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Regeneration of olfactory axons and synapse formation in the forebrain after bulbectomy in neonatal mice

(neuroplasticity/synaptogenesis/olfactory glomeruli/neurogenesis/telencephalon)

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ABSTRACT **We removed the right olfactory bulb in neonatal mice, leaving the bulb on the left side intact as an internal control. At 5 days of survival time, we observed that the right cerebral hemisphere was displaced forward to occupy the region made vacant by removal of the bulb. The frontal cortex was, consequently, in close proximity to the lamina cribrosa. As a result of bulb ablation and severance of the fila olfactoria, the sensory perikarya underwent total retrograde degeneration, which peaked at 8 days. New neurons differentiated in the neuroepithelium from basal stem cells and, at 30 days of survival, mature sensory neurons were reconstituted. These new elements sent their axons through the lamina cribrosa to reach the protruding cerebral hemisphere, penetrating it and forming glomeruli-like structures directly in the host tissue. The "glomerulization" of the sensory fibers persisted and actually expanded between 60 and 120 days. The new glomeruli were organized intimately within the brain tissue, and large neurons of the cortex were observed to be in close proximity. Ultrastructural observations of the newly formed glomeruli demonstrated that typical sensory axon terminals profusely branched and synapsed with unidentified postsynaptic processes that penetrated the glomeruli from the surrounding cerebral tissue.**

Over the last several years evidence has accumulated documenting the turnover of the olfactory sensory neurons in the intact neuroepithelium of vertebrates, including mammals (1–4). We have shown that, when their axons are cut, these neurons undergo rapid retrograde degeneration, and that stem cells in the neuroepithelium differentiate into mature neurons to replace the degenerated elements (4–6). Reconstitution of a mature population of neurons in the neuroepithelium is subsequently followed by re-establishment of their connections with the brain centers, i.e., the olfactory bulb glomeruli. These observations have been supported by both morphological and biochemical data in mice and rats (4, 7) and have been confirmed with behavioral tests in pigeons (8) and in mice (9, *).

From these results it appears that the olfactory sensory neurons have distinct attributes unique within the nervous system of mammals and other vertebrates. This capacity of the olfactory sensory neurons to both turn over and re-establish morphological and functional connections leading to a complete *restitutio ad integrum* is interesting with respect to the possibility of recovery of the system after damage. In addition, the characteristics of these neurons may allow us to pursue new lines of research presently unavailable with other elements of the nervous system.

It is the purpose of this communication to describe the results of the first in a series of experiments demonstrating the capacity of the olfactory sensory neurons to differentiate from stem cells and of their axons to regrow in the absence of their main target, namely, the olfactory bulb. We also intend to show the capacity of the regrowing axons to make spurious connections with brain centers other than the olfactory bulb.

MATERIALS AND METHODS

Fifty neonatal mice, ranging in age from 1 to 6 days, underwent surgery. Animals were housed with the dam under environmentally controlled conditions (20–22°C; 12 hr:12 hr light-dark cycle) until 25 days, at which time they were weaned. Then they were allowed ad lib access to food and water. Food and water were replenished and bedding was replaced regularly.

Mice were immobilized for surgery by placing them, three at a time, on a bed of crushed ice. While the mice were quiescent, a midline scalp incision was quickly made and the skull overlying the olfactory bulb was cleared of periostium. The right olfactory bulb was exposed by carefully cutting away the soft skull overlying the olfactory bulb with a finely sharpened Graefe microdissection knife. The bulb was then aspirated with a glass pipette using gentle suction. Only unilateral bulbectomy was performed, the contralateral side serving as an internal control. Care was taken to avoid unintentional traction on the adjacent cerebrum. In many cases this was ensured by carefully severing the olfactory bulb along its peduncle prior to aspiration. Care was also taken to avoid damaging either the lamina cribrosa or the bone underlying the bulb. However, in a few cases, especially in younger animals, some bone damage was observed. After removal of the bulb, the cranial cavity was packed with Gelfoam and the incision was closed. After surgery the animals were placed under a 100-W bulb for about 1 hr and then returned to the dam. The surgical survival rate was usually better than 90%. Animals were permitted to survive for periods ranging from 5 to 120 days. In addition, two groups of animals (three in each group), chosen randomly, were killed either directly or within 24 hr of surgery to confirm the totality of the bulbectomy.

