EXPERIMENTAL NEUROLOGY 134, 35–48 (1995)

B-50 (GAP-43) Immunoreactivity Is Rarely Detected within Intact Catecholaminergic and Serotonergic Axons Innervating the Brain and Spinal Cord of the Adult Rat, but Is Associated with These Axons Following Lesion

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The persistence of high levels of B-50 (GAP-43) in fibers innervating various regions of the adult central nervous system is generally thought to characterize neuronal systems capable of undergoing morphological plasticity. In a recent series of *in situ* hybridization studies, it has been shown that most catecholaminergic and serotonergic neurons of the adult rat brain express high levels of B-50 mRNA. The present study addresses the question whether high expression of B-50 mRNA in the catecholaminergic and serotonergic perikarya corresponds with detectable high levels of the B-50 protein in the efferent axonal fibers that innervate various regions of the adult rat brain and spinal cord. For this purpose, vibratome sections were doubly immunostained for B-50 and for tyrosine hydroxylase or serotonin and were analyzed by laser scanning confocal microscope. Colocalizations were investigated either (1) in regions of intact rat brain and spinal cord in which particular concentrations of B-50 immunoreactive fibers appeared codistributed with catecholaminergic or serotonergic fibers or (2) in intrahypothalamic portions of the medial forebrain bundle in which a surgical lesion was made. In the intact brain, frequent colocalizations of B-50 and tyrosine hydroxylase were detected in fibers innervating both the mediobasal hypothalamus and the neurointermediate hypophysial lobe. In all the other regions examined, the analysis of thin optical sections demonstrated that immunoreactivity to B-50 was only rarely associated with axonal profiles immunoreactive to tyrosine hydroxylase or to serotonin. By contrast, in the lesioned medial forebrain bundle B-50 immunoreactivity was found to be associated with numerous catecholaminergic and serotonergic axonal sprouts that regenerate around the surgical lesion. These data indicate that the majority of intact catecholaminergic and serotonergic axons innervating the adult rat brain and spinal cord contains low levels of B-50. However, following axotomy, B-50 is immunocytochemically detectable in the regenerating sprouts produced by both types of axonal fibers. This suggests that under basal conditions the relatively high content of B-50 mRNA in monoaminergic perikarya does not lead to appreciable accumulation of B-50 within corresponding axonal fibers and terminals, whereas conditions of morphological reorganization induce increased production of B-50 that accumulates within monoaminergic axonal sprouts. © 1995 Academic Press, Inc.

INTRODUCTION

The protein B-50 was originally described as a phosphoprotein located in the presynaptic membrane of adult rat brain (24, 65). B-50 is a prominent substrate for protein kinase C which modulates several second messenger systems: it affects the formation of phosphatidylinositol biphosphate from which the second messenger inositol triphosphate is generated (24); it is able to bind to calmodulin (1) and it enhances the binding of GTP to G-proteins (55). The identical protein GAP-43 was described as a growth-associated protein synthesized at strikingly high levels during neurite outgrowth (52).

In early postnatal brain and in neuronal tissue culture, B-50 (GAP-43) is abundantly localized in all neural growth cones (19, 37). It is thought that this protein plays a role in neurite outgrowth and synaptogenesis, by translating extracellular signals to the growth cone (25, 47). In adult brain in which synaptogenesis is completed and axonal elongation no longer occurs, significant amounts of the mRNA and protein continue to be expressed by specific neuronal systems. Although the reason why certain adult neurons continue to express high levels of B-50 is unknown, it has been hypothesized that such neurons may maintain a capacity throughout life for plasticity of axonal connections and axonal growth. Indeed, high levels of B-50 have been detected in areas of the adult brain thought to exhibit a high degree of synaptic plasticity, i.e., the olfactory bulb, the hippocampus, and the neostriatum (8, 41, 44, 48, 61). Moreover, B-50 has been shown to

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play a role in presynaptic signal transduction and neurotransmitter release (20–22). Lastly, a striking correlation has been found between increased synthesis and transport of B-50 following axotomy of peripheral and central neurons and the ability of these neurons to regenerate (16, 53, 57, 62).

The nature of the central neurons that continue to express high levels of B-50 during adulthood is largely unknown. From the anatomical organization of these neurons delineated in earlier immunocytochemical and *in situ* hybridization studies (8, 33, 36, 44, 49, 64), it can be assumed that some of them utilize glutamate as a neurotransmitter. More recently, a series of studies has strongly suggested a relation between B-50 and monoamines. Indeed, from *in situ* hybridization studies, it has been established that, in the adult rat brain, most neurons located within noradrenergic, adrenergic, serotonergic, and caudal dopaminergic nuclei exhibit a relatively high B-50 mRNA content (7, 13, 33, 64). Moreover, it has been shown that in the hippocampus, intra-axonal B-50 is related to the regulation of noradrenaline release (20–22, 28–30), and that antisense B-50 mRNA blocks dopamine release from PC12 cells (32). It is thus reasonable to assume that B-50 detected in the adult rat brain is preferentially associated with monoaminergic fibers in which it may play a role in neurotransmitter release or in some form of axonal plasticity.

Using immunocytochemistry, the protein B-50 has only been detected within axonal fibers and terminals, and rarely in the protein-synthesizing cell bodies. Until now, attempts to identify those axonal fibers exhibiting high levels of B-50 immunoreactivity (B-50-IR) are limited to the spinal cord of the adult rat and monkey in which B-50-IR has been reported to be localized within some serotonergic terminals (5, 12). Although axonal B-50-IR fibers and terminals have been shown to be distributed in various regions of the adult central nervous system that are densely innervated by catecholaminergic and/or serotonergic fibers, the immunocytochemical characterization of these B-50-IR fibers has not been carried out to our knowledge.

In the present study, we have used confocal microscopy combined with double fluorescence immunostaining of B-50 and of immunocytochemical markers of either catecholaminergic or serotonergic neurons. The possible association of B-50-IR with monoaminergic fibers was examined (1) in various regions of the intact brain and spinal cord that receive a dense innervation from those monoaminergic neurons that have been shown to express relatively high levels of B-50 mRNA (i.e., noradrenergic and/or adrenergic neurons of the locus coeruleus and/or the medulla oblongata, dopaminergic neurons of the substantia nigra, and serotonergic neurons of the raphe nuclei) and (2) in regions surrounding a surgical lesion placed through the medial forebrain bundle, which contains most catecholaminergic and serotonergic axonal fibers afferent to the forebrain (15, 40).

