EXPERIMENTAL NEUROLOGY 131, 93–107 (1995)

Immunolocalization of B-50 (GAP-43) in Intact and Lesioned Neurohypophysis of Adult Rats

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The persistence of high levels of B-50 in the adult brain is generally assumed to characterize neuronal systems capable of undergoing some form of plasticity such as axonal sprouting and regeneration. Since adult hypothalamo-neurohypophysial neurons are known to rapidly regenerate after being transected, the present study was undertaken to determine if such a capacity for regeneration could be related to the expression of this protein. Adult rats were killed by intraaortic perfusion of fixative either without lesion or at different delays after a surgical transection of the hypophysial stalk. Electron microscopy and laser scanning confocal microscopy were used to examine the regenerating axons after single or double immunocytochemical labeling of vibratome sections for B-50 and for various neuronal markers characterizing different types of neurohypophysial axons. In intact neurohypophysis, B-50 immunostaining was frequently associated with fibers immunoreactive to GABA or to tyrosine hydroxylase, whereas it was not detected within peptidergic neurohypophysial axons. In the lesioned neurohypophysis, B-50 was again frequently localized within axonal fibers immunoreactive to tyrosine hydroxylase or GABA. On the other hand, B-50 immunostaining was never detected within the numerous vasopressinergic or oxytocinergic axonal sprouts that regenerate all along the median eminence proximal to the lesion. These data indicate that persistence of high levels of B-50 within the neurohypophysis of adult rats is a specific feature of catecholaminergic and/or GABAergic axons innervating this region and that, contrasting to other neuronal systems, B-50 is not involved in the remarkable capacity for regeneration exhibited by the vasopressinergic and oxytocinergic neurohypophysial axons. © 1995 Academic Press, Inc.

INTRODUCTION

In the adult mammalian central nervous system, in contrast to the peripheral nervous system, transected axons generally fail to regenerate. Interestingly, however, neurons of the hypothalamo-neurohypophysial system have long been shown to present remarkable capacity for postlesional regeneration (2, 13, 27). In the rat, these neurons are mainly composed of magnocellular neurons of the paraventricular and supraoptic nuclei producing oxytocin or vasopressin, the axons of which project to the neural lobe of the hypophysis by running through the internal layer of the median eminence. When these axons are transected in their terminal portion, i.e., at the level of the median eminence or of the hypophysial stalk, they undergo dramatic reorganization leading (i) to massive sprouting of axon collaterals that penetrate the external layer and project into the perivascular space of the median eminence rostral to the lesion and (ii) to the partial reinnervation of the neural lobe by regenerating axons that cross the lesion (for review see 13, 31). During the past few years a series of studies has provided clear indications that B-50 (also called GAP-43 or neuromodulin) is involved in the mechanisms of axonal regeneration of adult neurons. Indeed, this membrane phosphoprotein, which is highly expressed by immature neurons that accomplish active axonal outgrowth, is again highly expressed in specific adult neuronal systems capable of undergoing axonal regeneration (4, 6, 28, 36, 37). In order to better understand the basis of the remarkable capacity for regeneration of the hypothalamo-neurohypophysial neurons, the present study was undertaken to determine if B-50 could be detected within these specific axon types in both intact and lesioned neurohypophysis of adult rats.

MATERIALS AND METHODS

Animals

Male adult Sprague–Dawley rats were used. They were kept in light (12L–12D) and temperature (24 ± 1°C) controlled rooms and had free access to standard dry food and tap water.

Surgical Lesions

After deep anesthesia with equithesine (3 ml/kg), animals were fixed in a stereotaxic device. Surgical lesions were performed by means of a rectangular metallic knife 2 mm large and 18 mm long (made from standard surgical blades) that was fixed in the specimen holder of the stereotaxic apparatus micromanipulator.

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The knife was placed 5 mm anterior to the interaural line and lowered through the midline to 11 mm below the dorsal surface of the skull (i.e., until touching the ventral surface of the cranial cavity). As attested by the microscopic examination of sagittal sections of fixed brains, such a surgical operation was found to induce a vertical lesion running through the different regions above the hypothalamus (namely including the cortex and the thalamus) and transecting the neurohypophysis at the level of the hypophysial stalk (Fig. 1).

Preparation of Tissues

Animals used included either control rats ($n = 5$) or rats which have received a lesion of the hypophysial stalk 5 days ($n = 5$), 10 days ($n = 5$), 15 days ($n = 5$), and 30 days ($n = 3$) before the fixation. After deep anesthesia with pentobarbital (60 mg/kg), they were perfused through the ascending aorta with phosphate-buffered saline (PBS), followed by 500 ml of fixative composed of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 *M* phosphate buffer at pH 7.4. The forebrain was dissected and fixed by immersion in the same fixative for 2 to 4 days. Pieces of ventromedial hypothalamus including the median eminence or the neurointermediate lobe were then cut frontally or sagittally with a vibratome into 30- to 40-μm-thick sections. These were carefully rinsed in PBS and subsequently treated for fluorescence or peroxidase immunostaining.

Immunocytochemistry

The antibodies used included (i) mouse IgG monoclonal antibodies against B-50 (NM4, see 26) and GABA (Chemicon) and (ii) rabbit polyclonal antibodies (IgG) against tyrosine hydroxylase (Jacques Boy Laboratories, Reims, France), corticotropin releasing hormone (kindly provided by Pr. C. Oliver, Marseille, France), B-50, vasopressin, oxytocin, and somatostatin (raised in our laboratories). During the immunocytochemical procedures, all these antibodies were diluted from 1/1000 to 1/5000 in PBS at pH 7.4 containing 0.1% saponin and 1% normal goat serum. The specificity of the antibodies used has been described and documented elsewhere (see 1 for antioxytocin, antivasopressin, anticorticotropin releasing factor, and antisomatostatin; 26, 29 for monoclonal and polyclonal anti-B-50; the specificity of antibodies to tyrosine hydroxylase and GABA were guaranteed by commercial firms). The method controls consisted of omitting the primary antibodies or applying each primary antibody sequentially and then reacting them with the inappropriate secondary antibody.

Fluorescence Microscopy

The vibratome sections were treated either for single or for double immunofluorescence labelings by incubating them for 48 h at 4°C with the primary antibodies. For double immunofluorescence labeling, they were incubated with two primary antibodies including a mouse monoclonal antibody ascites and a rabbit polyclonal antibody. For each neurointermediate lobe treated, some sections were additionally incubated with the monoclonal B-50 antibody and a mixture including polyclonal antibodies to vasopressin and oxytocin. After rinsing in PBS, sections were incubated for 2 h at 4°C with either one (single labeling) or two (double labeling) secondary antibodies corresponding to the primary antibodies used (i.e., anti-rabbit and/or anti-mouse IgG), respectively, conjugated with rhodamine and with fluorescein. The secondary antibodies were diluted 1/200 in PBS containing 1% of normal goat serum and 0.1% saponin. After careful rinsing, sections were mounted in Mowiol (Calbiochem, La Jolla, CA) and observed under a MRC-600 confocal laser scanning microscope (Bio-Rad) equipped with a krypton/argon-mixed gas laser. Two laser lines emitting at 488 and 568 nm were used, respectively, for exciting the fluorescein- and the rhodamine-conjugated secondary antibodies, providing a minimum overlap of the emission spectra of the two fluorochromes. The background noise of each confocal image was reduced by averaging six image inputs. In some cases, the organization of immunostained structures was studied on reconstructed thick sections made by projecting *Z* series of 20 to 30 consecutive confocal images 1 μm apart, collected through the thickness of the vibratome section.

