

# Expression of acidic and basic fibroblast growth factors in the substantia nigra of rat, monkey, and human

(coexistence/dopamine/mRNA/tyrosine hydroxylase/neurotrophic factor)

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Contributed by Tomas Hökfelt, August 16, 1991

**ABSTRACT** The distribution of acidic (aFGF) and basic (bFGF) fibroblast growth factor mRNA and protein were examined in mesencephalon by immunohistochemistry, immunoblot analysis, *in situ* hybridization histochemistry, and RNA analysis. Coexistence of aFGF or bFGF with tyrosine hydroxylase protein in nigral cells was observed with immunohistochemistry. Both aFGF and bFGF mRNAs were found in the substantia nigra. Unilateral 6-hydroxydopamine lesions of nigrostriatal neurons resulted in a loss of aFGF and tyrosine hydroxylase [L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] mRNA-positive neurons on the lesioned side. The distribution of aFGF mRNA in monkey brain was similar to that seen in the rat. RNA and immunoblot analysis confirmed the presence of both aFGF and bFGF mRNAs and proteins in the substantia nigra of rat, monkey, and human.

The discovery that neurotrophic factors present in the central nervous system can support the development and maintenance of various neuronal populations has led to the hypothesis that these factors may be related to the etiology, or useful in the treatment, of neurodegenerative disorders (1–3). Parkinson disease is characterized by a selective loss of mesencephalic dopamine (DA) neurons, and DA replacement therapy does not address the underlying neurodegeneration (4). Several recent studies have reported increased survival of mesencephalic DA neurons when exposed to various growth factors or extracts of target tissue *in vitro* (5–7) and *in vivo* (8, 9). However, the relationship between the source and target for many neurotrophic factors in discrete regions of the central nervous system remains to be established.

Fibroblast growth factors (FGFs) are a family of related peptides that have been characterized by their ability to induce cellular differentiation as well as by their mitogenic and angiogenic properties (10–12). Complementary DNAs encoding acidic (aFGF) and basic (bFGF) FGF have been cloned and sequenced and are found to share 55% total sequence homology on the amino acid level (10). aFGF and bFGF share a distinctive feature among FGFs by lacking a signal sequence, suggesting that they are not secreted through the classical exocytotic pathway (10–12). FGFs have been shown to bind to heparin and to a recently identified group of high-affinity cell-surface receptors that contain regions possessing tyrosine kinase activity (refs. 10 and 13–15 and the refs. cited therein). aFGF and bFGF have been hypothesized to leak out of damaged cells and bind to heparin sulfate proteoglycans in the extracellular matrix (16). The extracellular matrix may act as a reservoir or may be actively involved in interactions of FGFs with their cell-surface

receptors (17, 18). Thus, the trophic effects of aFGF and bFGF may be directed toward cells that produce these factors.

Both aFGF and bFGF have been purified from brain and have trophic effects on primary cultures of neurons from various central nervous system regions as well as on neurally derived cell lines *in vitro* (10–12, 19–24). In the present study we have examined the distribution of aFGF by immunohistochemistry, immunoanalysis, *in situ* hybridization, and RNA analysis, with focus on aFGF in the substantia nigra of rat, monkey, and human. bFGF was studied for comparison.

## MATERIALS AND METHODS

**Immunohistochemistry.** Male Sprague–Dawley rats (150–250 g; Alab, Stockholm) were pretreated with intraventricular colchicine (120  $\mu$ g/20  $\mu$ l) 24 hr before pentobarbital sodium (60 mg/kg i.p.) anesthesia and intracardiac perfusion with picric acid-containing formalin. After postfixation and rinsing, sections (14  $\mu$ m) through the substantia nigra were cut on a cryostat, thaw-mounted onto gelatin-coated slides, and incubated with antibodies to either affinity-purified aFGF [4  $\mu$ g/ml (25)] or bFGF [1:200; prepared against amino acids 1–24 of bFGF (J. McAvoy, Department of Histology, University of Sydney, Sydney, Australia)] either alone or in the presence of aFGF or bFGF protein (20  $\mu$ g/ml). Monoclonal anti-tyrosine hydroxylase [TyrOHase; L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] antibodies (1:3200; Incstar, Stillwater, MN) were used for double-labeling studies. After incubation in primary antiserum sections were rinsed and processed for indirect immunofluorescence, as described (25).

