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Beneficial effects of x-irradiation on recovery of lesioned mammalian central nervous tissue

(central nervous system regeneration/axotomy/gliosis/neuronal rescue/reactive astrocytes)

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ABSTRACT **We examined the potential of x-irradiation, at clinical dose levels, to manipulate the cellular constituents and thereby change the consequences of transection injury to adult mammalian central nervous tissue (rat olfactory bulb). Irradiation resulted in reduction or elimination of reactive astrocytes at the site of incision provided that it was delivered within a defined time window postinjury. Under conditions optimal for the elimination of gliosis (15–18 days postinjury), irradiation of severed olfactory bulbs averted some of the degenerative consequences of lesion. We observed that irradiation was accompanied by prevention of tissue degeneration around the site of lesion, structural healing with maintenance of the typical cell lamination, and rescue of some axotomized mitral cells (principal bulb neurons). Thus radiation resulted in partial preservation of normal tissue morphology. It is postulated that intrusive cell populations are generated in response to injury and reactive astrocytes are one such group. Our results suggest that selective elimination of these cells by irradiation enabled some of the regenerative processes that are necessary for full recovery to maintain their courses. The cellular targets of these cells, their modes of intervention in recovery, and the potential role of irradiation as a therapeutic modality for injured central nervous system are discussed.**

Injury in most adult mammalian tissues initiates a cascade of cellular events leading eventually to partial or complete recovery from the trauma. A similar cascade emerges in the adult mammalian central nervous system (CNS)—i.e., removal of cellular debris by macrophages and microglia, proliferation of glial and mesodermal cells (1–4), and axonal outgrowth (5, 6). Nevertheless, the neural tissue fails to regain structural integrity, the severed axons cannot manage to reestablish their synaptic connectivity, and consequently no resumption of functional tissue activity occurs (7).

It appears that the different cellular components of the adult mammalian CNS respond differently to injury; whereas damage to the neurons can be reversed with the support of the appropriate cellular milieu, such as transplanted Schwann cells and immature astrocytes (8, 9), trauma to neural tissue results in permanent and apparently irreversible changes of the properties of some of the glial cells. Axotomized CNS neurons (e.g., mitral cells) can regenerate with the support of Schwann cells; they extend axons into and across peripheral nerve grafts (10), and they succeed in reestablishing some synapses and physiological function with their target cells (11, 12). Astrocytes, however, in response to injury undergo biochemical and physiological changes transforming them into a population of cells termed reactive astrocytes (13–15). A most pronounced feature of reactive astrocytes is the increased level of the intermediate filaments, particularly of the glial fibrillary acidic protein (GFAP). Reactive astrocytes represent a major constituent of the scar tissue usually formed at the site of injury (16), and their accumulation is also termed glial scar or gliosis.

The working hypothesis of this study is that CNS response to injury differs from the response of other tissues due to the properties of the astrocytic population. Reactive astrocytes either are incapable of supporting or may actively suppress, directly or indirectly, axonal elongation and other regenerative processes. Hence, experiments were designed to examine to what extent the regeneration process in an injured CNS tissue is affected by the elimination of resident reactive astrocytes. Transection in the olfactory bulb (OB) served as a model system for CNS injury, a system in which the axotomized mitral cells (the OB principal neurons) degenerate (10). Ionizing irradiation, which, at the appropriate dose levels, selectively kills dividing cells (17, 18), was chosen as a procedure for elimination of reactive astrocytes. The objectives of this part of the study were to identify the time window after injury at which GFAP-positive astrocytes generated at the site of lesion are radiosensitive and to examine whether the elimination of the reactive astrocytes in the transected OB is accompanied by preservation of structural integrity and by rescue of axotomized neurons. A short account of this study appeared in an abstract form (19).