To prepare the histological material, we anesthetized the animals with Nembutal and then perfused them through the heart with approximately 25 ml of Ringer's solution containing 0.1% (wt/vol) procaine, followed by at least 100 ml of 10% (vol/vol) phosphate-buffered, neutralized formalin. The heads were then severed and cleared of muscle, and the lower jaw, teeth, and zygomatic arches were removed. The material was then postfixed in alcoholic Bouin's fluid for 2–3 days, washed in 70% (vol/vol) alcohol to remove picric acid, and decalcified in a 3% (vol/vol) solution of nitric acid. Decalcification was determined to be complete by both chemical testing and mechanical inspection. After 24–48 hr of washing in 70% ethanol, material was dehydrated, cleared, and routinely embedded in paraffin. Serial sections were cut at 12 μm and stained with iron hematoxylin.

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* Harding, J., Donlan, K., Chen, H. & Wright, J. (1977) *Proceedings of the Society for Neurosciences*, Seventh Annual Meeting, Anaheim, CA (abstr. 56.11).

For ultrastructural studies, mice were killed 30–120 days after operation. The animals were anesthetized with an intraperitoneal injection of Nembutal. The heart was exposed, the descending aorta was clamped, and perfusion was started with a solution containing 2% glutaraldehyde (Ladd Research, purified) and 0.6% formaldehyde, buffered with 60 mM sodium cacodylate. The amount of fixative used for each animal was approximately 100 ml. After perfusion, the forebrain was removed, kept in fixative for 2 more hr at 4°C, then transferred to a solution containing 7.5% sucrose and 60 mM sodium cacodylate, and left in the solution overnight. The forebrain was sagittally sectioned into slices of approximately 0.5 mm and then postfixed with 2% osmium tetroxide in 0.2 M sodium cacodylate. The slices were dehydrated in ethanol and embedded in Araldite 506. In order to have an overall view of the structures under study, sections of 1 μm thickness were cut coronally from every slice, mounted on subbed slides, and stained with toluidine blue for light microscope inspection. Selected areas of interest were properly trimmed and thin sections were prepared and stained for observation with the electron microscope.

RESULTS

Light microscopy

An examination of animals killed within 24 hr of surgery confirmed the totality of the unilateral bulbectomy. By 5 days, the cranial cavity appeared devoid of olfactory bulb tissue as well as the Gelfoam introduced at the time of surgery to prevent bleeding. However, a loose connective tissue was apparent within the cavity. Also at this time, the cerebral hemisphere on the operated side exhibited what appeared to be a forward displacement (Fig. 1). This was most clearly observable in horizontal sections where, on the operated side, prominent landmarks such as the rostral end of the lateral ventricle and the genu of the corpus callosum were greatly displaced in an anterior direction. This general morphological pattern of altered, previously symmetrical, structures was maintained at all longer survival times.

As a result of the axonal damage suffered during removal of the bulb, the sensory neurons' perikarya, in the neuroepithelium covering the nasal turbinates, underwent total retrograde degeneration. At 20 days of survival, new neurons were reconstituted in the neuroepithelium from their basal stem cells and new sensory fibers could be seen crossing the lamina cribrosa and entering the cranial cavity. Furthermore, the animals at 20 days of survival exhibited the first formation of glomeruli within the cranial cavity, proximal and within the cerebral hemisphere. By 30 days after bulbectomy, the olfactory epithelium showed complete restoration of its neural components and was indistinguishable from that of the unoperated side. Axonal fibers originating from the base of the neuroepithelium reconstituted the characteristic fila olfactoria and were conspicuously visible coursing through the connective spaces of the turbinates and crossing the lamina cribrosa. Upon reaching the level of the cranial cavity, several axons formed glomeruli-like terminal structures in close proximity or directly inside the cerebral tissue (Fig. 1*B*). We also observed fibers and their glomeruli-like formations proximal to the exposed ependymal lining of the cerebral ventricle. Indeed, examination of the histological preparations gave us the distinct impression of a migration of ependymal cells from the ventricular wall to the newly formed glomeruli. The same preparations clearly showed glomeruli directly enmeshed within the brain matter among populations of large neurons. As shown in Fig. 2*A*, these

