what appeared to be a fused main OB, but it is unclear whether these were formed by the fusion of two OBs or from a centrally-located, single OB. Three of these animals had a single accessory OB and two ventricles (Figure 3.4c). The other two animals, which were the result of the operations in which the entire sensory plate was removed and another OP was transplanted in its place, had two accessory OBs and a single ventricle (Figure 3.4d). While these animals had a single olfactory nerve, it is unclear whether the nasal capsule was a result of the development of the transplanted OP or a result of regeneration of one of the excised placodes.

**Quantitative Analysis of Partially-Deafferented Olfactory Bulbs**

To determine the effects of removing one OP on the number of axons innervating the OB and the number of output neurons of the OB, I estimated the number of olfactory axons and the number of cells in the mitral-cell/plexiform layer as described in Materials and Methods. Table 3.1 shows the data for 8 animals with partial deafferentation, their age- and stage-matched controls, and animals
TABLE 3.1 Data for Partially-Deafferented and Control Animals

<table>
<thead>
<tr>
<th>Description</th>
<th>Bulbs fused?</th>
<th>Total axons</th>
<th>Total OB cells</th>
<th>Convergence ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 no right nc</td>
<td>No</td>
<td>174,468</td>
<td>42,057</td>
<td>4.1</td>
</tr>
<tr>
<td>D2 right nc doesn't project to OB</td>
<td>No</td>
<td>187,437</td>
<td>36,719</td>
<td>5.1</td>
</tr>
<tr>
<td>D3 right nc doesn't project to OB</td>
<td>No</td>
<td>152,935</td>
<td>34,575</td>
<td>4.4</td>
</tr>
<tr>
<td>D4 single nc to midline</td>
<td>Yes</td>
<td>170,648</td>
<td>62,717</td>
<td>2.7</td>
</tr>
<tr>
<td>D5 no right nc, left nc to midline</td>
<td>Yes</td>
<td>160,344</td>
<td>46,550</td>
<td>3.4</td>
</tr>
<tr>
<td>D6 right nc not to OB left nc to midline</td>
<td>Yes</td>
<td>145,641</td>
<td>60,316</td>
<td>2.4</td>
</tr>
<tr>
<td>D7 single nc to midline</td>
<td>Yes</td>
<td>108,192</td>
<td>30,888</td>
<td>3.4</td>
</tr>
<tr>
<td>D8 single nc to midline</td>
<td>Yes</td>
<td>65,798</td>
<td>38,368</td>
<td>1.7</td>
</tr>
<tr>
<td>C1 control for D1</td>
<td>Yes</td>
<td>273,538</td>
<td>80,385</td>
<td>3.4</td>
</tr>
<tr>
<td>C2 control for D2</td>
<td>Yes</td>
<td>318,712</td>
<td>97,380</td>
<td>3.3</td>
</tr>
<tr>
<td>C3 control for D3</td>
<td>Yes</td>
<td>330,432</td>
<td>57,150</td>
<td>5.8</td>
</tr>
<tr>
<td>C4 control for D4</td>
<td>Yes</td>
<td>198,440</td>
<td>47,625</td>
<td>4.1</td>
</tr>
<tr>
<td>C5 control for D5</td>
<td>Yes</td>
<td>449,684</td>
<td>95,178</td>
<td>4.7</td>
</tr>
<tr>
<td>C6 control for D6</td>
<td>Yes</td>
<td>325,558</td>
<td>69,894</td>
<td>4.7</td>
</tr>
<tr>
<td>C7 control for D7,D8</td>
<td>Yes</td>
<td>342,502</td>
<td>77,597</td>
<td>4.4</td>
</tr>
<tr>
<td>NT1 NT removed on right side</td>
<td>Yes</td>
<td>327,729</td>
<td>81,846</td>
<td>4.0</td>
</tr>
<tr>
<td>NT2 NT removed on right side</td>
<td>Yes</td>
<td>328,288</td>
<td>69,945</td>
<td>4.7</td>
</tr>
<tr>
<td>NT3 NT removed on right side</td>
<td>Yes</td>
<td>447,488</td>
<td>98,250</td>
<td>4.6</td>
</tr>
</tbody>
</table>

1 nc = nasal capsule; OB = olfactory bulb
2 Determined from horizontal sections through olfactory bulb, see Fig. 3.4.
3 Total axon number in one nerve of experimental animals and two nerves of control animals.
4 All cells in the mitral cell/plexiform layer were counted, see Methods; this number includes the right, left, and accessory olfactory bulbs.
5 Convergence ratio = total axons ÷ total OB cells
with partial neural-tube removal. The number of olfactory axons and the number of mitral/tufted cells in the OB of animals with part of the neural tube removed early in development was not significantly different from control values (P>0.4 for both).

For the first set of analyses, animals with both types of morphologies of partially-deafferented OBs were grouped together. Statistical analysis revealed that all animals with partial deafferentation were significantly different from controls in total number of olfactory axons (P<0.0001) and total number of OB output neurons (P<0.001) (Figure 3.5). The number of axons in the single olfactory nerve of the experimental animals was not significantly different from the number of axons in one of the olfactory nerves of the control animals (P>0.7). Because experimental animals had one olfactory nerve, and control animals had two olfactory nerves, it is not surprising that the total number of axons in experimental animals was less than the number in control animals.

I have previously shown that olfactory-axon number and mitral/tufted-cell number are correlated throughout larval stages of development, and the convergence ratio remains the same during these stages (Chapter 2; Byrd and Burd, 1991a). In the current study, olfactory-axon number and mitral/tufted-cell number were both affected by partial deafferentation, and although the convergence ratio was not significantly different (P>0.1) according to the significance level chosen for these analyses, there appeared to be somewhat of a difference in convergence ratio
FIGURE 3.5. Quantitative analysis of olfactory-axon and mitral/tufted-cell numbers in all animals with partial deafferentation and control animals. Animals with partial deafferentation have significantly fewer olfactory axons and mitral/tufted cells than their control animals.
Effects of Partial Deafferentation on Olfactory–Axon and Mitral/Tufted–Cell Numbers

- Controls
- Partial deafferentation

(axonom)
between the two groups. The ratio of the number of mitral/tufted cells in the main OB to those in the accessory OB was also not significantly different from controls (P>0.4), even though some of the animals with partial deafferentation had two accessory OBs and others had only one.

For additional analyses, the two morphologies of partially-deafferented OBs were analyzed separately. In animals with a single OB and an olfactory nerve that projected to only one side of the rostral telencephalon (D1-D3 in Table 3.1), the total number of olfactory receptor-cell axons was approximately one-half of the value of their matched controls (56%; Figure 3.6a). This was significantly different from control values (P<0.02). The number of mitral/tufted cells was also significantly reduced compared to controls (48%; P<0.02; Figure 3.6b). This finding was not simply due to differences in size since experimental animals did not appear to be significantly smaller than their matched control animals in weight (P>0.8) or length (P>0.8).

Animals with a fused OB resulting from innervation by one nerve to the midline of the telencephalon (D4-D8 in Table 3.1) had 40% of the control value of number of olfactory axons (Figure 3.6a). This is a significant decrease compared to control values (P<0.02). The number of mitral/tufted cells in the OB was 66% of the control value (Figure 3.6b), a significant reduction (P<0.04). These animals also did not appear to be significantly smaller than their control animals in weight (P>0.3) or length (P>0.3).
FIGURE 3.6. Quantitative analysis of olfactory-axon and mitral/tufted-cell numbers in the two morphologies of animals with partial deafferentation, their control animals, and animals with partial neural-tube removal. A: Both morphologies of partially-deafferented olfactory bulbs have significantly fewer olfactory axons than their age- and stage-matched controls. Animals with a single bulb have about one-half the control value (56%), while animals with a fused bulb have even fewer (40%). Olfactory-axon number in animals with a portion of the neural tube removed is not significantly different from controls. B: In animals with a single bulb, the mitral/tufted-cell number is 48% of the value in control animals; in animals with a fused bulb, this number is 66% of control value. Mitral/tufted-cell number in animals with a portion of the neural tube removed is not significantly different from controls.
OLFACTORY AXON NUMBER (x1000)

- control
- deafferented
- neural tube removal

MITRAL/TUFTED CELL NUMBER (x1000)

- control
- deafferented
- neural tube removal
DISCUSSION

Removal of one OP early in development, before any development of the neural tube is apparent and before any synaptic connections are made with the neural tube, has dramatic effects on development of the OB. In cases where one side of the rostral telencephalon is innervated by the remaining olfactory nerve, a single OB develops on the innervated side and no OB develops on the uninnervated side. In cases where the midline of the rostral telencephalon is innervated by the single olfactory nerve, a smaller-than-normal (in output-neuron number), fused OB develops but is often lacking one of the accessory OBs. These morphological analyses further define previous observations. Other investigators have reported that removal of the OP in amphibians results in either no OB formation (Clairambault, 1971, 1976; Stout and Graziadei, 1980) or formation of a poorly-organized structure (Piatt, 1951). When Graziadei and Monti-Graziadei (1992) removed an OP from *Xenopus* embryos at stages 22-23 and analyzed the resulting OB at stages 47-60, they found that the single nerve produced a cone-like forebrain with a single ventricle, a single main OB, and a single accessory OB. This is similar to those in my fused-bulb, nerve-to-midline group. My morphological results support these previous observations. Unlike the previous studies, however, my study also attempted to quantify the apparent reduction.