METHODS

Surgery. Sprague–Dawley female rats (Charles River Breeding Laboratories), weighing ≈250 g, were anesthetized with 7% chloral hydrate injected intraperitoneally (0.6 ml per 100 g of body weight), and the left OB was exposed by drilling a hole in the skull and by cutting a slit in the dura. Two types of lesions were performed, stab wound and transection. Stab wound was inflicted with a miniblade (1 mm wide × 3 mm long) mounted on an electrode micromanipulator; penetration into the OB was 5 mm deep at a fixed angle. A total of 37 stab wounded animals, irradiated (n = 28) and control (n = 9), were examined. Transection of the OB at about the center of the rostral–caudal axis was performed with a microblade. Of the 21 centrally transected OBs (11 control and 10 irradiated), 9 were completely transected as determined by histological analysis of serial sections of the OBs.

Radiation. Irradiation was delivered by topical application of a sealed source of iridium-192, by a modification of an afterloading system (20). At the time of surgery, a nylon catheter was glued to the skull with cyanacrylate glue, both anterior and posterior to the opening, aligned directly above the injury area. A single sealed source of irridium-192 (activity = 1.9–2.3 mg of Ra equivalent; Best Industries, Springfield, VA) was introduced into the nylon catheter of anesthesized rats and was removed at respective time periods

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Abbreviations: CNS, central nervous system; GFAP, glial fibrillary acidic protein; MCL, mitral cell layer; OB, olfactory bulb; PI, postinjury.

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(20–24 hr), allowing a total dose of 20 grays (Gy) at a distance of 5 mm from the source. The dose distribution gradient along the dorsal–ventral axis of the OB was calculated by using computer-generated isodose curves (21, 22). The computed doses at the dorsal and ventral surfaces of the OB were 80 Gy and 9 Gy, respectively. In control treatment, animals were loaded with nonradioactive sealed sources.

Histology. At the end of the experiments, anesthetized animals were perfused with a phosphate-buffered saline solution (137 mM NaCl/2.7 mM KCl/0.9 mM $CaCl_2$/0.5 mM $MgCl_2$/1.5 mM KH_2PO_4/6.4 mM Na_2HPO_4) and then with a solution of 6% formaldehyde in the phosphate-buffered saline. After removal from the skull, the OBs were further fixed and frozen (23). Frozen tissues were cryostat sectioned (15 μm thick) in a plane parallel to the midline; the serially collected sections were kept at −20°C until processed. In the stab wound experiments, 80–100 sections included the entire pathway of the blade from the point of entry. Sections were stained with thionin for routine histological analysis. GFAP-positive astrocytes and neurite outgrowth were identified by indirect immunostaining of sections with antibodies against GFAP (24) and against neurofilaments, respectively, as described (23). Monoclonal antibodies (IgG fractions) against GFAP and against the 68-kDa and the 160-kDa neurofilaments (Boehringer Mannheim) as well as rabbit anti-mouse IgG conjugated to fluorescein (Cappel Laboratories) were used. Samples were examined with a Zeiss microscope equipped with epi-illumination optics with an exciter-barrier filter combination for fluorescein.

RESULTS

Irradiation Within a Specific Time Window Postinjury (PI) Prevents Gliosis. The effectiveness of the timing of radiation in reducing the population of reactive astrocytes along the site of a stab wound was examined at time points of delivery from 1 to 49 days PI and was compared with various control samples that were injured but not irradiated (Fig. 1). Analysis was performed on serial sections of each of the injured tissue samples, which were examined for the presence of GFAP-positive cells along the cut. The OB responds to a stab wound by the formation of a glial scar (Fig. 1 *A* and *E*). Irradiation, when applied within the time window of 2–3 weeks PI (n = 15), resulted in reduction of gliosis around the wound (Fig. 1*B*). The most effective elimination of reactive astrocytes along portions of the site of incision was obtained when irradiated at 15–18 days PI (Fig. 1*C*). Irradiation before day 6 PI or beyond day 29 PI (n = 11) had no detectable effect on scar formation (Fig. 1*D*) as compared with the unirradiated injured bulbs (Fig. 1*A*). The effectiveness of irradiation is dose dependent; even when delivered during the optimal period of 15–18 days PI, the reactive astrocytes were not completely eliminated as could be seen in deeper regions along the incision (below 5 mm from the source) (Fig. 1*F*), presumably due to increasing distance from the radiation source.