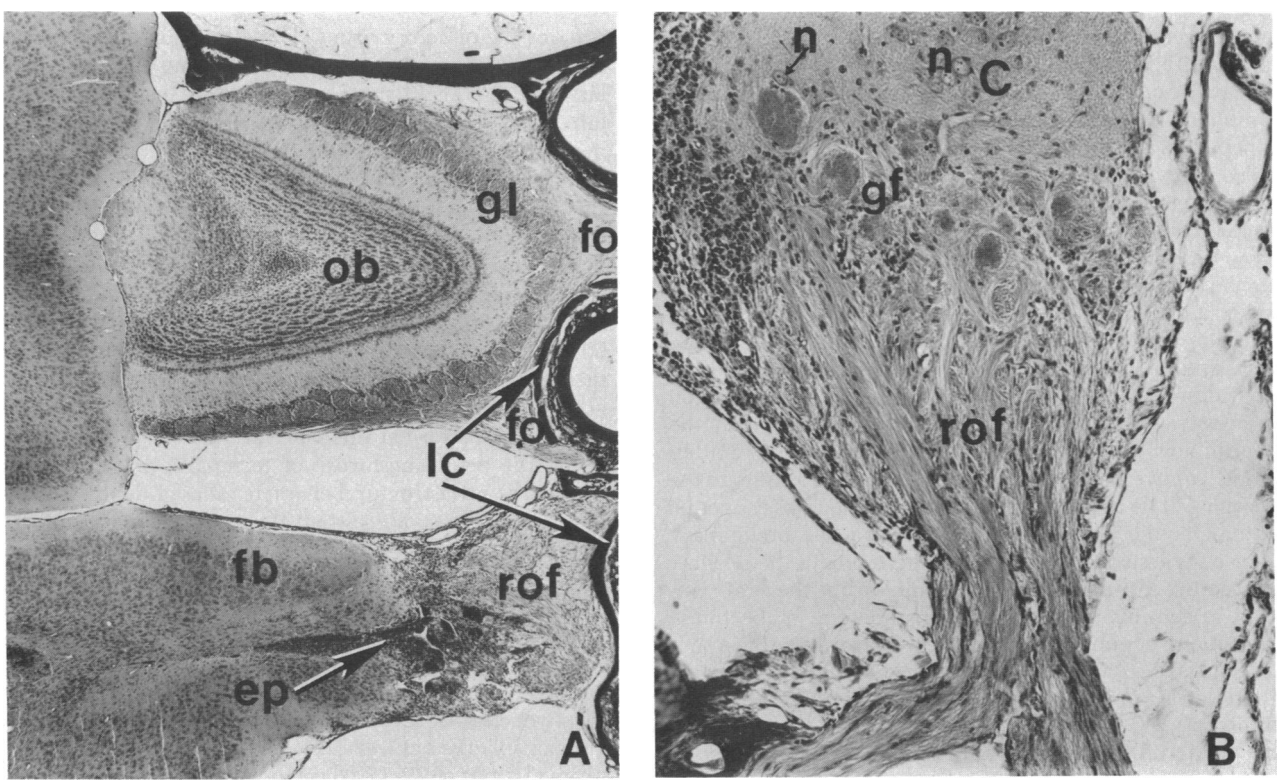

FIG. 1. (*A*) Horizontal section through the region of the olfactory bulb at 30 days of recovery in a unilaterally bulbectomized mouse. The intact olfactory bulb (ob) with its glomerular layer (gl) and the fila olfactoria (fo) crossing the lamina cribrosa (lc) is shown in the upper portion. In the lower portion (the operated side), the forebrain (fb) protrudes. Regenerated olfactory fibers (rof) reach the level of the forebrain (fb). The ependyma (ep) appears to expand towards the regrowing fibers. (Iron hematoxylin stain; ×40.) (*B*) Horizontal section along the medial aspect of the forebrain illustrating regenerated olfactory fibers (rof) and their glomeruli-like formations (gf) in close proximity and within the cerebral matrix (C). Large neurons (n) are displaced close to the glomeruli. Thirty days of survival. (Iron hematoxylin; ×200.)