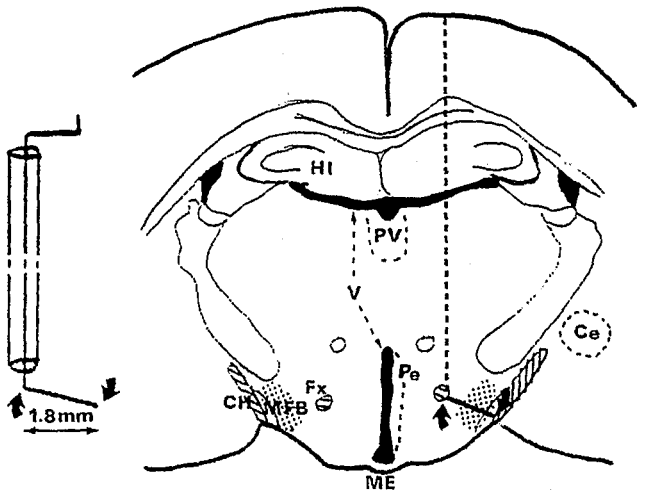


FIG. 1. Schematic representation of the knife used and of the surgical cut through the medial forebrain bundle (arrows) in a frontal plane including the lesion produced by lowering the knife (dotted vertical line) and containing some forebrain regions that have been investigated. Ce, central nucleus of the amygdala; CH, optic chiasma; Fx, fornix; HI, hippocampus; ME, median eminence; MFB, medial forebrain bundle; Pe, periventricular nucleus of the hypothalamus; PV, paraventricular nucleus of the thalamus; V, third ventricle.

MATERIAL AND METHODS

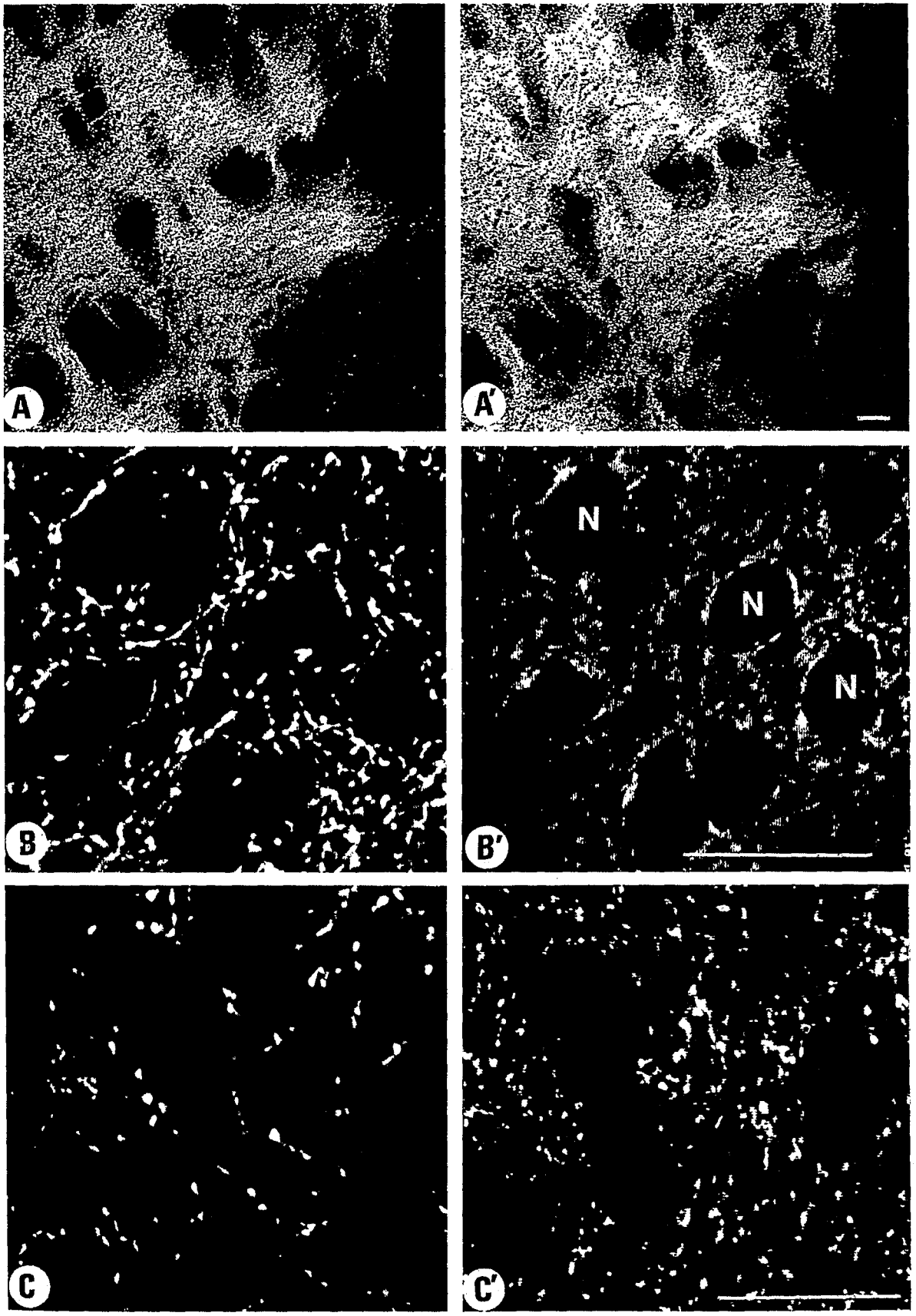
Animals

Male adult Sprague–Dawley rats (250–300 g) 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

Lesions were performed according to the method originally described by Halàsz and Pupp (27) (Fig. 1). After deep anesthesia with pentobarbital (60 mg/kg),

FIG. 2. Confocal paired images of sections immunostained for TH (left) and for B-50 (right). (A–A′) Through the neostriatum, the observation of a 10-μm-thick optical section obtained at low direct magnification (objective ×10), shows that TH and B-50 immunostainings appear closely codistributed. (B–B′) The observation of a thinner optical section obtained at higher direct magnification (about 1 μm thick, objective ×60), however, clearly shows that TH-IR and B-50-IR are associated with distinct axonal profiles closely surrounding neuronal perikarya (N) dispersed throughout the striatum. (C–C′) Through the periventricular nucleus of the hypothalamus, a thin optical section about 1 mm thick) shows that TH-IR and B-50-IR are associated with distinct axon-like profiles. A, ×110; B and C, ×660. Bars, 50 μm.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animals were secured to a stereotaxic device. An L-shaped knife (with a radius of 1.8 mm and an obtuse angle of 110°) was placed unilaterally into the lateral hypothalamus, according to the stereotaxic atlas of Paxinos and Watson (45) (6.7 mm anterior to the interaural line; 2 mm lateral to the midline; 9 mm below the surface of the skull). Turning the knife 180° to one side produced a lesion that extended between the fornix and the optic tract, transecting the anterior portion of the medial forebrain bundle.