Morphological Preservation by Irradiation of Transected Bulbs. We next examined whether radiation under conditions optimal for the elimination of gliosis can change the pattern of degenerative processes induced by the injury and whether it can affect the fate of axotomized neurons. In these experiments, the middle of the OB was transected from top to bottom, thereby severing the projections of the mitral cells into the brain (Fig. 2). Parameters of irradiation were kept the same as in the previous experiments, and radiation was delivered at 15–18 days PI. Irradiation of lesioned bulbs resulted in prevention of tissue degeneration and cavitation, healing of the wound (i.e., fusion of the two tissue stumps into one continuous structure), partial elimination of the glial scar, preservation of the typical cell lamination, and rescue of axotomized mitral cells as illustrated in Figs. 3–7.

Unirradiated severed OBs (n = 3) had markedly shrunk anterior to the cut (Fig. 3*B*). In contrast, severed and irradiated OBs (n = 4) were preserved in size (Fig. 3*A*), showing an almost-normal appearance similar to their uninjured, intact counterpart. Histological analysis comparing sections

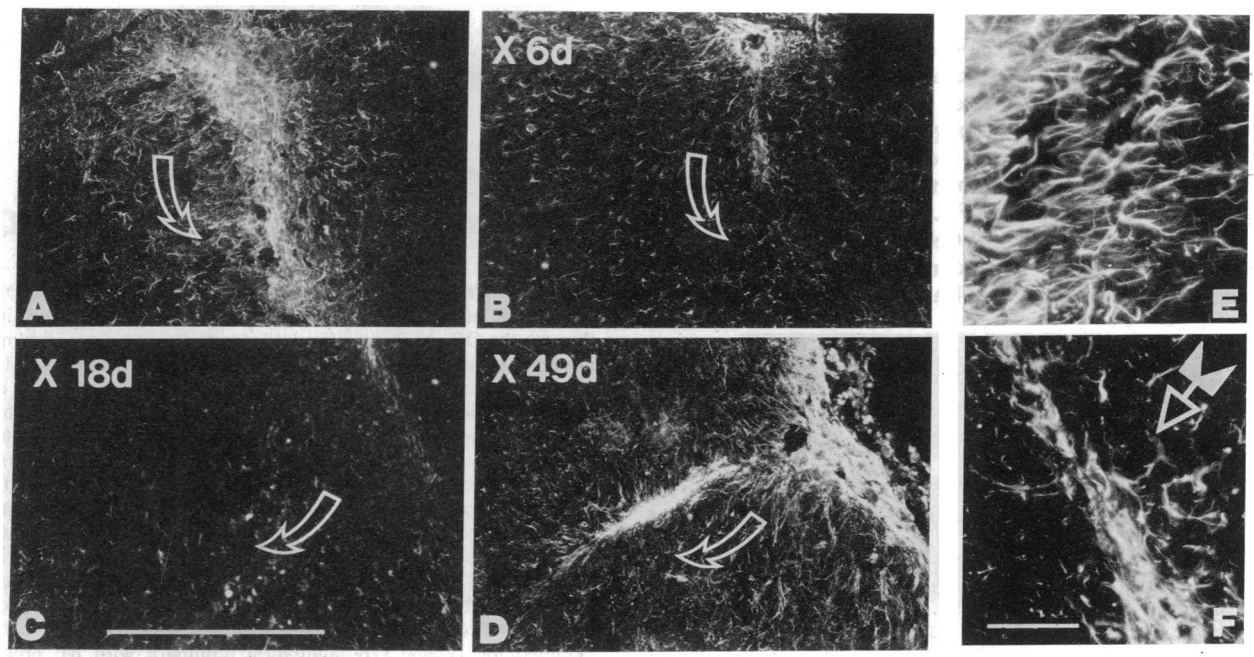

FIG. 1. Prevention of glial scar formation by PI irradiation: a temporal function. Distribution of GFAP-positive astrocytes along the stab wound in a control sample 69 days PI (*A*) and in samples that were irradiated (X) at 6 (*B*), 18 (*C*), and 49 (*D*) days PI and analyzed 50 days after the irradiation is shown. Arrows (in *A–D*) point to the pathway of incision from the site of entry into the OBs; the apical zone surrounding the penetration site showed varying degrees of gliosis (*B–D*). In comparing *A–D*, note that the sample irradiated 18 days PI (*C*) (at 3–4 mm from the irradiation source) contained virtually no GFAP-positive astrocytes along the cut. High-magnification micrographs of GFAP-positive astrocytes around the incision site in sections of control sample (*E*) and of the ventral portion of the stab-wounded OB irradiated (tailed arrow) 18 days PI (*F*) are shown; sections from the same samples are shown in *A* and *C*, respectively. (*A–D*, bar = 500 μm; *E* and *F*, bar = 50 μm.)

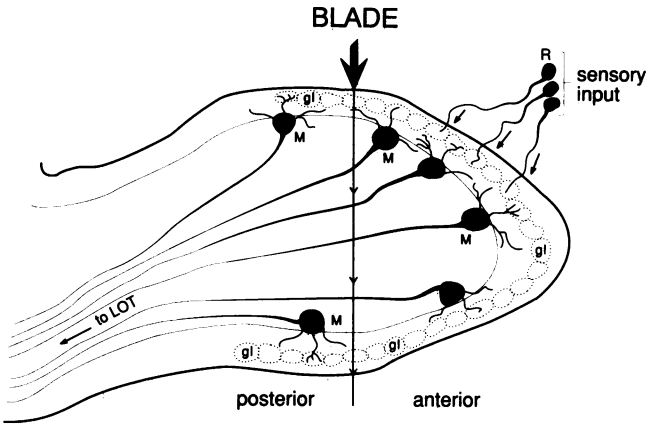