Electron Microscopy

Vibratome sections were successively incubated (i) for 48 h at 4°C with the polyclonal anti-B-50 antibody (diluted 1/1000), (ii) for 8 h at 4°C with a peroxidase-labeled Fab immunoglobulin fragment of goat anti-rabbit globulin (Biosys, Compiègne, France) diluted 1/1000, and (iii) with 0.1% 3,3′-diaminobenzidine diluted in 0.05% Tris buffer at pH 7.3, in the presence of 0.2% H_2O_2. After rinsing in 0.1 *M* cacodylate buffer at pH 7.3, sections were postfixed in 1% OsO_4 in the same buffer containing 1.25% potassium ferricyanide. They were then dehydrated in graded concentrations of ethanol and embedded in araldite. Punches of 1.5 mm in diameter were cut through the external layer of the median eminence proximal to the lesion. These punches were mounted on araldite blocks and cut into ultrathin sections that were counterstained with 5% uranyl acetate and observed in a EM 900 ZEISS electron microscope.

RESULTS

Confocal Microscopy

Single Immunostaining

Intact neurohypophysis. The organization of the different types of axonal fibers innervating the different

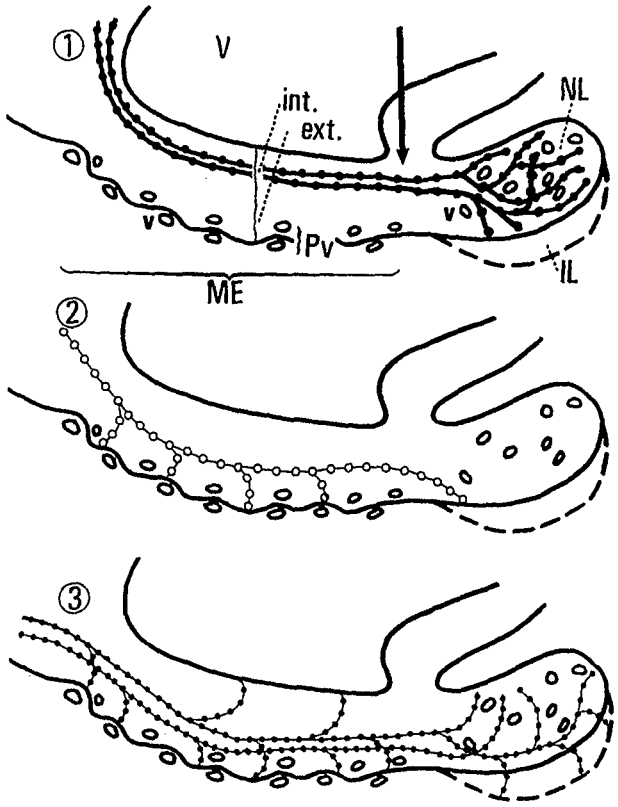


FIG. 1. Schematic representation of sagittal sections through the neurohypophysis showing the anatomical organization of the different types of neurohypophysial axons considered in the present study. (1) Vasopressinergic or oxytocinergic axons originating in the magnocellular neurons of the supraoptic and paraventricular nuclei; they form a dense pathway that run all along the internal layer of the median eminence and terminate around the blood vessels of the neural lobe. (2) Somatostatin- or corticotropin releasing hormone-IR axons, respectively, originating in parvicellular neurons of the periventricular and paraventricular nuclei; they project all along the external layer of the median eminence. (3) GABAergic and/or dopaminergic axons originating in parvicellular neurons of the arcuate nucleus; they densely project to the external layer of the median eminence and, to a lesser extent, to the neural and the intermediate hypophysial lobes. ext and int, external and internal layers, respectively, of the median eminence; IL, intermediate lobe of the hypophysis; ME, median eminence; NL, neural lobe of the hypophysis; Pv, perivascular space of the ME; v, blood vessels; V, third ventricle. Arrow indicates the location of the surgical lesion at the level of the hypophysial stalk.

portions of the neurohypophysis conformed to previous observations (represented in Fig. 1).

In agreement with previous observations (5, 9), strong B-50 immunostaining was detected throughout the hypothalamus. Remarkably, however, the use of either polyclonal or monoclonal anti-B-50 antibody always showed that B-50 immunostaining was much more intense throughout the neurohypophysis including the median eminence (Fig. 2) and the neurointermediate hypophysial lobe. The observation of thin optical sections clearly indicated that, in all these regions, B-50 immunostaining essentially was associated with fiber-like structures (Figs. 2–5). Moreover, after incubating vibratome sections with both the polyclonal and the monoclonal anti-B-50 antibodies for double immunofluorescence labeling, the observation of thin optical sections demonstrated that both immunostainings were always colocalized within such fibers throughout the neurohypophysis (not shown).

Lesioned neurohypophysis. As soon as 5 days after the transection of the hypophysial stalk, spectacular changes were observed in the anatomical organization of two types of neurohypophysial axons studied here. In agreement with previous studies (13, 27, 31), the more dramatic modifications concerned the organization of vasopressinergic and oxytocinergic axons throughout the median eminence rostral to the lesion. Indeed, whereas in the intact rats these axons were mostly localized to the internal median eminence layer (Fig. 6A), in lesioned animals numerous large, highly immunoreactive fibers were observed to extend either (i) ventrally toward the external layer from where they penetrate the perivascular spaces or (ii) dorsally toward the ependymal surface of the organ (Fig. 6B). Although less marked than for vasopressinergic and oxytocinergic fibers, lesioning the hypophysial stalk was also found to induce modifications of the anatomical organization of axons immunoreactive to tyrosine hydroxylase or to GABA. At the different postlesional times studied, numerous GABA- or tyrosine hydroxylase-IR axons were indeed observed to project toward the ependymal surface and throughout the perivascular space of lesioned median eminences (Figs. 6C and 6D). In all the lesioned neurohypophysis examined, no marked modifications were observed concerning the anatomical organization of peptidergic axons immunoreactive to corticotropin-releasing hormone or to somatostatin that project throughout the external layer of the median eminence proximal to the lesion.

Whatever the postsurgical delay, the lesioned median eminences were found to contain numerous, intensely immunostained B-50-IR fibers that, similar to the intact median eminence, were detected throughout the different layers of the organ. Due to the dense network formed by these fibers, it was difficult to determine any changes in terms of immunostaining intensity or number of fibers immunostained as compared to the intact immunostaining intensity or number of fibers immunostained as compared to the intact median eminence. However, in the caudal portions of the organ (i.e., those located close to the lesion), numerous B-50-IR fibers were found to project through the perivascular space or to run along the ependymal surface (Fig. 7), two locations in which they were rarely observed in the intact median eminence.