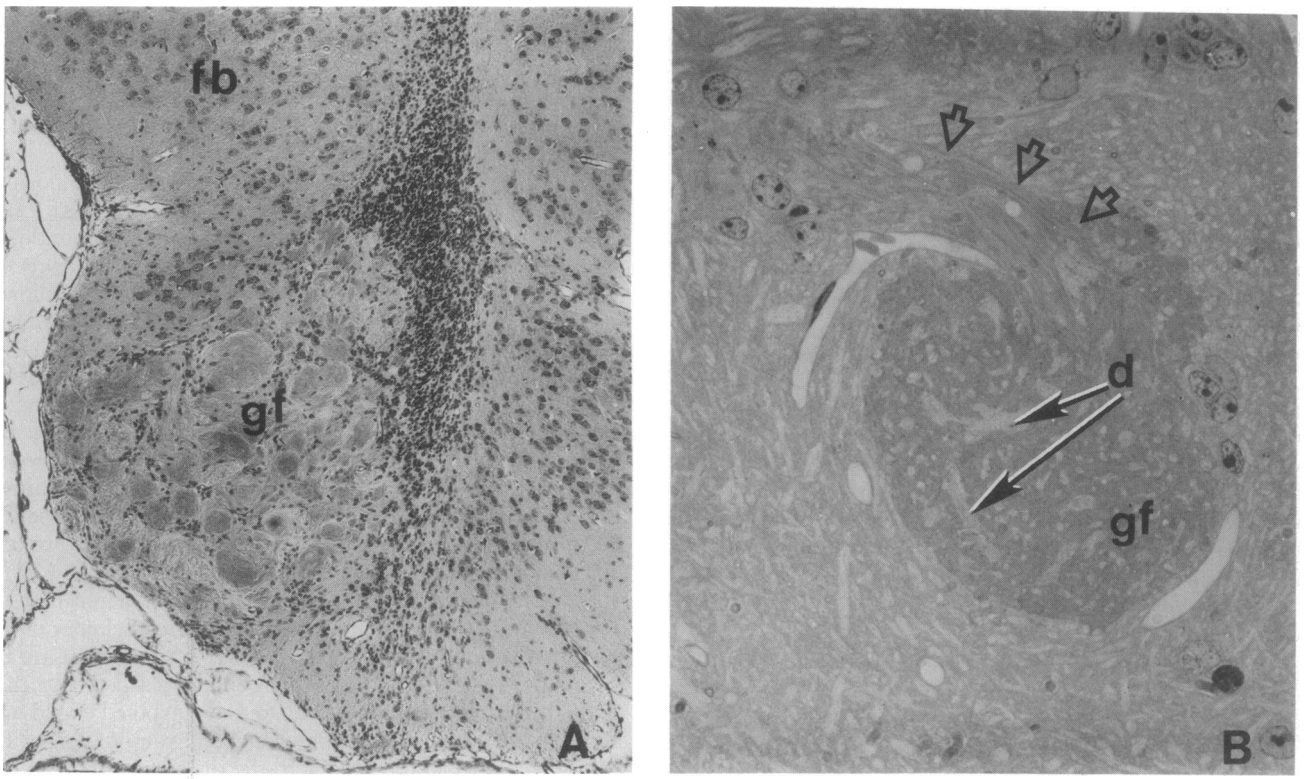

FIG. 2. (*A*) Coronal section of the rostral portion of the telencephalon on the operated side of a mouse after 30 days of survival, illustrating a marked penetration of the glomeruli-like formations (gf) and their intimate spatial relationship with the forebrain (fb). (Iron hematoxylin stain; ×125.) (*B*) Section of Araldite-embedded material (1 μm thick) illustrating a more detailed view of the glomerular pattern at 60 days of survival. Clear dendritic processes (d) are interspersed among the sensory terminals (see Fig. 3) of the glomerulus (gf). Open arrows indicate a primary bundle of olfactory sensory fibers characterizing the glomerulus. (Toluidine blue staining; ×750.)

glomeruli were in direct continuity with the surrounding neural tissue. A critical examination of the newly formed glomeruli at 30 days of survival, in both paraffin-embedded and Araldite-embedded material, revealed a characteristic structure reminiscent of the normal bulbar glomeruli. However, a more detailed analysis revealed a significant difference. Although clearly containing the presynaptic elements (namely, the terminals of the sensory axons), the pattern of the glomeruli, at this survival time, appeared devoid of the large dendritic processes characteristic of the normal glomeruli. Consequently, they acquired a finer and more homogeneous structure. At 60 days, the development of these glomeruli-like structures was more obvious and their penetration of the cerebral hemisphere was more widespread. At this time there was a marked penetration of processes with the characteristics of dendrites, and the glomeruli now began to show less homogeneity (Fig. 2*B*). In addition, large neurons were observed in close proximity to the glomeruli. While the identity of these large neurons cannot be determined on the basis of our observations so far, preliminary evidence suggests that they may be nerve cells of the telencephalic cortex. Formation of glomeruli within the brain appeared to continue at 90 days after unilateral bulbectomy. Furthermore, our observations of these preparations, together with those at 120 days of survival, indicated that the glomerulization persists and also suggested that there may be a relationship between the length of the survival and the expansion of the glomeruli within the brain.

