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Ventral mesencephalic and cortical transplants into the rat striatum display enhanced activity for neutral endopeptidase 24.11 ('enkephalinase'; CALLA)

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A role for neutral endopeptidase 24.11 (NEP) in growth and development is supported by the demonstration that NEP hydrolyses and inactivates a number of peptide growth factors including atrial natriuretic peptide, endothelins, bombesin-like peptides, and opioid peptides, including the enkephalins. In the present study, suspensions of cells obtained from the ventral mesencephalon or cortex of rat embryos (ED14) were implanted into the striatum of the adult rat brain. Three to 15 weeks after transplantation the relative distribution of NEP-positive cellular elements was visualized histochemically. NEP staining in the transplants consistently appeared before NEP staining in the surrounding host striatum supporting a relative increase in NEP activity in the transplants. The NEP staining richly visualized cells of varying size and morphology which lacked the normal organization of the host striatum. The histochemical staining in the transplants and the surrounding host tissue was completely blocked by a 100 nM concentration of the selective NEP inhibitors phosphoramidon or JHF-26, supporting the exclusive localization of NEP by this method. NEP localization in the embryonic (ED14) cortex and ventral mesencephalon was also confirmed, suggesting one possible origin for the NEP-positive cells visualized in the transplants. Fluorescent double-labeling studies for NEP and glial fibrillary acidic protein (GFAP) or transforming growth factor alpha precursor (TGFαp) revealed the presence of rich glial labeling within the transplants for both GFAP and TGFap. NEP-labeled cells in the transplants were closely associated with glial elements, however, only occasional glial elements in the transplants stained for NEP; supporting a non-astrocytic localization for the NEP in the transplants. The marked enhancement of NEP staining in the transplants may have significance for controlling the rate or pattern of growth of the transplanted cells through inactivation of peptide growth factors produced by, or in response to, the transplants.

INTRODUCTION

Neural transplantation research has demonstrated that implants of fetal tissue into adult brain can survive and establish connections with the host[33]. It is now clear from a variety of transplant systems that growth dysregulation can also occur, leading to a marked overgrowth of the transplant to form a benign mixed tumor[1]. Agents which support the survival of the transplant may include a variety of growth factors derived from either the host or transplant tissue. The potential mechanisms which terminate the action of these growth factors may include receptor-mediated endocytosis and enzymatic inactivation.

An enzyme which may serve to inactivate one or more peptide growth factors is neutral endopeptidase 24.11 (NEP). NEP has been widely localized in normal brain where it appears to inactivate a number of neuroactive peptides including the enkephalins and substance P[8]. A broader role for NEP in growth and development has been supported by the demonstration that NEP hydrolyses and inactivates a number of pep-

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tide growth factors including bombesin-like peptides[28], atrial natriuretic peptide[7,29], endothelins[31], and opioid peptides[12,21], including the enkephalins. NEP activity is also selectively enhanced in a number of malignant tissues including acute lymphocytic leukemia[14,16] and melanoma[13]. Inhibitors of NEP enhance the growth of squamous cell carcinoma in culture, further implicating the enzyme in abnormal growth and differentiation[28].

NEP activity is also elevated in a variety of human glioma cell lines[23]. In human glioma biopsies, NEP appears to be selectively associated with higher grade gliomas and rarely with low grade tumors[24]. The upregulation of NEP in glial tumors is surprising given that in normal rat brain NEP localizes to many neuronal populations[2,3,22,32] and rarely localizes to astrocytes[4,15,22]. The present study, thus, examined the expression of NEP in homotypic transplants of both cortical and ventral mesencephalic fetal cells into the adult rat striatum, because both types of transplants give rise to progressively expanding masses within the transplant site which behave like benign mixed brain tumors. These transplant systems were examined as a model system to characterize the cell types and their distribution which express NEP in a state of growth dysregulation. Like the host tissue, NEP was found to localize largely to non-astrocytic cellular elements further supporting the apparent benign nature of the transplant-derived tumors. The present study indicates that an apparent up-regulation of NEP occurs following transplantation which may play a role in the regulation of neuronal or glial growth in the transplants via the inactivation of endogenous peptide growth factors.

MATERIALS AND METHODS

General

Male Sprague–Dawley rats (250–350 g) were used in all studies. Animals were sacrificed under Rompum-Ketamine general anesthesia according to NIH guidelines for the care and handling of laboratory animals.

Neurotransplantation protocol

Under sterile conditions, the dorsolateral neocortex or ventral mesencephalon was dissected from 4–5 E-14 embryos and incubated for 30 min at 37°C in sterile Earle's Basic Salt Solution containing 0.025% (w/v) trypsin. The suspension was washed 3 times with sterile Earle's Basic Salt Solution containing 15 mM $MgSO_4$, 0.0125% (w/v) trypsin inhibitor, and 0.004% DNAase. A volume of 100 μl of tissue and washing solution was transferred to a glass microvial prior to trituration of the tissue. A total of 6 μl of the cell suspension was stereotaxically injected into the striatum of adult male rats with injection coordinates relative to bregma of anteroposterior: 0.7 mm; mediolateral: 3.0 mm; and dorsoventral: 2, 4 and 6 mm. Four animals received transplants of cells from ventral mesencephalon from one of two separate litters and had survival times ranging from 3 to 9 weeks (3, 4, 8, and 9 weeks). Five animals received transplants of cortical cells from one of three separate litters and had survival times ranging from 3 to 15 weeks (3, 4, 6, 9, and 15 weeks).

The boundaries of the regions of tissue prepared for transplantation were determined during microdissections of the fetal brain, based upon the protocol of Björklund et al.[5]. Briefly, for the cortical transplants, the cerebral vesicle was identified and dissected free at its anterior pole proximal to the striatum and hippocampal neuroepithelium. For the midbrain transplants, the ventral mesencephalon was identified and dissected free anterior and posterior to the mesencephalic flexure. Viability of the dissociated cell suspensions used for transplants was determined by staining aliquots of cells with an aqueous solution (1 μg/ml) of the vital stain acridine orange or the non-vital stain, ethidium bromide. All cell suspensions were used within 2 h after trituration of the tissue at which time greater than 90–95% cell viability was confirmed.