Preparation of Tissues

The animals used in this study include intact rats ($n = 5$) and rats killed 5 ($n = 3$) and 20 ($n = 3$) days after lesioning the lateral hypothalamus. After deep anesthesia with pentobarbital (60 mg/kg), rats were perfused through the ascending aorta with phosphate-buffered saline (PBS), followed by 500 ml of fixative composed of 4% paraformaldehyde in 0.1 *M* phosphate buffer at pH 7.4. The brain and spinal cord were dissected and fixed by immersion in the same fixative for 2 to 4 days before being cut with a vibratome into 30- to 40-μm-thick coronal sections. These were carefully rinsed in PBS and subsequently treated for double-fluorescence immunostaining.

Immunocytochemistry

The antibodies used included (1) two mouse monoclonal antibodies (IgG) against B-50 (clone NM4, see 38 and clone GAP-7B10, Sigma) and (2) rabbit polyclonal antibodies (IgG) against B-50 (43), tyrosine hydroxylase (TH; Jacques Boy Laboratories, Reims, France), or serotonin (5HT; Immunotech, Marseille, France). These antibodies were diluted 1/800 (anti-5HT), 1/2000 (anti-B-50), or 1/5000 (anti-TH) in PBS at pH 7.4 containing 0.1% Triton X-100 and 1% normal goat serum. The specificity of the antibodies used has been described and documented elsewhere (see 39 for NM4 monoclonal anti-B-50, 38 for GAP-7B10 monoclonal anti-B-50, 43 for polyclonal anti-B-50, 4 for anti-TH, 54 for anti-5HT). The method controls consisted of omitting the primary antibodies or applying each primary antibody sequentially and then reacting them with the inappropriate secondary antibody.

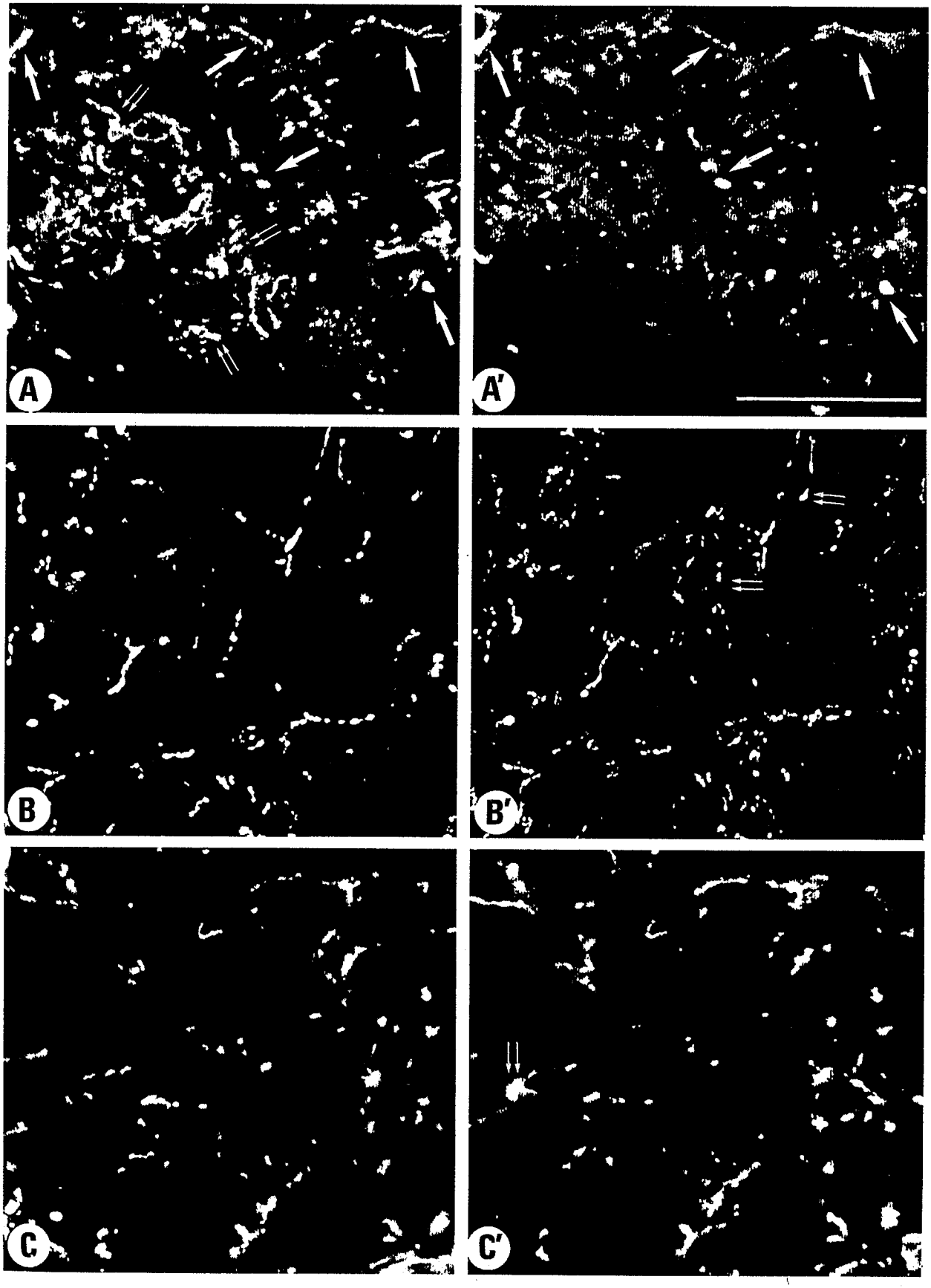
The vibratome sections were treated for double-immunofluorescence labeling by incubating them for 48 h at 4°C with two primary antibodies including the mouse monoclonal NM4 antibody anti-B-50 and a rabbit polyclonal antibody (anti-TH or anti-5HT). After rinsing in PBS, sections were incubated for 2 h at 4°C with two secondary antibodies corresponding to the primary antibodies, including goat anti-rabbit and goat anti-mouse IgG respectively conjugated with fluorescein and with rhodamine (Sigma). The secondary antibodies were diluted 1/200 in PBS containing 1% normal goat serum and 0.1% Triton X-100. 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. A minimum overlap of the emission spectra of the two fluorochromes was obtained by using two laser lines emitting at 488 and 568 nm for exciting the fluorescein- and the rhodamine-conjugated secondary antibodies, respectively. The background noise of each confocal image was reduced by decreasing the size of the confocal aperture and by averaging six image inputs. Depending on the objective used and the size of the confocal aperture, the thickness of the optical sections varied between approximately 10 μm (objective ×10 with a numerical aperture of 0.2) and less than 1 μm (objective ×60 with a numerical aperture of 1.4).