FIG. 2. Mitral cell organization in the OB and the experimental paradigm for their axotomy. The olfactory receptor cells are situated in the periphery; they send their axons into the bulb and these terminate in the glomeruli (gl) (25). The mitral cell bodies (M) are arranged in a layer below the glomeruli; these cells (the principal OB neurons) send a primary dendrite into the glomeruli and receive the olfactory input there, while they project their axons posteriorly into the olfactory cortex (25). On their course to the cortex, these axons traverse the bulb converging into the lateral olfactory tract (LOT). Within the 2 mm width of the OB, in the lateral-to-medial axis in the tissue segment of 0.6–1.1 mm, the mitral cell layer (MCL) runs parallel to the contour of the bulb (e.g., Figs. 4 and 5); thus, incision at the center of the OB (blade arrow) penetrates the MCL and leads to axotomy of the mitral cells anterior to the plane of the cut.

of three different samples, normal, severed, and severed and irradiated (Fig. 4), revealed that the overall cellular organization in the irradiated OB tissue (Fig. 4*A*) was similar to normal tissue (Fig. 4*B*); however, the cellular organization in the treated tissue was slightly deformed at certain portions along the cut (Fig. 4*A*). In contrast to the irradiated bulbs, in the untreated OBs (Fig. 4*C*), complete transection of the bulb resulted in cavitation and degeneration of tissue around the incision site in a manner similar to the response to extensive wounds seen in other CNS regions [e.g., cerebrum (7)]. In all untreated bulbs, transection resulted in disappearance of the mitral cells, the mitral cell layer (MCL), and the adjacent internal plexiform layer in the region anterior to the cut as illustrated in Fig. 5; in some of the samples degeneration of mitral fiber tracts was evident by the secondary gliosis seen along the rostral–caudal axis (Fig. 5*A*).

Rescue of Axotomized Mitral Cells. Irradiation of lesioned bulbs, both complete ($n = 4$) and incomplete ($n = 6$) transection, resulted in the rescue of at least some of the axotomized

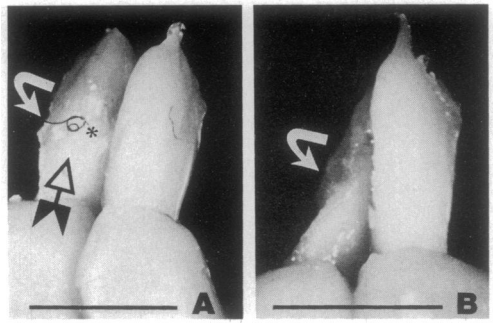

FIG. 3. Prevention of degeneration of severed OB by irradiation. The dorsal views of the OBs of two animals 59 days PI—an experimental OB (tailed arrow) irradiated 18 days PI (*A*) and a control untreated OB (*B*)—are shown. The left OB in both samples was transected (white arrows), and a monofilament nylon was sutured to the dura as a marker for the cut (asterisk in *A*). The dark areas on the OBs are artifactual due to the illumination. (Bars = 5 mm.)

