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The Expression of B-50/GAP-43 and GFAP after Bilateral Olfactory Bulbectomy in Rats

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Summary

In the present study we investigated the effect of a two-stage bilateral lesion of the olfactory bulb (OB) in rats on the regeneration ability of peripheral olfactory neurons and their reinnervation capacity in the spared OB. The outgrowth of newly-generated olfactory axons as well as the maturation of their terminal synaptic field was detected by immunohistochemistry of the growth-associated phosphoprotein B-50/GAP-43. In addition, the glial response to the surgery was monitored by an immunohistochemical marker for astrocytes, glial fibrillary acidic protein (GFAP). In neonatal rats (P3-P5), the right OB was removed, then three months later the contralateral side was ablated. Six days after the second operation the animals were transcardially perfused. Their brains were embedded in paraplast, serially sectioned and processed for histological and immunohistochemical observations. After neonatal OB ablation, homogeneous B-50-immunoreactivity (BIR) was found in the forebrain, olfactory axons and ectopic glomeruli localized in the small OB remnant-like structures and in the regenerated neuroepithelium. A strong GFAP response was revealed in the brain cortex as well as in the newly-formed olfactory axons and glomeruli-like structures of the OB remnants. After adult OB ablation strong BIR was observed in olfactory axons, while remaining glomerular structures were only faintly stained. The neuroepithelium revealed signs of massive degenerative processes with a substantial decrease in BIR. The GFAP-positive astrocytes were scattered throughout the entire OB remnant and were prominent in the glomeruli-like structures and adjacent frontal cortex. In the present study, we applied GAP-43 and GFAP immunohistochemistry to characterize the responses of individual olfactory components after two-stage olfactory bulbectomy. Furthermore, this model of OB ablation characterized by two immunohistochemical markers could elucidate certain molecular mechanisms involved in the regeneration and/or plasticity of the olfactory system.

Key words
Olfactory bulbectomy – Growth-associated proteins – Glial reaction – Regeneration

Introduction

Olfactory receptor neurons represent one of the few neuronal pools undergoing continual turnover throughout animal's life (Graziadei and Monti-Graziadei 1978). This is largely due to the unique ability of the adult olfactory epithelium to generate new neurons from a population of precursor cells present in the basal cell layer. However, this process can be accentuated after surgical or chemical damage to the mature neurons, resulting in their death and subsequent replacement from the progenitor cell compartment (Costanzo and Graziadei 1983). Two regeneration stages were described after peripheral or central lesions of the olfactory bulb. The first stage occurs independently of the type of lesion and is characterized by the formation of immature olfactory neurons expressing B-50/GAP-43. The second stage appears only after peripheral deafferentation, when the developing neurons have access to the targeted OB and the formation of a full complement of OMP-expressing neurons occurs (Verhaagen *et al.* 1990). These data indicate that OB provides an important target for reconstituted olfactory axons, however, the replacement of degenerated neurons from progenitor cells does not require the presence of the OB. It has

been shown in previous experiments concerning e.g. partial or total olfactory bulbectomy (Monti-Graziadei and Graziadei 1992) or OB transplantations (Žigová *et al.* 1990) that OB undergoes considerable remodelling as a consequence of its reinnervation. However, olfactory nerve penetration occurs preferably when the OB lesion is performed in neonates. Previous findings (Žigová *et al.* 1992, Hendricks *et al.* 1994a,b) supported the notion that OB transplantation may, to some extent, reestablish neuronal circuits affected by surgery, but the immunohistochemical response of individual OB components could be delayed (Čížková *et al.* 1995). To extend our previous findings after homotopic transplantation, we employed the same immunohistochemical markers, growth-associated protein and glial fibrillary acidic protein, to detect responses of the regenerated tissue after bilateral bulbectomies. The first marker, the growth-associated protein (B-50/GAP-43), is generally regarded as a plasticity marker protein for neurite outgrowth, since it is highly expressed during both nervous tissue development and regeneration (Strittmatter *et al.* 1992). There is also evidence that this protein plays a crucial role in neuritogenesis (Benowitz and Routtenberg 1987), axonal regeneration (Curtis *et al.* 1993), synaptic plasticity (Oestreicher *et al.* 1983, Benowitz *et al.* 1990) and neurotransmission (Coggins and Zwiers 1991, Gispen *et al.* 1991).