RESULTS

Throughout the different regions of the brain and spinal cord that were examined, the immunostaining pattern obtained with the NM4 monoclonal antibody against B-50 conformed to previous descriptions (see 8) and was very similar to the immunostaining patterns obtained with other antibodies to B-50 that were tested, including a monoclonal (GAP-7B10, Sigma) and a polyclonal (43) antibody. Double immunostaining combining the polyclonal and either the NM4 or the GAP-7B10 monoclonal antibodies to B-50 indicated that the three antibodies label the same axonal fiber- or terminal-like structures throughout various areas extending from the olfactory bulb to the spinal cord. Similarly to B-50-immunostaining, the distribution of structures immunostained with the antibodies against TH or 5HT fully conformed to previous descriptions (see 54 for 5HT immunostaining and 31 for TH immunostaining).

Scanning through successive vertical planes of immunostained vibratome sections showed that, as previously reported by others (12), the immunostaining obtained with the NM4 monoclonal anti-B-50 antibody was generally limited to the 10- to 15-μm-thick superfi-

FIG. 3. Confocal paired images of sections through the neurohypophysis immunostained for tyrosine hydroxylase (left) and for B-50 (right). The observation of 1-μm-thick optical sections clearly shows that, in contrast with the other brain regions, B-50-IR is associated with a number of TH-IR fibers innervating the median eminence (A–A', arrows) and with a large majority of these fibers innervating (B–B') the neural and (C–C') the intermediate hypophysial lobes. Note that throughout these neurohypophysial regions, the immunostaining patterns of these doubly immunostained fibers are similar for both antibodies, although a number of B-50-IR fibers appear TH negative (double arrows). A–C, ×700. Bar, 50 μm.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cial layers of the sections, whereas the immunostainings induced by the polyclonal IgG antibodies used here (i.e., anti-TH and anti-5HT) penetrated the whole thickness of the vibratome sections. The detection of possible colocalizations of B-50 and either TH or 5HT within axonal fibers and varicosities was, thus, limited to the superficial layers of the immunostained sections exhibiting intense B-50 immunostaining. Moreover, since the different types of immunostained fibers appear closely intermingled in most brain or spinal cord regions investigated, the present study was based mostly on the observation of thin optical sections (about 1 μm thick) obtained by using a planatochromat oil immersion objective ×60 with a numerical aperture of 1.4 and by decreasing the size of the confocal aperture.

Intact Brain and Spinal Cord

The possible occurrence of B-50-IR within monoaminergic fibers has been investigated in specific regions that are known to receive a particularly dense innervation from the main catecholaminergic or serotonergic cell groups.

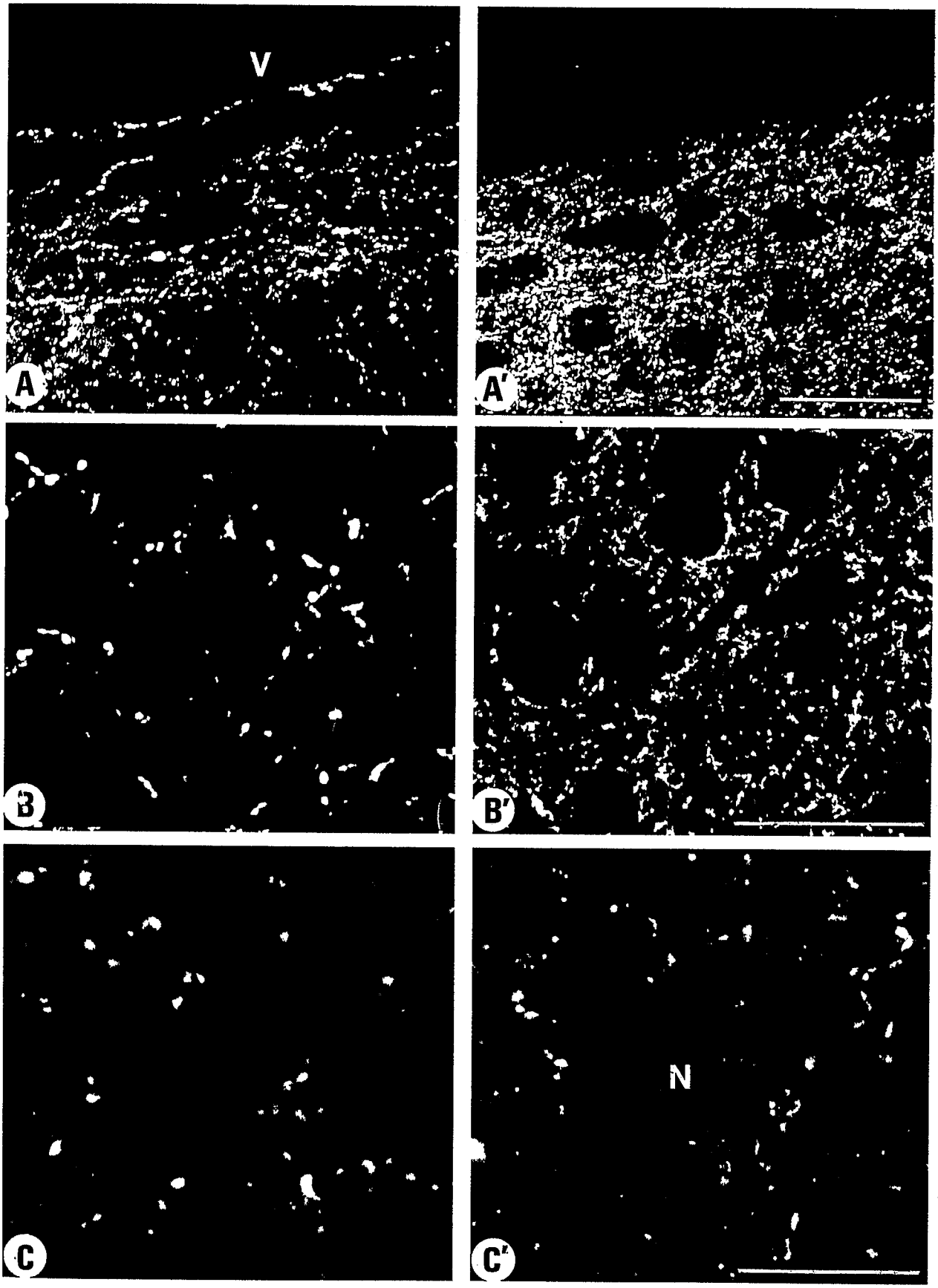
Double immunostaining for B-50 and for TH. When double-immunostained sections were observed at low magnification (objective ×10 or ×20), high concentrations of fibers immunoreactive to B-50 or to TH were frequently observed to be codistributed in brain regions extending from the olfactory bulb to the medulla oblongata (Fig. 2A). The possible colocalizations of both immunostainings within the same axonal fibers were examined at high magnification (objective ×60) within some of these areas, including the glomerular region of the olfactory bulb, the striatum, the lateral septum, the central nucleus of the amygdala, the hypothalamus, the dorsal thalamus, the hippocampus, the nucleus tractus solitarius in the medulla oblongata, and the spinal cord. Among these, the mediobasal hypothalamus was the only region where frequent colocalizations of B-50-IR and TH-IR could be detected. In this region, as previously reported (3), B-50-IR was associated with numerous TH-IR fibers innervating the median eminence and with the large majority of the TH-IR fibers innervating the neural and the intermediate hypophysial lobes (Fig. 3). The immunostaining patterns obtained with both antibodies were generally very similar within the doubly immunostained structures: intense immunostainings to TH or B-50 appeared homogeneously distributed along axon-like fibers or within isolated varicosities (Figs. 3B–B′ and 3C–C′). This was confirmed by the color superposition of both immunostainings which showed that in these regions, TH (green) and B-50 (red) immunostainings were mostly colocalized within the same axon-like structures (Fig. 6A). In all the other regions examined however, the observation of thin optical sections indicated that TH-IR and B-50-IR were associated with axon-like structures that exhibit clearly different anatomical patterns (Figs. 2B–B′ and 2C–C′). In these regions, the color superposition of both immunostainings indicated that TH-IR and B-50-IR were mostly associated with distinct, respectively green or red, axon-like structures. In regions like the striatum or the lateral septum that were found to contain a large number of densely intermingled TH-IR and B-50-IR fibers, color superpositions (yellow) were occasionally observed. However, contrasting to the neurohypophysial fibers where such color superpositions extended all along the immunostained fiber, they were here essentially limited to small portions of the immunostained fibers (Figs. 6B and 6C).