Electron microscopy

Ultrastructural observations provided a complementary view to that seen in light microscopy. The fiber bundles, originating from the neuroepithelium and running towards the brain via the lamina cribrosa, had the typical ultrastructural characteristics of the olfactory axons. These fibers had an average diameter of 0.2 μm, were unmyelinated, and were contained in large pockets of Schwann cell cytoplasm, each of which contained large numbers of axons. At 30 days of survival, the glomerular formations contained a spiral network of fine axons and their terminals had the characteristic vesicular content. At this survival time synaptic contacts were sparse and restricted to a few areas of the formation where clear processes, which we identify as dendrites, were located. However, the glomeruli were composed mainly of a compact mass of fibers and their terminals, with a very limited presence of other processes. At 60 days of survival, the pattern of the glomeruli changed markedly at the ultrastructural level, as was previously observed at the light microscopic level. The most obvious change in the glomerular pattern at this survival time was the appearance, inside the dense meshwork of terminals, of clear processes having the ultrastructural characteristics of dendrites. It is on these dendritic processes that the sensory axon terminals established synaptic contacts (Fig. 3). These were of the "polarized" type, the sensory axon terminals always acting as presynaptic and the dendrites as postsynaptic. In ultrathin sections it was possible to observe clear dendritic processes from the surrounding brain tissue penetrating into the glomeruli. However, the specific neurons from which these dendrites arose is not yet clear. Golgi preparations will help elucidate the origin and possibly the nature of these dendritic processes. At longer survival times of 90–120 days the glomerular pattern showed little change. The dendritic processes, however, became more conspicuous and the frequency of synaptic contacts increased. In addition, small neurons with a perikaryal diameter of 5–10 μm, together with larger neurons, were often seen in close proximity to the glomeruli.