Four of the animals with cortical transplants (4, 6, 9 or 15 weeks survival) also were found to have an extra-axial granulomatous mass which was examined using frozen cryostat sections (20 μm) which were stained histochemically for NEP prior to post-fixation with 4% formaldehyde for 1 h at 20°C for immunocytochemical localization of GFAP or TGFαp (see below).

Histochemical localization of NEP in transplants

Animals were perfused with 1.5% paraformaldehyde and 35- to 50-μm-thick brain sections were cut on a Vibratome, as previously described[2]. NEP was localized by a fluorescent histochemical method using a solution of 50 mM Tris-HCl, pH 7.4, containing 0.5 mM glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide (GaapMNA; Enzyme Systems Products, Livermore, CA), 6 mM nitrosalicylaldehyde (NSA; Eastman-Kodak, Rochester, NY), and 5 μg of endopeptidase-free aminopeptidase M (APM). The incubation time required to initially observe enzyme histochemical staining was a function of the incubation temperature. All staining was conducted at about 4–8°C which improved the solubility of NSA relative to cooler temperatures (i.e. 2–4°C). At this temperature, staining in the transplants was typically first visualized by 24–36 h. In the normal striatum, NEP staining was commonly first visualized by 48–72 h.

The specificity of the histochemical method to exclusively localize NEP in transplants or embryonic tissue was determined by staining in the presence of a specific NEP inhibitor: phosphoramidon or JHF-26, as previously described for brain tissue[2]. Tissue was preincubated for 30 min at 22°C in the presence of inhibitor, prior to staining in histochemical staining solution containing the same concentration of inhibitor as in the preincubation. Inhibitors were tested in a log-dose-dependent manner (1 nM-1 μM) to determine the minimum concentration of inhibitor which completely blocked all histochemical staining. No non-specific staining was observed as assessed by staining tissue in which endogenous NEP was inactivated by post-fixing tissue for 1 h in 4% paraformaldehyde.

NEP localization to fetal rat brain

Rat embryos (ED14) were collected by Caesarean section from two timed pregnant Sprague–Dawley females under deep pentobarbital anesthesia and rapidly frozen at −20°C in isopentane. Frozen sagittal sections from six embryos were obtained on a Cyrocut 1800 cryostat at −25°C with the embryo embedded in a 50:50 mixture of Tissue-Tek and Aqua-Mount. Sections were desiccated at 2–4°C for 6 h prior to storage at −20°C (1–3 weeks). Thereafter, thawed sections were post-fixed in 4% paraformaldehyde for 5 min prior to histochemical staining for NEP. Stained sections were either counterstained with ethidium bromide or further processed for immunocytochemical localization of GFAP (see below).

Immunocytochemistry

Antisera. (1) GFAP: rabbit polyclonal antiserum raised against bovine GFAP (DAKO). (2) TGFα 1296 rabbit polyclonal antiserum raised against human TGFαp was provided by Dr. Larry Gentry and previously characterized[9].

Methodology. Brain tissue was obtained from animals perfused as previously described[2] using 4% paraformaldehye. For all tissue, non-specific staining was blocked by incubation in 50 mM Tris-HCl, pH 7.4 containing 1% bovine serum albumin (BSA; fraction V) for 1

h prior to incubation for at least 18 h at 2–4°C in the primary antisera (1:1,000 for GFAP; 1:750 for TGFαp). Thereafter, the excess primary antiserum was removed over 30 min by four washes with ice-cold Tris buffer. Primary antisera were visualized by incubating with a goat anti-rabbit FITC-conjugated secondary antiserum (1:50; Chemicon) for 1 h at room temperature. Following 4 washes with ice-cold Tris (30 min) the tissue was coverslipped with 6:1 glycerol and PBS, pH 8.0. Analysis was done using a NIKON Type 104 microscope with epifluorescence illumination.

Combined fluorescent histochemical localization of NEP and immunocytochemical localization of GFAP or TGFαp

Immunocytochemical localization of glial-associated antigens in tissue sections of rat brain was combined with fluorescent histochemical localization of NEP in order to examine the expression of NEP in glial cells. Staining for NEP was done as described above. Thereafter, tissue was post-fixed for 1 h at 20°C in 4% paraformaldehyde prior to processing the tissue for immunocytochemistry, as described above. Analysis was done using a NIKON type 104 epifluorescent microscope employing a narrow band blue (FITC) filter to visualize the NEP fluorescent reaction product and the FITC-label or an ethidium bromide fluorescent counterstain using the same excitation filter.

RESULTS

Specificity of NEP histochemical localization

Fig. 1 demonstrates the specificity of the histochemical method used to localize NEP. A representative example of NEP staining is shown within a midbrain transplant into the adult striatum which was allowed to survive for three weeks (Fig. 1A,B). In the presence of a 1 μM concentration of two selective NEP inhibitors, phosphoramidon (Fig. 1C,D) and JHF-26 (not shown), all staining in the transplants was blocked. Similarly, at a concentration of inhibitor as low as 100 nM, essentially all staining was blocked, whereas at 10 nM staining appeared more similar to tissue stained without inhibitor. The effective concentrations of these inhibitors which block all NEP staining in the transplants is very similar to the concentrations required to block

