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Elevated Expression of B-50 (GAP-43)-mRNA in a Subpopulation of Olfactory Bulb Mitral Cells Following Axotomy

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Neurons in the central nervous system regenerate poorly or not at all. In contrast neurons of the peripheral nervous system have the ability to regrow their nerve fibers over considerable distances. Previously it has been suggested that the absence of the reinduction of the expression of growth associated proteins such as B-50 (GAP43) may be an important factor in the differential response of CNS and PNS neurons to injury. We studied B-50(GAP43) mRNA expression following lesioning of a class of CNS neurons, the olfactory bulb mitral cells. Expression of B-50 mRNA in approximately 40% of the mitral cells was upregulated in response to transection of their axons in the lateral olfactory tract (LOT). Enhanced expression persisted for 10 days postlesion but had virtually declined to control levels by 4 weeks after the lesion. A large proportion of the mitral cells gradually degenerated subsequent to LOT transection. Thus a subpopulation of mitral cells maintains their ability to upregulate B-50, a protein characteristic of growing axons, but enhanced B-50 expression is not accompanied by regeneration of the severed LOT.

Key words: growth associated protein B-50, central nervous system lesion, lateral olfactory tract, in situ hybridisation

INTRODUCTION

Neurons of the adult mammalian central nervous system (CNS) usually fail to regenerate following injury. Axonal regeneration of injured peripheral neurons, however, occurs vigorously. The dichotomy between the capacity of CNS and peripheral nervous system (PNS) neurons to regrow their axons is due to a variety of factors affecting the postlesion repair process. The inability of CNS neurons to regenerate is at least in part caused by environmental constraints in the lesion area. Extensive glial scar formation may produce a physical barrier for thin regenerative sprouts in the CNS (Reier et al., 1983). In addition the lack of appropriate neurotrophic factors (Bailey et al., 1992), the expression of axon growth inhibitory molecules (Schnell and Schwab 1990), and the failure to reinitiate expression of growth-associated proteins (Skene and Willard 1981; Benowitz et al., 1981; Skene, 1984) may also contribute to the diminished capacity of CNS neurons to regenerate.

A protein that has been suggested to have a role in axonal outgrowth is the growth-associated protein B-50 (also known as GAP-43, F1, and neuromodulin; reviewed in Skene, 1989). Following a peripheral nerve lesion, B-50 mRNA and protein expression are upregulated 5- to 10-fold (Skene and Willard, 1981; Hoffman, 1989, Van Der Zee et al., 1989). In contrast, several studies on the postlesion response of a number of CNS neurons (retinal ganglion cells, corticospinal neurons) failed to detect elevated levels of this protein (Skene and Willard, 1981; Redshaw and Bisby, 1984; Kalil and Skene, 1986; Reh et al., 1987). These observations suggest that the failure of CNS neurons to regenerate may to some extent be due to their inability to activate transcription of the B-50 gene. Thus, increased B-50 expression could be one of the prerequisites for successful axonal regeneration (Skene and Willard, 1981; Benowitz et al. 1981).

Recent studies, however, provide evidence for upregulation of B-50 expression in the CNS following ischemia or mechanical injury (Ng et al., 1988; Tetzlaff et al., 1991; Doster et al., 1991) and during reactive synaptogenesis (Benowitz et al., 1990). The induction of B-50 expression following mechanical CNS injury was not accompanied by axonal regeneration. This indicates