Prior to my quantitative analysis of olfactory axons and OB output neurons (mitral/tufted cells), it was expected that mitral/tufted cells might be affected by
the olfactory axons because they are the first neurons to differentiate and because they receive direct synaptic input from the olfactory axons. Other elements of the OB might be affected by the olfactory axons, but the current study was limited to the analysis of the potential affect on mitral/tufted-cell number. This quantitative analysis of the relationship between olfactory afferent axons and OB output neurons revealed that the difference in convergence ratio between animals with partial deafferentation (when grouped together) and controls was not statistically significant. This appears to be due to the fact that experimental manipulation of one of these elements (olfactory-axon number) results in a corresponding change in the other element (mitral/tufted-cell number). This supports the hypothesis that these two elements of the olfactory system are quantitatively related.

For additional statistical analyses, the two groups of experimental animals were analyzed separately. In both groups, the number of olfactory axons was significantly decreased to about one-half of the value in control animals. This is not surprising since the animals with partial deafferentation had only one olfactory nerve, while control animals had two olfactory nerves, and the number of axons in the single nerves of experimental animals was not significantly different from the number of axons in one of the nerves of control animals. In addition, in both groups of experimental animals, the number of output neurons of the OB appeared to be reduced significantly.
In the case of animals with a single OB, the number of olfactory axons was one-half of control values, and the number of mitral/tufted cells was one-half of control values. Therefore, partial deafferentation in these animals led to the formation of one-half of the normal sensory innervation to and efferent fibers from the OB. In the case of the fused OB, these animals had only 40% of the normal number of olfactory axons. The number of mitral/tufted cells was reduced compared to controls (to 66% of control value), but by less than would be predicted based on the number of olfactory axons innervating the OB. These results do not appear to follow our hypothesis about the relationship between olfactory axons and mitral/tufted cells. These animals had less than one-half of the normal number of olfactory axons innervating the OB, and the resulting OB had more than one-half of the normal number of output neurons. One explanation for finding more mitral/tufted cells than predicted could be that the incoming axons, in cases where they enter the midline of the rostral telencephalon, have access to both sides of the neural tube, and, therefore, have access to two sets of neuronal precursors (the cells lining both of the ventricles). In the other case, the axons grow into one side and have access to only the precursors on that side of the neural tube, so that only the OB on the innervated side is formed. Another possibility is that a limited number of olfactory axons are necessary to stimulate development of the observed number of mitral/tufted cells. A third possibility is that the variability among animals and the small numbers that were available for
analysis created some discrepancy in the exact numerical relationship between olfactory axons and mitral/tufted cells in experimental animals with a fused OB.

Exact matching of innervating axons and target cell numbers does not always occur (see Oppenheim, 1991). Perhaps, in my study, exact numbers of olfactory axons and output neurons is not crucial in bulb development. Other factors may be more important such as number of synapses, strength of synapses, size of neurons, or branching of processes. These factors have not yet been examined, but these types of analyses might lead to interesting results. In a quantitative study of aging in the rat olfactory system, Hinds and McNelly (1981) found that both olfactory receptor-cell number and mitral-cell size decrease in aging animals. Since the decline in receptor-cell number precedes the decline in mitral-cell size, the authors suggest that the changes in the OB are due to the changes in the olfactory epithelium. In addition, these authors found that the number of synapses in the glomeruli declined less with age than the number of receptors, and in the oldest group they analyzed (33 months), there was actually a significant increase in the number of synapses per receptor cell. Thus, perhaps there is an increase in the number of synapses per receptor cell to compensate for the decreased number of receptor cells. The findings from the Hinds and McNelly study suggest that different conclusions can be made based on which element or parameter is analyzed.
I found that when one OP is excised between stages 28 and 31, the placode regenerates 93% of the time. This had been shown in other studies in which an OP was removed. Clairambault (1976) reported that 90% of excised OPs in Rana regenerated, and Stout and Graziadei (1980) found 80-90% reinduction in Xenopus at the same stages used in this study. Piatt (1951) removed an OP in Ambystoma and then grafted belly ectoderm over the wound to prevent reorganization of the surrounding ectoderm; the placode still often regenerated. Why the OP did not regenerate in the cases examined here is unclear. It is unlikely that damage to the neural tube during the surgery was the reason that regeneration did not take place since the control experiments on rostral neural-tube removal show no effect on the number of olfactory axons or mitral/tufted cells. Perhaps the placode regenerated because some of the placodal cells were not excised. In many instances, however, the entire one-half of the sensory plate was removed with the placode, and regeneration still occurred. This is an interesting phenomenon and may have implications for the normal induction of the OP.

Extensive regeneration occurs in the neural tube of amphibians at early stages of development. I found that removal of a portion of the neural tube without disruption of the OP leads to complete regeneration of the neural tube and normal development of that area. Quantitative analysis supported this result and showed that these animals do not differ from control animals. These results are consistent with the qualitative reports of others (Burr, 1916b; Graziadei and Monti-
Graziadei, 1992). Burr (1916b) reported that regeneration of the rostral neural tube in *Ambystoma* depends upon the presence of the OP, but this result has been discounted by other studies (Spirito as reported in Cowan and Finger, 1982; Graziadei and Monti-Graziadei, 1992). The mechanisms responsible for regeneration of these tissues have not been elucidated.

In two of the animals examined, where a single nasal capsule resulted from experimental manipulation that included removing the entire sensory plate and transplanting one OP in its place, the resulting olfactory nerve contained fewer axons than in the other animals with a single olfactory nerve. It is possible that a single, smaller-than-normal nasal capsule developed from the grafted tissue because olfactory nerves from transplanted tissue can never reach the size of normal olfactory nerves (Byrd and Burd, submitted). On the other hand, it is possible that the transplanted tissue did not survive, and the single nasal capsule is the result of regeneration of one of the normal OPs. In this case, the small size of the nerve may have resulted from extensive damage that occurred during removal of the entire sensory plate; perhaps this negatively influenced complete regeneration of the nasal capsule. Performing this same experiment with donor tissue that is different from host tissue (using a fluorescent label or a different species that can be distinguished histologically, such as *Xenopus borealis*) would allow distinction between these two possibilities.
In my attempt to examine the interaction between the OP and the neural tube, I removed the OP at stages before and during the time when olfactory axons are first observed in the neural tube and no differentiation of the OB is apparent (Chapter 2; Byrd and Burd, 1991a). This was done to examine specifically the possible inductive role of the olfactory axons. It is known that, in mammals, olfactory axons penetrate into the neural tube to the ventricular zone before they retract to the upper layers (Hinds, 1972b). It is possible that the olfactory axons influence neurogenesis and/or neuronal maturation in the undifferentiated neural tube. My results are certainly consistent with this hypothesis, although neurogenesis and neuronal maturation were not specifically examined. It is also possible that the OB differentiates to some extent in anticipation of incoming axons, and then degenerates when axons fail to innervate it. I do not know if this occurred, since I examined the animals late in larval development.

Other studies that analyze the effects of sensory afferent axons on the development of their target have been performed on the invertebrate visual system. In this system, afferent axons have profound effects on the development of their central target. Mutations or lesions that disrupt the photoreceptor axons from innervating the first optic ganglion result in malformation of that ganglion in *Drosophila* (Power, 1943; Meyerowitz and Kankel, 1978) and *Daphnia* (Macagno, 1979). In *Drosophila*, the photoreceptor axons have been shown to induce neuronal precursors in the ganglion to divide (Selleck and Steller, 1991) by regulating the
G1-S transition in the cell cycle (Selleck et al., 1992). Development of the vertebrate visual system might be regulated in a similar manner since removal of the eye in the frog prior to axon outgrowth results in hypoplasia (Kollros, 1953) and a decrease in the number of mitotic figures in the developing tectum (Currie and Cowan, 1974; Kollros, 1982).

Many investigators have examined the effects of afferent innervation on a central structure after that target has begun to develop. Decreasing the afferent input to neurons prior to or during the period of cell death that naturally occurs in development can significantly increase the amount of cell death in many systems (see Oppenheim, 1981, 1991). In the auditory system of the chick, early addition and initial differentiation of neurons in the brainstem are not affected by otocyst removal, but completion of differentiation and maintenance of neurons is prevented (Levi-Montalcini, 1949; Parks, 1979). Similarly, Sohal (1976) found that lesions of the optic tectum did not affect cellular proliferation and migration in the target isthmo-optic nucleus, but differentiation of target neurons and maintenance of cell number, size, and processes in the nucleus were prevented. The present study is different from these studies because no mitral/tufted cells are present when the afferent axons are removed. Preliminary studies from our laboratory, however, examined the effect of olfactory axons on later development and maintenance of the OB by removing the nasal capsule after the bulb has begun to develop (stage 45/46) (Herrera and Burd, 1991). Results of that study suggest that olfactory axons
are indeed required for continued development of the OB and for maintenance of the OB structure during early larval periods. Whether these afferent effects are on decreased cell genesis or on increased cell death has not yet been specifically addressed.

In summary, the results of my study extend the observations of other investigators by providing quantitative analysis on the relationship between sensory afferent fibers and target output neurons in the OB. Together my study and those of other investigators support the hypothesis that olfactory axons play an inductive role in the development of the OB. Without this input, an OB does not form. In an attempt to elucidate the mechanism by which olfactory axons influence OB development, I quantified the olfactory axons and the mitral/tufted cells of the OB. A decrease in the number of olfactory afferent axons innervating the OB results in a decrease in the number of output neurons of the OB. The fact that the difference in the convergence ratio of olfactory axons onto mitral/tufted cells between animals with partial deafferentation and control animals was not statistically significant supports the hypothesis that the interaction between these two elements could be important for normal development of the OB.
CHAPTER 4: EFFECTS OF TRANSPLANTATION OF AN EXTRA PLACODE

In my quantitative study of normal development of the OB, I found that the number of olfactory axons and the number of mitral/tufted cells is positively correlated throughout larval stages of development (Chapter 2; Byrd and Burd, 1991a). In a study of partial sensory deafferentation during development, I found that removal of olfactory axons in embryos prevents the normal development of the OB. In partially-deafferented animals, a reduction in the number of afferent axons was paralleled by a reduction in the number of mitral/tufted cells (Chapter 3; Byrd and Burd, 1991c, in preparation). These studies support the hypothesis that there is an important relationship between these two elements of the olfactory system and are consistent with the hypothesis that olfactory axons play a critical role in the development of the OB.