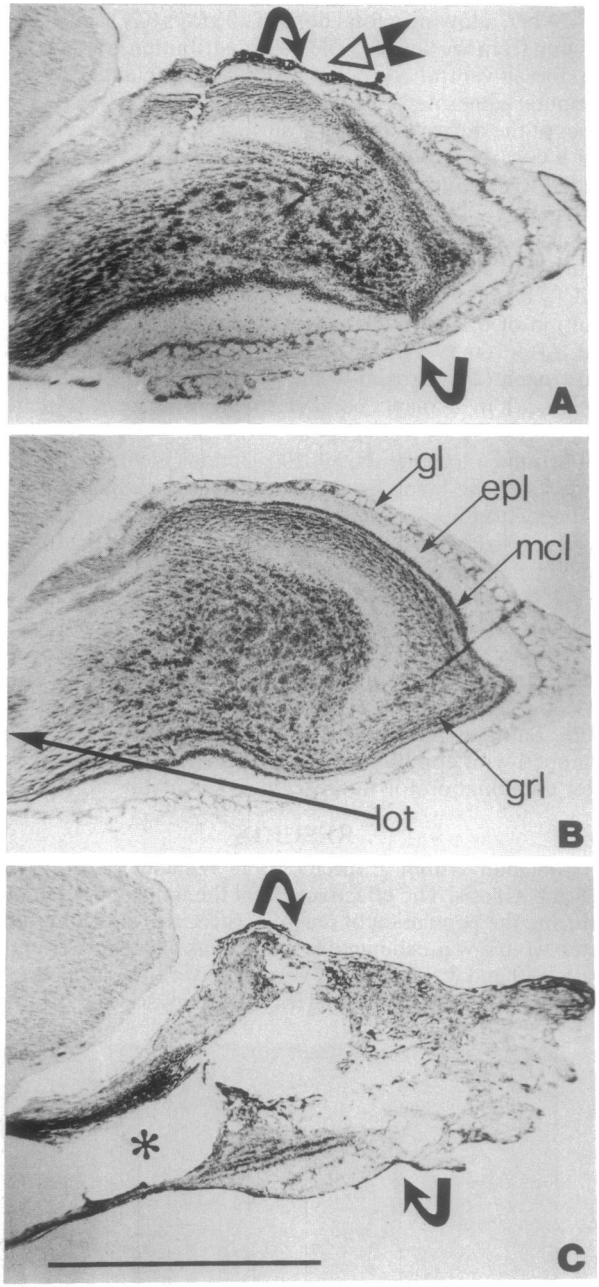

FIG. 4. Preservation of the cytoarchitecture of severed OB by irradiation. Micrographs of sagittal sections, stained with thionin, through the OBs of three differently treated animals are shown. (*A*) Severed, irradiated OB (the one seen in Fig. 3*A*). (*B*) Normal, intact OB. (*C*) Severed, untreated OB (the one seen in Fig. 3*B*). The entry and exit of the blade are indicated by curved arrows. Neuronal cell lamination in a normal OB (*B*) consists of glomeruli (gl), external plexiform layer (epl), MCL (mcl), and granule cell layer (grl); the direction (arrow) of the lateral olfactory tract (lot) is indicated. In the severed, untreated OB (*C*), most of its cellular components anterior to and around the cut had degenerated leaving behind numerous cavities (e.g., asterisk); posteriorly to the cut, a fraction of the MCL survived, but most of the tissue lacked the typical cell lamination. In contrast, in the irradiated OB (*A*, tailed arrow), anterior to the cut, the cellular components were preserved and overall the tissue retained the typical OB lamination (compare with *B*); however, neuronal laminae anterior to and at the cut had shrunk. (Bar = 3 mm.)