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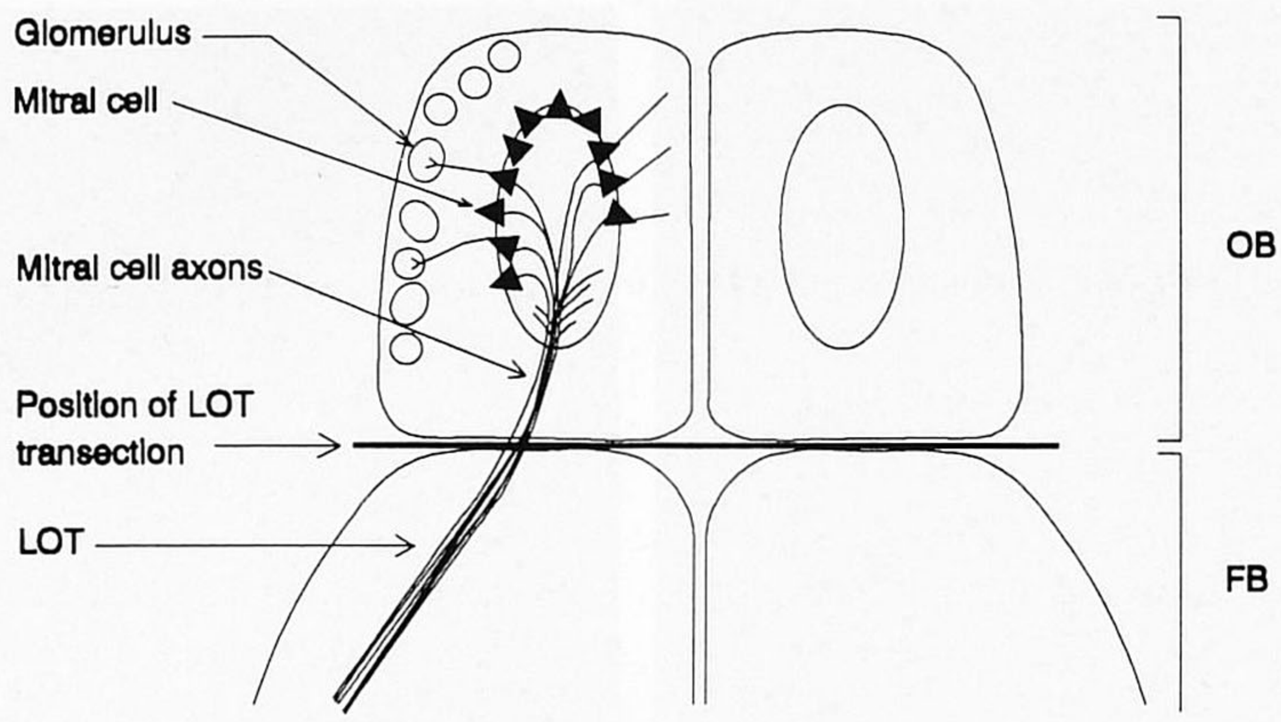


Fig. 1. Illustration of the anatomical relationships and the LOT lesion in the olfactory system with particular reference to the olfactory bulb mitral cells. LOT, lateral olfactory tract; OB, olfactory bulb; FB, forebrain.

that enhanced B-50 expression is not sufficient to endow certain CNS nerve cells with the capacity to regenerate their axons.

Here we investigate the response of B-50 mRNA in a subset of mature CNS neurons, olfactory bulb mitral cells, following lesioning of their axons in the lateral olfactory tract (LOT). Mitral and tufted cells are the output neurons of the main olfactory bulb. Their axons relay olfactory information through the LOT to higher brain centers, including the primary olfactory cortex, amygdala, olfactory tubercle, and entorhinal cortex (Scott and Harrison, 1987). In neonatal rodents the LOT has a considerable regenerative capacity (Devor, 1976). Following a transection of the LOT during the first week of life, mitral cell axons penetrate the lesion and reinnervate caudal target areas, resulting in neuroanatomical and behavioral recovery (Grafe, 1983; Small and Leonard, 1983). In adult animals, however, the regenerative ability of axons across a LOT lesion is significantly diminished, concomitant with extensive glia scar formation (Sijbesma and Leonard, 1986). We will show that a proportion of mature mitral cells in the rat olfactory bulb transiently upregulates B-50 mRNA following lesioning of their axons in the LOT. Enhanced expression of B-50 mRNA is not, however, accompanied by axonal regeneration. The failure of lesioned B-50 positive mitral cells to regenerate their severed axons may be due to the absence of a permissive environment in the region of the lesion.

MATERIALS AND METHODS

Animals and Surgery

A total of 44 male Wistar rats (200–250 g; TNO, Zeist, NL) were used in this study. Twenty-seven animals were subjected to LOT lesion and 8 were sham operated. Nine animals served as unoperated controls. The anatomical relationships in the olfactory bulb and the position of the lesion are depicted in Figure 1. Bilateral transection of the LOT was performed under deep anesthesia with Nembutal (1 ml/kg rat containing 60 mg/ml natriumpentobarbital and 9 mg/ml benzylalcohol, injected intraperitonially). A small groove 6 mm long and 0.5 mm wide was made in the skull with a dentist's drill at 5.7 mm rostral from bregma. The left and right LOT were cut with a no. 11 scalpel blade. The scalpel blade was lowered into the left side of the groove until it touched the base of the skull. Subsequently the scalpel blade was moved from the left to right, cutting both LOTs. Care was taken to avoid damage to the frontal poles of the cortex. Post mortem inspection of the lesion showed that this procedure resulted in complete transection of the left and right LOT in all animals. In the sham operated rats the scalpel blade was lowered into the groove to 4 mm below the upper surface of the skull. As in the lesioned animals this usually resulted in some bleeding, but in sham operated animals the LOT always remained intact.

In Situ Hybridisation

At postoperation days 2, 5, 10, 30, and 60 a number of lesioned, sham and control animals was anesthesized with Nembutal and perfused through the heart with 2% paraformaldehyde in 0.1M phosphate buffer at pH 7.4. Olfactory bulbs were dissected and cryoprotected by immersion in 7.5% and 15% sucrose in 100 mM phosphate buffer at pH 7.4. Tissues were snap-frozen in liquid nitrogen cooled isopentane and stored at −80°C until further processing for in-situ hybridisation (Verhaagen et al., 1990a).