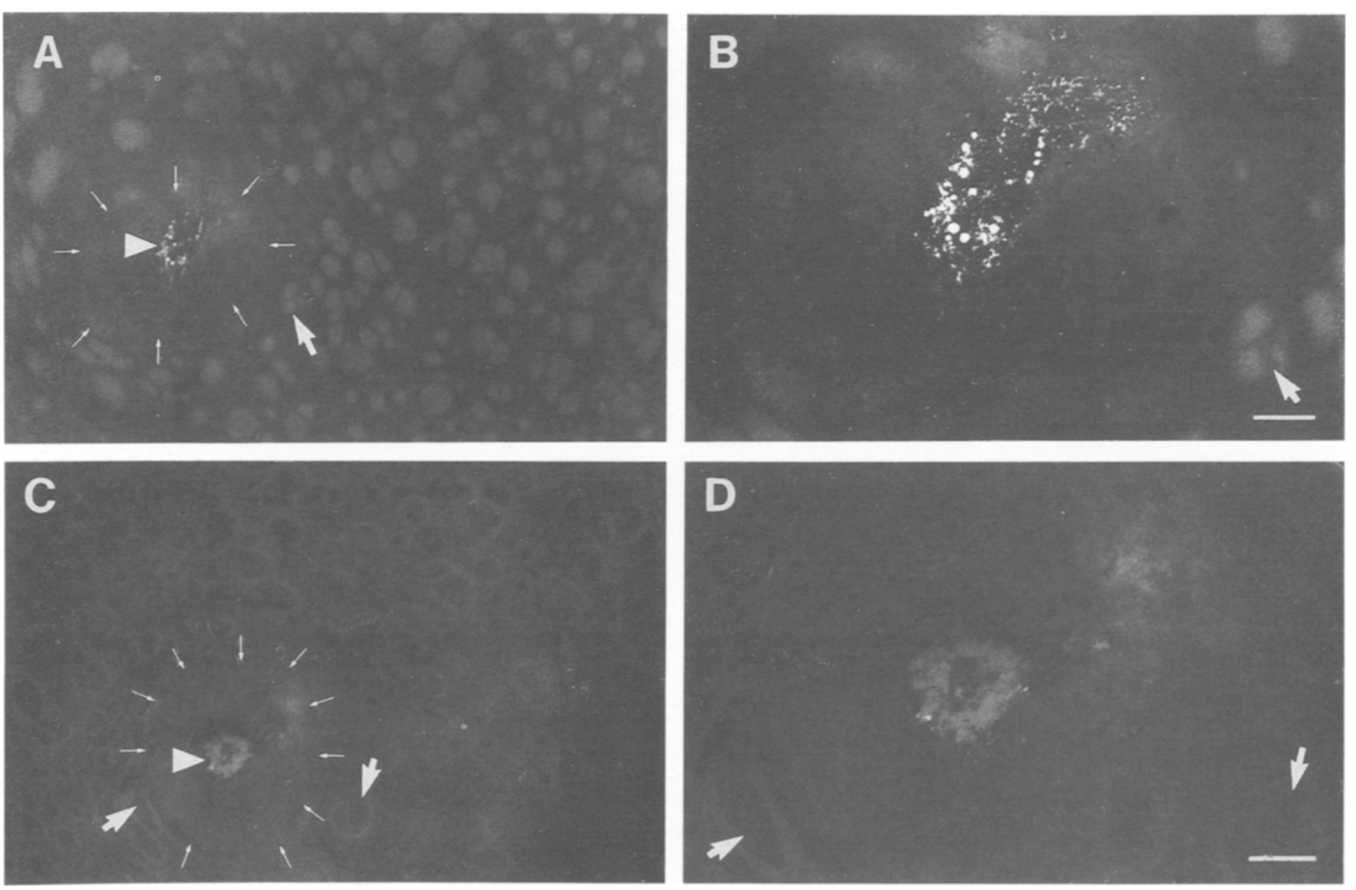
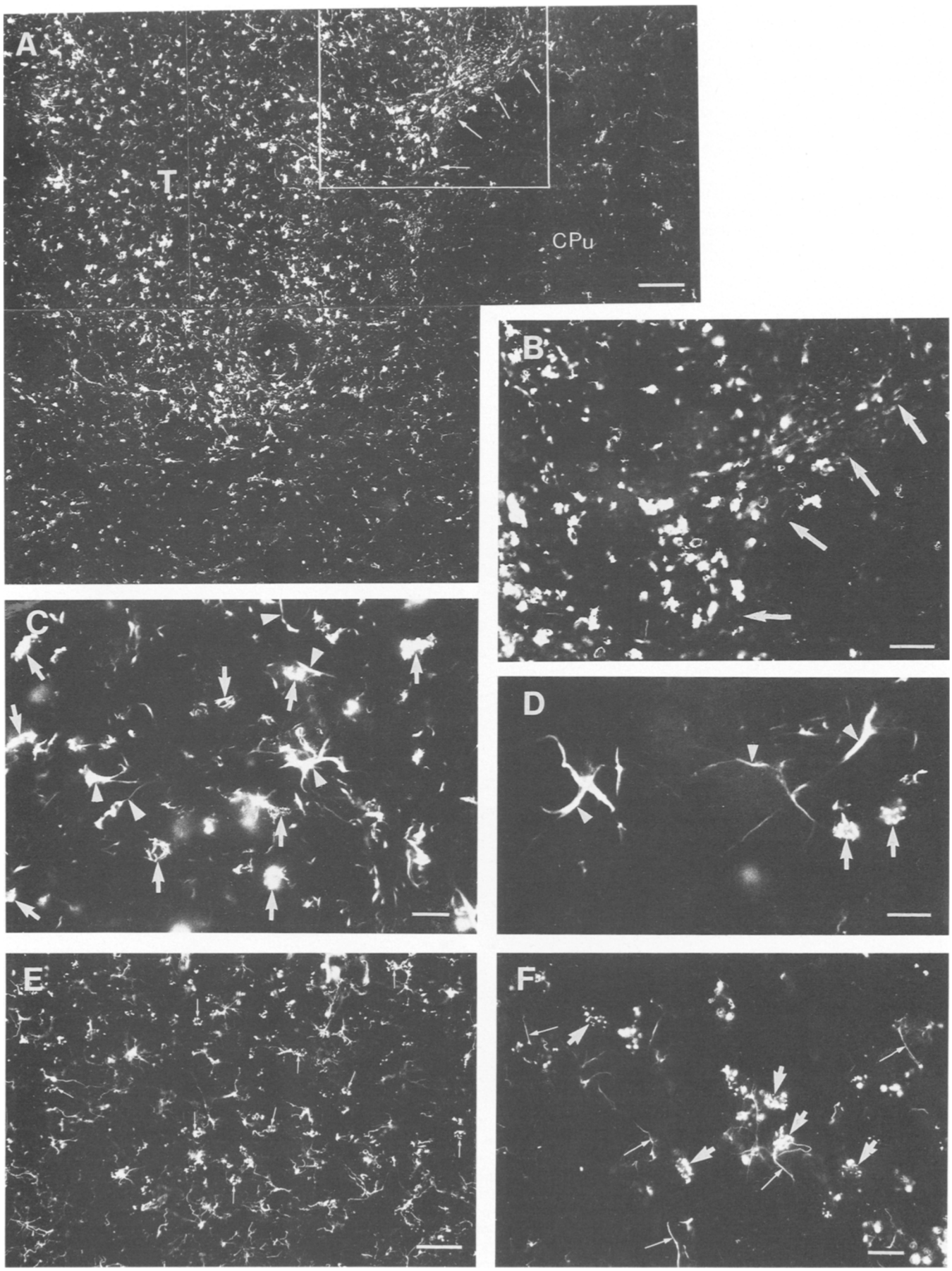