cells and preservation of the MCL (Fig. 4*A*). Survival of axotomized mitral cells, induced by irradiation, was assessed by counting mitral cells at the MCL in comparable sections from normal OB (Fig. 6*B*) and from completely transected, irradiated OB (Fig. 6*A*) and by plotting their mean values

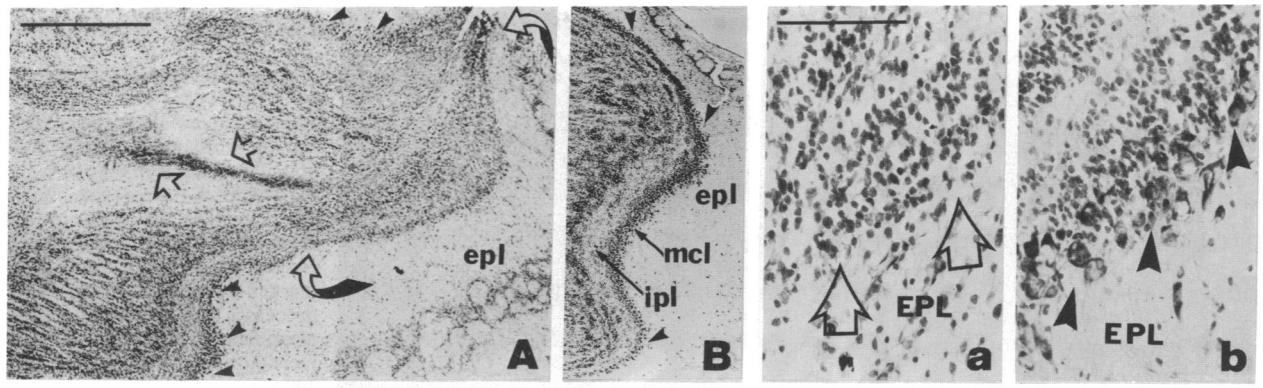

FIG. 5. Morphological consequences of transection. Thionin-stained sections, at a similar level, through partially transected OB (*A* and *a*) and normal OB (*B* and *b*). (*A*) The blade penetrated only through the dorsal portion of the OB (its pathway is in between the curved arrows). In the two OB sections (*A* and *B*), the MCL (mcl, arrowheads) can be seen extending between the external plexiform layer (epl) and the internal plexiform layer (ipl). Note that in the lesioned OB (*A*) the MCL and the internal plexiform layer are absent in the dorsal portions of this tissue section, in the region anterior to the cut; in the center of this section, in a posterior direction, a heavy band of secondary gliosis is seen (open arrowheads). (*a* and *b*) Enlarged views of MCL regions taken from the corresponding tissue samples shown in *A* and *B*. Note the distribution of mitral cell bodies (arrowheads) in the normal OB (*b*) and their absence (open arrowheads) from the region anterior to the cut (*a*). (*A* and *B*, bar = 1 mm; *a* and *b*, bar = 100 μm.)

(Fig. 6*C*). By this analysis, the estimated degree of rescue of axotomized mitral cells in this irradiated OB is about 32% (Fig. 6*C*). In contrast, in the unirradiated severed OBs all mitral cells proximal to the cut had vanished (Fig. 4*C*).

Analysis of the irradiated, severed OBs by neurofilament staining showed that the mitral and granule cell layers had an intense network of neurites and some of these neuronal processes had crossed the incision site (Fig. 7). Even though these samples showed neurite outgrowth, at present the study does not provide any experimental evidence that the rescued mitral cells managed to regrow their severed axons, let alone extend axons beyond the lesion site.

DISCUSSION

This study shows that the glial scar formed as a consequence of CNS injury can be eliminated or reduced by irradiation. This finding was expected and does not represent a novel concept in the mechanism of wound healing (26). Our main observation is that irradiation treatment—namely, the manipulation of the cellular environment—enables the injured CNS tissue to regain some normal structural features. Most importantly, along with the structural preservation, the axotomized principal neurons of the OB, which otherwise would have degenerated, were rescued. This study supports the idea that in response to injury a population of nonneuronal cells is generated, which obstructs and impairs the morphological recovery processes in the lesioned CNS. However, the study does not conclusively identify these cells as reactive astrocytes.