The preparation of ^{35}S-labelled sense and antisense

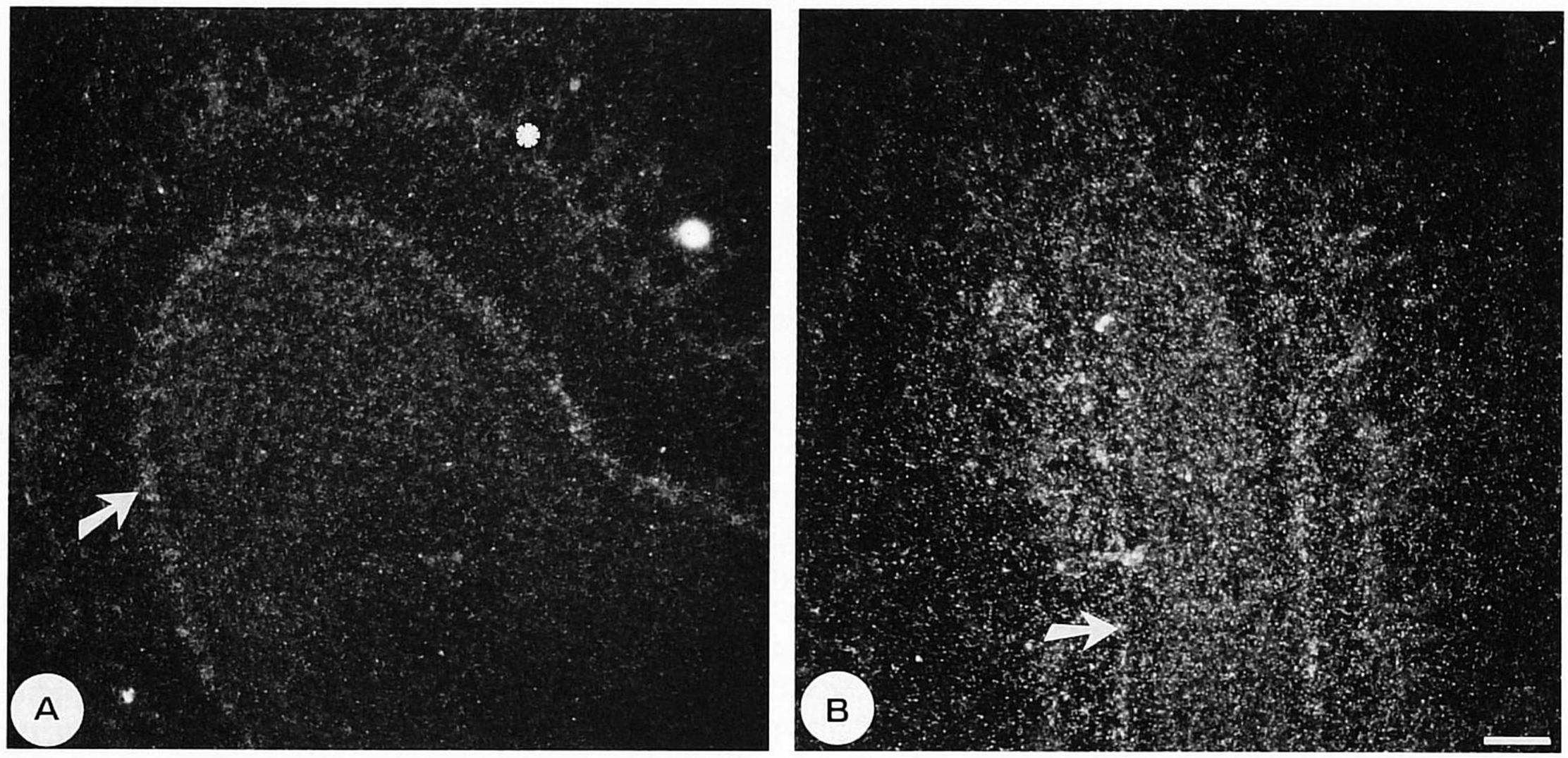


Fig. 2. Expression of B-50 mRNA in control olfactory bulb and in olfactory bulb 4 weeks following LOT transection. Low-power darkfield photomicrograph of control olfactory bulb (**A**) and of an olfactory bulb 4 weeks following LOT transection (**B**) reveals hybridisation signals in the mitral cell layer (arrow) and in the juxtaglomerular cells surrounding the glomeruli (*). Note that the bulb has shrunken dramatically 4 weeks postlesion. Scale bar is 100 μm.