To examine further the role of olfactory axons, I added an extra OP to *Xenopus* embryos. This study involved repeating the transplantation experiments performed by Stout and Graziadei (1980) in which the transplant innervates the OB in an attempt to quantify the putative enlargement of the OB reported by that group. At two stages of larval of development, quantitative data on the total number of olfactory axons innervating the OB, the total number of mitral/tufted cells in the OB, and the volume of the OB were collected. A morphological examination of the transplanted tissue and the OBs that received innervation from
this extra tissue was also performed. Here I report answers to the following questions: Does the transplanted OP retain the characteristics of olfactory epithelium in an ectopic location? Are the projections of the nerve from the transplanted placode similar to those of normal olfactory nerves? Do animals with a transplant have supernumerary innervation to their OBs? Does the nerve from the transplanted OP influence the number of mitral/tufted cells? These results appeared in Byrd and Burd (1989, submitted).

MATERIALS AND METHODS

Transplantation Experiments

Embryos were obtained from several gonadotropin-induced matings of *Xenopus laevis* breeders (obtained from Nasco or from a natural breeding population). Transplant surgeries were performed on 457 embryos between stages 28 and 32 (Nieuwkoop and Faber, 1956) by a technique similar to that described by Stout and Graziadei (1980). During these stages, the OP is pigmented (which aids in complete removal) and the olfactory axons are just beginning to contact the neural tube (Chapter 2; Byrd and Burd, 1991a). Embryos were anesthetized with 0.02% MS222 (tricaine methanesulfonate, Sigma, St. Louis, MO) in Steinberg's solution (see Chapter 2) and positioned in holes in clay dishes. One OP was removed from each donor embryo, inserted into a wound made between the normal
OPs of the recipient embryo, and held in place with a small piece of a siliconized, glass coverslip. After the wound was allowed to heal for 15 minutes, the embryo was transferred to a 100 ml plastic beaker with 50% Steinberg's solution and 50% rearing solution (Burd, 1991). The next day, the embryos were put into individual plastic trays in 400 ml of 100% rearing solution; they were fed boiled nettle (Wunderlich-Diez Corp, Hasbrouck Heights, NJ) daily after stage 40, and the water was changed weekly. Only animals with an obvious extra piece of tissue between the normal nasal capsules and with a separate nerve projecting to the OB were included in this study. Of the 35 animals that satisfied these criteria, 4 animals at stage 50 and 9 animals at stage 57 (late prometamorphosis) or stage 58 (onset of nictamorphic climax) were processed for morphological and quantitative analysis; 2 animals were used to map the distribution of olfactory axons from the transplant using DiI. Stage-matched controls from the same populations were processed with the experimental animals.

**Tissue preparation**

When they reached the appropriate stages, the 13 animals with a transplant and their stage-matched controls were anesthetized with 0.02% MS222, immersed in 4% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (stage 50) or perfused transcardially with 2% glutaraldehyde and 1.6% paraformaldehyde in 0.035 M sodium cacodylate buffer (stage 57/58), and left in the fixative solution
overnight. The next day, the nasal capsules, transplanted tissue, and telencephalon of each animal were postfix fixed in 2% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a graded series of alcohols, and embedded in Epon (nasal capsules and transplant) or Durcupan (brains). One-micron sections were obtained from the nasal capsules, transplants, and olfactory nerves; at selected areas of these tissues, thin, 70-nm sections were collected on Formvar-coated slot grids (Formvar, Ted Pella), stained with lead citrate and uranyl acetate, and viewed with a JEOL 1200 EX electron microscope. For the OBs, serial, one-micron, horizontal sections were taken and stained with toluidine blue (1% toluidine blue, 1% sodium borate).

Quantitative Analysis

The olfactory receptor-cell axons and the mitral/tufted cells were counted in stage 50 and 57/58 animals with a successful transplant and controls as previously described (Chapter 2; Burd, 1991; Byrd and Burd, 1991a). Olfactory receptor-cell axons were quantified from electron-micrographic montages of the olfactory nerves. The axons in all three nerves were counted from animals with a transplant. For control animals, the axons in one nerve were counted, and the number was multiplied by two to get the total number, since there is no significant difference between the two olfactory nerves in control animals (Burd, 1991). This number includes axons from the olfactory epithelium and the vomeronasal organ, since the two nerves run together and are indistinguishable in *Xenopus*.
An estimate of the number of mitral/tufted cells in the OB was determined from serial, one-micron, horizontal sections through the entire OB (including main and accessory OBs) of animals with a transplant and control animals as previously described (Chapter 2; Byrd and Burd, 1991a). It should be noted that the procedure used for estimation of the number of mitral/tufted cells includes the glial cells of the mitral-cell/plexiform layer and is, therefore, an overestimate of the actual number of output neurons. Glial cells make up only a small percentage of the cells in this layer (see Chapter 2), and the percentage did not differ significantly between control animals (3%, n=3) and experimental animals (2%, n=3).

The volume of the OBs of experimental and control animals was estimated in stage-50 (n=3) and stage-57/58 (n=5) experimental animals and their matched controls. To do this, I drew both the main and accessory OBs from every 40th, one-micron, serial section with a camera lucida drawing tube. The SigmaScan computer program (Jandel Scientific, Corte Madera, CA) was used to measure the area of the OB (both main and accessory bulbs combined) in each section. Then, the volume of the whole structure was estimated with the following formula:

\[
\frac{\text{area } 1 + \text{area } 2}{2} \times 40 \, \mu m + \frac{\text{area } 2 + \text{area } 3}{2} \times 40 \, \mu m + \ldots
\]

Statistical analyses were performed by the SAS System for personal computers (SAS Institute, Inc., Cary, NC). To determine whether stage-57 and stage-58 data could be grouped together, a Student's t-test on ranked data was performed, which is comparable to the non-parametric Wilcoxon-Mann-Whitney
two-sample test (Conover and Iman, 1981). Since the number of olfactory axons in stage-57 control animals was not significantly different from stage-58 control animals ($P>0.8$), both stages were grouped together for late-stage analyses. For comparisons of experimental larvae with controls, Student’s $t$-tests were performed. This type of statistical analysis is more powerful for small sample sized than other tests (Conover and Iman, 1981). For comparisons of paired data, such as comparison of one side of the animals to the other side, the paired-difference $t$-test was performed. For these analyses, the statistical tests were performed on the differences between the two sides and not on the actual data values. A significance level of 0.05 was used for all analyses.

Analysis of projections

The projections of the transplanted olfactory epithelium were compared to the projections of control olfactory epithelium with Dil (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Molecular Probes, Eugene, OR), by a method similar to that described by Godement and colleagues (1987). Two animals with a transplant and two controls were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer at stage 58; then a solution of 0.1% DiI in 90% ethanol and 10% dimethyl sulfoxide was injected into the closed transplant or into one nasal capsule of control animals. The animals were left in vials of fixative solution at room temperature for one month. After that time, the brains, with olfactory nerves
and nerves from the transplanted tissue attached, were embedded in gelatin-albumin (11 grams gelatin, 75 grams albumin in 250 ml water), sectioned on a Vibratome, counterstained with bisbenzimide (Hoechst 33258; Beltz and Burd, 1989), temporarily mounted with 0.1 M phosphate buffer onto glass slides, coverslipped, and photographed with a Zeiss fluorescence microscope.

RESULTS

Histology of the Transplant

The transplanted OP developed into a closed structure with a nerve that entered the OB between the two normal olfactory nerves (Figure 4.1). From external criteria, the transplants differed from normal nasal capsules in that they were smaller and less pigmented and had no opening to the external environment. The morphology of the transplanted tissue, however, was similar to control olfactory epithelium. At the light-microscope level, most areas of the transplanted tissue consisted of pseudostratified, columnar epithelium. Transplanted tissue differed from control olfactory epithelium, however, in that it was thinner and seemed to contain fewer cells across the apical to basal extent of the epithelium (Figure 4.2). While the cells tended to be oriented toward the lumen of the transplant, occasionally they were not as well organized. In some cases, the lumen of the transplanted tissue appeared to be covered with a thin layer of cuboidal cells.
FIGURE 4.1. Dorsal view of the heads of a control animal and animals with a transplant (at stage 58).  A: Control animal showing the two nasal capsules (N), two olfactory nerves (arrow) and the olfactory-bulb region of the brain (boxed region).  B-D: Heads of experimental animals showing the transplanted tissue (*), nerves from the transplant (arrowheads in C and D), and olfactory bulb (boxed region). Note that in B, the side of the olfactory bulb innervated by the nerve from the transplant appears larger than the other side, but in C and D, there is no apparent difference between the two sides. Scale bar = 2 mm.
FIGURE 4.2. Coronal sections through normal olfactory epithelium and transplants at stage 58. A: Control olfactory epithelium is a pseudostratified, columnar epithelium with bundles of olfactory axons along the basal surface (A). B and C: Sections through transplanted tissue from two animals shows a similar pseudostratified, columnar appearance with bundles of axons from the transplant (A). The tissue differs from normal olfactory epithelium, however, in that it is a closed structure with a lumen (L) in the center. Scale bar = 50 µm.
At the electron-microscope level, it was apparent that the transplanted tissue had the same cell types as normal olfactory epithelium (Figure 4.3). There were light cells with ciliated knobs at the surface that resembled olfactory receptor cells. There also appeared to be some receptor cells with microvilli, similar to the receptor cells of the vomeronasal organ in controls; however, we were not able to follow the cell processes down to their cell bodies. Dark and light cells without apparent processes were present just above the basal lamina and appeared to be basal cells. Two types of cells with characteristics of supporting cells were observed; their nuclei were in the region above the receptor cell nuclei, and each cell had a thick process that extended to the surface of the epithelium. One of these cell types (Figure 4.3b) had microvilli at the surface and many secretory vesicles throughout the apical process, features common to supporting cells in most vertebrates. The other cell type (Figure 4.3b) was ciliated, had rootlets extending from the basal bodies of the cilia, had numerous mitochondria throughout the apical portion of the cell, and resembled the ciliated supporting cells that have been described in fish (Bertmar, 1973; Andres, 1975; Ichikawa and Ueda, 1977). Some parts of the transplanted epithelium, as well as the cells covering the surface of some parts of the epithelium, consisted of columnar cells that resembled cells of the respiratory epithelium.