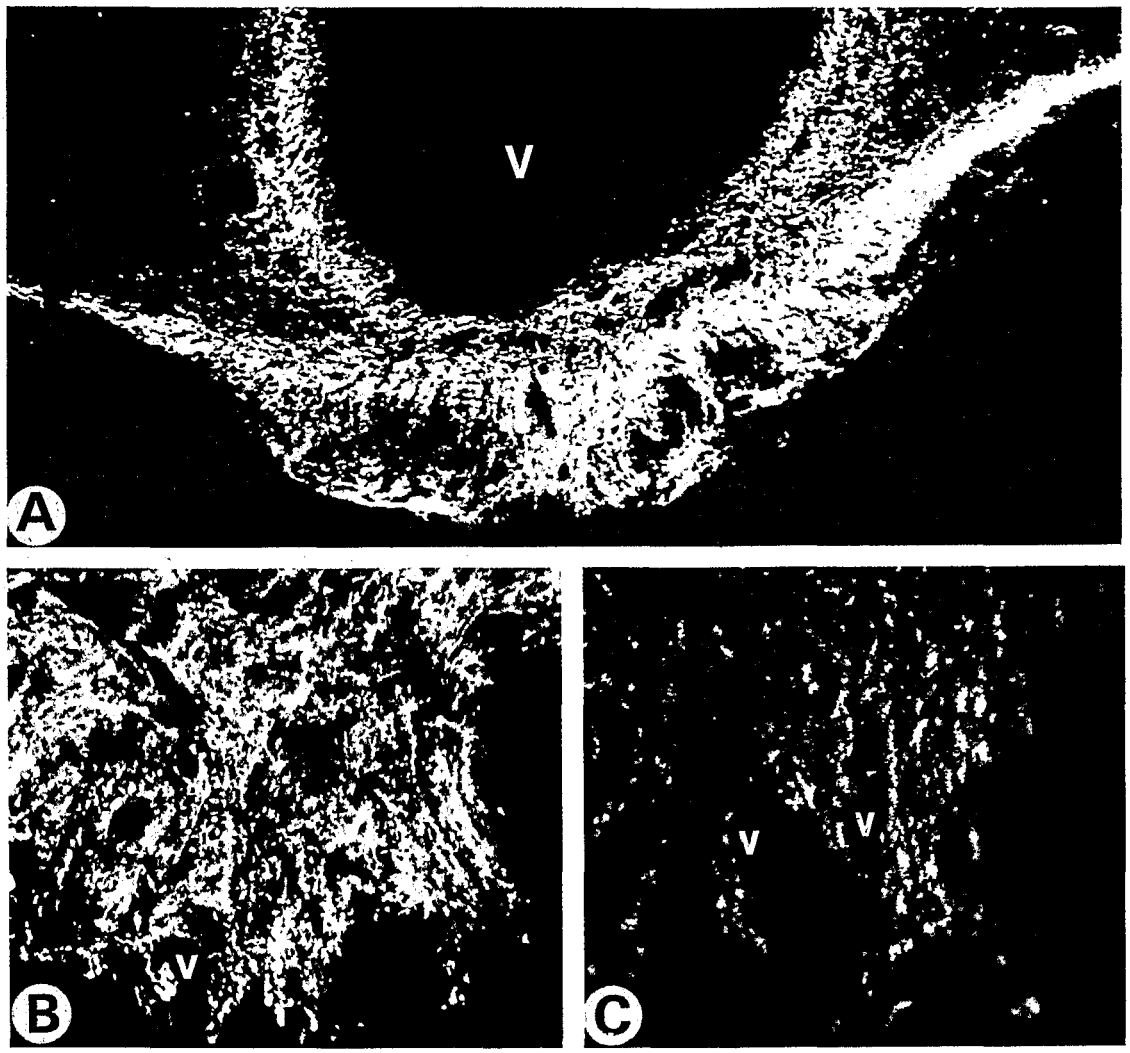


FIG. 2. Intact neurohypophysis. Confocal images of frontal sections through the median eminence immunostained for B-50. (A) Low magnification (objective 20× with a numerical aperture of 0.5) showing the distribution of intense B-50 immunostaining along the periventricular hypothalamus and throughout the different layers of the median eminence. (B and C) Higher magnifications obtained by using objective lens with increasing numerical aperture (objective 40× with a numerical aperture of 1.0 for B, objective 60× with a numerical aperture of 1.4 for C), providing optical sections of decreasing thickness. (C) Clearly shows that B-50 immunostaining is mostly localized within fibers-like structures located in the external layer of the median eminence. V, third ventricle; v, blood vessel. A, ×200; B and B′, ×400; C and C′, 600.

Double Immunostaining for B-50 and for Tyrosine Hydroxylase or GABA

Intact neurohypophysis. Throughout the different layers of the median eminence, but more frequently in the external layer, B-50-IR was frequently associated with fibers immunoreactive to tyrosine hydroxylase or to GABA (Figs. 3A and 3A′; 3B and 3B′). Such colocalizations were also observed throughout the neural lobe (Figs 4A and 4A′; 4B and 4B′). In these two regions, however, some B-50-IR fibers were found to be tyrosine hydroxylase- or GABA-negative. Remarkably, in the intermediate lobe, B-50-IR was systematically colocalized with tyrosine hydroxylase- or GABA-IR within large radiating fibers dispersed throughout the organ (Figs. 5A and 5A′; 5B and 5B′). Double immunostaining for tyrosine hydroxylase and GABA further indicated that both labelings were colocalized in the majority of, but not all, the fibers innervating the median eminence and the neural lobe (not shown), whereas such colocalizations were systematic within fibers innervating the intermediate lobe (Figs. 5C and 5C′).

Lesioned neurohypophysis. As observed in the nonlesioned median eminence, a majority of the B-50-IR fibers detected in the different layers of the lesioned median eminence was also immunoreactive to tyrosine hydroxylase or to GABA. Such colocalizations were also observed for those B-50-IR fibers projecting to the ependymal surface (not shown) or the perivascular