B-50 mRNA probe and the procedure used for in-situ hybridisation have been described in detail previously (Verhaagen et al., 1990a). Eight micrometer cryostat sections from fixed olfactory bulbs were mounted on poly-L-lysine coated microscope slides. Sections were kept in 70% ethanol overnight and were subsequently rinsed in phosphate buffered saline (150 mM sodium chloride, 10 mM phosphate buffer, pH 7.4), in 2× SSC (SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) and acetylated in 0.25% acetic anhydride/0.1 M triethanolamine. After additional washes in 2× SSC and PBS, sections were incubated in 0.1 M glycine/0.1 M Tris-HCl pH 8.0, rinsed briefly in 2× SSC, and dehydrated in 70% and 95% ethanol. The hybridisation was performed on the air dried, dehydrated sections for 5 hr at 62°C in hybridisation solution (50% formamide, 10% dextransulfate, 25 μg salmon sperm DNA/ml, 10 μg tRNA/ml, 10 mM dithiothreitol, and 4× SSC) containing the radiolabelled probe. Each microscope slide with 5 olfactory bulb sections was incubated in 50 ul hybridisation solution containing 7×10^5 cpm B-50 mRNA probe. Following the hybridisation sections were rinsed in 2× SSC/50% formamide and in 0.1× SSC/20 mM 2-mercaptoethanol. Sections were dehydrated, and then rinsed in xylene and 100% ethanol. Sections were coated with NTB-2 emulsion (Kodak) and developed after an exposure time of 4 days. Cells were visualized by counterstaining with hematoxylin.

Quantification of In Situ Hybridisations

The autoradiographic signal on mitral cells was quantitated by determining the relative fraction of the surface area of individual mitral cells covered with silver grains. Mitral cells were visualised on a television screen with a 100× oil immersion objective using a microscope (Olympus BH-2) and a videocamera linked to a microcomputer equipped with image analysis software (DIFA Measuring Systems, Breda, The Netherlands). For each animal 5 horizontal sections were cut through the midplane of the olfactory bulb approximately 50 μm apart. The third section was used for counting the total number of mitral cells in this section and for quantification of the autoradiographic signal on 65 mitral cell bodies. In order to avoid biased sampling, the first mitral cell chosen for quantification was always located most rostrally in the section. Subsequently, going in the clockwise direction, the next 64 mitral cells were used for quantification. Only mitral cells in which a nucleus was visible were used for quantification. Since the cell surface area covered by silver grains is measured, rather than the actual number of grains, the measured fraction of mitral cell surface area covered with silver grains has been cor-

rected for silver grain overlap according to a mathematical compilation previously used to quantitate autoradiographically detected NGF receptor densities in individual sensory neurons (Richardson et al., 1989). Thus the term *hybridisation signal* as used in the figures and text represents the fraction of mitral cell surface area covered with silver grains corrected for silver grain overlap and is expressed in arbitrary units (AU). Statistical analysis of the results was performed with ANOVA followed by Student's t-test.

RESULTS

B-50 mRNA Distribution in Normal Adult Olfactory Bulb

In control olfactory bulb B-50 mRNA was expressed at low levels in mitral cells and juxtaglomerular cells (Figs. 2A and 4A). Some large neurons in the external plexiform layer, probably tufted cells, also expressed B-50 mRNA. Low levels of labelling are present in the granule cell layer. These observations are consistent with previous reports (De la Monte et al., 1989; Verhaagen et al., 1990b). Dark-field microscopy revealed B-50 mRNA expression in the mitral cell layer as an ovoid-shaped band of autoradiographic silver grains (Fig. 2A). High magnification bright-field microscopy and quantification showed that the labelling of mitral cells was heterogeneous, ranging from virtually no labelling in most cells to some cells with an expression level up to 15 AU (Figs. 4A and 5B).

Reaction of B-50 mRNA to LOT Transection

B-50 mRNA hybridisation signals in mitral cells increased after cutting of their axons in the LOT. As early as 2 days following lesioning, a small fraction of the total population of mitral cells expressed elevated levels of B-50 mRNA (Fig. 5B), but the mean hybridisation signal on mitral cells had not yet increased significantly (Fig. 5A). At 5 and 10 days following LOT transection about 40% of the mitral cells had increased their B-50 mRNA levels 2- to 5-fold (Figs. 3 and 5A,B). At these postlesion times, a patchy groupwise induction of B-50 mRNA was observed in mitral cells throughout the mitral cell layer. At 1 and 2 months following the lesion, olfactory bulbs had become considerably smaller in size, and a large number of mitral cells had degenerated (Fig. 5C). In the majority of the remaining mitral cells, B-50 mRNA expression was virtually as low as in mitral cells in control animals (Figs. 2B, 4C, and 5A,B). B-50 mRNA expression in the juxtaglomerular and granule cells was not increased in the lesioned animals. These neurons do not extend an axon into the LOT and are therefore not directly injured. The tufted cells, however, do project their axons into the LOT, but these cells do not upregulate their B-50 mRNA expression in response to the LOT lesion.