***In Situ* Hybridization Histochemistry.** Male Sprague–Dawley rats (150–250 g; Alab), grey monkeys (*Macaca fasciculata*), and human postmortem material were used for analysis. Tissues were frozen, sectioned at 14  $\mu$ m in a cryostat, thaw mounted onto slides (ProbeOn, Fisher), and stored at  $-20^{\circ}$ C until used. A 48-base-pair oligonucleotide complementary to nucleotides 284–331 within the coding region of human aFGF [100% sequence homology with rat aFGF (26); 33% sequence homology with the corresponding region of bFGF (27)] was synthesized on an Applied Biosystems model 380B DNA synthesizer. An oligonucleotide probe complementary to rat/human TyrOHase [nucleotides 121–157 (28)] was also used. Each oligonucleotide probe was 3'-end-labeled using terminal deoxynucleotidyltransferase (Amersham) and adenosine 5' [ $\alpha$ - $^{35}$ S]thio]triphosphate (DuPont/NEN) to a specific activity of  $1.0$ – $3.5 \times 10^9$  cpm/ $\mu$ g and purified on NENsorb 20 columns (DuPont/NEN). A

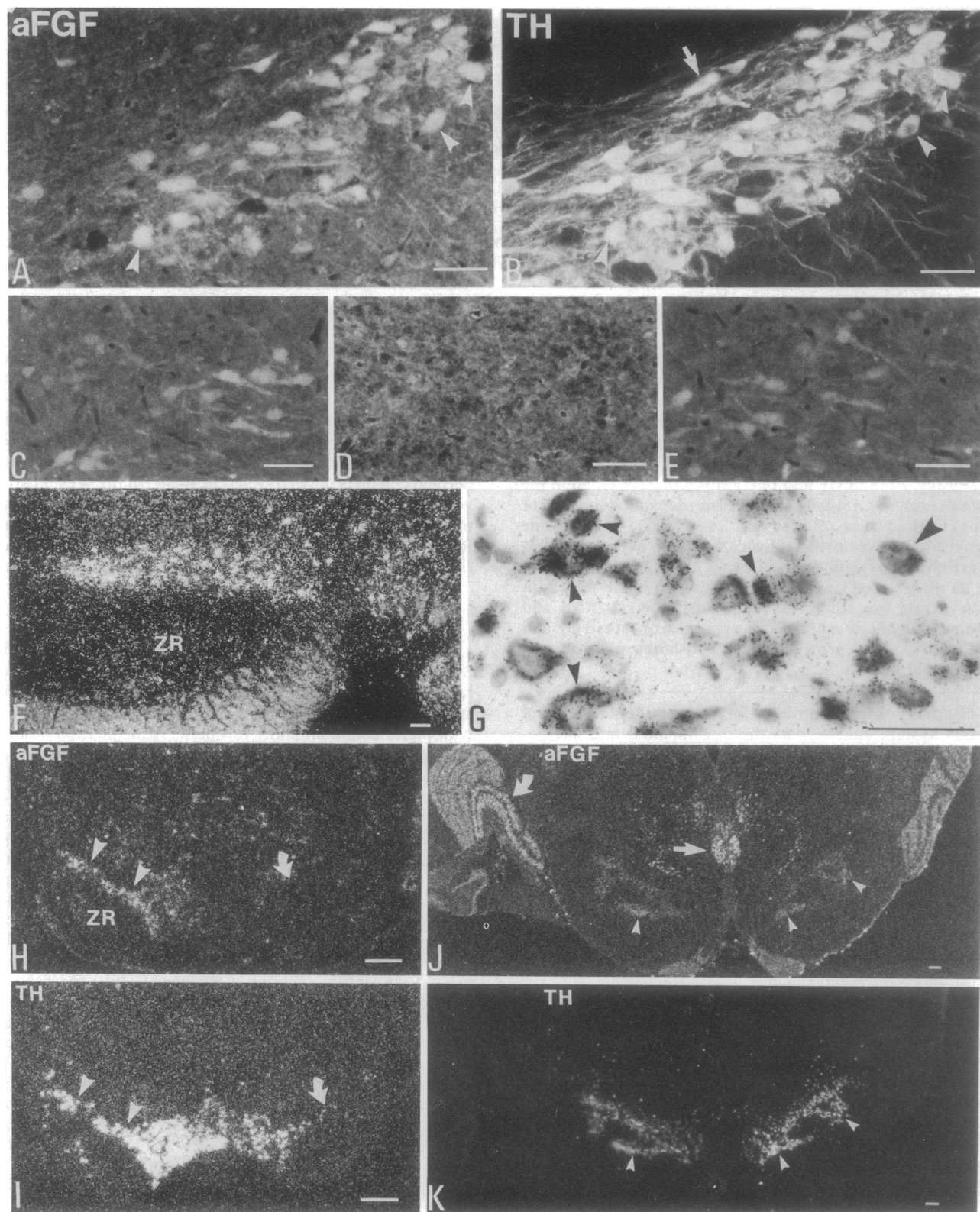


FIG. 1. Expression of aFGF protein and mRNA in the substantia nigra of rat (A–I) and monkey (J and K). (A) Immunofluorescence localization of aFGF in the substantia nigra. (B) Same section as in A stained with anti-TyrOHase (TH). Arrowheads show cells also stained for aFGF in A; arrow in B points to a cell positive for only TyrOHase. (C–E) aFGF immunohistochemistry shows section incubated with aFGF antiserum (C), after antibody absorption with aFGF protein (20  $\mu$ g/ml) (D), and after absorption with bFGF protein (20  $\mu$ g/ml) (E). (F) Dark-field micrograph of aFGF mRNA in rat substantia nigra. ZR, substantia nigra zona reticulata. (G) Bright-field micrograph of rat substantia nigra showing aFGF mRNA-positive (small arrowheads) and -negative (large arrowhead) cells. (H) Loss of aFGF mRNA after unilateral 6-hydroxydopamine lesion in rat (arrowheads, control side; curved arrow, lesioned side). (I) TyrOHase mRNA in the adjacent section to H. (J) aFGF mRNA in monkey mesencephalon is found in substantia nigra (arrowheads), oculomotor nucleus (arrow), and lateral geniculate nucleus (curved arrow). (K) TyrOHase mRNA in adjacent section to I. (Bars = 50  $\mu$ m.)

2.2-kilobase (kb) rat cDNA probe to bFGF (29) was labeled with  $^{32}\text{P}$  by the random-primer method and denatured before use. Hybridizations were conducted by following described protocols (30).