Fig. 1. Fluorescent photomicrographs demonstrating the specificity of the localization of NEP to a transplant arising from an E 14 ventral mesencephalic cell suspension implanted into the adult rat striatum. The animal survived 3 weeks after the transplantation. A: low-power photomicrograph showing the localization of NEP labeled cellular elements (arrowhead) in a representative coronal section through the transplant. The transplant (delineated by small arrows) lacks the normal morphology of the surrounding striatum including the axon fasicles of the internal capsule (large arrow). B: high-power detail of A showing a cluster of round cell bodies intensely labeled for NEP. The large arrow indicates several fasicles of the internal capsule also seen in A. A low-power photomicrograph in C and the corresponding detail in D of a region from the same transplant (small arrows) as in A,B show the effect of the NEP inhibitor phosphoramidon (1 μM) which has completely blocked all specific NEP fluorescent staining in the same region (arrowhead) as seen in A and B. Note the presence of nonspecific autofluorescent staining by nitrosalicylaldehyde seen in C and D which appears as a light gray indistinct fluorescence. The large arrows show axon bundles of the internal capsule in the normal striatum. Bar = 100 μm in B and D.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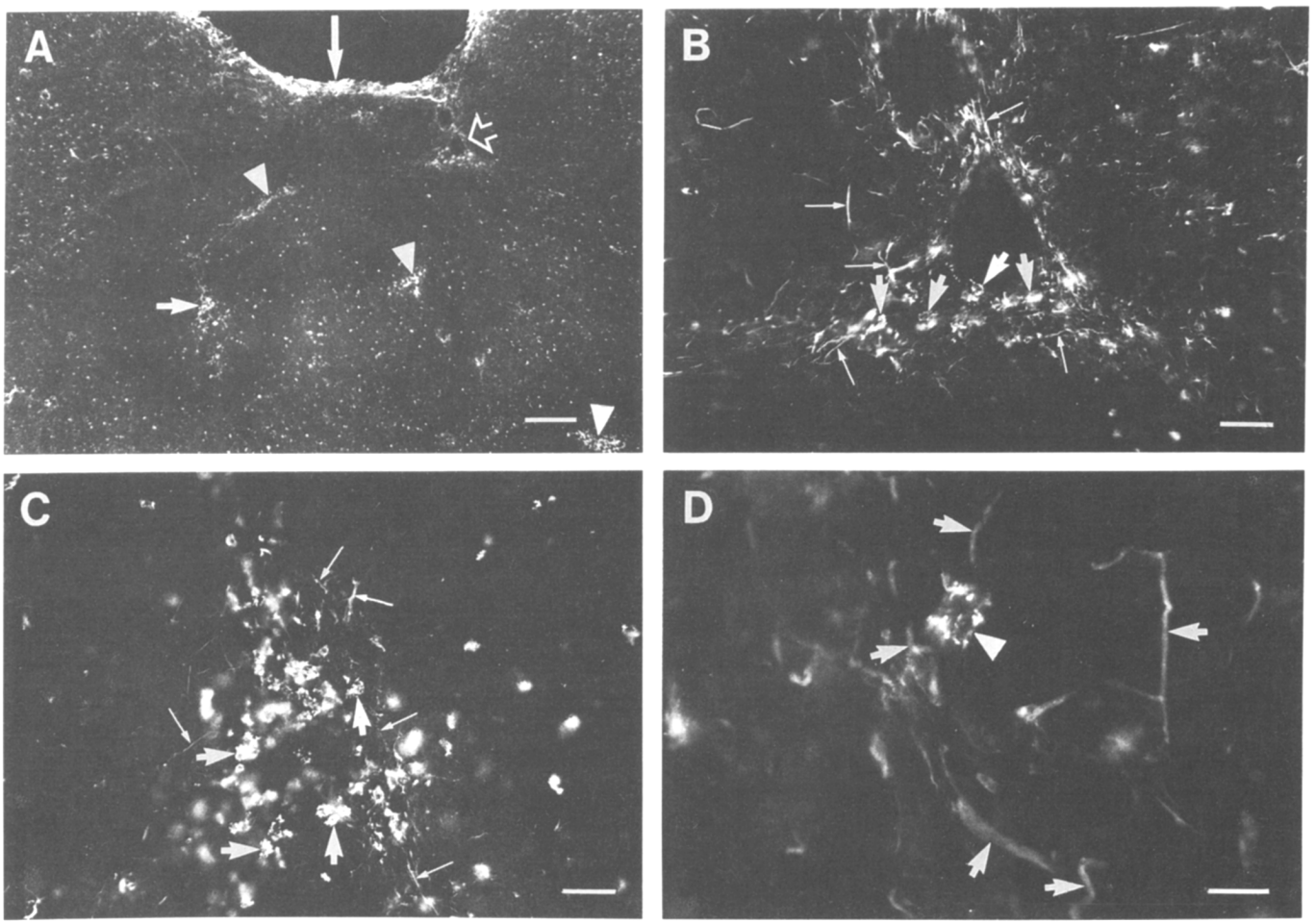

Fig. 3. Fluorescent photomicrographs showing the relative distributions of NEP and TGFαp-immunoreactive elements in an intrastriatal transplant of cortical cells (ED 14) 6 weeks after the transplant. A: the various small arrows and arrowheads indicate several collections of cells richly labeled for NEP. The large arrow indicates the injection site which is demarcated by intense NEP labeling. Two regions associated with blood vessels in the transplant are indicated by the open arrow and small arrow and are shown in detail in B and C, respectively. In both B and C small tight clusters of cells intensely labeled for NEP (large arrows) are interspersed with numerous fine glial processes (small arrows) labeled for TGFαp. D: high-power photomicrograph showing a single cell labeled for NEP (arrowhead) relative to glial processes (arrows) stained for TGFαp. Bar in A = 200 μm; B, C = 40 μm; D = 10 μm.