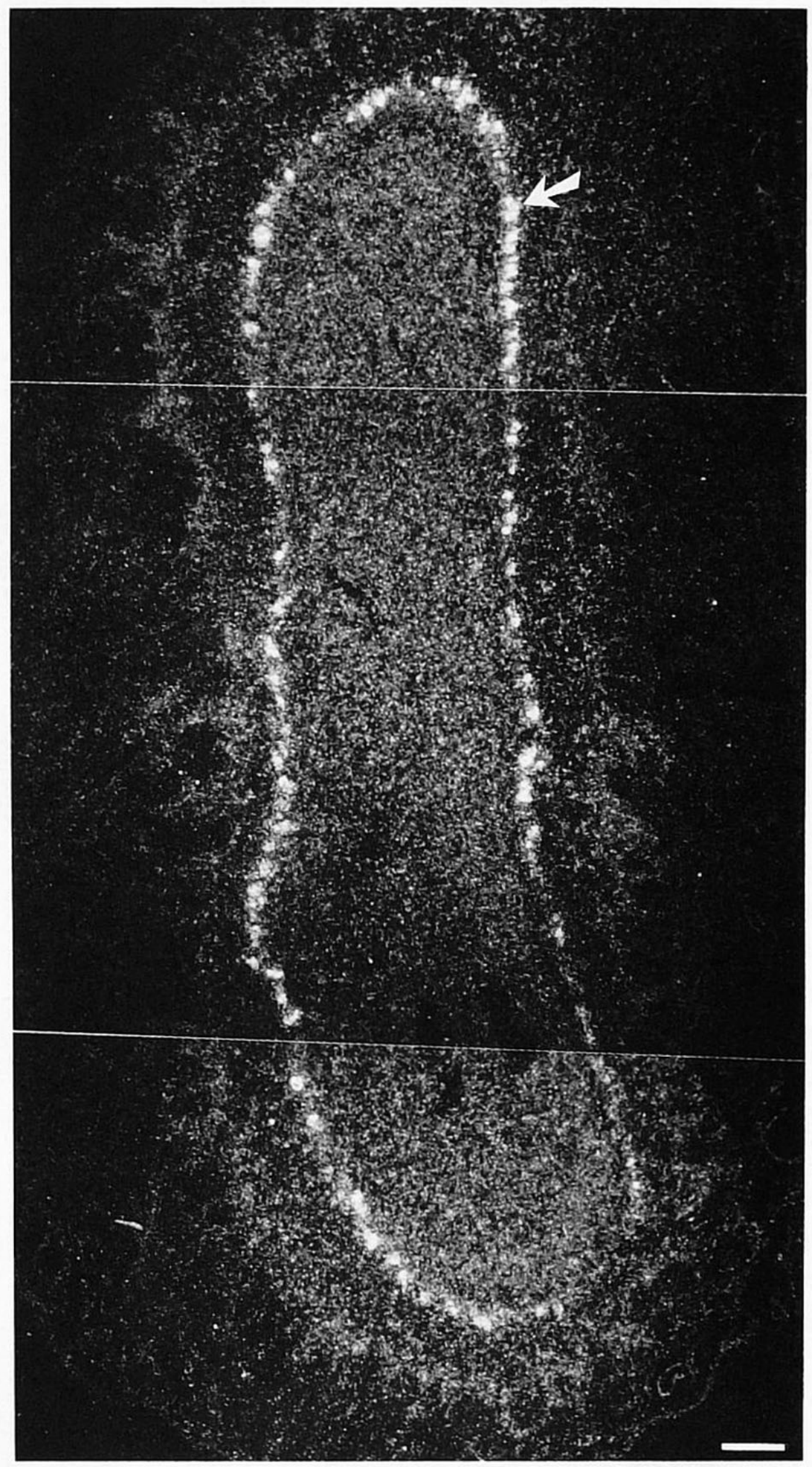


Fig. 3. Increased expression of B-50 mRNA in olfactory bulb 5 days following LOT transection. This low-power darkfield photomicrograph of an olfactory bulb 5 days postlesion demonstrates an induction of B-50 mRNA expression in groups of mitral cells (arrow) throughout the mitral cell layer. Scale bar is 100 μm.

B-50 mRNA hybridisation signals in the control unoperated animals and in the sham-lesioned animals were not statistically different. In addition no differences became apparent between control and sham animals sacrificed at a particular postlesion time point. Thus, for statistical analysis control and sham animals were considered as a single experimental group.