The second marker, glial fibrillary acidic protein (GFAP) is also involved in some restorative processes. It was found that some glial cells, especially astrocytes, are highly active in ion regulation, production of neurotrophic factors as well as in the reuptake of some neurotransmitters and in their recycling (Nieto-Sampedro *et al.* 1985). However, glial-neuronal interactions may play an important role during the regeneration process after OB lesion by forming a glial scar (Smith *et al.* 1986), which potentially blocks the olfactory axonal outgrowth in adulthood. On the contrary, immature glial cells in two-week-old young rats can promote regrowth of lesioned nerve fibres (Sijbesma *et al.* 1986). A recent study by McKeon *et al.* (1991) suggested that this change in the permissiveness of astrocytes is due to an expression of inhibitory molecules by mature astrocytes. Other investigators demonstrated the existence of astrocytic subtypes (Bailey and Shipley 1993) in the olfactory bulb. In addition, astrocytes associated with a single layer of the OB can differentially express enzymes for the synthesis and degradation of excitatory amino acids (Poston *et al.* 1991); in the cerebellar cortex, a subpopulation of astrocytes differentially express glutamate dehydrogenase activity (Aoki *et al.* 1987). These findings suggested that the morphological diversity and differential distribution of astrocytes in the OB might correlate with their functional diversity.

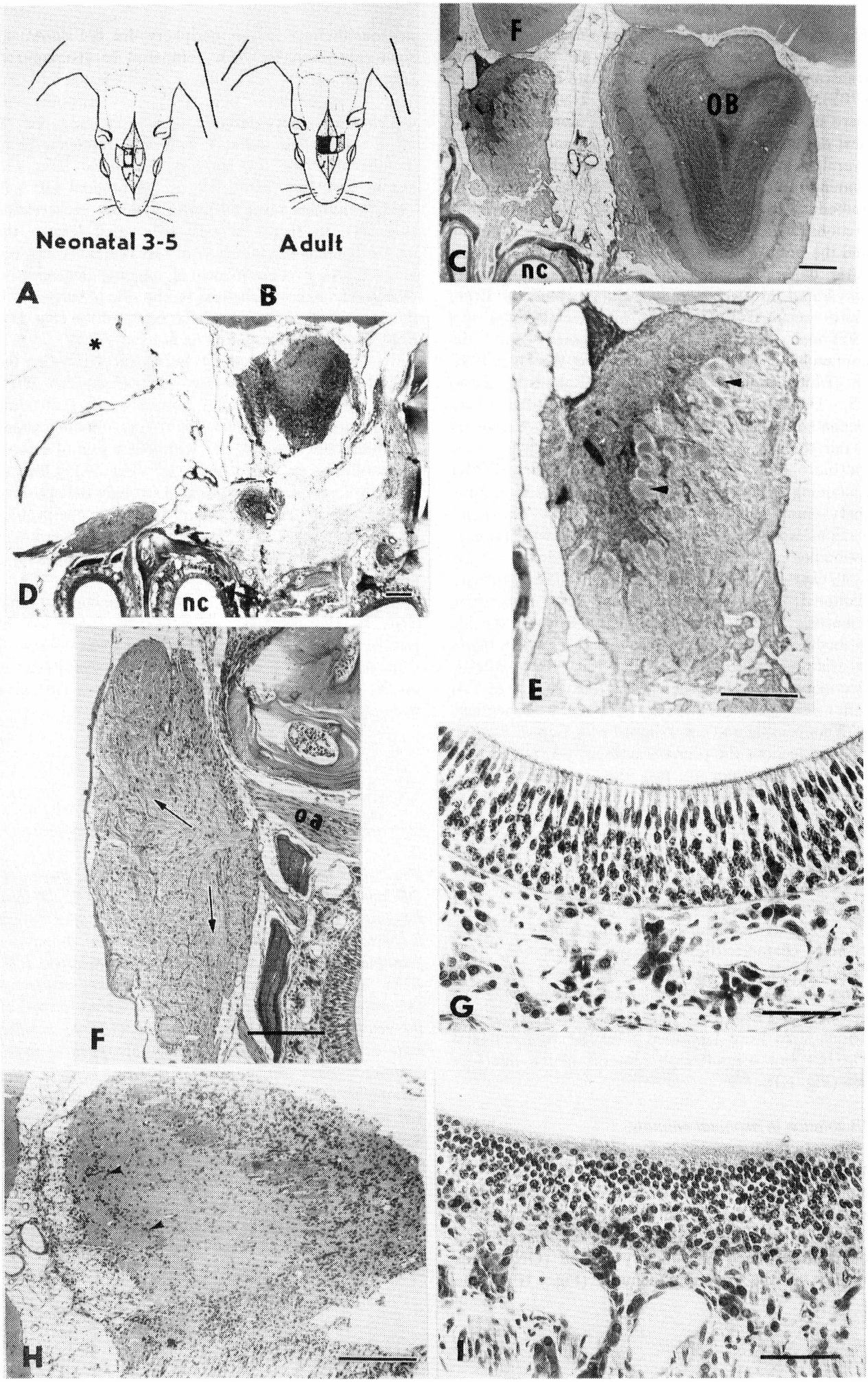
In the present study we applied B-50/GAP-43 immunohistochemistry in order to characterize the two-stage bilateral bulbectomy in rats, namely the response of the neuroepithelium at the periphery and regrowth of olfactory axons into the OB remnants. At the same time, the extent and the pattern of astrocytic reaction was detected by GFAP immunohistochemistry.