NEP staining in normal brain[2]. Thus, two structurally and chemically distinct NEP inhibitors potently blocked all histochemical staining in both mesencephalic and cortical transplants indicating that the histochemical method employed localized a single enzymatic activity corresponding to NEP.

NEP localization within ventral mesencephalic transplants

Three weeks after transplantation, small nests of transplanted cells were typically observed in the striatum which lacked the normal architecture of the surrounding tissue. Clusters of cells intensely labeled for

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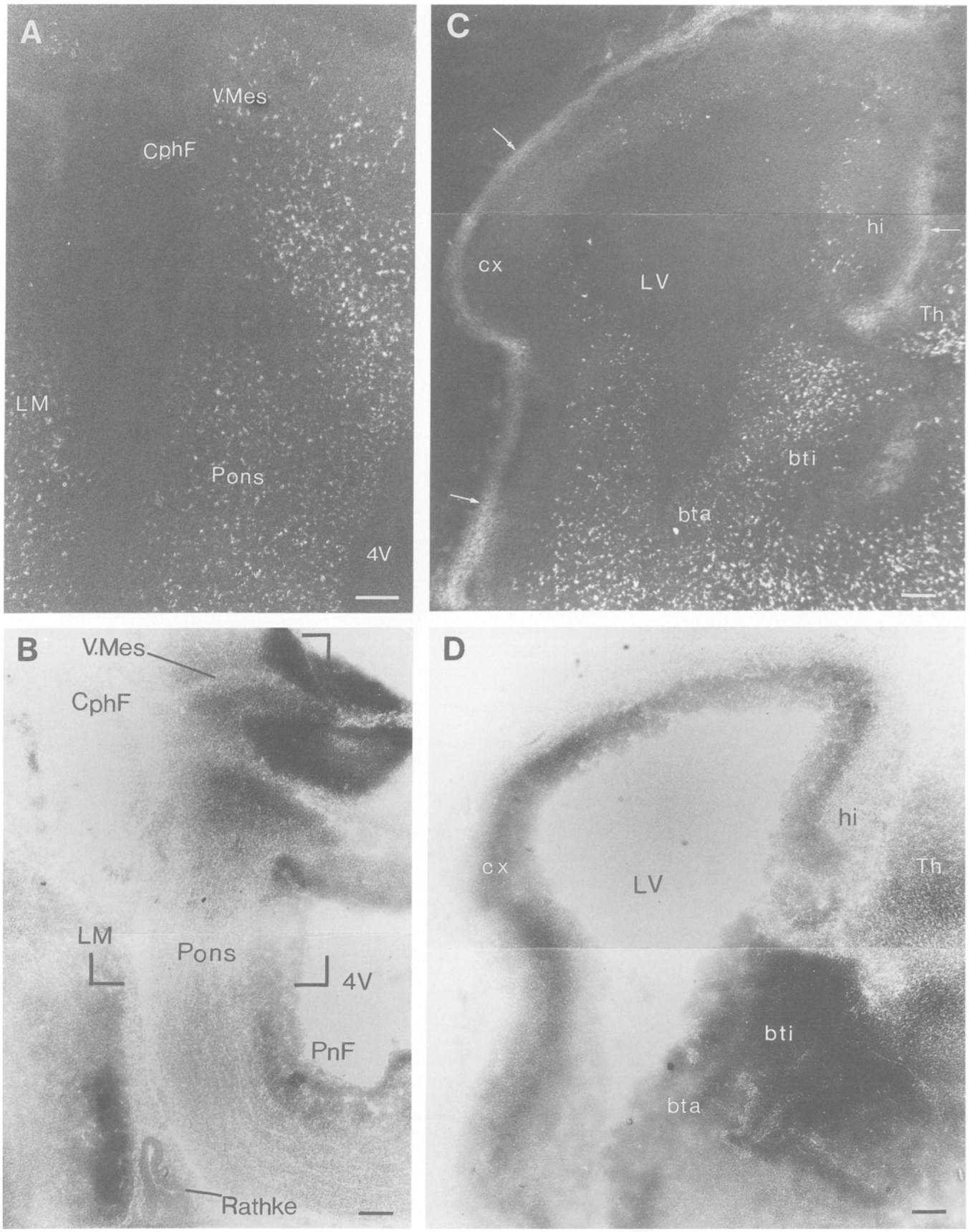
Fig. 2. Distribution of NEP staining in ventral mesencephalic (A–D) and cortical (E,F) transplants. A: fluorescent photomicrograph of a montage of a coronal section through a transplant (T) of ventral mesencephalic cells and the adjacent caudate putamen (CPu) at right and below, from an animal allowed to survive 8 weeks after transplantion, showing the increased cellularity of the transplant. The section was double-labeled for GFAP and NEP which were co-visualized using a fluorescein filter. Numerous cell bodies intensely labeled for NEP are interspersed among extensive fine glial processes. B: detail of the boxed area in A photographed to show the morphology and distribution of only the NEP-labeled cell bodies at the border of the transplant (arrows) which was visualized using a catecholamine filter. Note the relative lack of staining in the adjacent host tissue. C,D: high-power details of the relative distribution of GFAP- and NEP-labeled elements in the middle of the midbrain transplant. The glial cells (arrowheads) and NEP-positive cells (arrows) have close but non-overlapping distributions. E,F: intermediate (E) and high (F)-power details of the relative distribution of GFAP- and NEP-labeled elements in a transplant of cortical cells which survived for 6 weeks. The labeling for GFAP and NEP were co-visualized using a fluorescein filter. The arrows in E show a number of NEP-positive somata. In F the glial cells (thin arrows) and NEP-positive cells (large arrows) are seen to have close but non-overlapping distributions. Bar in A = 100 μm; B,E = 50 μm; C,F = 20 μm; D = 10 μm.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NEP were visualized within the transplant (Fig. 1A,B). At the incubation times required to optimize the histochemical staining within the transplants (24–36 h), there was little or no NEP staining in the surrounding host striatum. With longer staining periods, reaction product was localized to the striatum which was typical of that previously reported[2]. Typically, at the incubation temperatures employed (i.e. 4–8°C), NEP staining in the transplants preceded staining in the surrounding normal host striatum by 24–48 h. This finding suggested that the NEP activity within the transplants was significantly enhanced relative to the host striatum.