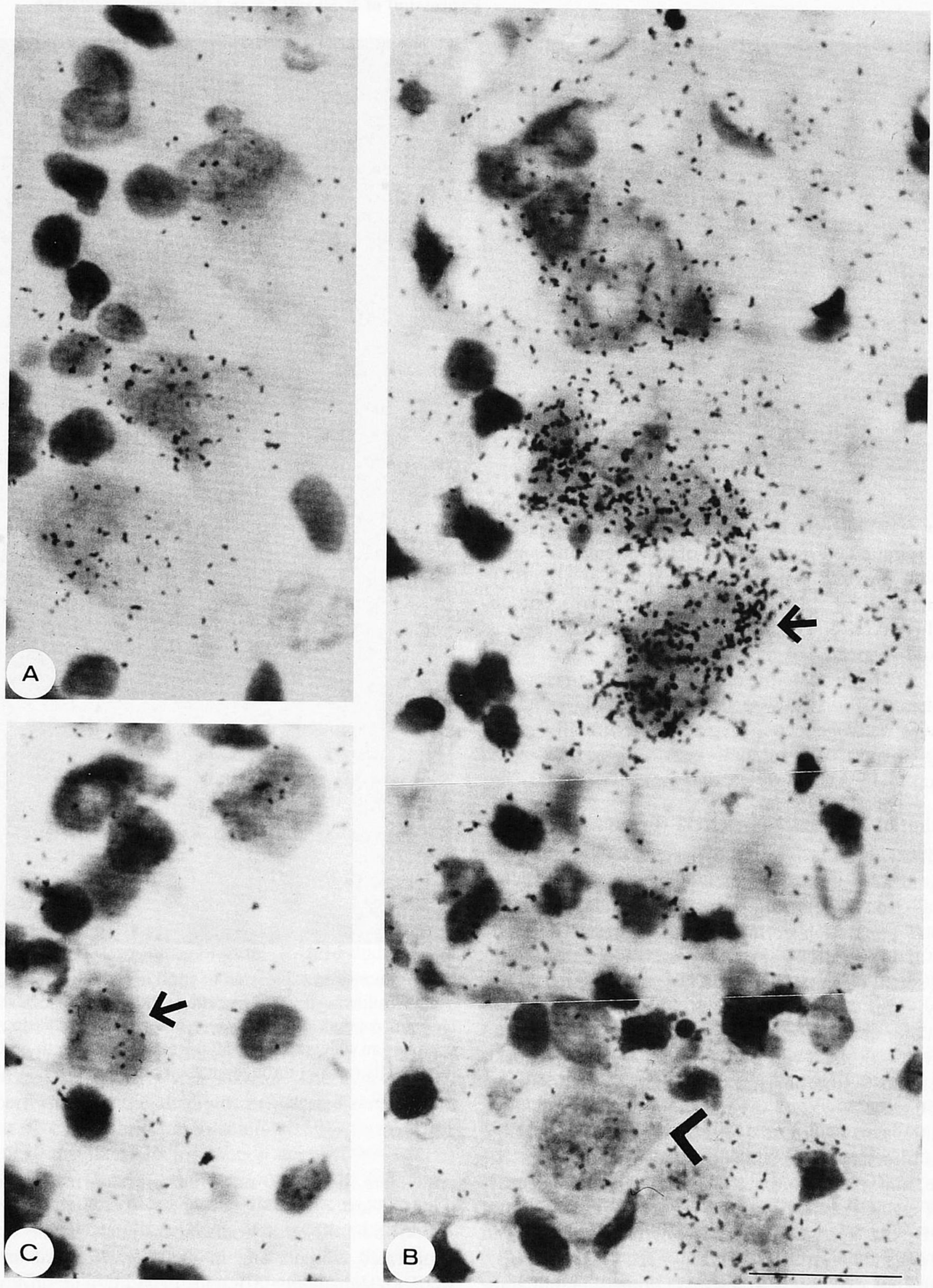
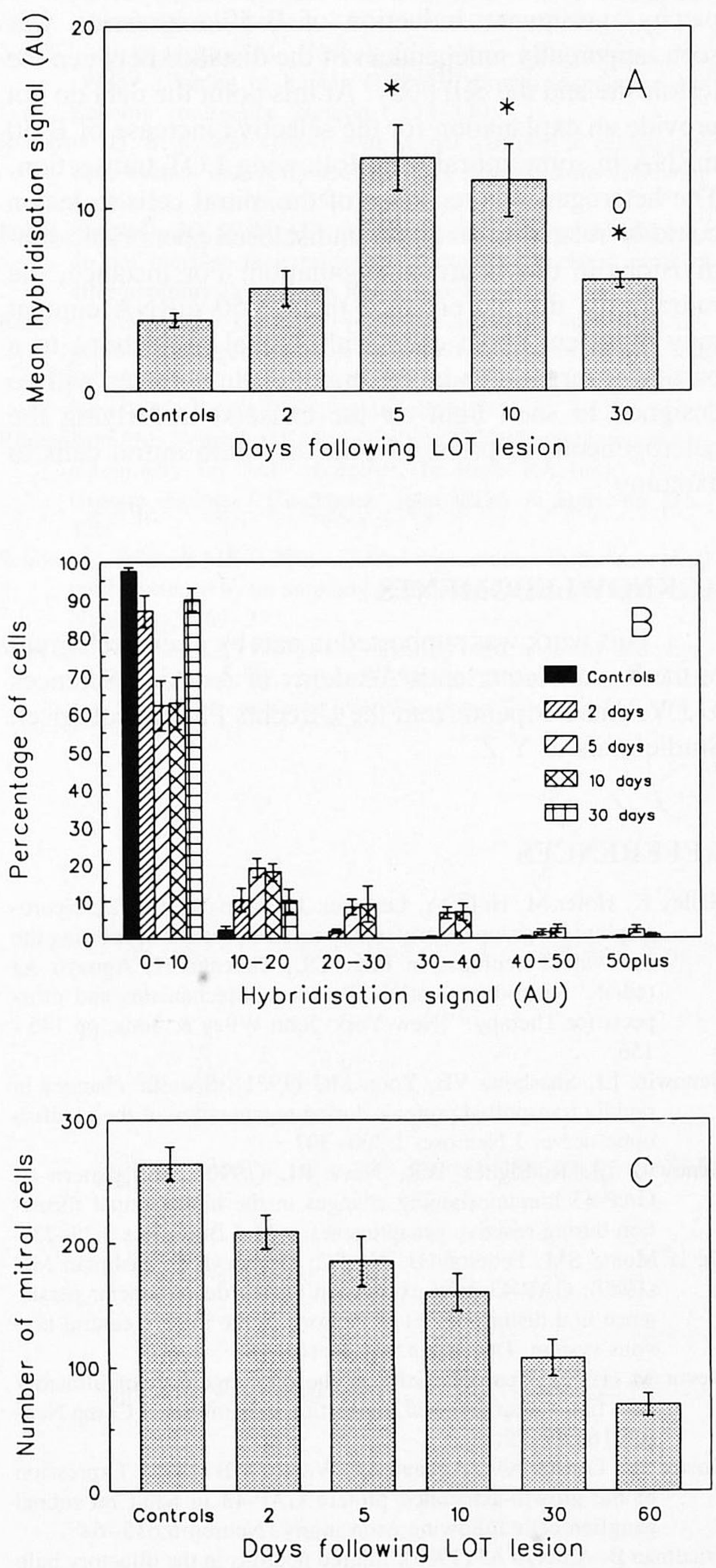