Methods

Surgery

Thirteen Albino Wistar rats were housed and handled according to the approved guidelines. Ten of them were cold-anaesthetized at the age of 3–5 days and subjected to the removal of the right OB, while the left OB was left intact (Fig. 1A). Three sham-operated animals served as controls. Three months later the animals were anaesthetized with an i.m. injection of ketamine hydrochloride (Narcamon, Léčiva, ČR, 10 mg/100 g body weight). A small opening was drilled in the frontal bone over the left OB. The OB was removed by aspiration and the completeness of bulbectomy was confirmed visually. To control the bleeding, the cavity was gently filled with Gelaspon and the skin was closed with sutures. Postoperatively applied Gentamicin sulfate (2.5 mg/animal) was given i.m.

Fig. 1. *Diagram of the two-stage OB lesion. A: OB ablation in neonatal animal. B: OB ablation in adult animal. C: Horizontal section through the forebrain (F) and OB remnant after first-stage OB lesion; (nc) nasal cavity. D: Horizontal section through the forebrain and OB remnants following two-stage OB lesion, the part of the forebrain on the neonatal lesioned side was missing (*). E: Remnant on the neonatal OB lesioned side with deranged laminar organization, glomeruli-like structures (arrowheads) F: The structure composed of differently cut profiles of olfactory axons (arrow). Visible line of primary olfactory axons incoming from the neuroepithelium to this structure (oa). Horizontal section through the olfactory neuroepithelium after neonatal (G) and adult OB lesion (I). G: The pattern of regenerated neuroepithelium. H: OB remnant after adult OB lesion with visible line of the glomerular structures (arrowheads). I: The damaged neuroepithelium with reduced number of disorganized cells. C,D,E,F,G,H,I Haematoxylin staining Bars: C, D = 300 μm; E, F, H = 200 μm; G, I = 50 μm.*


Neonatal 3-5
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Histological preparation and immunohistochemistry

Six days after surgery, all animals were transcardially perfused with phosphate-buffered saline (PBS), pH 7.4, followed by Bouin's fixative. The heads were stored overnight at 4 °C in the same fixative. The next day they were rinsed in 50 % ethanol, decalcified overnight in RDO (Sigma), later extensively rinsed in running water, dehydrated, cleared in toluene and embedded in Paraplast (Monoject, USA). Horizontal sections (20 μm) through the olfactory neuroepithelium and the brain were mounted on gelatine coated slides. After deparaffinization in xylene the sections were rehydrated through degraded ethanol solutions. Every fourth section was stained with haematoxylin and additional sections were processed for the immunohistochemical procedure using the Duet ABC kit (Biotinylated Goat-anti-Mouse/Rabbit Ig, Dako AS, Denmark). The rehydrated sections were incubated in 0.3 % H_2O_2 diluted in 20 % methanol for 30 min to destroy endogenous peroxidase activity. Non-specific binding was blocked by incubation with PBS containing 2 % bovine serum albumin, 4 % normal goat serum and 0.3 % Triton X-100 for one hour. Sections were then incubated overnight with primary antibodies at 4 °C. We used monoclonal mouse B-50/GAP-43 (Oncogene Sciences, USA) antisera dilution 1:200 and polyclonal rabbit GFAP (Oncogene Sciences, USA) dilution 1:800. Bound specific antibodies were visualized by the avidin-biotin peroxidase procedure using 0.05 % 3,3,4,4-diaminobenzidine hydrochloride (DAB) in 0.05 M Tris buffer containing 0.01 % H_2O_2. Finally, all sections were dehydrated and coverslipped with Depex (Sigma). In some sections the primary antibody was replaced by normal goat serum to test the specificity of the immunolabelling procedure.