At longer survival periods after transplantation (6 weeks or longer), the size of the transplants was markedly increased and typically formed a well circumscribed mass within the striatum. Fig. 2A shows a representative example of such a transplant which grew to fill a large region within the striatum. Numerous cell bodies, intensely labeled for NEP, were visualized throughout the transplant. In these older transplants, as well, the intensity of histochemical staining was markedly higher than the surrounding normal striatum (Fig. 2A; Fig. 5A,B). In addition, the size and morphology of the NEP-stained cells in the transplant was variable. In general, these cells were round or ovoid in appearance, ranging in size from 5–20 μm. Grossly, the transplants lacked the organization of the surrounding host striatum. At the periphery of the transplants, as seen in Fig. 2A, the NEP-labeled cells formed circular arrays which appeared to arise due to the growth of the transplant around the fascicles of the internal capsule of the adjacent host striatum.

Fig. 2 panels C and D show the results of fluoresent double-labeling studies in which NEP reaction product was co-visualized in the same tissue section with GFAP localized immunocytochemically. At short survival times (3–4 weeks), clusters of somata intensely labeled for NEP (e.g. Fig. 1A,B) often were surrounded by processes intensely labeled for GFAP (not shown). In general, at all survival times, the NEP staining did not overlap with GFAP-labeling and the morphological appearance of the cellular elements labeled by the two fluorophores was distinctly different (Fig. 2D; Fig. 5E). Whereas the NEP staining was evenly distributed throughout the transplant, the astrocyte staining was markedly increased at the boundaries of the transplant where numerous reactive astrocytes appeared to wall-off the transplant.

NEP localization within cortical transplants

In general, the cortical transplants grew rapidly and in 4–6 weeks formed a large mass within the striatum which appeared to be well-circumscribed and non-invasive. At longer survival periods (15 weeks), the transplants grew quite large, resulting in a pronounced mass effect, causing marked compression of the striatum and ventromedial displacement and compression of the lateral ventricle and septum. These transplants extended, as well, into the overlying cerebral cortex.

As was observed for the mesencephalic transplants, NEP staining in the cortical transplants (Fig. 5C) displayed richer staining than in the adjacent host or corresponding contralateral control striatum. This staining also appeared consistently at earlier incubation times than the staining in the host tissue. Throughout the cortical transplants, numerous cell bodies of varying size and morphology were intensely labeled for NEP (Fig. 2E,F; Fig. 5C,D). These cells were round or ovoid in appearance and ranged in size from about 5–15 μm.

Immunocytochemical localization of GFAP showed that there was a prominent zone of reactive-appearing astrocytes which surrounded the periphery of the transplant and a lighter glial infiltrate within the core of the transplant (Fig. 5C). As observed for the mesencephalic transplants, the NEP staining generally did not overlap with GFAP-labeled cellular elements (Fig. 2E,F) and the morphological appearance of the cells labeled by the two fluorophores was distinctly different (Fig. 2F). Nevertheless, in many areas, NEP-positive cells were closely associated with astrocytes (Fig. 5C,D) and occasional glial elements within the zone of reactive astrocytes at the periphery of the transplant showed light NEP labeling (Fig. 5D).

An unexpected finding which was consistently observed with the cortical transplants was the presence of an extra-axial mass which grew on the surface of the skull. When paraffin-embedded sections of these masses were stained with hematoxylin and eosin, the morphology was found to be characteristic of an infec-