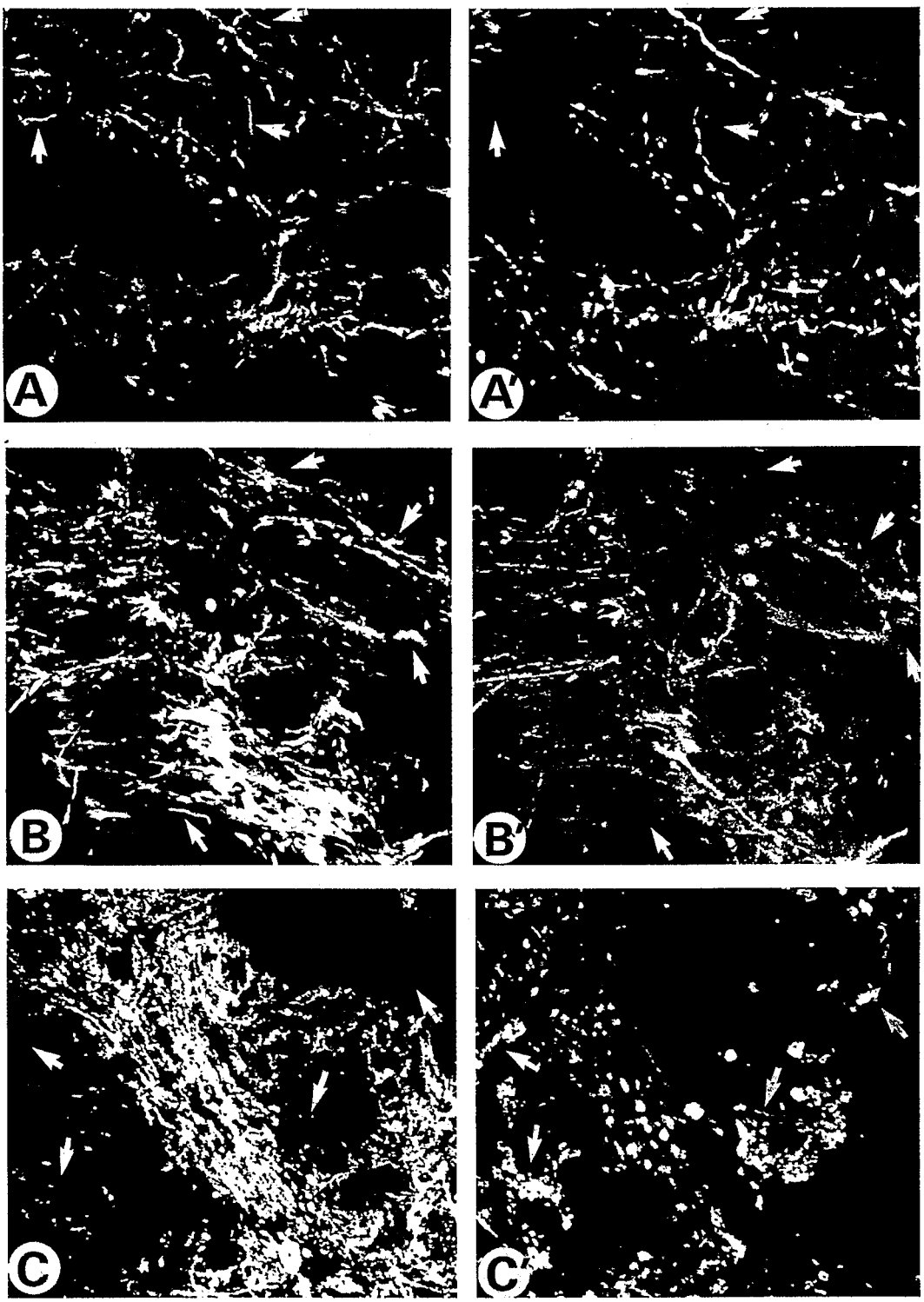


FIG. 3. Intact neurohypophysis. Confocal paired images of sections through the median eminence immunostained for B-50 (A–C) and for tyrosine hydroxylase (A'), GABA (B'), and corticotropin releasing factor (C'). Thin optical sections showing that throughout the external layer the majority of the fibers immunoreactive to B-50 also exhibit immunoreactivity to tyrosine hydroxylase (A, A') or to GABA (B, B'), although some B-50-IR fibers appear tyrosine hydroxylase-negative (arrows, A, A') or GABA-negative (arrows, B, B'). On the other hand, most corticotropin releasing hormone-IR fibers appear B-50-negative (arrows, C, C'). A–C, ×600.

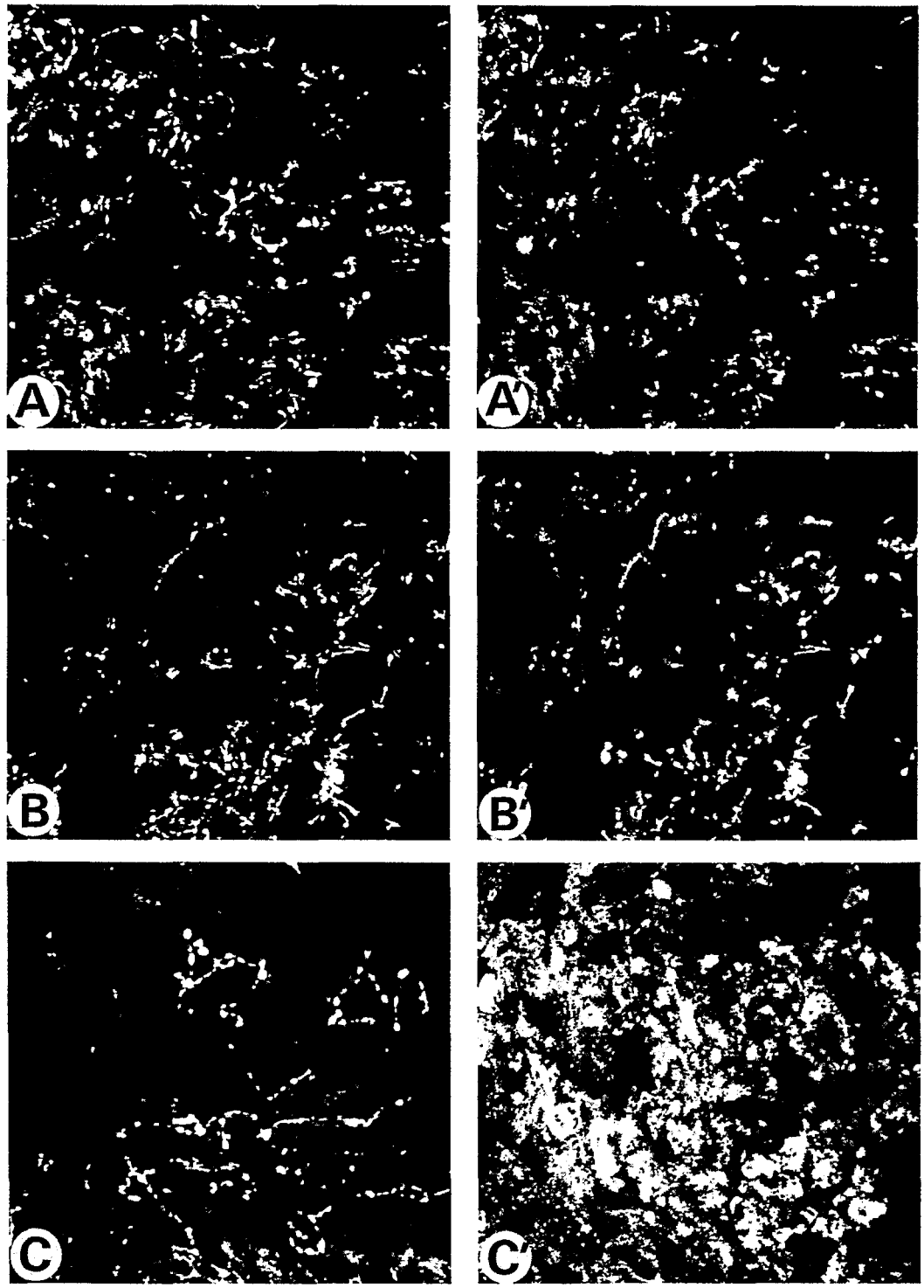


FIG. 4. Intact neurohypophysis. Confocal paired images of sections through the neural lobe immunostained for B-50 (A–C) and for tyrosine hydroxylase (A'), GABA (B'), or vasopressin + oxytocin (C'). Thin optical sections showing that B-50-IR is associated with thin fibers that are also immunoreactive to tyrosine hydroxylase (A, A') and to GABA (B, B'), but are clearly distinct from the large axonal profiles immunoreactive to vasopressin and oxytocin (C, C'). A–C, ×600.