Gross examination of animals with a transplant suggested that the nerve from the transplanted tissue in some animals might be as large as the normal
FIGURE 4.3. Electron-microscopic view of transplanted epithelium and control olfactory epithelium at stage 58. The transplanted tissue (A and B) has ciliated olfactory receptor neurons (R), supporting cells with microvilli (M) or cilia (C), and both light (L) and dark (D) basal cells; the basal lamina is marked with arrowheads. Note in B that the basal bodies in the ciliated supporting cells have long rootlets extending from them (arrowhead), unlike the basal bodies in the ciliated receptor cells (arrow). The control epithelium (C and D) has the same cell types (marked as in A and B) and also has receptor cells with microvilli (R'). Scale bar = 4 μm (A, C, and D) or 1 μm (B).
olfactory nerve. Microdissection and tissue sections revealed, however, that even in these animals, the nerve from the transplant was always smaller. Ultrastructurally, these nerves were very similar to control nerves; the only differences were that their cross-sectional areas were obviously smaller than normal olfactory nerves and the axon bundles were smaller and were not as tightly packed as they were in normal olfactory nerves.

**Morphology of Olfactory Bulbs in Animals with a Transplant**

By external observation, the OB of most of the animals with a transplant appeared identical to normal OBs (Figure 4.1), and serial sections revealed that the OBs of animals with a transplant developed the layers of the normal OB. At stage 50, the OBs of experimental animals were very similar to control OBs except that the side innervated by the transplant appeared to be larger than the contralateral side (Figure 4.4). Comparison of the volumes of the OBs, however, revealed that there was a difference between the side innervated by the nerve from the transplant and the contralateral side that was not statistically significant (P>0.07; compare 0.016 mm$^3$ to 0.013 mm$^3$; 0.016 mm$^3$ to 0.014 mm$^3$; 0.035 mm$^3$ to 0.036 mm$^3$). It appears that there is a trend toward an increase on the side innervated by the nerve from the transplant, and perhaps analysis of larger numbers of animals would allow detection of a statistically-significant difference between the sides. Comparison of the total OB volumes revealed that the OBs of stage-50 animals
with a transplant seemed to be larger than control OBs (Figure 4.6; compare 0.043 ± 0.014 (SEM) mm$^3$ for experimental animals to 0.030 ± 0.008 (SEM) mm$^3$ for controls). Statistical analyses, however, did not detect a significant difference between experimental animals and control animals at stage 50 (P>0.3).

In most of the animals examined at stage 57/58, the OBs appeared identical to controls in both gross examination and serial, thick sections (Figure 4.5a and b). The OBs were fused, as normal OBs in *Xenopus* are after stage 50 (Chapter 2; Nieuwkoop and Faber, 1956; Byrd and Burd, 1991a), and the side innervated by the transplant was not obviously different from the contralateral side. In two of the nine animals at stage 57/58, however, gross examination and microdissection suggested that the side of the OB innervated by the transplant might be larger than the other side. Sections through the OB of these two animals revealed that the ventricle was pushed forward, so that the volume of the OB actually appeared equal on both sides (Figure 4.5c). Comparison of the volumes of the two sides in these two animals confirmed that the side innervated by the transplant did not appear to be significantly larger than the contralateral side (compare 0.152 mm$^3$ to 0.134 mm$^3$ in the first case; 0.089 mm$^3$ to 0.080 mm$^3$ in the second case). Also, in these two animals, the two sides of the OB did not fuse. The OBs of stage-58 animals with a transplant were not larger than control OBs (Figure 4.6; compare 0.278 ± 0.040 (SEM) mm$^3$ for experimental animals to 0.286 ± 0.036 (SEM) mm$^3$ for controls; P>0.7).
FIGURE 4.4. Olfactory bulbs of a control and animals with a transplant at an early-larval stage. These are horizontal sections through the olfactory bulb of a stage-50 control animal (A) and two stage-50 animals with transplants (B and C). All are unfused olfactory bulbs with the typical laminated structure, ventricles (v), and accessory olfactory bulbs (aob); onl=olfactory nerve layer, gl=glomerular layer, m/pl=mitral-cell/plexiform layer, gcl=granule cell layer. Note that in the animals with transplants, the side of the bulb receiving the extra nerve (*) appears larger than the contralateral side in these sections. This was true for all stage-50 animals with transplants. Scale bar = 100 μm.
FIGURE 4.5. Olfactory bulbs of a control and animals with a transplant at a late-larval stage. These are horizontal sections through the olfactory bulbs (boxed regions in Figure 3.1) of stage-58 animals. A: Control olfactory bulb showing the typical laminated structure, ventricles (v) and accessory olfactory bulbs (aob); onl=olfactory nerve layer, gl=glomerular layer, m/pl=mitral-cell/plexiform layer, gel=granule cell layer. B: Olfactory bulb from one of the animals with a transplant showing the similarity to the control olfactory bulb. Even with the addition of an extra olfactory nerve (which entered at *), both sides of the bulb appear to be similar in structure and size. Two of the nine animals examined had an olfactory bulb that appeared to be larger on the side innervated by the transplant; the olfactory bulb from one of these animals is shown in C. Note that the transplant-innervated side (*) extends more rostral than the other side, but so does the ventricle (arrowheads), and the two sides of the bulb are not fused as they are in controls. Scale bar = 250 μm.
FIGURE 4.6. Olfactory-bulb volumes in animals with a transplant. Volumes of olfactory bulbs of control animals and animals with a transplant were compared at stage 50 and stage 57/58. Bar graphs of total olfactory bulb volume (from serial sections) show that there is no difference between experimental animals and control animals at either stage.
OLFACTORY BULB VOLUME (mm$^3$)

- Control animals
- Animals with transplant

Stage 50

Stage 57/58
Projections of the Transplanted Tissue

The projections of the nerve from the transplant were compared to the projections of normal olfactory nerves. When one nasal capsule of stage-58 control animals was filled with DiI, the olfactory nerve was found to project mainly to the ipsilateral side of the OB, but many axons extended to the contralateral side (Figure 4.7a). Transplanted tissues labeled with DiI were found to have a nerve that entered one side of the OB, but the axons from that nerve projected to both sides of the OB (Figure 4.7b). In addition, as with the normal olfactory axons, the axons from the transplant were restricted to the olfactory nerve layer and glomerular layer of the OB. The projections of the nerve from the transplant were not examined in stage-50 animals, since the OBs are not fused in these animals and, therefore, the axons are confined to the side of the OB they entered.

Quantitative Analysis

Stage 50. To determine if the nerve from the transplanted tissue increased the total number of afferent fibers projecting to the OB, the total number of axons in the three nerves of the animals with a transplant and in the two nerves of the control animals was estimated. The total number of olfactory axons in stage-50, control animals was 25,475 ± 6,023 (SEM); in animals with a transplant, the total number of olfactory axons was 38,585 ± 7,755 (SEM). Despite this apparent increase in total axon number in animals with a transplant, no significant increase
FIGURE 4.7. Projections of an olfactory nerve and the nerve from a transplant labeled with Dil. **A:** Dil follows the olfactory axons from the nasal capsule of a stage-58 control animal to the olfactory bulb. The axons are confined to one olfactory nerve (ON) and distribute mainly to one side of the olfactory bulb, but some fibers cross the midline (arrow) and project to the other side of the fused olfactory bulb. **B:** Dil labeling of the olfactory bulb from a stage-58 animal with a transplant also shows that fibers reach both sides of the olfactory bulb (midline is at arrow). The nerve from the transplant is not in this plane of section, but it innervates the right OB. **C** and **D:** Hoechst staining of the same sections in **A** and **B,** respectively, labels the nuclei for better identification of the olfactory-bulb layers (labeled as in Figure 4.5). Scale bar = 250 μm.
could be detected with the statistical methods used (P>0.3; Figure 4.8a). The nerve from the transplanted tissue appeared to have fewer olfactory axons compared to control olfactory nerves, although this difference was also not statistically significant (P>0.1). Nonetheless, the nerve from the transplant contributed only an average of 18% of the total number of axons in young animals (Table 4.1). Even though the nerve from the transplant was rather small, when added to the normal olfactory nerve that innervated the same side of the OB, the number of olfactory axons was significantly larger than the number of olfactory axons innervating the contralateral side (from the normal olfactory nerve on that side only) (P<0.002). In addition, because the two sides were not fused at stage 50, the axons were confined to the side they innervated. This meant that one side of the OB actually received more afferent innervation than the other side of the bulb.