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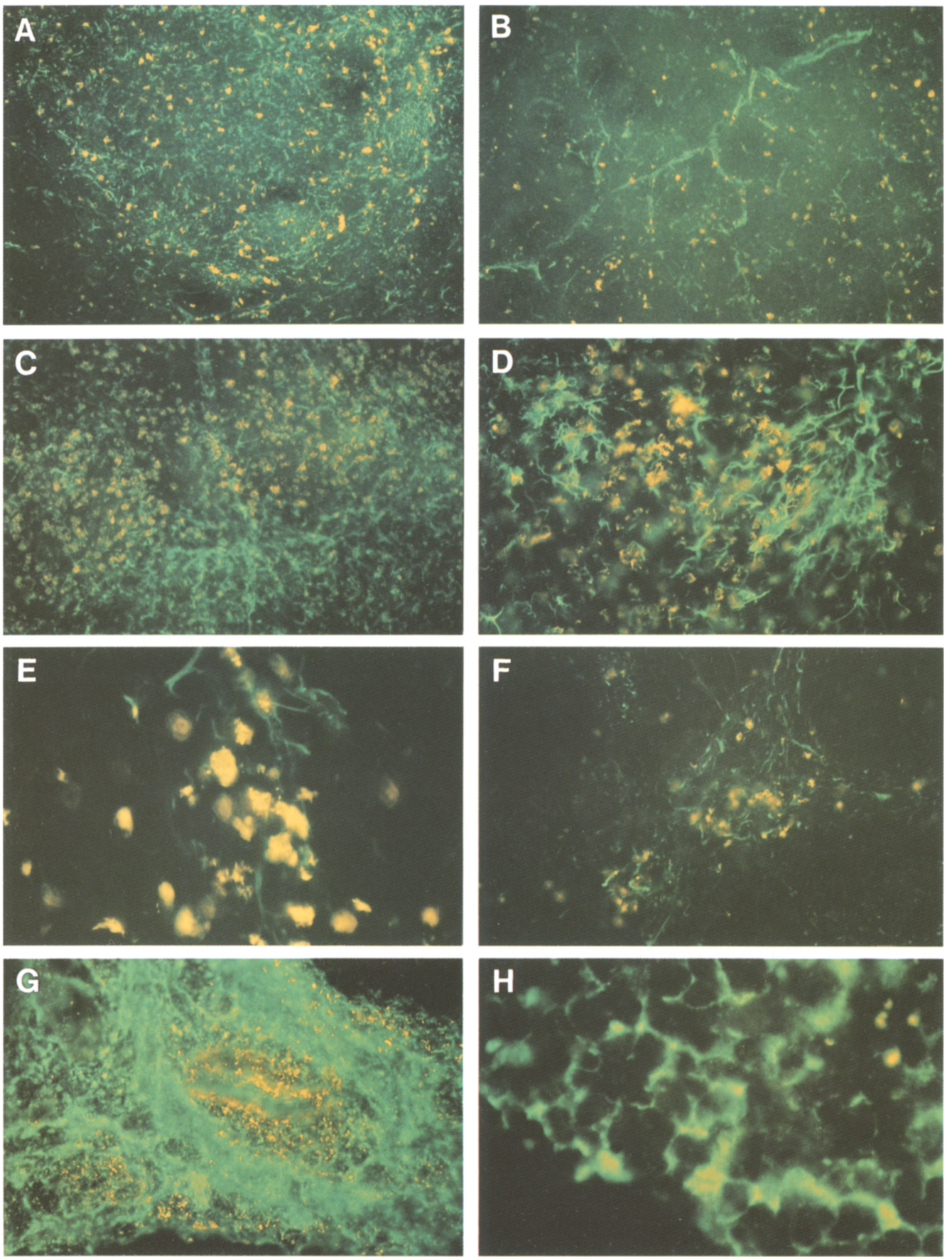
Fig. 4. Fluorescent photomicrographs from an E-14 rat embryo showing the relative distribution of NEP staining in representative regions of the ventral mesencephalon (V.Mes) and cortical neuroepithelium (cx) which were transplanted. A,B: higher-power detail of NEP staining in the brainstem (A) and the corresponding lower-power photomicrograph of the same section visualized by Nissl stain (B). The brackets in B demarcate the area represented in A. C,D: NEP staining is enriched in the deeper layers of the cortical neuroepithelium (cx) and the adjacent basal telencephalon (bta, bti), as verified by the corresponding photomicrograph of the same section visualized by Nissl stain (D). The arrows indicate non-specific labeling of the tissue by nitrosalicylaldehyde at the pial surface and adjacent to the hippocampal neuroepithelium (hi). Bar in A = 100 μm; B,C,D = 200 μm.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tious granuloma containing regions of calcification surrounded by bands of fibroblasts and infiltrates of macrophages, lymphocytes, and neutrophils (not shown). The granulomas stained richly for NEP (Fig. 5G) which appeared to be associated with infiltrates of white blood cells identified by staining with hematoxylin and eosin (not shown). In addition, rich TGFαp immunocytochemical labeling was closely associated with many of the NEP-positive regions (Fig. 5G) and labeled numerous intensely immunoreactive processes (color plate, panel H) which appeared, on staining with hematoxylin and eosin to correspond to fibroblast-rich regions (not shown).

Localization of NEP and transforming growth factor-alpha

The staining for TGFαp was restricted to a subpopulation of astrocytes which were scattered throughout both the mesencephalic and cortical transplants, as previously described[19]. Labeled astrocytes were often found to be closely associated with blood vessels. Double-labeling studies for NEP and TGFαp revealed that areas enriched in TGFαp were often richly labeled for NEP, as well (Fig. 3A–C). Fig. 3B,C shows two examples of TGFαp staining of apparent astrocytic processes adjacent to a blood vessel. Closely associated with these processes were numerous small somata intensely labeled for NEP which may be endothelial cells of vessels supplying the transplant. The TGFαp and NEP staining did not appear to overlap and cellular elements of differing morphology were revealed by the two fluorophores (Fig. 3D; Fig. 5F).

Localization of NEP to fetal brain

To investigate the source of the NEP staining in the transplants, cryostat sections of rat embryos (ED14) were stained for NEP. These studies confirmed that NEP localizes to the regions of the ventral mesencephalon (Fig. 4A) and the cortical neuroepithelium (Fig. 4C) that were dissected to prepare the suspensions of fetal cells for the transplants. In general, the embryos displayed rich NEP staining which was widely distributed in the CNS and appeared to bear a similar distribution to that which we have previously reported in the adult rat CNS[2,3]. Staining was particulary enriched in the basal telencephalon, including the striatum, the hypothalamus, the pituitary/Rathke's pouch, and throughout the pons, medulla, and spinal cord. Rich NEP staining was also observed in a number of peripheral tissues including the thyroid, lungs, liver, intestines, and metanephros and light NEP staining was observed over the atria and ventricles of the heart (not shown).

It was not possible to characterize, in most regions, the cellular localization of the NEP reaction product due to limitations in the tissue morphology encountered with the frozen sections. NEP staining was optimized with a brief post-fixation in 4% paraformaldehyde (5 min). Prolonged fixation with paraformaldehyde (> 20 min) or brief fixation with cold acetone (> 5 s) gave inconsistent staining, often destroying all enzymatic activity.

All histochemical staining throughout the embryos (both CNS and peripheral tissues) was blocked in a dose-dependent manner by the NEP inhibitors, JHF-26 and phosphoramidon (data not shown) confirming the exclusive localization of NEP by the histochemical method. Staining was completely blocked at a concentration as low as 100 nM, but did not differ from control when treated with a dose of 1 nM.