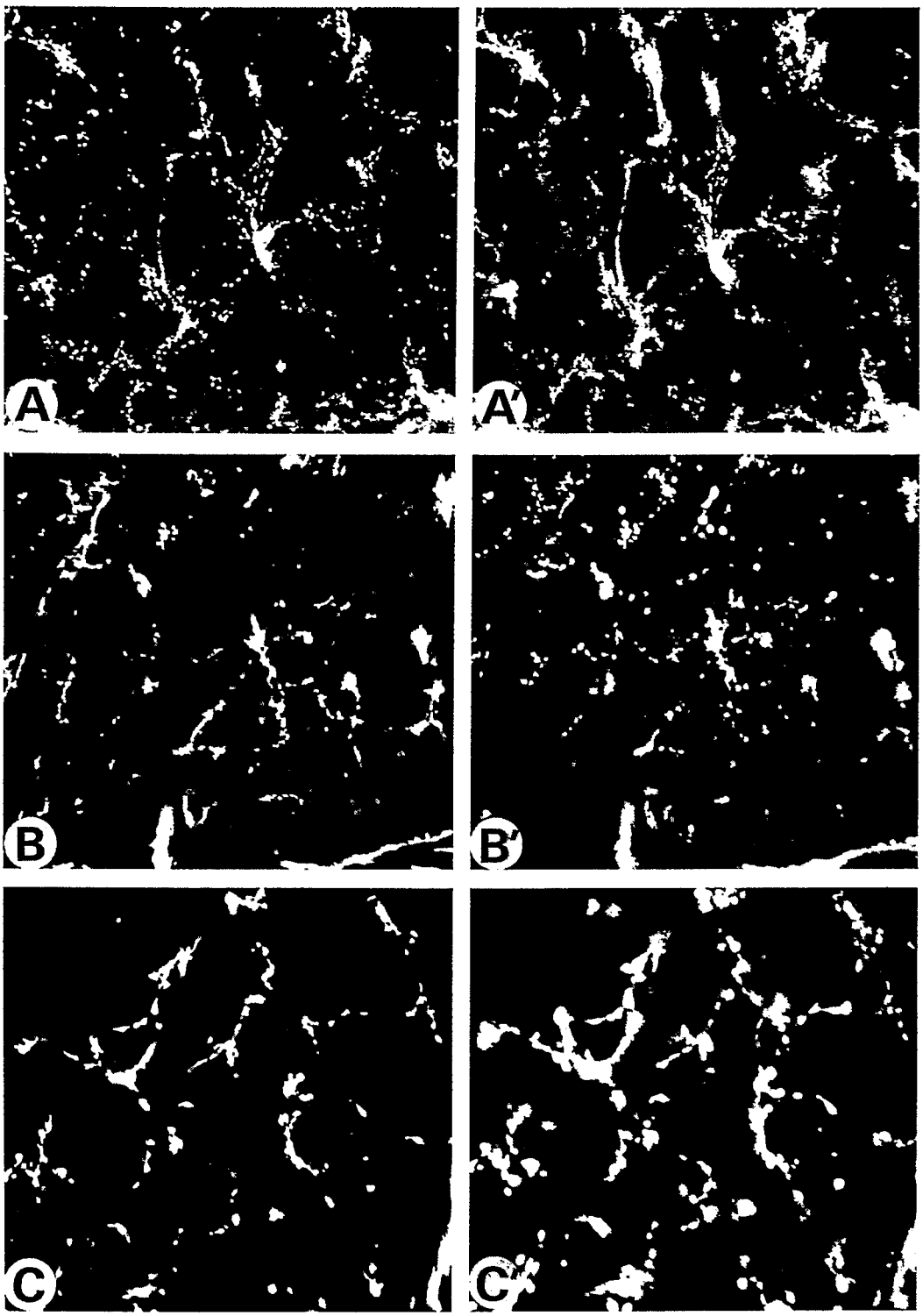
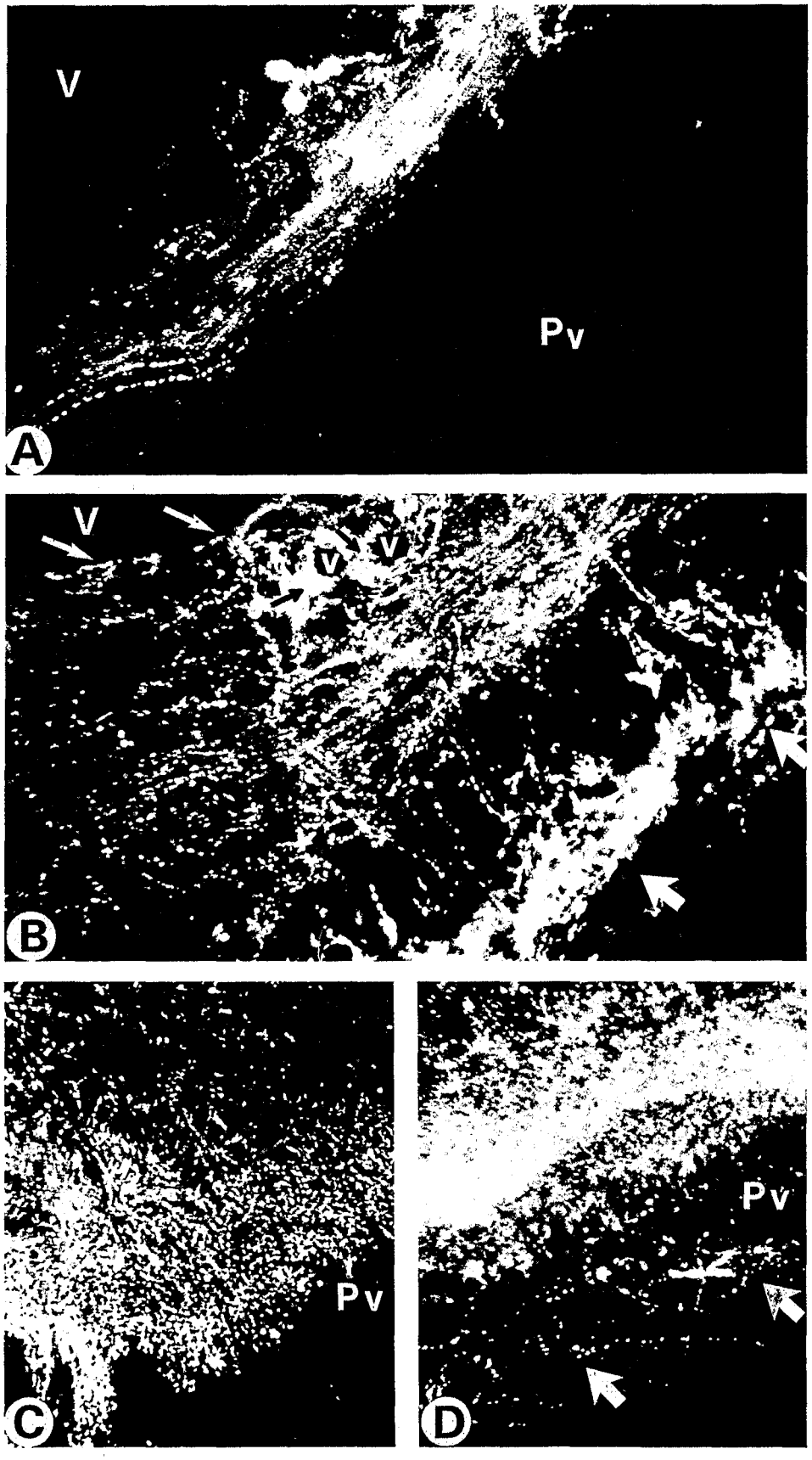


FIG. 5. Intact neurohypophysis. Confocal paired images of sections through the intermediate lobe immunostained for B-50 (A, B) and for tyrosine hydroxylase (A') or GABA (B') and for GABA (C) and tyrosine hydroxylase (C'). Thin optical sections showing that B-50-IR is systematically associated with fibers immunoreactive to tyrosine hydroxylase (A, A') or to GABA (B, B'). Similarly, the fibers innervating this organ are systematically immunoreactive to both GABA and tyrosine hydroxylase (C, C'). A–C, ×600.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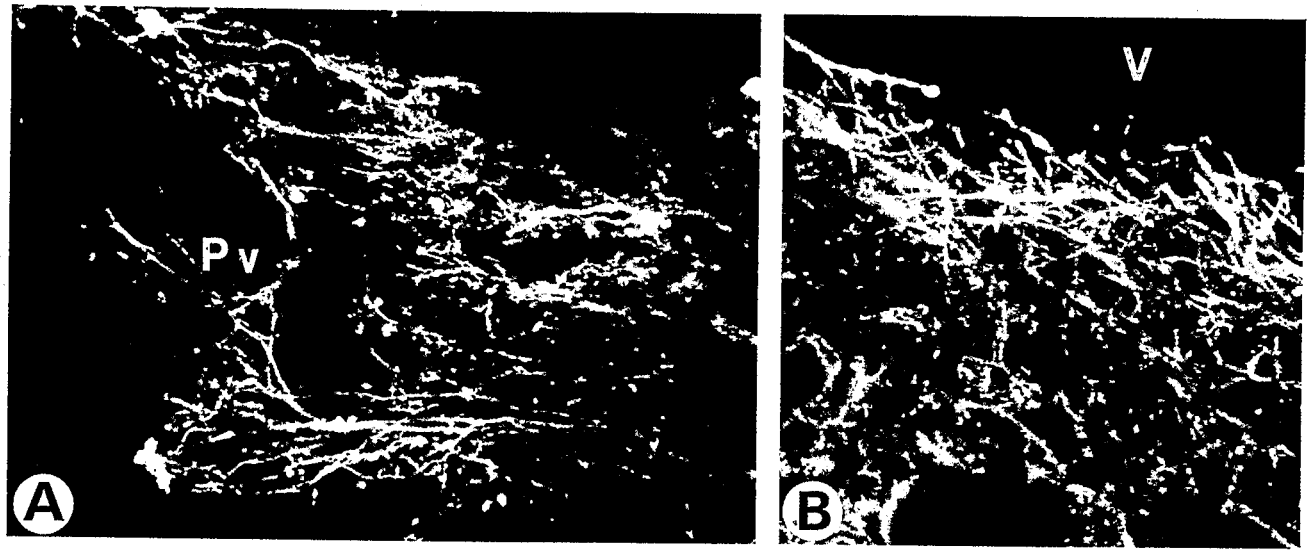