Results

General morphology

Intact olfactory bulb in control animals showed characteristic laminar organization (not shown). In the experimental animals, the neonatal OB lesion was followed by contralateral ablation of the OB three months later (Fig. 1A,B). The OB remnants of various sizes were identified after the first neonatal (Fig. 1C) and second adult removal of the olfactory bulb (Fig. 1D).

OB ablation in neonatal animals

In the neonatally bulbectomized animals, large lesions of the OB were found in 4 cases. The OB remnants revealed signs of laminar organization only in restricted areas (Fig. 1E). In six animals with a subtotal lesion, OB structures close to the lamina cribrosa contained a bunch of olfactory neuron (ON) fibres arising in the neuroepithelium (Fig. 1F). The neuroepithelium in the periphery did not show any significant changes when compared to the controls (Fig. 1G).

OB ablation in adult animals

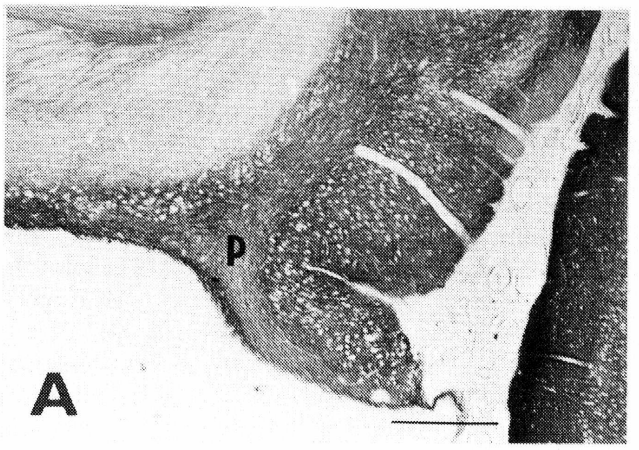
In seven animals with OB removed three months after the first surgery, we found large OB lesions with less than 50 % of the original OB left. These remnants revealed partial laminar preservation (Fig. 1H). In three animals with small lesions the typical laminar organization of OB remnants was not affected. As a consequence of ongoing degenerative changes the neuroepithelium on the side of surgery was thinner with disarranged cellular composition (Fig. 1I).

B-50/GAP-43 immunohistochemistry

On the neonatally bulbectomized side, the rostral part of the forebrain was aspirated in a few cases. The damaged tissue formed small protruding prominences that displayed a homogeneous B-50-immunoreactivity (BIR) without a sign of ectopic glomeruli-like structure formation (Fig. 2A). Close to the cribriform plate we identified strongly BIR-positive structures composed mainly of differently cut profiles of olfactory fibres (Fig. 2B,C). In these remnant-like structures few GAP-43-like structures were also present.

In the regenerated neuroepithelium situated close to the remnant, activation of B-50/GAP-43 positive cells in the medial layer was observed (Fig. 2D). The BIR in the nerve bundles was similar as on the contralateral side with visible axons emerging from the neuroepithelium (Fig. 2D).