Immunocytochemical localization of GFAP in four animals revealed few astrocytes in either the ventral mesencephalon or the cortical neuroepithelium despite the presence of rich staining for GFAP in dorsal root

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Fig. 5. Fluorescent photomicrographs showing the relative distribution of NEP reaction product (bright yellow) and fluorescein-labeled GFAP- (panels A–E) or TGFαp-immunoreactive elements (panels F–H) visualized using a narrow band blue (fluorescein) filter by a fluorescent double-labeling technique (see text for details). A,B: low-power fluorescent photomicrograph of a coronal section through a transplant of fetal ventral mesencephalic cells (8 weeks after transplantation) and the adjacent striatum showing the increased staining for both NEP and GFAP in the transplant, at center, compared to the normal distribution of NEP and GFAP in the contralateral striatum (panel B). C: low-power fluorescent photomicrograph of a coronal section through a transplant of fetal cortical cells (6 weeks after transplantation). The dorsal extent of the transplant is at the left and the lateral extent at the top of the figure. Collections of somata intensely labeled for NEP are shown adjacent to the medial extent of the transplant where rich GFAP-labeling of reactive astrocytes is seen. D: detail of panel C showing the close association of NEP-labeled somata and GFAP-labeled astrocytes. The astrocytes appear to be largely unstained. E: high-power fluorescent photomicrograph showing the close, but non-overlapping, association of NEP-positive somata and GFAP-immunoreactive astrocytes in an intrastriatal transplant of ventral mesencephalic cells 8 weeks after implantation. F: high-power flourescent photomicrograph from an intrastriatal transplant of cortical cells 6 weeks after the implant showing a collection of small cells intensely labeled for NEP interspersed among numerous fine glial processes labeled for TGFαp. G: fluoresecent photomicrograph showing the relative distributions of NEP and TGFαp in a section taken from an extra-galeal infectious granuloma which grew in animal sacrificed 8 weeks after implantation of fetal cortical cells (see text for details). H: high-power detail showing the intense TGFαp immunoreactivity in the granuloma. A–C = 50×; D,F, G = 100×; E = 200×; H = 500×.

and other ganglia (data not shown). No NEP staining was found to colocalize with GFAP-labeled astrocytes in either the CNS or PNS.

DISCUSSION

The present study was undertaken to determine whether the predominantly non-glial localization of NEP in the adult rat brain[4] is also present during embryonic development at the age of the transplants (ED14) and to what extent this developmental pattern of expression is altered when a mixed fetal transplant into the adult rat brain gives rise to a progressively enlarging benign mixed tumor. To address this question, we developed a new fluorescent double-labeling technique to examine the distribution of NEP in astrocytes. The transplanted cells in this study derived from fetal brain regions which contained relatively few glia, despite the fact that numerous GFAP-positive astrocytes were visualized in peripheral nerves and ganglia. Despite the presence of NEP staining in the embryonic cortical neuroepithelium and the ventral mesencephalon, no NEP labeled astrocytes were observed. The transplants, by contrast, were greatly enriched in astrocytes. However, an examination of the cell types expressing NEP in the transplants supported an apparent localization of NEP predominantly to non-glial elements. Only occasional astrocytic elements colocalized NEP. Hence, the apparent non-astrocytic expression of NEP in the ED14 embryo persisted in the transplants and suggests that the neuronal localization of NEP characteristic of the adult rat brain may also be characteristic of the fetal brain and persist following transplantation.

The present study demonstrated that suspensions of cells from two different regions of the fetal brain gave rise to transplants which were apparently enriched in NEP activity. This was supported by the observation that under conditions in which rich staining for NEP was optimally visualized in the transplants, relatively low levels of staining were observed in the surrounding host striatum which displays one of the higher levels of NEP activity in the rat CNS[6,21,25]. In agreement with previous studies in the neonatal rat brain[6], rich NEP activity was found to localize to the striatum, as well as to the regions employed in the transplants, thus suggesting one possible source of the NEP localized to the transplants. The marked upregulation of NEP in the transplants may have significance for the regulation of transplant growth or differentiation via the cleavage of endogenous peptide growth factors produced by or in response to the transplants. NEP may inactivate peptide growth factors or, alternatively, convert them to an active form. NEP staining localized to many areas of the transplant rich in astrocytes where, NEP might, for example, serve to regulate glial proliferation. Endothelin is a potent glial mitogen[18] inactivated by NEP[31]. Atrial natriuretic peptide is also cleaved by NEP[7,29] and has a glial antiproliferative effect[17]. The enkephalins suppress the proliferation of glia in culture and increase the surface area of the cells[10,30]. The enkephalins also negatively modulate the growth of neuroblastoma cell hybrids[34]. Were enkephalins from the surrounding host striatum to have a similar growth-suppressing effect, the enriched NEP in the transplants might antagonize this effect via enkephalin hydrolysis. Such a role for NEP in the inactivation of the enkephalins or other peptide growth factors is reasonable given the recent finding that NEP peptidase activity antagonizes the growth-promoting autocrine effects of the bombesin-like peptides on squamous cell carcinoma growth[28].

One complication of transplantation as a potential therapeutic modality is the inherent potential of the transplants to give rise to tumors in the host[1,11,19,20]. In many instances, the tumors are cytologically benign, but, nevertheless, exert a progressive mass effect. In support of the benign nature of these mixed tumors, we observed NEP to be almost exclusively localized to non-glial cellular elements. This is consistent with the recent finding of Monod et al.[24] that only 10% of low grade gliomas express NEP immunoreactivity, whereas 96% of high grade gliomas stain for NEP.

We examined the localization of TGFαp in the transplants for several reasons. TGFα is a potent neurotrophic factor, supports the growth of neurons and is also a known glial mitogen[19,26]. TGFαp has been shown to be enriched in astrocytes, and is increased in fetal midbrain and cortical cell suspension transplants[19]. TGFα is also selectively elevated in high grade human gliomas[27]. The relative distribution of TGFαp and NEP was studied given that both have been identified as potentially useful tumor markers. The close association between TGFαp-labeled astrocytes and NEP in the transplants and the extra-axial granulomas suggests that the combination of these two markers may be useful to gauge the 'proliferative potential' of either benign or malignant tumors.

ABBREVIATIONS

4V	fourth ventricle
bta	basal telencephalon, anterior
bti	basal telencephalon, intermediate
CphF	cephalic flexure
cx	cortical neuroepithelium
GFAP	glial fibrillary acidic protein

hi	hippocampal neuroepithelium
LM	lateral mammillary nucleus
LV	lateral ventricle
PnF	pontine flexure
Rathke	Rathke's pouch
TGFαp	transforming growth factor-alpha precursor
Th	thalamus
V.Mes	ventral mesencephalon

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