**RNA and Immunoblot Analysis.** Discrete dissections of the substantia nigra were performed on rat, monkey, and human samples. Rat brain regions were dissected fresh from coronal slices (31), and monkey and human brains were dissected from frozen coronal slices. Total RNA was prepared from tissue samples, separated on agarose gels, and transferred onto Hybond-N nylon membranes (Amersham) as described (32). The ribosomal 18S and 28S bands were marked for use as size standards. Protein analysis was done as described (25, 33).

**6-Hydroxydopamine Lesions.** Male Sprague-Dawley rats (175–225 g, Alab) were pretreated with desmethylimipramine (25 mg/kg i.p., 30 min before surgery) and anesthetized with pentobarbital sodium (55 mg/kg i.p.) before stereotaxic injection of 6-hydroxydopamine into the posterior median forebrain bundle (10  $\mu\text{g}/2 \mu\text{l}$  of saline containing 0.2% ascorbic acid; 0.2  $\mu\text{l}/\text{min}$ ) (coordinates from Bregma: anterior posterior  $-4.5$ , lateral 1.8, ventral  $-8.2$ ). Rats used for *in situ* hybridization were sacrificed by decapitation 14 days after surgery and processed as described above.

## RESULTS

**Antibody and Probe Specificity.** The aFGF antibody used in these experiments has been characterized (25, 33). Anti-bFGF antiserum exhibited little cross reactivity with aFGF by immunoblot analysis (data not shown). The oligonucleotide probes used in these studies for TyrOHase and aFGF recognize single bands of 1.9 kb and 4.3 kb, respectively, on Northern (RNA) blots of total RNA from adult brain (data not shown). The rat cDNA probe to bFGF recognizes four bands on Northern analysis; a prominent 3.7-kb band, as well as minor 2.4-kb, 1.7-kb, and 1.5-kb bands.

**aFGF.** With immunohistochemistry, weak staining for aFGF was observed in neurons of the substantia nigra, and double staining showed that most aFGF-positive neurons contained TyrOHase, but not all TyrOHase-positive nigral neurons contained aFGF (Fig. 1 A–C). Absorption of aFGF antiserum with aFGF protein (Fig. 1D), but not with bFGF protein (Fig. 1E), blocked the immunostaining in the substantia nigra. Immunoblot analysis confirmed that detectable aFGF protein was expressed in substantia nigra but not in cerebellum, striatum, or prefrontal cortex (Fig. 2).