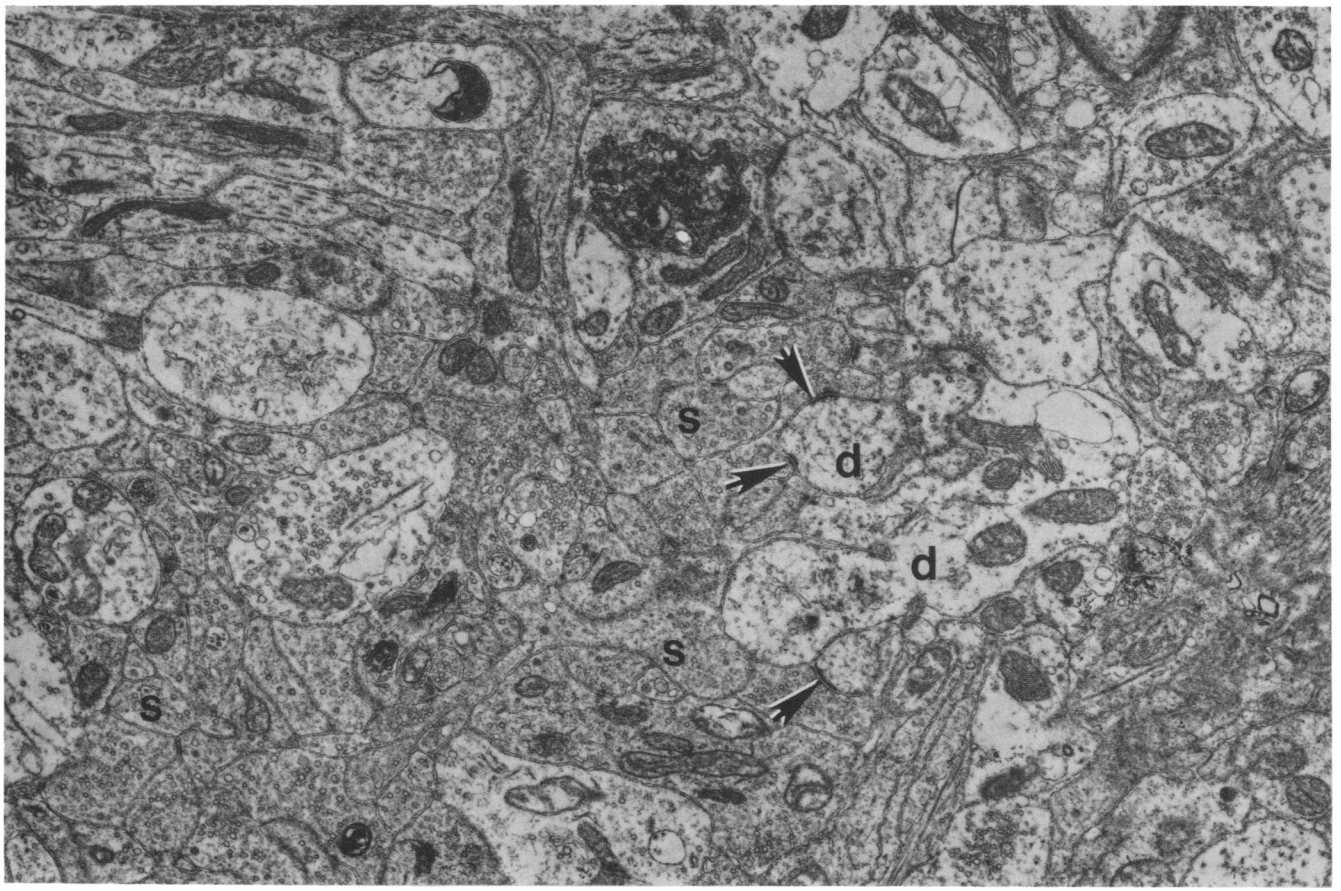

FIG. 3. Electron micrograph of the core of a glomerulus showing the typical sensory terminals (s), dendritic processes (d), and some synaptic contacts (arrows). Sixty-eight days survival. (×30,000.)