FIG. 7. Lesioned neurohypophysis, 10 days postlesioning. *Z* series projections of 20 consecutive confocal images of sagittal sections through the median eminence immunostained for B-50. Intense B-50-IR is associated with fiber-like structures that project both throughout the perivascular space bordering the median eminence (A) and along the ventricular surface of the organ (B). Pv, perivascular space; V, third ventricle; A and B, ×400.

space of the lesioned median eminences (Figs. 8A and 8A′; 8B and 8B′). In these regions, however, a number of B-50-IR fibers always appeared tyrosine hydroxylase- or GABA-negative. Double immunostaining for GABA and tyrosine hydroxylase further indicated that, as in the unlesioned median eminence, both neuronal markers were colocalized in a majority of, but not all, these fibers (not shown).

Double Immunostaining for B-50 and for Neuropeptides

Intact neurohypophysis. All along the rostro-caudal extension of the median eminence, B-50-IR fibers were frequently located in regions containing high concentrations of fibers immunoreactive to corticotropin releasing factor or somatostatin. However, although both types of immunostained fibers frequently appeared tightly intermingled, the observation of thin optical sections generally failed to indicate an association between B-50 and any of the peptidergic fibers (Figs. 3C and 3C′). Within the neural lobe, no colocalizations could be detected between B-50-IR, generally associated with thin elongated fibers, and vasopressin- or oxytocin-IR, generally associated with large axonal dilatations (Figs. 4C and 4C′).

Lesioned neurohypophysis. Whatever the postlesional delay, the observation of thin optical sections through the lesioned median eminences indicated that B-50-IR was not associated with the numerous vasopressinergic or oxytocinergic fibers detected on the ependymal surface or throughout the perivascular space proximal to the lesion, although both types of immunostained fibers frequently appear closely intermingled (Figs. 8C and 8C′).

Electron Microscopy

In this part of the study, the observations have been focused on the external layer and the perivascular space bordering of the median eminence. Indeed, the light microscopic observations clearly indicated that these regions contained the highest concentrations in B-50-IR fibers, in both the intact and lesioned neurohypophysis. Moreover, a large majority of regenerating vasopressinergic or oxytocinergic axons was found to project into this region of the organ. Simple morphological criteria based on the granular content of axonal profiles were

FIG. 6. *Z* series projections of 20 consecutive confocal images of sagittal sections through unlesioned (A and C) and lesioned (B and D) median eminences immunostained for oxytocin (A and B) or tyrosine hydroxylase (C and D). In an unlesioned median eminence, fibers immunoreactive to oxytocin are restricted to the internal layer where they form a dense bundle running parallel to the ventricular surface (A), whereas fibers immunoreactive to tyrosine hydroxylase densely terminate along the external layer of the organ (C). Ten days after hypophysial stalk transection, numerous regenerating fibers immunoreactive to oxytocin or to tyrosine hydroxylase are present throughout the perivascular space bordering the external layer (large arrows B and D). Note that oxytocin-IR regenerating fibers are also detected around blood vessels (v) within the internal layer and at the surface of the ventricular border of the internal layer (small arrows B). Pv, perivascular space; v, blood vessels; V, third ventricle. A–D, ×590.

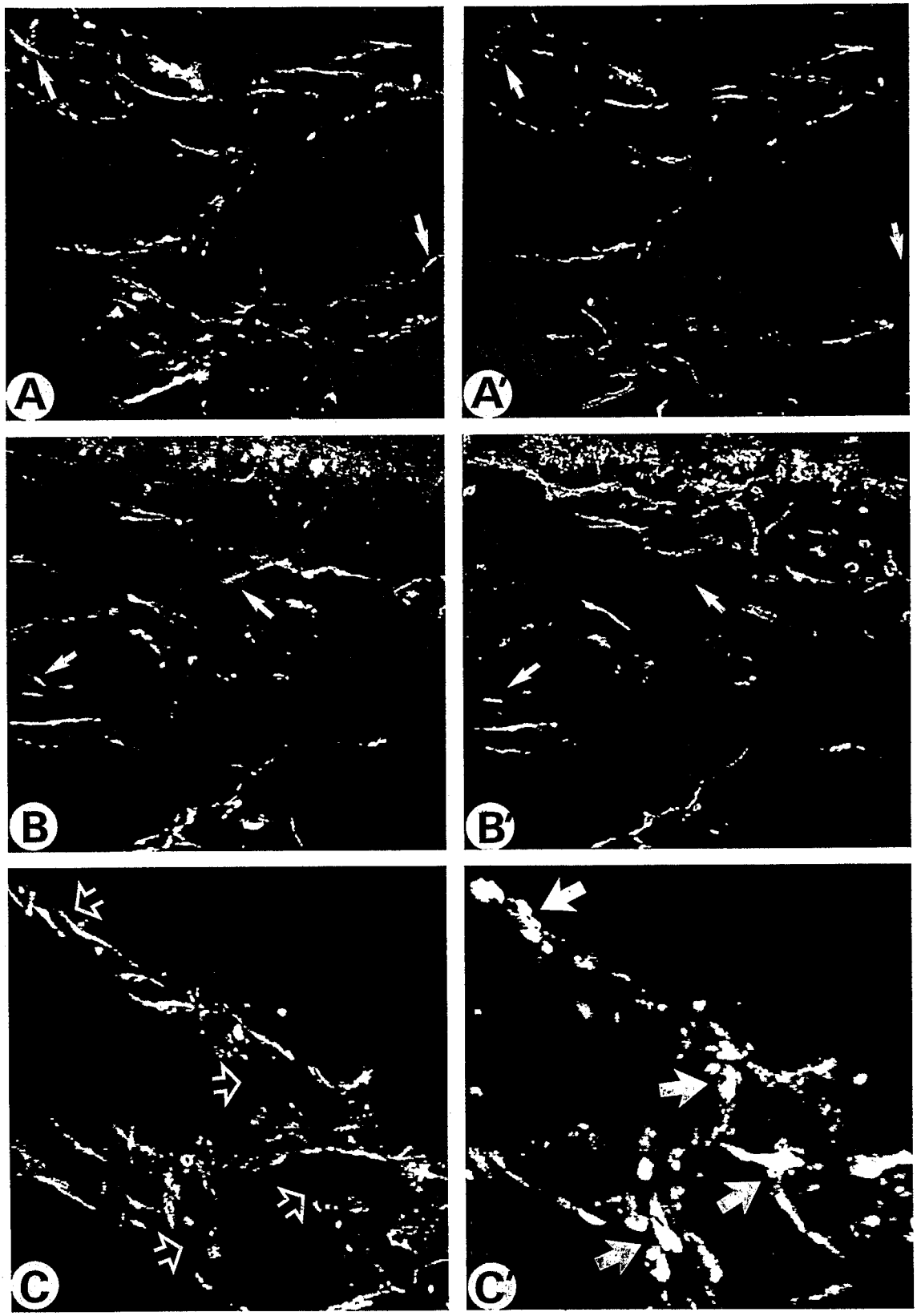

FIG. 8. Lesioned neurohypophysis, 10 days postlesioning. Confocal paired images of sections through the median eminence immunostained for B-50 (A–C) and for tyrosine hydroxylase (A'), GABA (B'), or oxytocin (C'). Thin optical sections showing that the majority of the thin elongated B-50-IR fibers projecting throughout the perivascular space are also immunoreactive to tyrosine hydroxylase (A, A') or to GABA (B, B'). On the other hand, the large varicose oxytocinergic fibers present in this region (arrows, C') are deprived of B50-IR (open arrows, C). Note that as in the external layer of intact median eminences (Fig. 3), a number of B-50-IR fibers projecting through the perivascular space appear tyrosine hydroxylase- or GABA-negative (arrows, A, A'; B, B'). A–C, ×600.