Fig. 4. High-power brightfield photomicrographs of the response of B-50 mRNA to LOT lesion. Note the induction of B-50 mRNA in individual mitral cells 10 days postlesion (arrow in **B**). The arrowhead identifies a mitral cell that does not respond to lesion. Mitral cells in controls (**A**) and 4 weeks following the lesion (arrow in **C**) exhibit low B-50 mRNA expression. Scale bar is 50 μm.

The observed changes in B-50 mRNA expression following LOT lesioning could be affected by shrinkage or swelling of the injured mitral cells. Therefore the in-situ hybridisation signal per unit mitral cell surface area was plotted as a function of the postlesion time. This gave an increase in the B-50 mRNA hybridisation signal similar to the increase that was observed when the quantitative mRNA analysis was performed per individual cell, demonstrating that the lesion effect is not caused by a change in the mitral cell size. Thus, it is legitimate to use the expression level of B-50 mRNA in individual mitral cells as a measure of their B-50 mRNA content following lesioning. In control tissue and in olfactory bulb of lesioned animals, virtually no hybridisation signal was observed with a sense B-50 probe. This demonstrates the specificity of the hybridisation procedure.



DISCUSSION

The results presented here demonstrate an increase in the expression of B-50 mRNA in a class of CNS neurons, the olfactory bulb mitral cells, due to transection of their axons in the LOT. An increased B-50 mRNA level was seen in approximately 40% of the mitral cells and persisted for up to 10 days after the lesion but had declined significantly 1 month postlesion. LOT transection caused a massive progressive mitral cell degeneration. Apparently the induction of B-50 mRNA expression is not sufficient to endow these neurons with the capacity to regrow their axons. Axotomized mitral cells in the adult olfactory bulb are still capable of regeneration when they are exposed to a peripheral nerve graft (Friedman and Aguayo, 1985). Thus, the potential for axonal growth is present in a proportion of olfactory bulb mitral cells, both in terms of their ability to upregulate expression of a growth-associated protein and successful extension of nerve fibers in a peripheral nerve graft. The failure of the B-50 positive mitral cells to regenerate may be related to the previously described nonpermissive CNS environment in the lesioned LOT of adult rats (Sijbesma and Leonard, 1986).