At the level of the rat mesencephalon aFGF mRNA was observed in several nuclei, including strongly positive neurons in the oculomotor nucleus, the mesencephalic nucleus of the fifth nerve, lateral mammillary nucleus, ventral tegmental area, and substantia nigra (Fig. 1 F and H). Unilateral 6-hydroxydopamine lesions reduced the number of cells positive for aFGF (Fig. 1H) and TyrOHase mRNA (Fig. 1I) in the substantia nigra on the lesioned side. Northern analysis confirmed the presence of a 4.3-kb transcript in the rat substantia nigra (Fig. 3). No detectable aFGF mRNA was present in neurons or extracts of rat cerebellum, striatum, or prefrontal cortex (Fig. 3).

Neurons expressing aFGF mRNA were also observed in the monkey mesencephalon. The lateral geniculate nucleus, substantia nigra, the oculomotor nucleus, and the mesencephalic nucleus of the fifth nerve contained hybridization-positive cells (Fig. 1J). aFGF protein and a 4.3-kb transcript were detected in extracts of monkey substantia nigra with immunoblot analysis (Fig. 2) and Northern analysis (Fig. 3). In the human substantia nigra some neurons were hybridization-positive for aFGF mRNA (data not shown). All aFGF mRNA-positive neurons contained melanin pigment, although there were many melanin-positive cells that did not

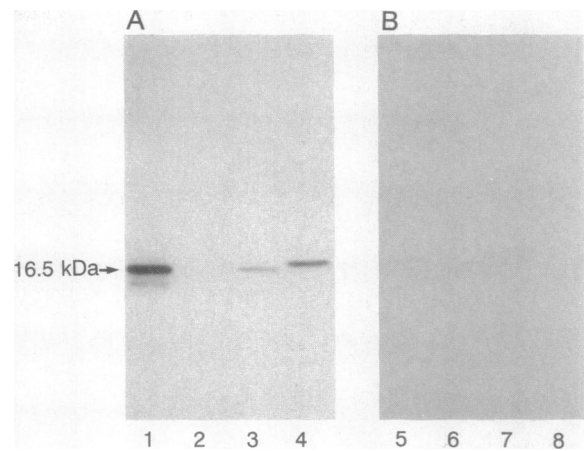


FIG. 2. Immunoblot analysis of protein extracted from rat, monkey, and human brain. Lanes: 1, recombinant human aFGF; 2, recombinant bFGF; 3, human substantia nigra; 4, monkey substantia nigra; 5, rat substantia nigra; 6, rat cerebellum; 7, rat striatum; and 8, rat prefrontal cortex.

appear to be hybridization positive. Northern and immunoblot analysis confirmed the presence of aFGF mRNA (Fig. 3) and aFGF protein (Fig. 2) in the human substantia nigra.

**bFGF.** Strong bFGF immunostaining was seen in all parts of the substantia nigra (Fig. 4 A, C, and D). bFGF was also observed in glial cells (Fig. 4C) and was generally more widely distributed throughout the mesencephalon than aFGF. Analysis of adjacent sections showed a partial overlap of bFGF and aFGF, but colocalization was not directly demonstrated. Double staining showed that almost all bFGF-positive nigral (Fig. 4 A and D) neurons contained TyrOHase and vice versa (Fig. 4 B and E). Absorption of bFGF antisera by bFGF protein resulted in loss of the evenly distributed cytoplasmic staining but revealed a staining pattern with an apparent nuclear localization (Fig. 4F) (34).

bFGF mRNA was widely distributed within the rat mesencephalon. Hybridization-positive neurons were located in the substantia nigra, although many other hybridization-positive cells were observed throughout the region (data not shown). Northern analysis confirmed the presence of bFGF in the rat substantia nigra (Fig. 3). In contrast to aFGF, bFGF mRNA was present in extracts of cerebellum and striatum but absent from prefrontal cortex (Fig. 3).

Neurons expressing bFGF mRNA were observed in the monkey mesencephalon. The substantia nigra contained hybridization-positive cells (data not shown). A 3.7-kb transcript was detected in extracts of monkey substantia nigra

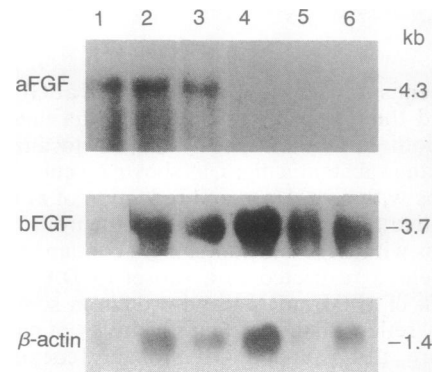


FIG. 3. Northern analysis of total RNA extracted from rat, monkey, and human brain. Lanes: 1, human substantia nigra; 2, monkey substantia nigra; 3, rat substantia nigra; 4, rat cerebellum; 5, rat striatum; and 6, rat prefrontal cortex.