used to tentatively identify the different types of neurohypophysial axons observed (23): (i) the axonal profiles containing large dense granular vesicles of around 200 nm in diameter were identified as vasopressinergic or oxytocinergic axons originating in magnocellular hypothalamic neurons, (ii) axonal profiles containing numerous granular vesicles with smaller diameters varying between 80 and 120 nm were identified as peptidergic axons originating in various parvocellular hypothalamic neurons, and (iii) axonal profiles containing scarce dense vesicles of small diameter were identified as nonpeptidergic axons.

Unlesioned Neurohypophysis

In all the intact median eminence examined, intense B-50 immunostaining was associated with small diameter axonal profiles located throughout the external layer of the organ and containing scarce if any granular vesicles (Fig. 9A). Within these axons, B-50 immunostaining appeared dispersed within axonal profiles containing numerous, densely packed synaptic-like vesicles. On the other hand, putative peptidergic axons containing numerous granular vesicles of small size, as well as glial processes extending between the axonal profiles appeared deprived of B-50 immunostaining. In all the intact median eminences examined, vasopressinergic or oxytocinergic axons containing large granular vesicles could be detected in the internal, subependymal layer, but not in the external layer.

Lesioned Neurohypophysis

In the lesioned median eminences examined, B-50-immunostained axons were detected both within the external layer and throughout the adjacent perivascular space (Figs. 9B and 9C). As in intact median eminences these labeled axonal profiles contained numerous synaptic-like vesicles and only scarce granular vesicle of small size. In contrast with the intact median eminences, however, numerous vasopressinergic or oxytocinergic axonal profiles containing large granular vesicles were observed all along the external layer and throughout the perivascular space (Figs. 9B and 9C). They always appeared deprived of B-50 immunostaining. Throughout these regions, no B-50 immunoreactivity could be associated with the nonneuronal structures, including glial cells, vascular endothelial cells, and fibroblasts.

DISCUSSION

Methodological Considerations

During past years, combined immunocytochemical labeling techniques have been extensively used for characterizing the colocalization of various neuronal markers (including transmitters, neuropeptides, and enzymes) within the same neuronal systems (for review see 20). The more critical requirements of these techniques are the absence of cross-reaction between the different immunostainings and their clear discrimination. In the present study, such conditions were insured by using (i) two primary antibodies raised in two different species (mouse and rabbit) and (ii) a confocal microscope equipped with a krypton/argon-mixed gas laser providing two laser lines at 488 and 568 nm that unambiguously discriminates the two secondary antibodies respectively labeled by fluoresceine and rhodamine. Moreover, the use of a laser scanning confocal microscope allows the simultaneous observation of both immunostainings on 30- to 40-μm-thick immunostained vibratome sections. A first advantage of this approach concerns the absence of freezing of tissue for cryostat sectioning that may alter the cellular morphology. A second advantage of confocal microscopy is the considerable improvement of the optical resolution both in the lateral dimension and in depth. The confocal effect and the degree of optical resolution are known to be mainly determined by the quality and the numerical aperture of the microscope objective (39). The use of a lens objective with high numerical aperture (objective 60× with numerical aperture 1.4) allowed us to study the localization of the two immunofluorescent labelings on single optical sections of less than 1 μm thick. This obviously greatly diminishes the risk of superimposition artifacts that may occur when studying the colocalization of two immunocytochemical markers within axons, especially in the median eminence which contains a large variety of closely intermingled axons. On the other hand, differential penetrations of the various types of immunostaining within the thickness of vibratome sections may represent a major drawback of the approach used here, giving rise to false negative labeling artifacts (30). Immunostaining penetrations could, however, easily be controlled under the confocal microscope, by scanning through the successive vertical planes. This showed that, under the conditions used here, all the IgG antibodies penetrate the whole thickness of the vibratome sections.

Identity of the Neurohypophysial Axons Exhibiting B-50 Immunoreactivity

One of the more striking observations of the present study is that a majority of B-50-IR fibers detected throughout the intact and lesioned neurohypophysis was also immunoreactive to tyrosine hydroxylase or to GABA. It is known from previous neuroanatomical studies that the median eminence and neurointermediate lobe are densely innervated by fibers immunoreactive to both GABA and tyrosine hydroxylase (25, 35, 39) that originate in GABAergic/dopaminergic neurons located in the dorsomedial and periventricular subdivisions of the arcuate nucleus (7, 25). Interestingly, the present data clearly indicate that, within the intermedi-