I then determined if the transplanted tissue had an effect on the major output neurons of the OB, the mitral/tufted cells. To do this, I estimated the number of cells in the mitral-cell/plexiform layer in serial, one-micron sections through the OB. There was no significant difference between animals with a transplant and control animals in the total number of cells in the mitral-cell/plexiform layer in the main and accessory OBs at stage 50, (P>0.7; Figure 4.8b), although the animals with a transplant appeared to have slightly more cells.
### TABLE 4.1 Data for Animals with a Transplant and Control Animals

<table>
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<th>TRANSPLANTS</th>
<th>Stage</th>
<th>Axons R. nerve</th>
<th>Axons L. nerve</th>
<th>Axons T. nerve</th>
<th>T. Nerves % of total</th>
<th>Cells R. OB</th>
<th>Cells L. OB</th>
<th>Convergence ratio</th>
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<tr>
<td>T1</td>
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<td>15,620</td>
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1. R. nerve=right nerve; L. nerve=left nerve; T. nerve=nerve from transplant; R. OB=cells in the right main and accessory olfactory bulb; L. OB=cells in the left main and accessory olfactory bulb.
2. Convergence ratio is total axons + total OB cells.
3. For most control animals only one nerve was counted, since there is no significant difference between the number of axons in the two olfactory nerves (Gund, 1991).
4. This control animal was also age- and stage-matched with T4 for paired statistical analyses.
FIGURE 4.8. Quantitative analysis of olfactory-axon and mitral/tufted-cell numbers in animals with a transplant. A: Bar graphs of olfactory-axon number in control animals (n=3, stage 50; n=9, stage 57/58) and animals with a transplant (n=4, stage 50; n=9, stage 57/58) are shown. These numbers include the axons from the three nerves in animals with a transplant and two nerves in controls. Note that there appears to be an increase in olfactory-axon number at stage 50, but this is not a statistically-significant increase. At stage 58, there is no difference between control and experimental groups. B: Bar graphs of mitral/tufted-cell number in the main and accessory olfactory bulbs of experimental and control animals at both stages shows that there is no significant difference between the two groups.
OLFACTORY AXON NUMBER (x1000)

- Control animals
- Animals with transplant

MITRAL/TUFTED CELL NUMBER (x1000)

- Control animals
- Animals with transplant
The average number of mitral/tufted cells in animals with a transplant was 10,219 ± 1,962 (SEM), and in controls, it was 9,177 ± 2,552 (SEM). Even though the side with the transplant nerve was innervated by more afferent axons and appeared larger than the other side, it was not significantly different from the contralateral side in number of mitral/tufted cells (P>0.2; Table 4.1). In these young animals, with a transplant, the convergence ratio was higher than in controls, but again, no significant difference could be detected with statistical methods (P>0.06). It appears that there is a trend toward an effect on convergence ratio, but the current analyses could not demonstrate such an effect.

Stage 57/58. To determine if fusion of the two sides of the OB, which allows afferent fibers to reach both sides, as described above, had an effect on the quantitative results above, experimental animals and their controls were also analyzed at stage 57/58. The total number of olfactory axons in stage-57/58 controls was 282,711 ± 22,356 (SEM); in animals with a transplant, the total number of olfactory axons was 269,911 ± 21,165 (SEM) (Figure 4.8a). Statistical analysis of these older animals showed that animals with three nerves were not significantly different from controls (with two nerves) in total olfactory-axon number (P>0.9).

The nerve from the transplanted tissue was significantly smaller than control olfactory nerves (P<0.00001) and only contributed an average of 12.5% of the total axon number at stage 57/58 (Table 4.1). Together, however, the nerve from the
transplant plus the normal olfactory nerve that projected to one side of the OB were significantly different from the contralateral, normal nerve at stage 57/58 (P<0.0004; Table 4.1). As was the case with younger animals, one side of the OB received more afferent axons than the other side of the bulb. The axons, however, may not solely innervate the side of the bulb receiving nerve projections, since our DiI experiments showed that the innervating fibers have access to both sides of the fused OB in these older larvae.

The number of mitral/tufted cells in the OB of animals with a transplant was not significantly different from control values at late-larval stages (P>0.8; Figure 8b). The average number of mitral/tufted cells in stage-57/58 animals with a transplant was 53,016 ± 2,636 (SEM), and in controls, it was 53,827 ± 3,472 (SEM). In animals with a transplant, the two sides of the OB did not differ significantly in mitral/tufted-cell number (P>0.8; Table 4.1) even though more axons entered one side of the OB than the other side as shown above.

There was no statistical difference in the ratio of the number of olfactory axons to the number of mitral/tufted cells (convergence ratio) in animals with a transplant and their controls at stage 57/58 (P>0.8). In control animals, the convergence ratio was 5.3:1; in animals with a transplant at these same stages, the convergence ratio was almost the same, 5.1:1. Perhaps this is not surprising in light of the fact that there was no difference in either olfactory-axon number or mitral/tufted-cell number.
DISCUSSION

In the olfactory system, the number of sensory afferent axons innervating the OB has been related to the number of output neurons, the mitral cells (or mitral/tufted cells in cases where the two cannot be distinguished) in adults (Allison and Warwick, 1949; Gemne and Døving, 1969; Bhatnagar and Kallen, 1975) and during development (Chapter 2; Meisami, 1989; Derivot, 1984b; Byrd and Burd, 1991a). The ratio of the number of olfactory axons to the number of mitral/tufted cells (convergence ratio) does not appear to change throughout larval development in *Xenopus* (Chapter 2; Byrd and Burd, 1991a) and does not appear to be affected by partial deafferentation (Chapter 3; Byrd and Burd, 1991c, in preparation). This suggests that there is a close quantitative relationship between olfactory axons and bulb neurons throughout this developmental period. Therefore, it was predicted that transplantation of an extra OP that innervated the OB would increase the number of afferent axons entering the OB; increased numbers of olfactory axons were then expected to be paralleled by an increase in the number of mitral/tufted cells.

Transplantation experiments were performed before or during the stages when the olfactory axons have just innervated the neural tube; it was expected that this would allow the greatest effect on OB development. I found that the addition of an extra OP at embryonic stages of development in *Xenopus* appeared to increase the total number of olfactory axons at an early stage of larval development.
(stage 50), but statistical analyses showed that the increase was not significant. This is not surprising when one couples the fact that very few animals were available for analysis to the fact that there is considerable variability in olfactory-axon number between animals (even in control animals). Statistical methods are not very powerful given these two factors. Therefore, even though the increase was not statistically significant, analysis of the raw data shows that there is a trend toward an increase in olfactory-axon number at stage 50. The number of mitral/tufted cells also appeared to increase slightly, but not to a statistically-significant level.

This finding, contrary to the predicted results, has several possible explanations. While damage to the neural tube during transplantation was a possibility, I ruled this out in another study (Chapter 3; Byrd and Burd, 1991c, in preparation) when I looked at the effect of removing a portion of the neural tube at embryonic stages. The neural tube regenerated in 24 hours and formed a normal OB with numbers of olfactory axons and mitral/tufted cells that were not different from control values.

Another possible explanation is that the transplanted OPs develop into a closed piece of tissue which, most likely, does not respond to odors. Perhaps activity in response to odorants in the afferent fibers is necessary for them to have an effect on the OB. Even though there were more axons in animals with a transplant at stage 50, these axons might not have had normal activity patterns and may not have been able to elicit a response in the OB. Studies of the effect of
unilateral naris closure on OB development have suggested that odorant-response activity in receptor cells is important for normal OB development (Meisami and Safari, 1981; Benson et al., 1984; Meisami and Noushinfar, 1986; Frazier and Brunjes, 1988). Studies on the visual system, however, have shown that spontaneous activity may be just as important as normal sensory stimuli (Stryker and Harris, 1986). Therefore, even though the closed transplant probably does not respond to environmental odors, there may be spontaneous electrical activity in the transplanted tissue that would be sufficient to elicit the normal developmental effect of olfactory axons on their target neurons. In support of this, Burr (1930) found that when his OP transplants were buried beneath the surface and cystic, without an opening to the external environment, the result of hyperplasia in the cerebral hemispheres was the same as for open transplants.

Numerous studies have provided support for the hypothesis that olfactory axons provide a trophic factor that not only stimulates bulb development but also can instruct neurons in other central nervous system (CNS) areas to express OB characteristics, including the formation of glomerulus-like structures (Burr, 1924; May, 1927; Stout and Graziadei, 1980; Magrassi and Graziadei, 1985). In addition, olfactory axons have been reported to induce biochemical changes in ectopic CNS targets. Guthrie and Leon (1989) showed that when the OB is removed in rats, the olfactory axons project to the rostral forebrain. When this occurs, some neurons in the forebrain contacted by the olfactory axons expressed immunoreactivity for
tyrosine hydroxylase (the rate-limiting enzyme in catecholamine biosynthesis). While neurons in this region do not normally express tyrosine hydroxylase, a class of OB neurons that are postsynaptic to the olfactory axons are known to synthesize this enzyme. In addition, tyrosine hydroxylase immunoreactivity is regulated by olfactory-axon innervation in the developing and adult mammalian OB (Baker et al., 1983; McLean and Shipley, 1988). Together, these findings suggest that olfactory axons appear to induce biochemical programs in neurons, whether or not they are the normal postsynaptic target.

Stout and Graziadei (1980) examined the effect of OP transplants that innervated the OB. In a qualitative study, they reported that the resulting OB was larger in volume. In the present study, I generated some experimental animals that appeared similar to those reported by Stout and Graziadei (1980), and gross examination of the OBs at stage 50 might suggest that the transplant had an effect on the size of the OB. Serial sections through the OBs, volume measurements, and mitral/tufted-cell counts, however, showed that animals with a transplant that innervated the OB did not differ significantly from control animals in OB volume or in mitral/tufted-cell number.