Our study established in rat OB that irradiation in the third week PI is most effective in eliminating GFAP-positive astrocytes at the incision site. By assuming that the radiosensitive cells are those that proliferate, our data suggest that the mitotic activity of the astrocytes that form the scar starts at about day 6 PI, peaks at 15–18 days PI, and is completed by the fourth week PI. In the period in which mitotic activity of reactive astrocytes has been studied, primarily within 1–6 days PI, these cells proliferate very little (27). Our data fit well with observations made on the content of glial mitogens and inhibitors of glial proliferation in injured rat brain, in which in the second and third week PI the mitogens are maximal (28) and the inhibitors are minimal (29). It is worth noting that glial scar formation is delayed by chronic treatment of lesioned CNS with antimitotic agents (30).

Data in the literature are insufficient to support or rule out a direct primary role of glial scar and reactive astrocytes in

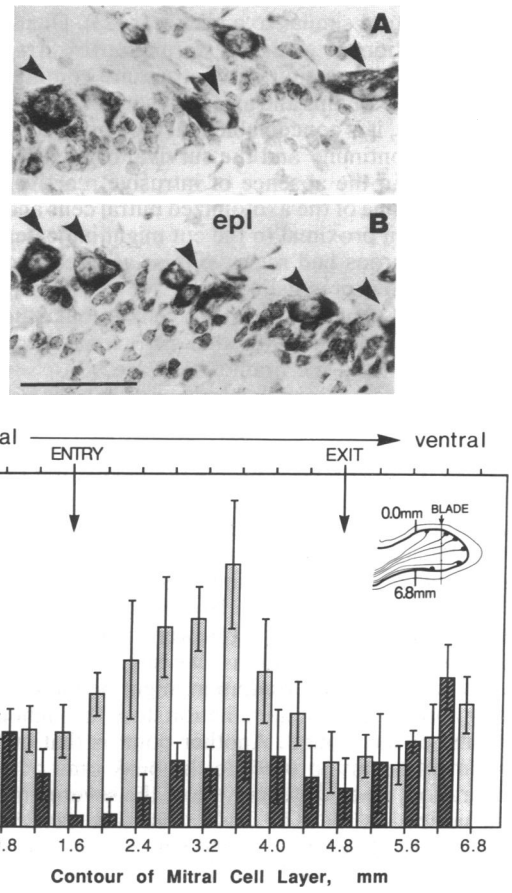

FIG. 6. Degree of rescue of axotomized mitral cells. Photomicrographs of the mitral cell bodies at the MCL in thionin-stained sections of severed and irradiated OB (*A*) in the region anterior to the cut (the sample in Fig. 4*A*) and of normal OB (*B*) (the sample in Fig. 4*B*) are shown. (Bar = 50 μm.) (*C*) Mitral cell body content at the MCL in single sections through normal OB (stippled bars) and irradiated, severed OB (hatched bars). Mitral cells along the contour of the MCL in a dorsal-to-ventral direction were counted in 6 alternate sections of 12 serial sections (as seen in *A* and *B*), and their numbers in corresponding fields across sections were averaged. The bars are the means of cell numbers in a field (400 × 80 μm^2), and error bars are the SD. Note that at 0–1.2 mm and 5.0–6.8 mm along the MCL (posterior to the cut), the numbers of mitral cells in treated and normal bulbs are similar, and anterior to the cut, 41 cells are found in the treated tissue section as compared with 125 in the normal.