Fig 2. *BIR in a horizontal section through the forebrain, OB remnants and neuroepithelium (A, B, C, D after neonatal bulbectomy and E, F, G after adult bulbectomy). A: Homogeneous BIR in the brain prominence (P) without glomeruli-like structures. B,C: BIR is highest in the glomeruli-like structures (arrowheads) and in the olfactory nerve axons (arrow) of the remnants. D: Activation of B-50/GAP-43 positive cells in the medial layer with visible positive nerve bundles (nb) and axons emerging from the neuroepithelium. E: The remnant structure after adult OB lesion revealed homogeneous BIR in the olfactory axon area (arrow) and glomeruli (arrowheads). F: Detail of the glomerular structures (arrowheads) with patchy pattern of B-50/GAP-43 immunoreactivity. G: Small number of persisting cells in the neuroepithelium exhibited slight BIR but high BIR in the nerve bundles (nb). Bars: A, B, C, E = 200 μm; F = 100 μm; D, G = 50 μm.*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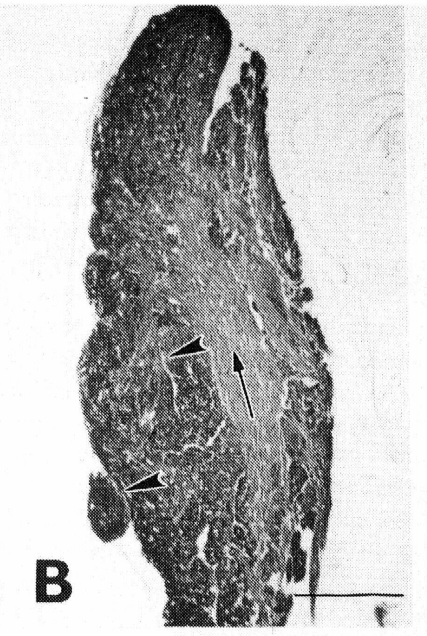
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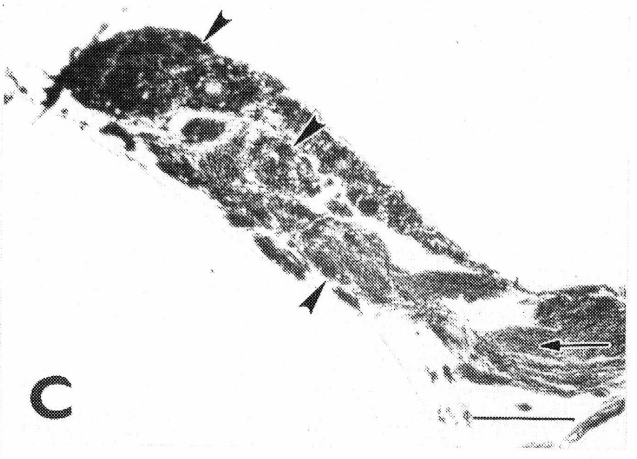
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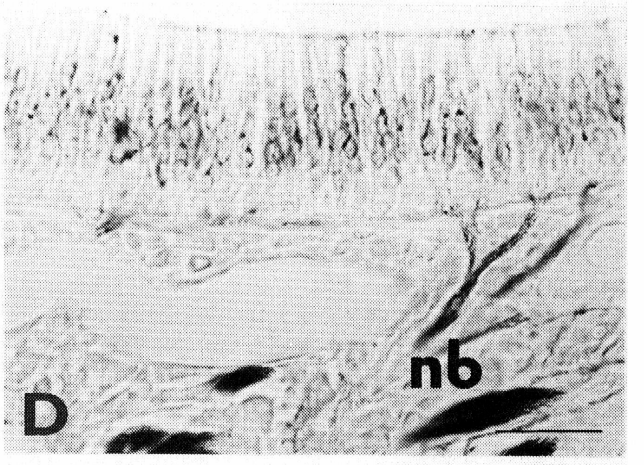
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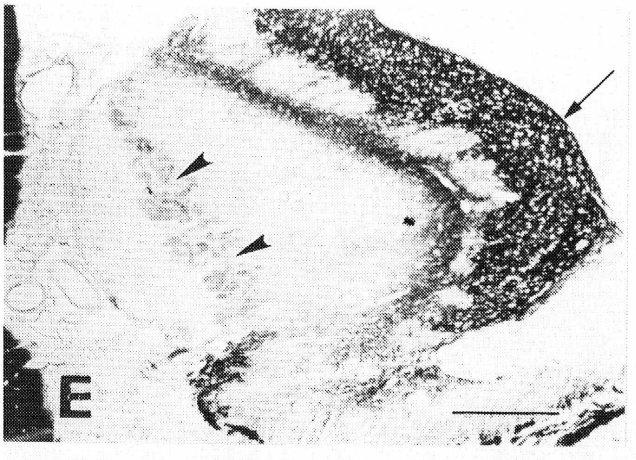
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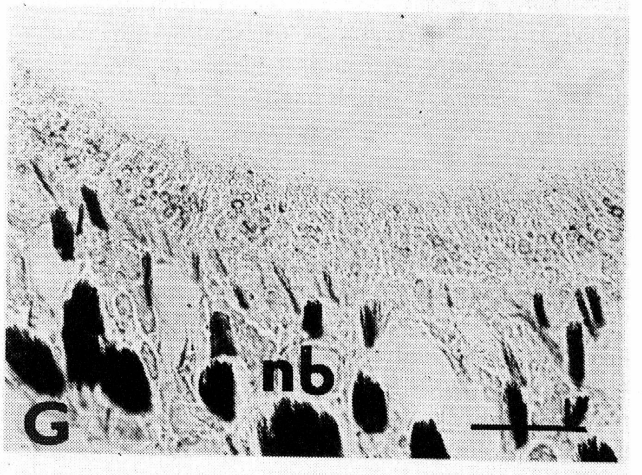
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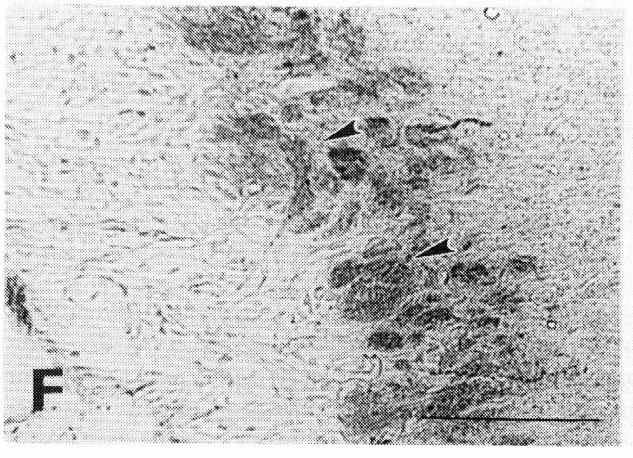
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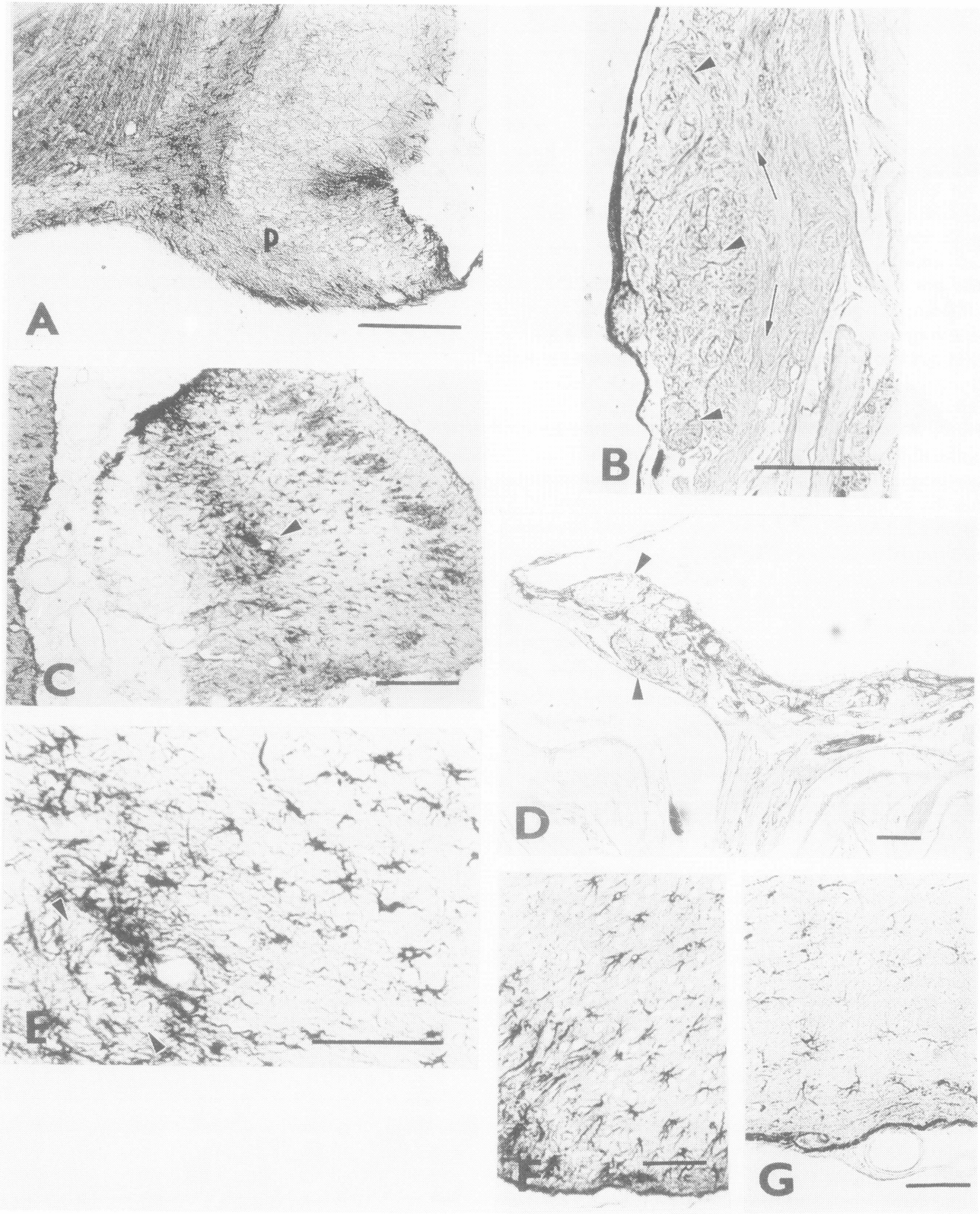