The number of animals with a transplant that satisfied my criteria for quantitative analysis (i.e., possessing a nerve to the OB) was quite small. I had a low success rate in my attempts to transplant an extra OP into the region between the two normal placodes. My success rate was much higher, however, when I
transplanted the tissue to other regions, including the back of the head or in place of the eye (C.A.B. unpublished observations). The reasons for this are unknown. Perhaps it is due to the fact that I was attempting to transplant the tissue into a small space without disturbing the normal placodes, while in the ectopic regions I was able to make a larger wound. Another explanation might be that there is inhibition of OP development in the regions of the sensory plate that do not normally form placodal tissue, and this inhibition prevented the transplants from forming or limited their growth when they did form. This might account for the fact that the number of axons in the nerve from the transplant was always smaller than normal. This fact coupled with variation in axon number and cell number may have masked subtle changes resulting from the transplants. Future studies examining transplants of nasal capsules at later stages of development may be beneficial. At stage 45, most of the attempted transplantation operations resulted in a successful transplant innervating the OB (C.A.B., unpublished observations). This may allow examination of the role of activity and evaluation of more extensive quantitative analyses.

It is clear that olfactory axons have many important functions in normal OB development (since removal of the axons early in development can prevent the formation of the OB; Chapter 3), but what factors they provide to the OB and how it is that they transmit their effect are unknown. If there are chemical factors released from the axons and these stimulate neuronal development or play a role
in maintaining neuronal survival, I would have expected that increasing the number of olfactory axons would also increase the amount of these putative factors. In young animals with a transplant, there were apparently more olfactory axons, and yet there was only a slight, if any, increase in mitral/tufted cells in the OB. Perhaps these factors are at maximum levels in a normal OB, and increasing the number of axons (and the factor) cannot increase the number of mitral/tufted cells.

Numerical matching of afferent and target cells is common throughout the nervous system (see Purves and Lichtman, 1985). In many regions, excess neurons initially develop and then are pruned back by cell death (see Oppenheim, 1991). For example, the number of motoneurons that survive through development appears to be related to the size of the peripheral target. Hollyday and Hamburger (1976) implanted a supernumerary leg in chick embryos and found a reduction in naturally-occurring cell death of motoneurons. In other systems, increasing afferent input can reduce the amount of normal neuronal death that occurs during development. For example, in the rat visual system, increasing the number of projections to the nucleus of the optic tract by ablation of the dorsal region of the lateral geniculate nucleus results in an increase in the number of cells due to the prevention of normal cell death (Cunningham et al., 1979). Competition for synaptic sites and availability of a trophic agent are two possible mechanisms for these target effects. There is, so far, no evidence for a normal period of cell death in OB development (Chapter 2; Byrd and Burd, 1991a), so the sparing of
neurons that normally would have died may not be a mechanism at work in this system, at least not during the larval stages examined.

In spite of the findings outlined above, size-matching of targets does not always work perfectly (see Oppenheim, 1991). For example, Lamb (1980) removed a hindlimb in *Xenopus* and allowed the motoneurons on that side to innervate the limb on the contralateral side. He found, contrary to his expected findings, that a single limb supported twice the usual number of motoneurons. Perhaps in his study and in mine, the wrong elements were measured. While increasing the number of afferent fibers (but not to a statistically-significant level) did not affect the number of output neurons in the OB, other factors such as other OB cells, number of synapses, branching of mitral/tufted-cell dendrites, or size of OB neurons may have been affected. To resolve these possibilities will require further analysis.

Another major finding of this study is that the apparent increase in olfactory axons that is seen at young stages is not found at older stages. One possible explanation for this finding is that, over time, the OB regulates the number of axons it will support. This would agree with the suggestion made by others (Hinds et al., 1984; Mackay-Sim and Kittel, 1991) that the adult OB regulates the size of the olfactory receptor-cell population. Several investigators have shown that new olfactory receptor cells are produced throughout the life of the organism (Thornhill, 1970; Moulton et al., 1970; Graziadei and Metcalf, 1971). Mackay-Sim and Kittel (1991) hypothesize that immature neurons fail to reach maturity and die if the
mature receptor cells maintain normal synaptic connections with OB neurons. Perhaps the target sites are limited; when these sites are occupied by synapses from mature receptor cells, the maturation of new receptor cells is suppressed. This mechanism may also be operational during development. Perhaps this is what prevents the transplant from attaining the size of a normal olfactory organ and blocks total axon number from increasing above control values.

It is well-established in the olfactory system that olfactory receptor-cell axons are important for the development of cells in the OB. But the OB may also regulate the receptor cells. During development, cilia are first seen in mouse olfactory epithelium when the olfactory axons reach the OB (Cuschieri and Bannister, 1975). This might suggest that maturation of receptor cells depends upon contact with the OB. To address this, Chuah and Farbman (1983) set up an organ culture system for explants of olfactory epithelium and analyzed the maturation of olfactory receptor cells. Synthesis of olfactory-marker protein (OMP) was used to measure maturation. OMP is found in olfactory receptor cells (Margolis, 1972), specifically mature or nearly mature receptor cells (Farbman and Margolis, 1980; Miragall and Monti Graziadei, 1982). In development, the appearance of OMP expression coincides with the formation of synapses in the OB (Monti Graziadei et al., 1980). In their organ culture studies, Chuah and Farbman (1983) found receptor cells that expressed OMP when cultured alone, but full maturation of all the receptors was not found. When cultured with OB, maturation
was enhanced (as measured by increased OMP expression). When the olfactory epithelium and OB were separated by a filter that allowed movement of diffusible factors, the maturation of receptor cells was not enhanced. These results imply that direct contact is important; the OB probably provides a limited source of factors and/or limited amount of available synaptic space.

An alternative explanation for finding an apparent increase in olfactory-axon number at early stages but not at older stages is that the transplanted tissue is inhibited by the nascent nasal capsules. As I mentioned before, transplantation of an olfactory placode to ectopic locations (outside the sensory plate) results in a successful transplant much more often than transplantation to areas within the sensory plate. The possibility exists that the nascent olfactory placodes inhibit the development of the transplanted olfactory placode. If this is true, then it is also possible that the nascent nasal capsules inhibit the development of successful transplants and prevent the number of olfactory axons in the nerve from the transplanted tissue from attaining that in normal olfactory nerves. The results of my study show that the nerve from the transplanted tissue was smaller than normal olfactory nerves, but it is not possible from the present study to determine whether regulation by the OB or by the nascent nasal capsules is responsible.

In the current study, I also examined the morphology of the transplanted tissue at the light- and electron-microscopic levels. I found that olfactory placodes can survive in ectopic locations and develop the normal cell types found in olfactory
epithelium. Similar findings have been reported for antennal sensory neurons. When *Manduca sexta* moths are debrained prior to the birth of the sensory neurons of the antennal imaginal disk, the antennal sensory neurons develop normally (Sanes et al., 1976). These results suggest that antennal sensory neurons, like the cells of the olfactory placode, do not depend on appropriate connections with their target for development of normal cell types.

In conclusion, I found that transplantation of an extra olfactory organ resulted in an initial increase in the number of olfactory axons at stage 50, after which the number of axons is reduced to control levels by stage 57/58, after fusion of the OBs has occurred. There does not appear to be a change in mitral/tufted-cell number or OB volume at older stages. At young stages, both mitral/tufted-cell number and OB volume are increased (but not to a statistically-significant level), but the increase is not as great as that for olfactory-axon number. One possible explanation for these results is that the OB regulates the number of axons that it can maintain and, thus, prevents supernumerary innervation from occurring. It is likely that target and afferent fibers interact to regulate the final number of neurons in both structures.
CHAPTER 5: GENERAL DISCUSSION AND FUTURE DIRECTIONS

Major Contributions of This Work

In this dissertation work, my results have confirmed that the axons of the olfactory receptor cells are necessary for normal development of their target structure, the OB. Olfactory axons are related quantitatively to the number of output neurons of the bulb, the mitral/tufted cells, in normal development, following partial deafferentation, and in late-stage larvae with supernumerary olfactory organs. These studies will, I hope, be interesting to investigators in the fields of neuroscience, developmental biology, cell biology, and sensory biology. The following paragraphs will outline some of the major contributions of this work.

The study on the normal development of the OB in *Xenopus laevis* was performed because no previous study had followed the development of this structure in *Xenopus*. In Chapter 2, I showed the morphology of the developing OB from initial differentiation through adulthood. My investigation provides important information that will serve as a background for future work in the olfactory system of *Xenopus*. This work also serves as a means to compare amphibian olfactory-system development to other animals. In addition, I established proof of a quantitative relationship between olfactory axons and mitral/tufted cells during development since the convergence ratio remains constant during normal larval stages. In doing so, I also determined the convergence ratio for adult
Xenopus, which can be used to compare this frog to other animals where ratio is known for adults.

My second study involved examination of the effects of partial deafferentation on the early events in bulb differentiation. This experiment was designed to analyze the possible role of olfactory axons in the initial events of OB differentiation. For this experiment, I attempted to quantify a phenomenon seen for a number of years in previous studies. These studies described the fact that olfactory axons are necessary for development of the OB. In my study, I analyzed, in a quantitative manner, the relationship between olfactory axons and mitral/tufted cells in animals with partial deafferentation. This was done to examine the effects of experimental manipulation on the correlation that was observed during normal development. These results showed that when both groups of morphologies of partially-deafferented OBs are analyzed together, the relationship did not appear to change significantly, even when a significant decrease in the number of afferent axons was created. With these results, I have provided proof of the strength of this association.