Double immunostaining for B-50 and for 5HT. The possible colocalization of B-50 and 5HT immunoreactivities was more closely examined in specific regions exhibiting a codistribution of both immunostainings, including the striatum, the suprachiasmatic nucleus in the hypothalamus, the dorsal thalamus, the hippocampus, the nucleus tractus solitarius in the medulla oblongata, and the spinal cord. The observation of thin optical sections at high direct magnification indicated that the fiber-like structures immunoreactive to B-50 or to 5HT detected in these regions exhibited clearly different anatomical patterns (Fig. 4). Moreover, no B-50-IR could be detected within the numerous supraependymal 5HT-IR fibers that were observed all along the surface of the cerebral ventricles (Fig. 4A). The color superposition of both immunostainings confirmed that in all the regions examined, B-50-IR and 5HT-IR were mostly associated with distinct structures, although limited color superpositions were occasionally detected in regions containing densely packed 5HT-IR and B-50-IR fibers (Figs. 6D and 6E).

Lesioned Brains

In all the regions of the lesioned brains examined, the immunostaining patterns observed with the antibodies used here were rigorously the same as those observed in the intact brains, except for the areas closely sur-

FIG. 4. Confocal paired images of sections immunostained for 5HT (left) and B-50 (right). The observation of a 5-μm-thick optical section (A–A′) through the dorsomedial thalamus (paraventricular nucleus of the thalamus) shows that 5HT and B-50 immunostainings exhibit clearly different patterns and that no B-50-IR is associated with the supraependymal 5HT-IR fibers running along the ventricular surface. The observation of 1-μm-thick optical sections through the nucleus of the tractus solitarius of (B–B′) the medulla oblongata and (C–C′) the ventral horn of the spinal cord indicates that 5HT-IR and B-50-IR are essentially associated with distinct axon-like profiles. A, ×470; B and C, ×705. Bars, 50 μm.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rounding the lesion. As early as 5 days after the lesion, numerous fibers intensely immunoreactive to B-50, TH, or 5HT were observed to accumulate throughout the lateral hypothalamus closely surrounding the lesion and, more especially, along the lateral portion of the cut that extends through the medial forebrain bundle. Occasionally, some of these fibers were found to project toward the core of the lesion consisting of cavities more or less filled with glial cells, macrophages, erythrocytes, and amorphous material. Twenty days after the surgical cut, the number of these fibers appeared to be dramatically increased both throughout the area closely surrounding the cut and within the core of the lesion. Examination of doubly immunostained material indicated that, at least until 20 days after the surgical lesion, numerous TH-IR or 5HT-IR axonal fibers located along the border of the lesion, or projecting within the lesion core, also exhibited intense B-50-IR (Figs. 5A–A′ and 5B–B′). In these regions, however, numerous B-50-IR fibers were found to be unstained either for TH or for 5HT. Conversely, a number of TH-IR or 5HT-IR fibers appeared to be devoid of B-50-IR. B-50-IR was rarely detected in TH-IR or 5HT-IR fibers located in hypothalamic areas at a distance of more than about 300 μm from the lesion border. Moreover, in all lesioned brains examined, B-50-IR was frequently found to be localized within 5HT-IR supraependymal fibers located along the ventricular surface bordering the dorsal thalamus (Fig. 5C–C′). In contrast, such colocalization was only rarely detected within the supraependymal 5HT fibers located along the ependymal borders of the third and fourth ventricles.

DISCUSSION

Although many studies using immunocytochemistry have demonstrated the occurrence of B-50-IR in various regions of the adult brain and spinal cord (8, 16, 17, 35, 44), little information has been provided concerning the nature of these fibers. As already mentioned by others (12), this is probably due to technical difficulties related to visualizing double-labeled fibers or varicosities using conventional light microscopy. Indeed, due to the small size of these structures that, moreover, are frequently densely intermingled, artifacts of false colocalizations resulting from the superposition of differentially labeled fibers are difficult to identify. In this regard the approach used in the present study, i.e., double-immunofluorescent labeling combined with confocal microscopy, certainly offers several advantages: (1) the application of double-immunofluorescent labeling is simple and the two secondary antibodies labeled with either fluorescein or rhodamine can be unambiguously discriminated by the two laser lines at 488 and 568 nm provided by the krypton/argon mixed-gas laser of the confocal microscope used here, (2) it allows the simultaneous observation of fluorescent-labeled structures on 30- to 40-μm-thick vibratome sections, thus avoiding freezing of tissue for cryostat sectioning that may alter the cellular morphology, (3) the penetration of the different immunostainings within the thickness of the vibratome section can easily be controlled under the confocal microscope by scanning through the successive vertical planes. This considerably decreases the risks of false-negative-labeling artifacts due to differential penetrations of the various types of immunostaining within the thickness of vibratome sections (46), and (4) compared to conventional fluorescence microscopy, confocal microscopy considerably improves the optical resolution both in the lateral dimension and in depth (63). The visualization of two immunofluorescent-labeled antigens on single optical sections of about 1 μm greatly diminishes the risk of superimposition artifacts, especially in regions which contain thin, closely intermingled axons. On the other hand, however, decreasing the size of the confocal aperture considerably decreases the amount of fluorescence detected, thus hindering the visualization of slightly immunoreactive structures.