Fig. 3. *GFAP immunoreactivity in the horizontal sections through the brain and remnants (A, B, D after neonatal and C, E, F after adult bulbectomy). A: Increased GFAP-IR in the protruded brain prominence (P). B, D: The most distinct GFAP-IR in the remnants was observed around glomerular formations (arrowheads) and occasionally in the nerve layer (arrow). C: Enhanced astrocytic reaction throughout the entire remnant. E: Detail of reactive astrocytes around remaining glomerular structures (arrowheads). F: Increased GFAP-IR in the frontal cortex in comparison with G: control cerebral cortex. Bars: A, B, C, D = 200 μm; E, F, G = 100 μm.*

On the side lesioned in adulthood, the remnants were situated in most cases close to the forebrain, in the position of the previous OB and revealed a strong pattern of BIR, predominantly in the olfactory nerve layer (Fig. 2E). The glomerular area of the OB remnants manifested faint immunoreactivity that defined the shape of the slightly stained glomeruli (Fig. 2F).

At this time period, a small number of persisting cell bodies in the neuroepithelium revealed slight BIR (Fig. 2G). The intensity of BIR in the nerve bundles was pronounced (Fig. 2G).

GFAP immunohistochemistry

On the neonatally bulbectomized side, the protruded prominence of the brain and cerebral cortex revealed an increase of astrocytic figures (Fig. 3A). In the variously shaped remnant-like structures situated close to the cribriform plate an astrocytic reaction was found throughout the whole remnant. However, the most distinct immunoreactivity was observed around glomerular formations and occasionally in the nerve layer (Fig. 3B,D). From the protruded brain tissue, large numbers of fibres emerged which seemed to bind and probably create connections with the remnant.

On the side lesioned in adulthood, the astrocytic reaction was enhanced in the OB remnant as well as in the periphery of the adjacent cerebral cortex (Fig. 3C). Astrocytes in the OB remnant were scattered throughout the entire remnant with a higher degree of immunoreactivity in the ependymal layer and around the remaining glomerular structures (Fig. 3E). These astrocytes appeared larger in size and had thicker processes. The increased astrocytic immunoreactivity was especially prominent throughout the cerebral cortex (Fig. 3F) as compared to the control sections (Fig. 3G).

Discussion

Our results have shown that central lesion performed in neonatal rats caused various structural rearrangement of the spared bulbar tissue and profound regeneration changes at the periphery. In this case of OB lesion the degeneration of the sensory neurons is followed by generation of new neuronal cells. These are able to regrow their axons into the remaining OB remnants and reestablish synaptic contact in the ectopic glomeruli (Graziadei and Samanen *et al.* 1980). A period of 20–30 days after the lesion is required for the characteristic morphological pattern of the glomeruli to develop. Our post-lesion period for possible regeneration of the olfactory neuroepithelium and reconstitution of glomeruli was 90 days. At this time point we detected reconstitution of the epithelium through the formation of new olfactory receptor neurons that express B-50/GAP-43. However, it should be mentioned that reconstituted peripheral sensory input does not fully regain its normal biochemical and morphological characteristic and remains in an immature state for longer time period (Verhaagen *et al.* 1990). Within the spared OB, the most obvious features were the presence of incoming olfactory axons from the periphery and the random location of glomeruli-like structures. The small remnant-like structures were usually disorganized and their new glomeruli-like structures were formed in ectopic locations. A notably high BIR was present in olfactory axons and glomeruli-like structures. BIR has been considered as a specific marker for neurite outgrowth, neuronal differentiation and neuroplasticity. In neonatal rats, B-50/GAP-43 is expressed in all olfactory neurons, while in adulthood this protein is restricted to a limited number of neurons located in the basal region of the neuroepithelium and olfactory axons in lamina propria mucosae. In the olfactory bulb, the expression of this protein can be found in the incoming primary olfactory axons, which form the nerve fibre layer. The glomerular structures reveal a patchy pattern of BIR, since most of axodendritic synapses in the glomeruli are already established (Verhaagen *et al.* 1989). In the present study, intense B-50/GAP-43 staining represents axonal outgrowth and remodelling, associated with the regrowth of newly formed olfactory axons and formation of the BIR-positive glomeruli after bulbectomy. Moreover, some potential functional recovery might be suggested. Since the olfactory peduncle, the target of the mitral and tufted cells of OB was mostly missing, olfactory nerve penetration into the frontal cortex could be observed only in a few cases. It is possible that the frontal cortex, which is considered to be a higher cognitive centre, may play a dominant role in recovery of olfaction. In such cases, we identified fibres emerging from the damaged brain traversing the whole distance and tending to grow towards the remnant. These fibres might play an important role in preserving the connections of the brain and periphery. However, only careful electron microscopic analysis can elucidate the actual synaptic organization of the glomeruli in the OB remnant.