My final study was an analysis of the effects of the addition of a supernumerary OP early in development. In the first part of my analysis, I examined the structure and cell types of transplants of placodes that developed in an ectopic region (i.e., in part of the sensory plate where OPs are not normally found) at the light- and electron-microscope level. Morrison and Graziadei (1983)
examined transplants of olfactory epithelium from young rats between post-natal day 5 and 10 into the fourth ventricle. Their analysis was different from mine because it was at the light-microscope level only and the tissue that was transplanted had already developed into an epithelium. In my study, the placode was transplanted before the olfactory epithelium had formed, and I analyzed the tissue at both the light- and electron-microscope levels. The fact that normal cell types develop from this tissue even in an ectopic location may have implications for studies on the factors contributing to the development of the olfactory epithelium from the OP.

In the transplant study, I also showed OBs of animals with a transplant at 50 and 58. Previous studies did not show sections through the bulbs, so it was not possible to tell if the structure was significantly altered. I measured OB volumes and showed that OBs in animals with a transplant were not significantly larger than controls, even when gross examination suggested that there may have been an increase. In this respect, the results of my study, which involved quantitative methods and a more thorough examination, contradict those of Stout and Graziadei (1980).

While the deafferentation study concentrated on the influence of the peripheral olfactory structure on its central target, the results of the transplantation study support the hypothesis that the OB plays a role in regulating the growth and maintenance of the size of the olfactory epithelium. In the transplantation
experiments, I attempted to increase the number of olfactory axons innervating the OB and determined the effects on the development of the OB. I was unable to increase the number of olfactory axons even though the animals had an extra piece of olfactory tissue and three olfactory nerves that entered the OB. One explanation for this result is that the OB regulates the number of afferent fibers it will receive. Alternatively, the development of the transplanted tissue might be suppressed by the nascent nasal capsules.

Finally, the results of my transplant study suggest that the hypothesis that the OP controls the development of the OB is too simplistic. In reality, there appears to be a more complicated relationship between the axons and the OB which may involve reciprocal influences. Clearly, the axons from the OP are crucial for OB differentiation. This interaction may not be one-sided. The OB seems to receive information from the olfactory axons and also may provide important information to the developing olfactory epithelium. Target and afferent interactions probably work together to regulate the final number of neurons in the developing animal. Future analyses of the interactions between the OP and the OB should involve investigation of the role of axons in maintenance of the OB. A long-term, future goal is to identify the molecular signals and responses communicated by sensory afferent axons and target neurons in this system.
Future directions that will extend the findings of this work

The results of my dissertation have provided answers to some questions about interactions between developing olfactory structures. This work has also, however, brought up many questions that could be answered in future analyses. For example, I did not address in detail the hypothesis that the olfactory axons may initially induce OB development, after which the OB is set on a developmental program. This program might regulate the size of the olfactory epithelium (by regulating the number of axons that have connections to the OB). Furthermore, it is also possible that the OB may develop to a limited extent in anticipation of the incoming axons. These possibilities will be analyzed in the experiments outlined below.

Transplantation experiments in embryos.

First, an attempt could be made to increase the number of animals with a transplant that had their surgery at the same stages used in my studies. This would involve increasing the success rate of transplants performed at stages 28-32. I believe that one of the problems that have resulted in this low success is the fact that only a small wound can be made between the host OPs without disturbing them, so the transplanted tissue is generally pushed in rather deep. One experiment I tried to deal with this problem was to remove the entire sensory plate, creating a large wound, before transplanting an OP. Since the host placodes
generally regenerate, I hoped that the transplanted tissue would grow, and the host OPs would regenerate; this might then result in the development of three placodes. Unfortunately, when I did this experiment, only one OP formed, and it was unclear whether it was from host or donor tissue. Other attempts could be made to make a larger wound, but I am not confident that it is possible to do this with the current surgical procedure. A larger wound might disturb the host placodes.

Another possible way to increase the number of animals with an embryonic OP transplant would be to include transplants that fuse with one of the host OPs. In previous transplantation studies, the transplanted OP often fused to one of the host placodes. Stout and Graziadei (1980) reported fusion of the transplant with the host OP in 95% of the cases when they transplanted an OP to the same region I did. Burr (1930) also mentioned that the OP often fused with the host OP in his transplantation experiments. I did not include fused transplants in my study because I wanted to have a separate nerve from the transplant for my quantitative analyses. Perhaps these animals should be included in future analyses, to increase the number of animals for study.

The observation that I made concerning the low success of embryonic transplants placed in the sensory plate region may indicate an interesting phenomenon. A transplant into head regions other than the sensory plate successfully formed a nasal capsule in 84% of the cases, but transplants into the
sensory-plate region developed in only 8% of the cases. Perhaps the transplant usually does not develop in the sensory plate because of some inhibitory factor that prevents the OP from forming except in the normal location within the sensory plate. The epithelium in the entire sensory-plate region is presumably capable of forming placodal tissue (P.P.C. Graziadei, personal communication). One hypothesis is that the non-placodal regions of the sensory plate are actively prevented from forming placodal tissue. Thus, in my experiments when I attempted to transplant an OP into the region where placodes are not supposed to be, it is possible that an inhibitory factor prevented growth of the transplant in all but a few cases. To begin to analyze this hypothesis, the skin between the placodes could be removed and transplanted to head regions outside of the sensory plate. This may release the inhibition on the transplanted, sensory-plate tissue and allow it to develop into olfactory epithelium.

This brings up the interesting questions of what induces the placodes to form and why do they almost always regenerate? Zwilling (1940) performed experiments in *Rana* that showed that belly ectoderm can be induced to form nasal tissue if head mesoderm is inserted beneath it. He proposed that the olfactory organ is induced by the mesoderm associated with the roof of the archenteron. These conclusions were supported by other experiments in *Rana* by Cooper (1943) and Emerson (1945) that showed that ectopic OP transplants or explants of late-gastrula tissue form nasal sacs more completely and quickly if part of the underlying
mesoderm is included. Alternatively, Haggis (1956) proposed that the neural plate is involved in the induction of the olfactory organ. He removed the anterior neural plate in *Ambystoma* and found that the development of the nose was hindered. Haggis (1956) also showed that placodal tissue differentiates into nasal tissue if it is cultured with neural tube, not head mesoderm. Perhaps the fact that these experiments have been performed in two different species can explain the contradiction in the results. Regardless of the reason, the fact remains that the inducing factor is still unknown.

If the success rate for the generation of animals with a transplant can be improved, or if transplants that have fused with the host OP are used for analysis, there are still many interesting questions that were not addressed in my studies. One question concerns the ingrowth of olfactory axons into the neural tube in normal animals and in animals with a transplanted OP. In the mouse, some olfactory axons penetrate deep into the neural tube before retracting to the more superficial layers where they are found normally (Hinds, 1972b). It is not known if this phenomenon occurs in frogs. This should be addressed first in unoperated animals. I would attempt to label the incoming olfactory axons in young embryos, beginning with stage 30, with immunocytochemistry, using antibodies to GAP-43 (a growth-associated protein found in growing axons; Skene and Willard, 1981) or E7 (a monoclonal antibody from Dr. Burd's laboratory that appears to label *Xenopus* olfactory receptor cells and their axons; Matheson and Burd, 1991). Alternatively,
the lipophilic dye, DiI, could be applied to the placode, where it would be picked up by the cells there and transported along the cell membrane. Once these experiments have been done and it has been discovered whether or not *Xenopus* olfactory axons behave like mouse axons, the axons from the transplanted epithelium can be labeled and compared to normal olfactory axons. To label the axons in the transplant, the tissue to be transplanted could be labeled with DiI before the transplantation surgery. Alternatively, the donor tissue could be labeled with a fluorescent dextran (by adding the dye to the egg, so that every cell would have it) so that the olfactory axons from the transplant could be distinguished from the tissue of the host. The dye is visible in living animals for at least two weeks (O’Rourke and Fraser, 1986), long after the embryonic stages important for this experiment.

Another question that could be asked in these animals with a transplant is why the nerve from the transplant is always much smaller than control olfactory nerves. The number of axons is never equivalent to the number in controls. One possibility is that since the transplant tissue is a closed structure and cannot respond to odors in the environment, the axons from the transplant lack the activity necessary to compete with the normal olfactory axons. To address this, first, I would analyze whether or not the closed nature of the transplant effects the size of the nerve by attempting to cut open the transplant to see if opening the structure allows the nerve to attain a larger size. Another method to address this
would be to cut one normal olfactory nerve, wait a period of time, then analyze the number of axons in the nerve from the transplant to see if that number is increased above the number of axons in nerves from closed transplants.

The results of my transplantation experiments have led to the suggestion that the OB influences the development of the olfactory epithelium. To address this phenomenon, it may be possible to transplant extra neural tube tissue and an extra placode to enlarge the target area for the olfactory axons. This may allow a larger transplant to be supported, so the nerve from the transplant may obtain the size of a normal nerve. Similarly, extra neural tube may be added without a transplant to see if the normal olfactory nerve will have an increased number of axons due to the increase in target area. This would be similar to findings in the chick where enlargement of the target area for motoneurons by addition of a supernumerary limb bud resulted in an increased number of motoneurons due to reduction in cell death (Hollyday and Hamburger, 1976).

The above experiments would continue the work that I started and deal with the results of transplanting an extra OP at stages before the OB has started to differentiate. Other interesting questions may be addressed by performing the surgeries at older stages, when OB development has already begun.
Transplantation experiments in larvae.