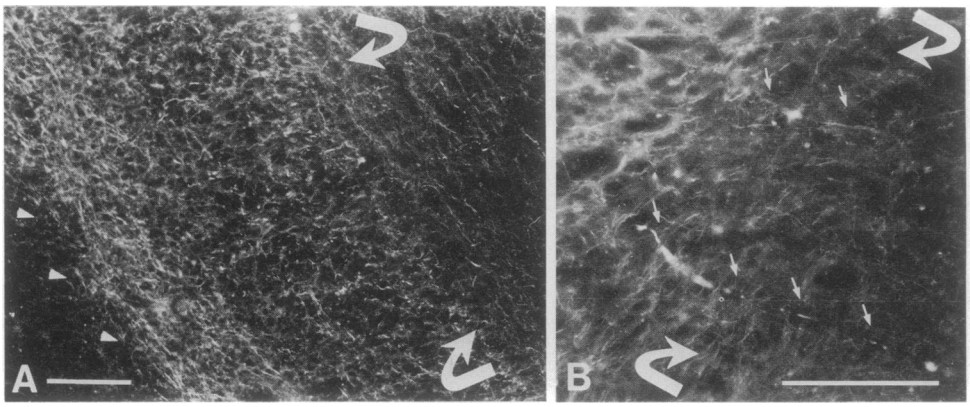

FIG. 7. Neuronal process network in severed and irradiated bulb anterior to and along the transection site. Neurofilament staining of a section of the sample in Fig. 4*A* is shown. (*A*) The MCL (arrowheads) and granule cell layer are lined up with a network of neurite processes, which is seen also around the incision site (curved arrows). (*B*) Some of these processes (arrows), seen at higher magnification, extend and cross the incision site (curved arrows). (Bars = 100 μm.)

the impairment of axonal regrowth and neural tissue regeneration (31). Grafting studies suggest an inhibitory role of some astrocytes in regenerative processes. Namely, astrocytes beyond a certain cell age, when inoculated into a silicone chamber between stumps of an adult rat sciatic nerve, completely inhibited the normally occurring nerve regeneration, and these cells encapsulated the nerve stumps with a structure similar to a glial scar (23). Our study shows that prevention of glial scar formation by irradiation was accompanied by restitution of structural continuity between the two tissue stumps and also by rescue of axotomized mitral cells. Hence, it is conceivable that the observed restitution of structural continuity and the survival of the mitral cells can ensue only in the absence of intrusive reactive astrocytes. Rescue of some of the axotomized mitral cells and the neurite network seen proximal to the cut might indicate that certain of these neurons had regrown their severed axons past the lesion site along preexisting tracts. Nevertheless, the analysis of rescue of axotomized mitral cells in our study does not address the question of whether any axonal regrowth toward the target cells in the brain took place.

Histologically, along the dorsal-to-ventral axis of the OBs, a reasonably good correlation existed in the irradiated bulbs between healing and rescuing of mitral cells and lack of gliotic scar. In the dorsal portions the wound did not heal, presumably due to the high irradiation doses at the region closer to the radioactive source; in deeper cell layers (0.25 mm), the wound healed completely with no visible sign of the incision and no glial scar (determined also by electron microscopy analysis; data not shown). However, below 3 mm from the OB surface, the lesion signs were apparent again. On the basis of these observations, we suggest that the degree of beneficial effects of irradiation might be increased by delivering a uniform optimal radiation dose throughout the entire volume of the wound. Another point is that our irradiation treatment seems to precede the peak time of neuronal degeneration, since degeneration of the severed mitral fibers is first noticed at 6 days PI, and it is maximal 20–25 days PI (32).

The role of reactive astrocytes in neuronal regeneration remains elusive. The presumed obstructive role of the reactive astrocytes in regeneration does not necessarily imply a direct effect of the cells on either axonal growth, neuronal maintenance, or tissue regeneration; rather, these might be exerted indirectly via the vascular system. Tissue regenerative processes, in general, are dependent on factors supplied by the vascular system. Reactive astrocytes might be involved in uncoupling an essential supply by either inducing an unscheduled formation of a blood–brain barrier (33) and/or by preventing angiogenesis and neovascularization. Regardless of the identity and the mechanism, it seems that effective elimination of the intrusive cells by irradiation enables restoration of normal morphological features in the mammalian CNS. The rescue of the axotomized neurons indicates that the door for functional recovery might be opened by the radiation treatment. If true, our data would suggest that radiation may have a potential use in the management of CNS injury, such as trauma to the brain and spinal cord.

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