By using confocal microscope analysis of doubly stained sections, we show here that most intact catecholaminergic and serotonergic axonal fibers or terminals detected throughout various regions of the brain and spinal cord of adult rats do not contain immunocytochemically detectable levels of B-50. Since previous studies have shown that the corresponding catecholaminergic and serotonergic neuronal cell bodies express relatively high levels of B-50 mRNA (7, 13, 33, 64), our results would appear surprising. There are several possibilities to explain the apparent discrepancy.

First, the immunocytochemical method used here is not sensitive enough for an optimum detection of B-50 or the markers of monoaminergic neurons (i.e., TH or 5HT). In previous ultrastructural studies, B-50-IR was detected in virtually all unmyelinated axon profiles of the adult rat hippocampus and central gray (58, 59). Although B-50-IR was found here to be associated with numerous axonal fibers innervating these regions, it is clear that all unmyelinated fibers were not labeled. Thus it is likely that, in contrast to specific ultrastructural immunocytochemical techniques, the conditions used here do not allow the visualization of those axonal profiles that contain very low levels of B-50. As mentioned above, this applies particularly to the observation of thin confocal images which were obtained by decreasing the size of the confocal aperture. However, B-50-IR axonal fibers and terminals were presently detected in all the regions in which they have been previously described by different studies using light microscope immunocytochemistry. Even for the thin-

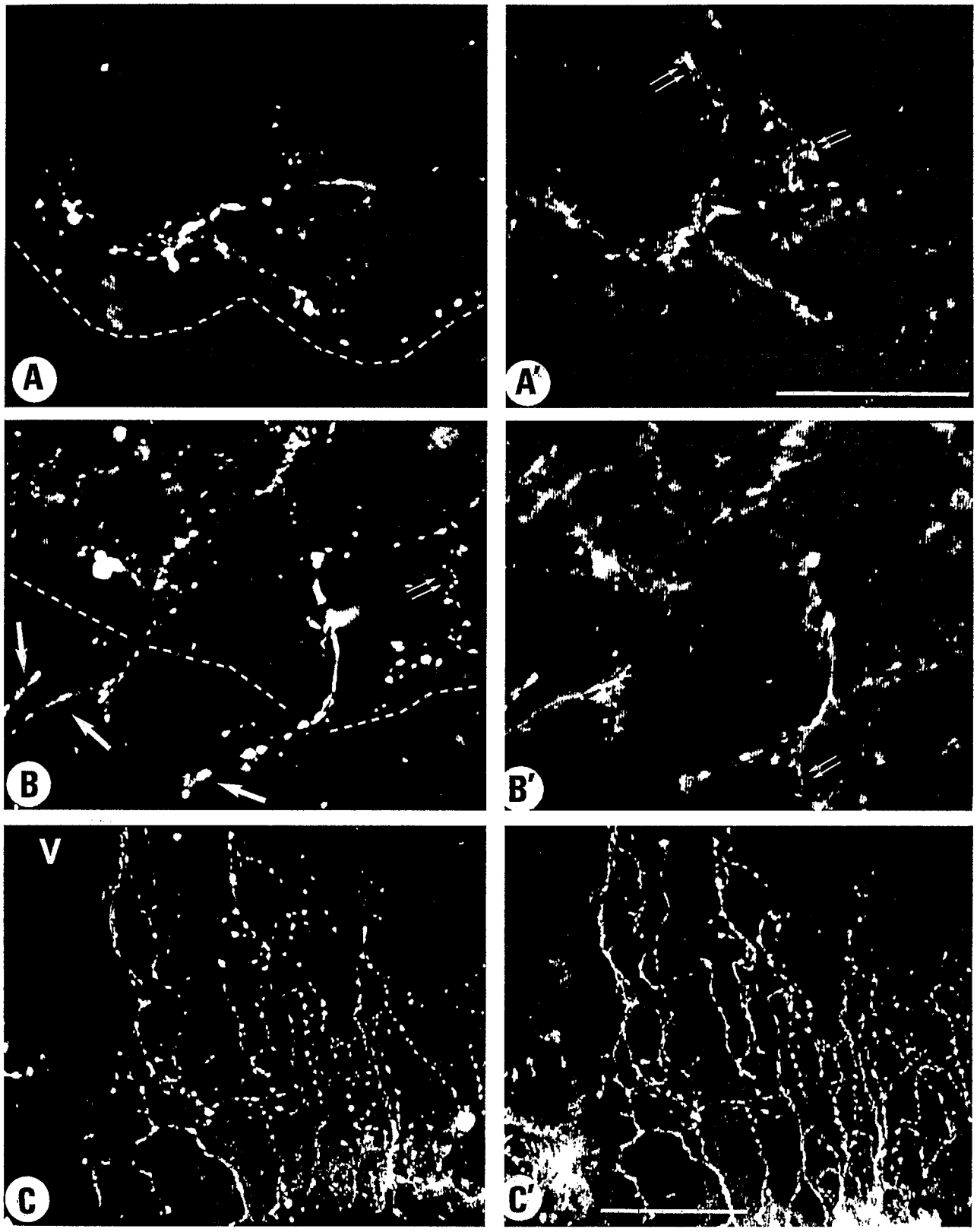


FIG. 5. Confocal paired images of sections through lesioned brains immunostained for B-50 (right) and TH or 5HT (left). (A–A′ and B–B′) Lateral hypothalamus at the level of the surgical cut through the medial forebrain bundle. The observation of 1-μm-thick optical sections shows that a majority of the (A–A′) TH-IR or (B–B′) 5HT-IR fibers located close to the border of the lesion (dotted line) or extending within the lesioned tissue (arrows, B) also exhibit B-50 immunostaining. Note that a number of B-50-IR fibers are negative to either TH or 5HT (double arrows, A′ and B′), and that a number of 5HT-IR fibers are negative to B-50 (double arrows, B). (C–C′) Ventricular border of the thalamus. The observation of a 5-μm-thick optical section clearly shows that the majority of the 5HT-IR supraependymal fibers also exhibits intense B-50-IR. A–B, ×720; C, ×480. Bar, 50 μm.