By stage 45, the nasal capsule has developed, and the olfactory axons make synapses with the OB. The OB will continue to develop to a considerable extent after this stage, but it already has rudimentary forms of all of the layers. Experimental manipulations at this stage can address questions about the transplant tissue itself (i.e. the turnover rate and activity patterns), the role of olfactory axons in the maintenance of the structure of the OB, the possible role of activity in the effect of olfactory axons, and the effects of olfactory axons on other elements of the OB. I have performed preliminary transplantation experiments on young larvae at stage 45. While I did not analyze the results of these surgeries, I did conclude that surgeries at stage 45 have a much higher success rate (89%) than surgeries at embryonic stages (8%). Therefore, more animals with a transplant could be generated and analyzed in a shorter period of time than it took for animals in my study.

The first step in the analysis of animals that received an OP transplant at stage 45 would be to compare the morphological and quantitative results to those in my study. Does the transplant still develop into a closed capsule? Is the nerve from the transplant still significantly smaller than a normal olfactory nerve? Does the presence of the transplant result in supernumerary innervation of the OB? Is the OB affected by the presence of the transplant? All of these questions could be answered by performing the same analyses that I have used.
Additional questions could be answered with different analyses of animals given an extra placode at stage 45. For instance, it would be interesting to know if the nerve from the transplant and the normal olfactory nerve segregate into separate glomeruli and if the right and left olfactory nerves normally segregate. My DiI analysis has shown that there is considerable crossing of axons through the fused midline at stage 58. I was not able to distinguish individual glomeruli. To answer these questions, DiI could be applied to the nasal capsules (or transplant tissue), and photo-oxidation of the DiI could be used to allow analysis of thinner sections in which glomeruli may be distinguished. This analysis could even be taken to the electron-microscope level, if necessary. Addition of an extra olfactory organ may lead to segregation by the axons of the normal olfactory organ and the axons from the transplant, whether or not the axons from the right and left olfactory nerves normally segregate. A similar study was performed in three-eyed frogs (Constantine-Paton and Law, 1978). In \textit{Rana} addition of extra eye early in development leads to innervation of a previously uninnervated tectal lobe by two sets of axons, those from the normal eye and those from the supernumerary eye. This results in the formation of eye-specific bands in the tectum. Perhaps the olfactory system will be similar. There is a major difference, however, between these two systems since the projections of the normal nerves overlap in the olfactory system, but the projections of the normal, optic nerves do not overlap.
These same types of experiments could also be used to compare closed and open transplants. In transplants done at stage 45 it may be possible to generate open transplants more successfully than it was in earlier stages; if open transplants cannot be generated, then the closed transplant could be cut open. It may be possible to use these experiments as a measure of the success of the transplant in competition with the normal olfactory nerves. Perhaps there will be a difference between open transplants (which can presumably respond to odorants) and closed transplants (which cannot respond to odorants). In the visual system, monocular eye-lid suture in monkeys (which deprives one eye of patterned visual experience) causes the closed eye to lose out in competition with the open eye, so the axons from the open eye take over some of the space in the visual cortex that normally would be occupied by the other eye (Hubel et al., 1977). It might be expected that similar results will be found in the olfactory system. Since the two normal olfactory nerves already have synaptic connections to the OB when the transplant is added, they may have an unfair advantage in the competition between normal and transplanted olfactory axons. If the two olfactory nerves are cut before the extra nasal capsule is transplanted, the competition between the normal and transplanted olfactory axons may be more equitable. It would be interesting to see if this allows the transplant to attain the size of the normal nasal capsules.

A question that I have been asked when I have given seminars is how the turnover rate in the transplanted tissue compares to normal olfactory epithelium.
I was unable to answer this question due to the low number of successful animals that I had. This question could more easily be addressed with a \(^3\)H-thymidine study in animals given a transplant at stage 45. Similarly, the turnover rate of closed and open transplants could be compared.

Finally, the transplanted epithelium could be compared to normal olfactory epithelium in terms of the presence of certain antigens. My work has shown that the transplanted tissue has the same cell types as normal olfactory epithelium by morphological criteria. Immunocytochemistry could be used to see if transplant epithelium has the same antigens recognized by the monoclonal antibody E7 (Matheson and Burd, 1991) and antibodies to keratins, intermediate filaments, as well as other antibodies.

Deafferentation in embryos.

Future studies could also concentrate on the effects of deafferentation on the OB. Partial deafferentation at early stages addresses the question of the possible role of olfactory axons in the induction of the OB. A major caveat encountered in these studies was the frequent regeneration of the OP removed at early stages. Only 7% of the attempted partial deafferentation surgeries resulted in the development of an OB innervated by a single nerve. This consistently occurred in my studies despite the fact that I tried to alter the surgical procedure so that very little tissue was removed in some cases and very large amounts of tissue were
removed in other cases. With this problem in mind, the following experiments are tentatively proposed.

The first analysis that I would like to see performed with these studies is a light-microscope investigation of the period just after the OP is removed. I have found that removal of the OP at stages 28-32 can result in the absence of an OB (if the remaining olfactory nerve innervates solely the ipsilateral side of the brain). It is unknown, however, if the OB differentiates to some extent in anticipation of the incoming axons, then degenerates when they do not appear. For this analysis, removal of one OP could be performed at stages 24-28, then the effects could be analyzed in animals between stages 32 (when differentiation of the OB begins to appear [Chapter 2]) and 48 (when the mature structure of the OB is present [Chapter 2]). Additionally, this study could be followed to older stages, to see how the fused OBs with a single ventricle in some of the animals with partial deafferentation occurred. At this point, it is unknown in those animals if two OBs begin to develop and then fuse, or if only a single OB is induced to form.

Several other types of analyses could be performed on animals with embryonic, partial deafferentation. A $^3$H-thymidine study of the neural tube could be performed in control animals and animals in which one OP was removed to compare the number of cells dividing on both sides of the OB and the number of OB neurons that are formed. Fewer incoming olfactory axons may cause fewer OB cells to be born. This would be an extension of my study in that I have shown that
there are fewer mitral/tufted cells in animals with partial deafferentation, but I did not address whether fewer cells were born or fewer cells survived.

The final experiment that I propose for examination of deafferentation at embryonic stages is an analysis of total deafferentation at stages 28-30. Presumably, no incoming olfactory axons would result in a failure of OB formation. I have very preliminary evidence (one animal) that suggests that some differentiation can occur in the absence of innervation by the olfactory axons, although in my studies, when one side of the forebrain was not innervated by olfactory axons, there was no indication of an OB. I examined one animal in which both OPs were removed at stage 30 and did not regenerate. The forebrain appeared to contain cells rostral to the cells that normally surround the ventricle. In addition, there were two laterally-positioned balls of cells that appeared quite similar to accessory OBs. I did not examine other animals with no nasal capsules, and I would not want to make conclusions from the preliminary analysis of one animal. While I think this would be an important study for the future, I know that it is extremely difficult to obtain enough animals for analysis since the OPs usually regenerate.

Deafferentation in larvae.

The above experiments would further analyze the role of olfactory axons in the initial stages of OB development. Other studies could address the role of olfactory axons in continued development and maintenance of the OB. This would
address the question of whether or not there is a critical period for the influence of olfactory axons. In most sensory systems, there is a critical period during which deafferentation has the most profound effects (e.g., visual system: Hubel and Wiesel, 1970; auditory system: Born and Rubel, 1985; somatosensory system: Woolsey and Wann, 1976; Belford and Killackey, 1980; Fox, 1992). Preliminary studies in the olfactory system of *Xenopus* involving deafferentation at stage 45 suggest that olfactory axons are indeed required for further development and maintenance of the bulb at least through this stage (Herrera and Burd, 1991). The structure of the OB is lost in these animals, but it is unclear if the cells died or just lost their normal organization. One way to distinguish between these two possibilities could be to perform the deafferentation at stage 45 and then look at the OB at given time periods following the surgery to follow the changes in the OB. In addition, if it is possible to perform a quantitative analysis on those animals, the results could be compared to my results for deafferentation at early stages. One possible way to distinguish the mitral/tufted cells from the other cells in the region (since they lose the typical organization and are difficult to identify after deafferentation) is to retrogradely label the output neurons at stage 45 with injections of fluorescent beads or horseradish peroxidase into the lateral pallium. Once the cells were labeled, the deafferentation procedure could be performed, and the mitral/tufted cells present at the time of deafferentation could be identified.
Additional analyses.

Another interesting question that has arisen from my studies is whether or not the ratio between olfactory axons and mitral/tufted cells that was found in larval development is observed when the olfactory axons innervate another part of the brain. I found that transplantation of an OP to ectopic regions such as the back of the head or in place of the eye are not very difficult and tend to be very successful. Transplants of OPs into the optic vesicle (after removal of the eye) tend to innervate consistently the dorsal thalamus (Stout and Graziadei, 1980). Perhaps it would be possible to count the number of incoming axons and the number of displaced thalamic neurons. The major caveat to this analysis is that it would require a good understanding of the structure of the diencephalon and an ability to distinguish between ectopic cells and normal thalamic neurons.

The final goal of most of these experiments is to discover the cellular interactions or molecular factors that mediate the interactions between olfactory axons and the OB. Probably the best way to definitively analyze this is to culture undifferentiated neural tube and OPs. While it would be a rather labor-intensive project to set up a culture system for these tissues, there are many interesting experiments that could then be performed. For example, the OP and the neural tube could be cultured together and separately to see if differentiation of neural tube is dependent upon the presence of the placode. If it is, then OP-conditioned medium could be added to cultured neural tube to see if contact or a diffusible
factor is involved. The results of some of these experiments should lead to a better understanding of the interactions between the OB and the OP during development. Eventually, it will be possible to identify the molecular factors involved in these interactions.
APPENDIX A: ANIMAL SUBJECTS APPROVAL

The work presented here involved research on live vertebrate animals. This research has been approved by the Institutional Animal Care and Use Committee (IACUC control number 89-0186). In addition, I have been certified by the IACUC after completing the University Laboratory Animal Medicine training program (certificate number 000012).

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