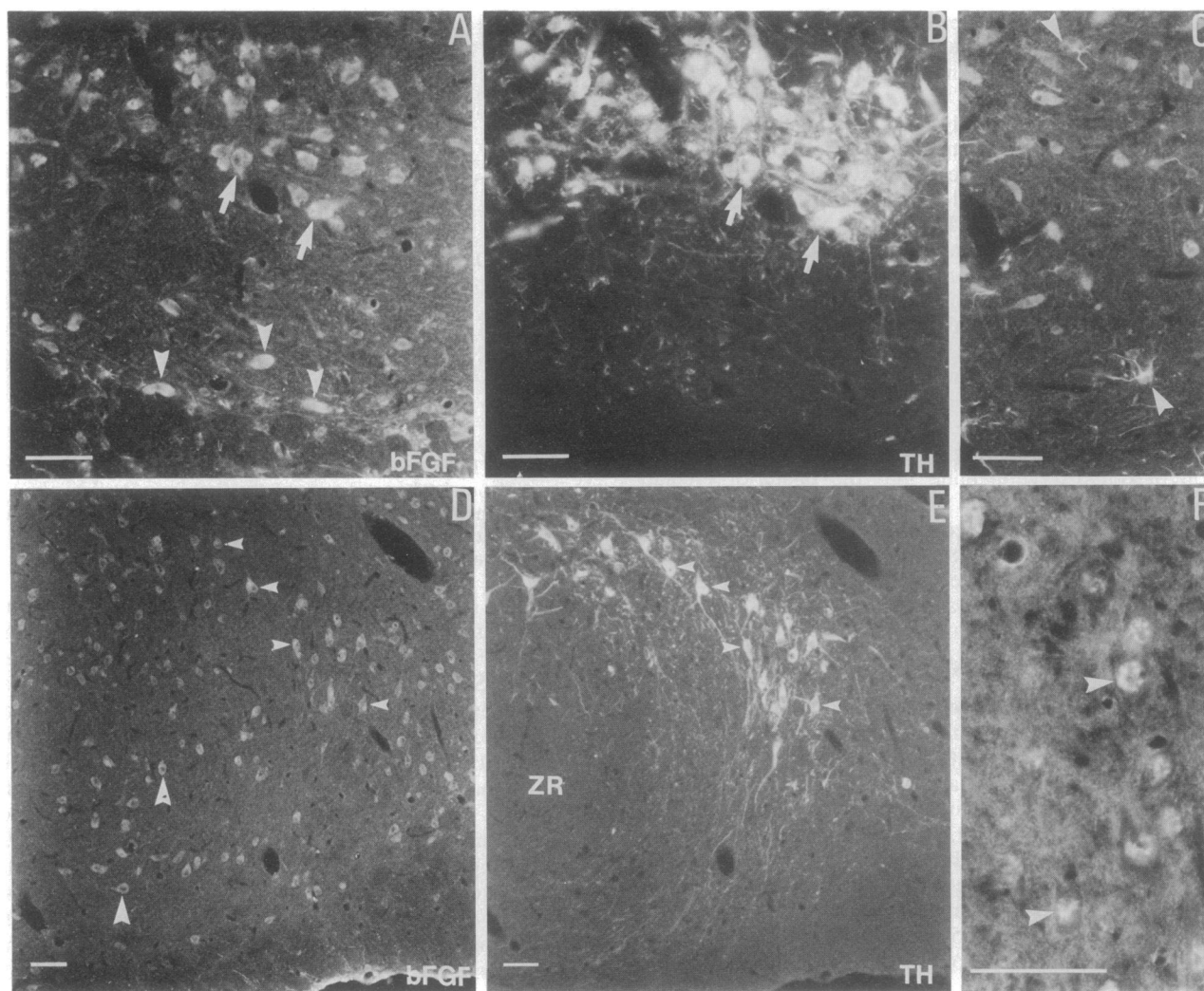


FIG. 4. Immunofluorescence micrographs of rat substantia nigra showing bFGF and TyrOHase. (A and B) Subpopulation of bFGF-positive neurons in substantia nigra contain TyrOHase (TH) (arrows, compare A with B), but some bFGF-positive nigral neurons do not contain TyrOHase (arrowheads). (C) bFGF is present in glia (arrowheads) within the ventral mesencephalon. (D and E) bFGF-positive cells are found in both substantia nigra zona compacta, where they may be colocalized with TyrOHase (small arrowheads, compare with E) and zona reticulata, where they are not colocalized with TyrOHase (large arrowheads). (F) Absorption of bFGF antiserum by bFGF protein results in apparent nuclear labeling. (Bars = 50  $\mu$ m.)

with Northern analysis (Fig. 3). bFGF protein was also observed in the monkey substantia nigra (data not shown).