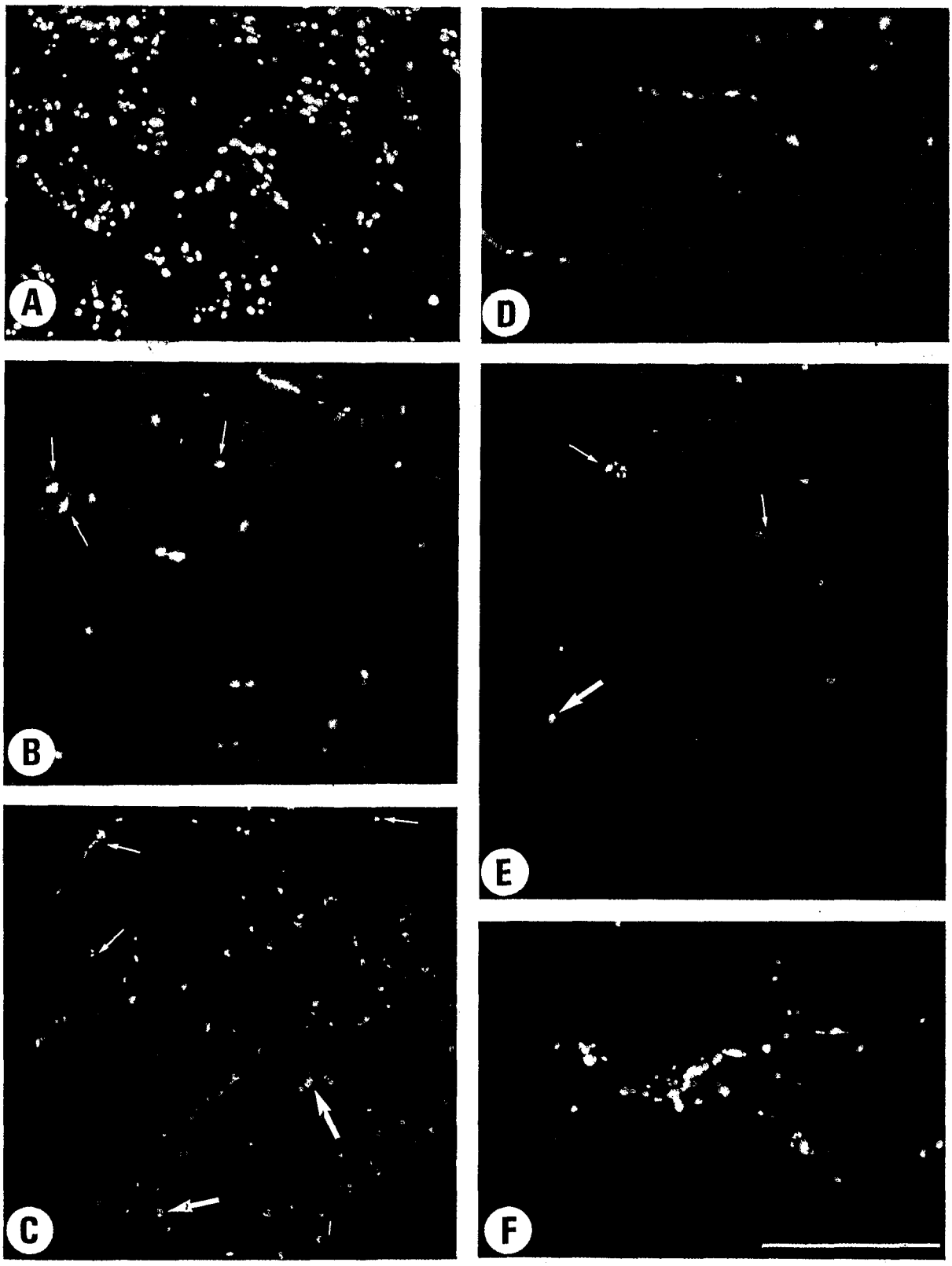
nest optical sections observed, all the brain and spinal cord regions investigated here were found to contain numerous highly fluorescent B-50-IR axonal profiles. It can thus be assumed that the conditions used here allow the detection of most axonal fibers containing high to moderate levels of B-50. Similarly, it can be assumed that the 5HT immunostaining performed here actually allowed the detection of the large majority of the serotonergic fibers and terminals originating in neurons of the raphe nuclei. Indeed, labeling of 5HT-IR axonal fibers and terminals could be observed both (1) in regions where they form dense concentrations like the suprachiasmatic nucleus of the hypothalamus, the nucleus tractus solitarius in the dorsal medulla, or the intermediolateral cell column and the ventral horn of the spinal cord and (2) in regions where they are more dispersed like the ependymal surface of the ventricles, the superficial cortical layers, or the dorsal horn of the spinal cord. Concerning the TH immunostaining, we have used a particularly sensitive polyclonal antibody to TH that was found to intensely label catecholaminergic fibers innervating various brain regions including: (1) the striatum, where TH-IR fibers mostly correspond to dopaminergic fibers originating in neurons of the substantia nigra (23), (2) the magnocellular nuclei of the hypothalamus where TH-IR fibers mostly correspond to noradrenergic and adrenergic fibers originating in neurons of the medulla oblongata (50), and (3) the hippocampus, where TH-IR fibers mostly correspond to noradrenergic fibers originating in neurons of the locus coeruleus (34). Thus, although it has been shown that the sensitivity of immunocytochemical detection of tyrosine hydroxylase varies considerably within catecholaminergic axon terminals according to the neuronal system of origin (6), it can be assumed that the TH immunostaining performed here allowed the labeling of a majority of the axonal fibers and terminals belonging to the main central catecholaminergic systems including the dopaminergic neurons of the substantia nigra, the noradrenergic neurons of the locus coeruleus, and the noradrenergic and/or adrenergic neurons of the medulla oblongata. These systems have been shown to express high levels of B-50 mRNA (see 7, 33, 64).

Second, instead of insufficient detection, an alternative explanation for the lack of detectable colocalization may be that B-50 and the markers of monoaminergic neurons are localized to different compartments of the axonal fibers or terminals. Indeed, TH or 5HT are located either in the cytoplasm or within vesicles, whereas B-50 is mainly bound to the plasma membrane (14, 26, 48, 58, 59). Such different intracellular localizations may explain that the two immunostained antigens are not strictly superposed all along labeled axonal fibers (see Figs. 3B–B′ and 3C–C′). However, the fact that this systematically leads to the spatial dissociation of the two immunostainings does not fit the present demonstration of clear colocalizations between B-50-IR and TH-IR or 5HT-IR, in both intact or lesioned brains (Figs. 3, 5, and 6). Moreover, in a previous study using the same methodological approach, axonal colocalizations could be clearly demonstrated between TH (which is located in the cytoplasm or in vesicles) and polysialylated neural cell adhesion molecule (which is bound to the plasma membrane) within fibers innervating the mediobasal hypothalamus (2).

The data reported here further appear to be in contradiction with previous studies in which a high degree of colocalization between B-50-IR and 5HT-IR has been found throughout the spinal cord (5, 12). Such colocalizations have been detected by conventional fluorescent microscopy in doubly stained sections 14 to 20 μm thick. However, the resolution of this procedure does not allow an unambiguous discrimination of differentially immunostained fibers that are superposed within the thickness of the section. Moreover, the present observations indicated that, even on 1-μm-thick optical sections, a number of apparent colocalizations could result from the superposition of adjacent fibers, namely in regions like the striatum or the intermediate cell column of the spinal cord, that contain strikingly high densities of closely intermingled differentially immunostained axonal fibers (Fig. 6). It is thus likely that such reported colocalizations between B-50-IR and 5HT-IR have been overestimated.