Our observations provide an example of upregulation of B-50 mRNA in CNS nerve cells that do not regenerate. The early studies on the response of B-50 in

Fig. 5. Quantitative analysis of B-50 in-situ hybridization following LOT lesion. **A:** The mean hybridisation signal in AU (± SEM) as a function of the postlesion time demonstrates increased B-50 mRNA expression in mitral cells at 5, 10, and 30 days following the lesion (* $P < 0.05$). At 30 days postlesion the mean expression level had decreased significantly (0 $P < 0.05$) as compared to the expression 5 and 10 days after the lesion. **B:** Differential increase in B-50 mRNA expression in mitral cells following lesioning. Mitral cells were divided in subclasses on basis of their B-50 mRNA hybridisation signal. A clear shift to classes with a higher expression level is apparent. **C:** Degeneration of mitral cells following LOT transection. The number of mitral cells (± SEM) in one horizontal section through the midportion of the olfactory bulb is shown at various postlesion times.

injured CNS neurons did not report an increase in the expression of B-50. Metabolic labelling studies showed that B-50 synthesis was not increased in axotomized retinal ganglion cells and in corticospinal neurons (Skene and Willard, 1981; Reh et al., 1987; Kalil and Skene, 1986). These neurons do not regenerate and their failure to reinitiate B-50 expression is in line with the notion that B-50 is one of the essential proteins necessary for successful regeneration of nerve tracts. Recent studies, however, provided the first evidence for an increased B-50 expression in injured CNS neurons (Ng et al., 1988; Benowitz et al., 1990; Tetzlaff et al., 1991; Doster et al., 1991). Tetzlaff et al. (1991) reported that injury to rubrospinal neurons results in an increase in the expression of the cytoskeletal proteins actin and alpha- and beta-tubulin, and in the growth-associated protein B-50. The postlesion response of rubrospinal neurons and olfactory bulb mitral cells as described here differs in 2 important aspects. First, in contrast to the transient increase in B-50 expression in mitral cells, enhanced expression of B-50 in rubrospinal neurons persisted for 7 weeks (the longest time point studied) following the lesion. Second, following a rubrospinal tract lesion, all rubrospinal neurons increased their B-50 mRNA expression, while LOT transection results in B-50 upregulation in a proportion of the mitral cells. Thus, although damaged CNS neurons can exhibit increased B-50 mRNA expression, additional regulatory mechanisms differentially affect the temporal and spatial patterns of this response in different classes of neurons in the CNS.

One of the factors that has recently been shown to be of importance to the upregulation of B-50 mRNA levels is the distance between the nerve cell body and the lesion site (Doster et al., 1991). This probably provides the explanation for the discrepancy between the early findings of a lack of induction of B-50 following lesioning of CNS neurons and the recent demonstrations of upregulation in traumatized neurons in the CNS. Intracranial lesions of the optic nerve failed to induce B-50 expression in retinal ganglion cells, but intraorbital lesions did induce B-50 expression (Skene and Willard, 1981; Doster et al., 1991). In analogy with these observations in the optic nerve, upregulation of B-50 expression in injured corticospinal tract appears to be also affected by the distance between the lesion and the cell body (Tetzlaff et al., 1990). The heterogenous expression of B-50 mRNA among damaged mitral cells is not due to variation in the distance between the lesion in the LOT and the mitral cell cell bodies in the olfactory bulb, since this would produce an entirely different pattern of expression. Mitral cells form a typical ovoid shaped band of cells in the olfactory bulb, projecting their axons through the granule cell layer into the LOT (Fig. 1). As a consequence of their neuroanatomical organization, axons of rostral mitral cells are cut at approximately 3 mm from their cell bodies, while axons of caudal mitral cells are injured at about 1 mm from their cell body. If this relatively small difference in the length of the remaining proximal axon stump would affect the upregulation of B-50 mRNA, the most rostrally located mitral cells would not respond to LOT lesion, while the caudal cells would increase their B-50 expression. No rostral-caudal gradient of B-50 mRNA expression in olfactory bulb mitral cells occurs following LOT lesion, but instead a patchy, groupwise induction of B-50 expression was seen, apparently independent of the distance between the lesion site and the cell body. At this point the data do not provide an explanation for the selective increase of B-50 mRNA in some mitral cells following LOT transection. The heterogeneous response of the mitral cells to lesion could be related to an as yet undisclosed phenotypic subdivisions in this neuronal population. For instance, the mitral cells that do enhance their B-50 mRNA content may represent nerve cells with axonal projections to a particular target area in the brain. Future studies will be designed to shed light on the cause(s) underlying the heterogeneous response of olfactory bulb mitral cells to axotomy.

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