Immunoblot and Northern analysis confirmed the presence of bFGF mRNA and bFGF protein in the human substantia nigra (Figs. 2 and 3).

## DISCUSSION

In the present study we have observed both aFGF and bFGF mRNA and their proteins in the substantia nigra of three species. Both aFGF and bFGF appear localized in DA neurons in the substantia nigra, as shown by colocalization of the proteins with TyrOHase and reduction of aFGF mRNA after selective lesions of rat nigral DA neurons. Almost all DA neurons within the substantia nigra contained bFGF, but aFGF appeared restricted to a subset of DA neurons, although lack of aFGF mRNA and protein in some DA cells may simply reflect an insufficient sensitivity of our methods. Furthermore, aFGF and bFGF probably coexist in some nigral DA neurons, although we have no direct double-labeling evidence concerning their colocalization.

Previous studies on the distribution of bFGF have noted its presence in brain. Northern analysis has suggested that bFGF mRNA is distributed widely throughout the brain (35),

and immunohistochemical studies have reported localization of bFGF in neurons of the cerebral cortex, hippocampus, cerebellum (36–38), and in neuroglia (39). These data are in accord with our present observations suggesting a widespread distribution of bFGF in neurons and glial cells within brain.

With regard to aFGF, we have found restricted neuronal expression of aFGF in brain (present study) and in motor neurons and sensory ganglia (25). Moreover, we have observed the distributions of aFGF mRNA and protein to be identical in our studies. In contrast, a previous immunohistochemical study (40) has suggested a predominantly glial localization of aFGF in brain. Additionally, widespread neuronal distribution of aFGF mRNA, including presence within hippocampus, cerebellum, and neocortex, has been suggested based on *in situ* hybridization studies (41). However, the localization of aFGF mRNA reported by these authors (41) more closely resembles the distribution of bFGF mRNA we have observed. For example, in the cerebellum we have noted no detectable aFGF mRNA, whereas abundant bFGF mRNA was observed in this structure (Fig. 3). In contrast, Wilcox and Unnerstall (41) have observed aFGF hybridization-positive cerebellar granular cells. These differences may be due to the low-stringency conditions used by these authors



and high homology (83%) between their bovine aFGF probe and the bFGF sequence.

The presence of both aFGF and bFGF in DA neurons of the substantia nigra suggests that these neurons, or afferents to these neurons, may be responsive to the trophic effects of these molecules. It will be important to establish the precise localization of receptors for aFGF and bFGF (refs. 13 and 14 and the references cited therein) in the mesencephalon to determine whether these molecules may be acting on nigral neurons. Additionally, the discrete localization of aFGF compared with bFGF argues for the possibility that these two molecules may have different trophic roles.

We thank K. Åman, K. Friberg, W. Hiort, and S. Nilsson for excellent technical assistance; Dr. J. McAvoy (University of Sydney, Australia) for supplies of bFGF antiserum; Dr. Judith A. Abraham (California Biotechnology, Inc., Mountain View, CA) for human recombinant bFGF; Drs. Kurokawa, Seno, and Igarashi (Takeda Chemical Industries, Osaka) for rat bFGF cDNA probe; and Dr. A. Härfstrand (Pharmacia) for donation of monkey tissue. Human tissue used in this study was obtained from the Maryland Brain Collection at the Maryland Psychiatric Research Center in collaboration with the division of Forensic Pathology, University of Maryland (supported, in part, by the National Institute of Mental Health Grant 44211). These studies were supported, in part, by the Scottish Rite Schizophrenia Research Foundation (A.J.B.), the Pharmaceutical Manufacturers Association Foundation (A.J.B.), the Curtis L. Carlson Visiting Professorship (R.E.), the Swedish Medical Research Council (04X-2887; T.H.), and the National Institute of Mental Health (43230; T.H. and M.G.).

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