On the contrary, adult bulbectomy served as effective peripheral deafferentation resulting in functional disruption (Račeková *et al.* 1994). The time required for possible recovery after partial bulbectomy is much longer period than 6 days after the lesion. However, olfactory bulbectomy appears to be an age-dependent phenomenon, since the olfactory axon penetration from the neuroepithelium is blocked by glial scar tissue in adulthood. On the bulbectomized side in adulthood, immunostaining for B-50/GAP-43 revealed similar immunoreactivity in the remaining remnants as in the control adult OB. However, more striking changes of BIR were recognized in the

periphery. The thickness of the olfactory neuroepithelium and the number of B-50/GAP-43 positive cells decreased in comparison to the controls. These degenerative processes in the neuroepithelium could be due to functional disruption.

Recent studies *in vivo* (Salm *et al.* 1985) and *in vitro* (Weinstein *et al.* 1991) suggest that changes in GFAP expression correlate with the morphological changes in astrocytes. Astrocytes are differentially distributed within the anatomical layers of the bulb. The highest levels of GFAP-IR in the OB are found in the subependymal zone (SZ) and the glomerular layer (GL) (Bailey and Shipley 1993). These are undoubtedly the places of highest neuronal plasticity in the bulb. Increased GFAP and hypertrophy of astrocyte processes are characteristic for reactive gliosis, which is generally thought to inhibit axonal regeneration. Previous reports have shown that activated immature astrocytes will permit axonal growth both *in vivo* (Smith *et al.* 1986) and *in vitro* (Rudge and Silner 1990), while the mature activated astrocytes appear to impede axonal growth.

In our experiment with two-stage bulbectomy the distribution of GFAP-IR provide information about the density and morphological changes of astrocytes on both lesioned sides. However, our attention was focused more on the spared olfactory bulb tissue after neonatal lesion, in which the immature astrocytes could play an important role during the reconstitutional process. According to the disorganized bulbar structure, the astrocytes are scattered throughout the entire remnant. Only in the part of remaining ectopic glomerular structures could astrocytes be found which were organized with respect to the glomeruli. However, the link between a high level of GFAP expression in the GL and the ability of the GL astrocytes to permit or impede axonal growth is not yet clear. The GL is, therefore, an excellent model for characterizing the role(s) of astrocytes and GFAP expression in axonal reinnervation and plasticity in the adult CNS.

The central lesion utilized in adult animals propose the inhibitory character of mature astrocytes impeding axonal outgrowth. Since most lesions led to the formation of reactive astrocytes within 2 days after a lesion, it was expected that the changes of GFAP would still be present 6 days after adult bulbectomy. The present findings support the idea that the time course of changes in GFAP labelling depends on the severity of the pathological insult. The increase of GFAP in the cerebral cortex as well as in the OB remnant was related to the formation of reactive astrocytes exhibiting enhanced synthesis of GFAP. This profound reactive gliosis together with degeneration changes in the periphery could predict functional disruption.

Our study of two-stage bilateral bulbectomy should be considered as a preliminary study which provides the anatomical basis for further behavioural and electrophysiological experiments. The goal of these future studies should be to determine the extent to which anatomical olfactory bulb reorganization is still compatible with functional recovery on the neonatal lesioned side (in the case when consequential contralateral adult bulbectomy is performed for unilateral functional elimination).

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