Why is B-50 undetectable in the intact monoaminergic axons when it has been shown that the corresponding cell bodies express relatively high levels of B-50 mRNA? The presence of B-50 mRNA under basal conditions may reflect either inactive translation or rapid turnover of the translation product. Another possibility is that, due to the widespread axonal projec-

FIG. 6. Superposed color confocal images of sections immunostained for B-50 (red, A–F) and for TH (green, A–C and F) or 5HT (green, D and E). (A–E) Intact brain and spinal cord. The observation of 1-μm-thick optical sections shows that (A) in the neural hypophysial lobe B-50-IR and TH-IR structures are mostly superposed (yellow), whereas the majority of the structures immunoreactive to B-50 (red) are distinct from the structures immunoreactive to TH (green) detected in the glomerular region of (B) the olfactory bulb and (C) the striatum and from the structures immunoreactive to 5HT (green) detected in (D) the suprachiasmatic nucleus of the hypothalamus and (E) the intermediate cell column of the spinal cord. Note that in these regions some color superpositions are observed on isolated profiles (large arrows) at the level of close appositions between two differentially immunostained structures (small arrows). (F) Lesioned brain, 20 days after the lesion. The observation of a 1-μm-thick optical section through the lateral hypothalamus shows that B-50-IR (red) and TH-IR (green) are colocalized (yellow) within fibers located along the lesion border through the medial forebrain bundle. C and F correspond to the color superpositions of the confocal paired images shown in Figs. 2B–B′ and 5A–A′, respectively. A–F, ×600. Bar, 50 μm.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tions of monoaminergic neurons, B-50 produced in the somata is getting largely diluted in the wide arbor of these cells' projections. Interestingly enough, however, our data clearly demonstrate that, in striking contrast to the intact fibers, intense B-50-IR is frequently associated with both catecholaminergic and serotonergic transected fibers closely surrounding a surgical lesion placed in the medial forebrain bundle. In the rat, this large axonal bundle has been shown to contain a large variety of monoaminergic afferents to the forebrain, including serotonergic axons from the raphe nuclei (15) and noradrenergic axons from either the locus coeruleus or the dorsal medulla oblongata (40). Since it has long been known that monoaminergic neurons possess a remarkable capacity for sprouting and regeneration (11, 42), it is assumed that the majority of 5HT-IR or TH-IR fibers that accumulate closely around or that project within the lesioned area actually represent axonal-regenerating sprouts of such serotonergic or noradrenergic axons.

The appearance of B-50-IR within these sprouts provides additional support to the concept that, also in adult central neurons, B-50 is involved in axon outgrowth or remodeling during postlesion recovery or adaptation (9, 10, 25, 53, 56). This further suggests that, when morphological plasticity of the monoaminergic axons is induced by axotomy, the relatively high levels of B-50 mRNA present in the corresponding cell bodies allow rapid upregulation of B-50 that is transported through the axonal fibers and accumulates in the regenerative sprouts adjacent to the lesion site. Interestingly, in all the lesioned brains examined, B-50-IR was also found to be associated with a majority of the 5HT-IR supraependymal fibers located along the ventricular surface of the dorsal thalamus. Since these supraependymal 5HT-IR fibers appeared to contain no B-50-IR in the intact brain, it is likely that B-50-IR characterizes regenerating axonal sprouts of serotonergic supraependymal fibers that have been severed by the knife. Accordingly, it is assumed that the rare catecholaminergic or serotonergic fibers that were found to contain B-50-IR in intact brains and spinal cords represent remodeling fibers. This interpretation may also apply to those intact TH-IR fibers innervating the median eminence and the neural hypophysial lobe or to those projecting into the intermediate hypophysial lobe that exhibit intense B-50 immunoreactivity. As reported in a previous study (3) these fibers mostly correspond to dopaminergic/GABAergic fibers originating in neurons of the dorsomedial and periventricular subdivisions of the hypothalamic arcuate nucleus which have been reported to contain neurons exhibiting moderate B-50 mRNA signal (64). Remarkably, these fibers present particular anatomical and functional organizations. They project in regions deprived of blood–brain barrier (namely the intermediate hypophysial lobe and the perivascular space bordering the median eminence and the neural hypophysial lobe). There, they form synaptic-like connections with nonneuronal cells, i.e., the glial cells of the neurohypophysis (G. Alonso, unpublished observations) and the endocrine cells of the intermediate hypophysial lobe (51).

In addition to its roles in neuritogenesis and axonal plasticity, B-50 has been implicated as an important regulator of neurotransmitter release (18, 25). Evidence for an involvement of B-50 in neurotransmission is derived largely from two types of study. First, it was shown that the extent of B-50 phosphorylation showed a correlation with stimulated neurotransmitter release in hippocampus slices (20, 21, 28) and in intact synaptosomes (22). Second, in studies with permeabilized rat cortex synaptosomes, antibodies against B-50 were shown to inhibit the calcium-induced release of [^{3}H]-noradrenalin, or endogenous noradrenalin and colecystokinin-8 (29, 30). Apparently, the findings that B-50-IR is not detected in most of the TH-IR-containing axonal fibers or terminals are not consistent with a direct role of B-50 in noradrenalin release. As mentioned above, however, it is possible that intact catecholaminergic fibers effectively contain B-50, but that the intraaxonal levels of the protein are very low and cannot be detected under the conditions used here. It seems then that further studies using more sensitive (preferably ultrastructural) immunocytochemical approaches are required to clarify the possible occurrence of B-50 within noradrenergic (and/or other monoaminergic) nerve terminals.

In conclusion, the present data demonstrate that, except for the mediobasal hypothalamus, the numerous axonal fibers and terminals exhibiting intense B-50 immunoreactivity that are detected in the central nervous system of the adult rat are not monoaminergic. It is possible that some of these fibers are glutamatergic. Indeed, large amounts of B-50 mRNA have been evidenced within neuronal systems like the hippocampal and cortical pyramidal cells (33, 36, 49, 64) that are known to utilize glutamate as a neurotransmitter. As yet, however, the nature of the large majority of intensely B-50-IR axonal fibers innervating various regions of the intact brain and spinal cord of adult rat remains unknown. The identification of these neuronal systems obviously will greatly help in better understanding the role(s) played by B-50 in adult neurons.

ACKNOWLEDGMENTS

This work was supported by IRME. Confocal microscopy was done using the facilities of CRIC. The authors thank Dr. M. Mercken and Innogenetics (Ghent, Belgium) for providing the monoclonal B-50 antibody (NM4). They also thank A. Legrand for her excellent technical assistance and J. R. Teilhac for artwork.

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