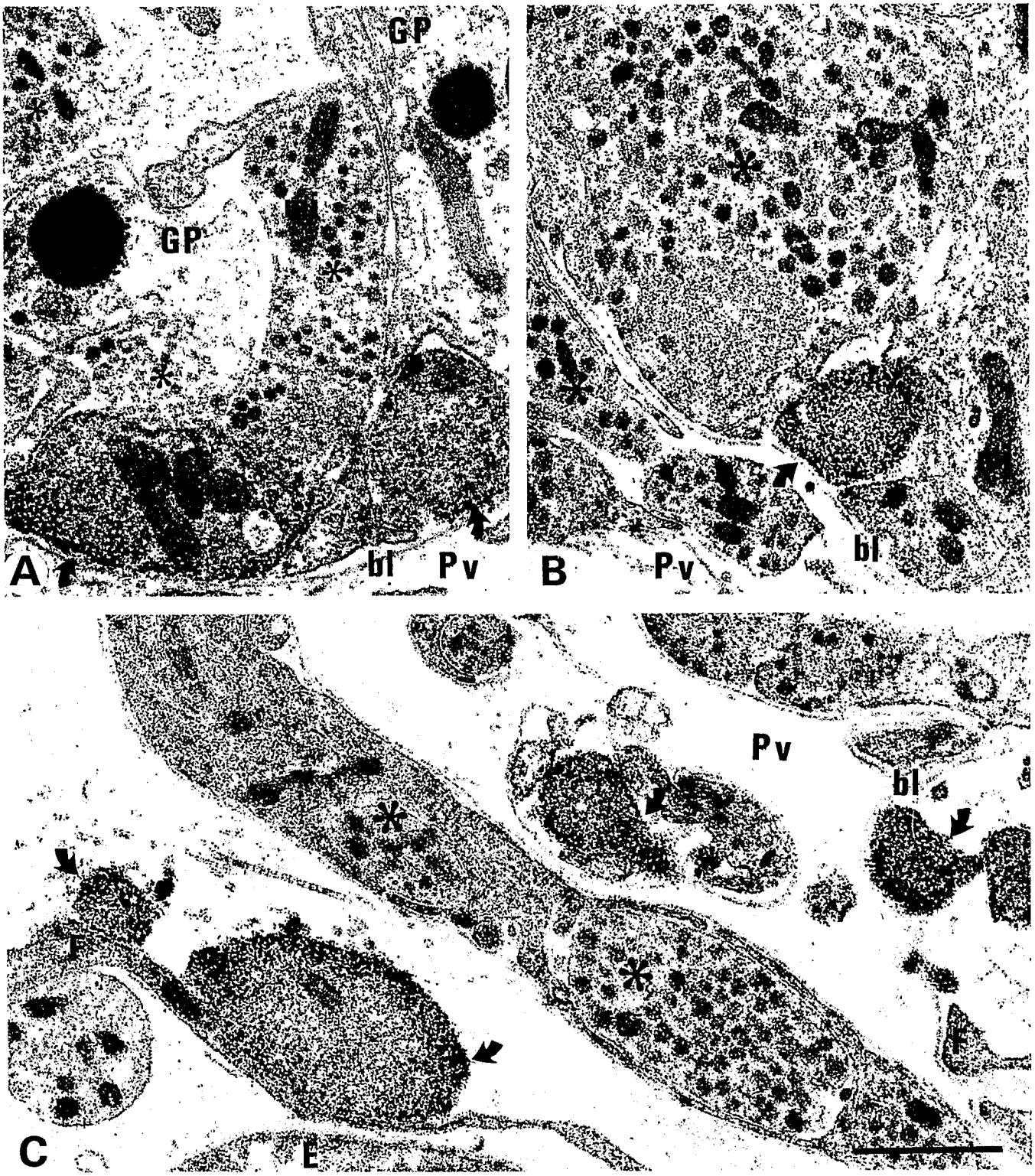

FIG. 9. Electron micrographs of sections through median eminences immunostained for B-50. (A) Intact neurohypophysis. B-50 immunostaining is associated with small axonal profiles terminating along the external layer and containing numerous, densely packed synaptic-like vesicles (arrows). On the other hand, B-50 immunostaining is absent from both putative peptidergic axonal profiles containing numerous small granular vesicles (80–100 nm in diameter, small asterisks) and glial processes (GP) containing lipid droplets. (B and C) Lesioned neurohypophysis, 10 days postlesioning. Within the external layer (B) and throughout the perivascular space (C), B-50 immunostaining is associated with axonal profiles that are devoid of granular vesicles (arrows). In both regions, no immunostaining product can be detected within axonal profiles containing large secretory granules (150–200 nm in diameter) that can be identified as regenerating vasopressinergic or oxytocinergic neurohypophysial axons (large asterisks). bl, basal lamina; F, fibroblast; E, endothelial vascular cell; GP, glial process; L, lipid droplet; mi, mitochondria; Pv, perivascular space. A–C, ×25,000. Bar, 1 μm.

ate lobe, B-50 was systematically associated with fibers immunoreactive to GABA or tyrosine hydroxylase. Since it is known that GABA and tyrosine hydroxylase are constantly colocalized within fibers innervating the intermediate lobe (39 and present data, Figs. 5C and 5C'), it can be assumed that the large majority of fibers double-labeled for B-50 and for GABA or tyrosine hydroxylase detected in the neurohypophysis corresponds to GABAergic/dopaminergic axons originating in neurons of the mediobasal hypothalamus. As compared to the vasopressinergic or oxytocinergic axons, only scarce information is provided on the capacity of these axons to regenerate after a lesion of the neurohypophysis. It was clearly shown here that their anatomical organization was modified all along the median eminence proximal to the lesion. Namely, numerous axonal fibers immunoreactive to GABA or to tyrosine hydroxylase were detected in the perivascular space and along the ventricular surface, two locations in which they were rarely observed in the intact median eminence. It is thus likely that such fibers represent regenerative sprouts of axons originating in the GABAergic/dopaminergic neurons of the dorsomedial and periventricular subdivisions of the arcuate nucleus that innervate the neurointermediate lobe. From our observations, it can be admitted that most of these regenerating axonal fibers do contain high amounts of B-50. The present data also demonstrate that in the intact as well as in the lesioned neurohypophysis, intense B-50-IR is associated with a number of fibers that appear tyrosine hydroxylase- or GABA-negative. At the moment, the nature of these fibers remains obscure, since besides the GABAergic/dopaminergic fibers, the neurohypophysis knowingly contains a large variety of monoaminergic (namely histaminergic and serotonergic) or peptidergic axonal fibers. In this regard, however, the present data indicate that B-50-IR could not be associated with various peptidergic fibers detected in the intact neurohypophysis. Namely, B-50-IR was not detected both within the axons immunoreactive to somatostatin or to corticotropin releasing hormone that project all along the external median eminence and within vasopressinergic or oxytocinergic axons that project within the neural lobe. This indicates that under basal conditions, peptidergic neurons innervating the neurohypophysis express very low if any B-50. A more surprising finding was, however, that B-50 could not even be detected within the numerous regenerating vasopressinergic or oxytocinergic axons that sprout throughout the lesioned neurohypophysis. The possibility that these regenerating fibers contain a slightly modified B-50 molecule appears unlikely since they could not be labeled by neither of the monoclonal and polyclonal anti-B-50 antibodies used here. It can thus be assumed that in these specific hypothalamo-neurohypophysial neurons, such a very active axonal regeneration is not associated with the reexpression of B-50.

The Functional Significance of B-50 in Neurohypophysial Axons

Since it is generally admitted that B-50 is involved in the mechanisms of axonal growth occurring during axonal sprouting or axonal regeneration (4, 5, 9, 15, 22, 41), it could reasonably be assumed that the occurrence of high levels of this embryonic marker within neurohypophysial axons of the adult rat was related to their capacity for regeneration. This idea is supported here by the detection of intense B-50-IR within a number of axonal sprouts projecting through the perivascular space or running at the surface of the ventricular wall. However, the present finding that this protein is not detectable within the numerous vasopressinergic or oxytocinergic axonal sprouts that very actively regenerate through the external layer and the perivascular space of the lesioned median eminence implies that in the adult rat, B-50 is not essential for the postlesional regeneration of these peptidergic neurohypophysial axons. Kruger *et al.* (24) have reported that in the brain of the adult rat, the supraoptic nucleus that essentially contains vasopressinergic and oxytocinergic cell bodies projecting to the neurohypophysis, does not reveal any expression of B-50 mRNA. Recently, Reh *et al.* (32) reported that in the retina, neurons develop without hardly expressing B-50. It would therefore be of interest to find out if the vasopressinergic and oxytocinergic neurons differentiate without expressing B-50. It has been recently reported that neurons and glial cells of the hypothalamo-neurohypophysial system continue to express high levels of PSA-NCAM throughout adulthood (8, 21, 38). Since it is generally admitted that membranes containing large amounts of PSA-NCAM favor the moving of cellular structures (19, 33, 34), it could be assumed that this embryonic molecule plays a role in the mechanisms of postlesional regeneration developed by these specific neurohypophysial axons.

The present findings clearly indicate that in the lesioned median eminence, B-50 is essentially associated with the same axon types as in the intact median eminence, i.e., the GABA- and tyrosine hydroxylase-IR axons innervating the median eminence and the neurointermediate lobe. Besides the role in axonal growth, other roles have been suggested for B-50 (14, 37). Particularly, for adult neurons it has been proposed that B-50 is involved in the mechanism of neurotransmitter release (10–12, 16–18). Moreover, recent studies have provided evidence that central monoaminergic neurons of adult rat always exhibit high expression of B-50 mRNA (3, 42). Besides a role in the mechanisms of postlesional regeneration, such a role can thus be considered for the high levels of B-50 detected in the GABAergic/dopaminergic fibers innervating the neurohypophysis of adult rats.

ACKNOWLEDGMENTS

This work was supported by IRME. Confocal microscopy has been realized using the facilities of C.R.I.C. The authors thank Dr. Merken and Innogenetic (Ghent, Belgium) for providing the monoclonal B-50 antibody (NM4). They also thank A. Legrand for her excellent technical assistance.

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