DISCUSSION

In previous studies, the olfactory sensory neurons of adult mammals have been shown to possess the capacity to turn over and to regrow their axons, even when experimentally sectioned, to their normal target, i.e., the olfactory bulb (4). Our present observations show that, despite the destruction of their normal target by bulbectomy and the ensuing retrograde degeneration of their perikarya, the olfactory neurons retain the ability to regenerate and, more significantly, to regrow their axons and form synaptic connections into a novel neural environment. The morphological appearance of these newly formed terminal structures is distinctly similar to that seen in the intact olfactory bulb. Indeed, because of their typical glomerular appearance and their persistence within the forebrain, we feel compelled to term these structures "glomeruli" and to label the process by which they form as one of "glomerulization." Our observations of the regenerative phenomena of the sensory olfactory neurons and their axons into the forebrain cannot be directly compared with the previous observations of other authors describing either regeneration of axons or collateral sprouting in different portions of the central nervous system (10–13). First, the phenomena described in the present study do not entail the regeneration of merely an axonal process, but rather they involve the differentiation and maturation of an entire neuron, and eventually the reconstitution of an entire cranial nerve, i.e., the olfactory nerve. Second, the distance traveled by the new axons is far greater than that noted previously by those authors that have detailed regenerative and sprouting phenomena in the central nervous system of mammals.

Our observations not only emphasize the unique ability of the sensory neurons to regrow, but they also indicate the capacity of a portion of the forebrain to accept a nonspecific input. Unfortunately, the extent of reorganization of the host cerebral tissue cannot be fully assessed on the basis of our present observations. While we have observed neurons surrounding the glomeruli, as well as dendritic processes acting as postsynaptic elements, the nature and functional connections of these cortical neurons remain to be determined. We are confident, however, that the target of the regrown olfactory axons is a remnant neither of the olfactory bulb nor of the accessory olfactory bulb. In this context our observations underscore the possibility of what may be an essential role of the ependyma for the acceptance of the new incoming olfactory fibers. However, the role of the ependyma, whether neural or supportive, must be clarified by further observations. Several authors have attributed a neurogenetic capacity to the ependymal layer, even in adult rodents (14, 15). The nature of the influence of the incoming olfactory axons upon the ependymal layer remains to be determined.

In all our preparations we have consistently observed the regrowing olfactory axons directed towards the homolateral telencephalic wall, even though, while on their course, they ran in the vicinity of the contralateral olfactory bulb. In the absence of the homolateral olfactory bulb, we considered the possibility that the regenerated olfactory fibers could have crossed the midline, where there was no appreciable anatomic barrier, and could have established connection with the contralateral bulb. However, we have never seen this in any of our preparations. Furthermore, in some preparations we have noted scar tissue appearing as loose connective tissue, adjacent to the lamina cribrosa on the operated side. However, whatever scar formation was induced by surgery provided a barrier neither to axonal regrowth nor to the penetration of these fibers into the central

nervous system. Thus, unlike the events described after spinal cord transection (13), scar formation induced by surgery did not seem to inhibit olfactory fiber regrowth.

On the whole, then, our observations seem to indicate either an unsuspected plasticity of the cerebral cortex, as shown by its capacity to accept foreign input, and/or they may indicate a unique property of the primary sensory neurons. These latter elements, which continuously turn over, may retain their ability to induce glomerular structures in the forebrain as they do in the primitive telencephalic vesicle during ontogenesis (16). Alternatively, they may be extremely plastic and adaptable to any "foreign" environment. In any event, our observations on the formation of glomeruli in the forebrain of bulbectomized mice provide us with a basis for examining the extent and limits of plasticity of a circumscribed population of neurons, both olfactory and cerebral. Furthermore, the interaction between renewable cells within a novel cellular matrix may provide us with a model system with which we may discern the complex relationships between neural specificity and environment. It is our future goal to examine these foregoing hypotheses and define the characteristics of the regrowing neurons and their axons and the regions of the brain in which they are accepted.

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