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# Immunocytochemical localization of basic fibroblast growth factor in the rat pineal gland

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Abstract: The immunocytochemical localization of basic fibroblast growth factor (b-FGF) during the postnatal development of the rat pineal gland was studied using a polyclonal antibody against the fraction 1-24 of bovine recombinant b-FGF. Basic FGF immunoreactivity was evident from day 20 after birth in the endothelial cells and perivascular spaces of the gland. Although b-FGF immunostaining showed its maximal expression at 30–45 days, it was maintained throughout the entire study period (up to 6 months), mainly in the distal zone of the gland. Pinealocytes did not show b-FGF immunoreactivity at any time. There were no differences in the localization patterns or the intensity of b-FGF immunostaining after the prenatal denervation with DSP-4, a neurotoxic amine. The physiological role of b-FGF in the adult pineal gland remains unknown; however, it does not seem to play a major role during the cytodifferentiation period of the parenchymal cells, or during the neovascularization in the early postnatal days. Furthermore, its immunocytochemical expression is not affected by the prenatal sympathetic denervation with DSP-4, in contrast with other neutrophic factors.

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## Introduction

Basic fibroblast growth factor (b-FGF), a polypeptide originally isolated from bovine pituitary extracts, has a broad series of biological effects on a wide spectrum of cell types. Numerous in vitro studies have demonstrated that b-FGF (1) stimulates the growth and proliferation of several mesoderm-derived cells (2) is a potent angiogenic factor, (3) increases the survival of cultured neurons and the growth of the neurite, (4) stimulates astrocyte proliferation and, in general, delays the ultimate senescence of cells in culture, promoting the stabilization of their phenotypic expression [Gospodarowicz et al., 1987].

In vivo studies have confirmed the pivotal role played by b-FGF in the processes of angiogenesis and tissue repair [Gospodarowicz et al., 1987; Rifkin and Moscatelli, 1989]. Furthermore, b-FGF is an inducer of the mesoderm during early embryogenesis [Slack et al., 1987] and probably has an effect on the proliferation and differentiation of astrocytes and oligodendrocytes in the central nervous system [Morrison et al., 1985; Eccleston and Silberberg, 1985].

In 1988, the presence of the so-called endothelial cell stimulating angiogenic factor was reported in extracts of bovine and human pineal glands in concentrations that were notably higher compared with those of other highly vascular tissues [Taylor et al., 1988]. Further evidence showed that this growth factor was actually b-FGF. We have found no data in the literature on the cellular localization of b-FGF in the pineal gland.

In the present work we studied, by immunohistochemistry, the presence of b-FGF and its postnatal development in the rat pineal gland. Moreover, since chemical denervation produces some effects on the expression of other growth factors in this gland [García-Mauriño et al., 1992], we included in the study protocol pineal glands from N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) denervated rats. DSP-4 is a neurotoxic amine that produces a selective axonal degeneration of noradrenergic neurons [Jonsson et al., 1981]; these

constitute the main innervation of the pineal gland in this species [Bowers et al., 1984; Calvo et al., 1990a].

## Material and methods

### Animals

Forty-eight pineal glands from Wistar rats of both sexes were studied. Animals were kept under standard lighting conditions (LD 14:10), with free access to food and water.

For the study of the postnatal development, three animals from the same litter were killed by decapitation under ether anesthesia on days 1, 3, 5, 7, 10, 15, 20, 30, 45, 60, 90, and at 6 months after birth. The procedure was carried out at 1800 between April and May. Two pineal glands from each interval (n = 24) were fixed by immersion in Methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid) for 24 hr at room temperature. Twelve pineal glands were fixed in Bouin's solution under the same experimental procedure and embedded in paraffin.

Chemical denervation was performed prenatally by the intraperitoneal injection of DSP-4 (Fluka AG, Buchs, Switzerland ; 25 mg/kg of body weight diluted in distilled water administered in a single dose to pregnant rats on gestational day 15 [Jonsson et al., 1981]). The pineal glands of the animals delivered were studied at the same intervals stated above (n = 12). Since in preliminary results we observed a much better preservation of the b-FGF immunostaining in methacarn-fixed tissues, all the pineal glands from denervated rats were fixed by immersion in this fixative and embedded in paraffin.

Four additional pineal glands from control rats 30 and 45 days old were fixed by immersion in glutaraldehyde 2%–paraformaldehyde 2% in phosphate buffer 0.1 M pH 7.4 at 4°C for the study in semi-thin sections at the light microscopic level. These glands were embedded in Epon without previous osmification.

### Immunocytochemical procedure

Paraffin-embedded tissues were immunostained according to the three-step peroxidase-antiperoxidase (PAP) technique [Taylor, 1986]. Briefly, after deparaffination and abolition of endogenous peroxidase activity with a solution of methanol containing 3% hydrogen peroxide, sections 7-μm thick were incubated in 3% nonimmune swine serum (NSS) for 30 min followed by anti-b-FGF antiserum for 16 hr at room temperature (diluted 1:200 in 3% NSS-PBS). This antiserum was developed in rabbits using the synthetic peptide sequence of bovine b-FGF[1–24] (Sigma Chemical Co., St. Louis, MO). The antiserum detects b-FGF and does not cross-react (< 1%) with acidic-fibroblast growth factor in Western blot analysis. Further incubations with swine anti-rabbit IgG and the PAP complex (both from Sigma, St. Louis, MO) were carried out at room temperature. The immunoreaction product was visualized with a freshly prepared solution of 3-3'-diaminobenzidine-tetrahydrochloride (DAB). A slight nuclear counterstaining with hematoxylin was applied.

Specificity controls consisted of (1) incubation with NSS instead of the primary antiserum and (2) incubation with the primary antiserum pre-adsorbed with recombinant bovine b-FGF (Böehringer, Mannheim, Germany). In the preadsorption test, the primary antiserum was incubated with 75 μg/ml of b-FGF during 3 hr at room temperature and later for 10 hr at 4°C. Final antibody dilution remained at 1:200. The pre-adsorbed antiserum was microfuged at 12,500*g* for 15 min and the supernatant was used to verify the specificity of the immunoreaction.

The intensity of the immunoreaction of the Bouin-fixed tissues was not increased after the digestion of the sections with 0.4% pepsin (Sigma, St. Louis, MO) in HCl, 0.01 N for 15 min.

Epon-embedded tissues were cut with a LKB ultramicrotome (0.5 μm). Sections were etched with sodium ethoxide, and a PAP technique was applied as described elsewhere [Calvo et al., 1990b]. In this case, the anti-b-FGF[1–24] antiserum was diluted 1:150.

## Results

Basic FGF immunoreactivity was not detected in the pineal gland of rats between 1 and 15 days after birth. At 20 days, a sparse immuno-positivity was first found in scattered endothelial cells and perivascular spaces. Basic FGF immuno-positive structures reached their maximal expression and stronger labeling in the pineal glands of rats 30 and 45 days of age (Figs. 1, 2). At these ages, a wide immunostaining in nearly all the endothelial cells and perivascular spaces of the gland was detected. Occasional scattered immuno-positive mesenchymal pericapillary cells were observed, although the level of resolution did not permit the precise identification of the exact cell type. Immunoreactive parenchymal pineal cells were never seen throughout the study period.

Immuno-positive b-FGF structures were still very prominent in 60-day-old rats, although a decrease in the number of positive capillaries was

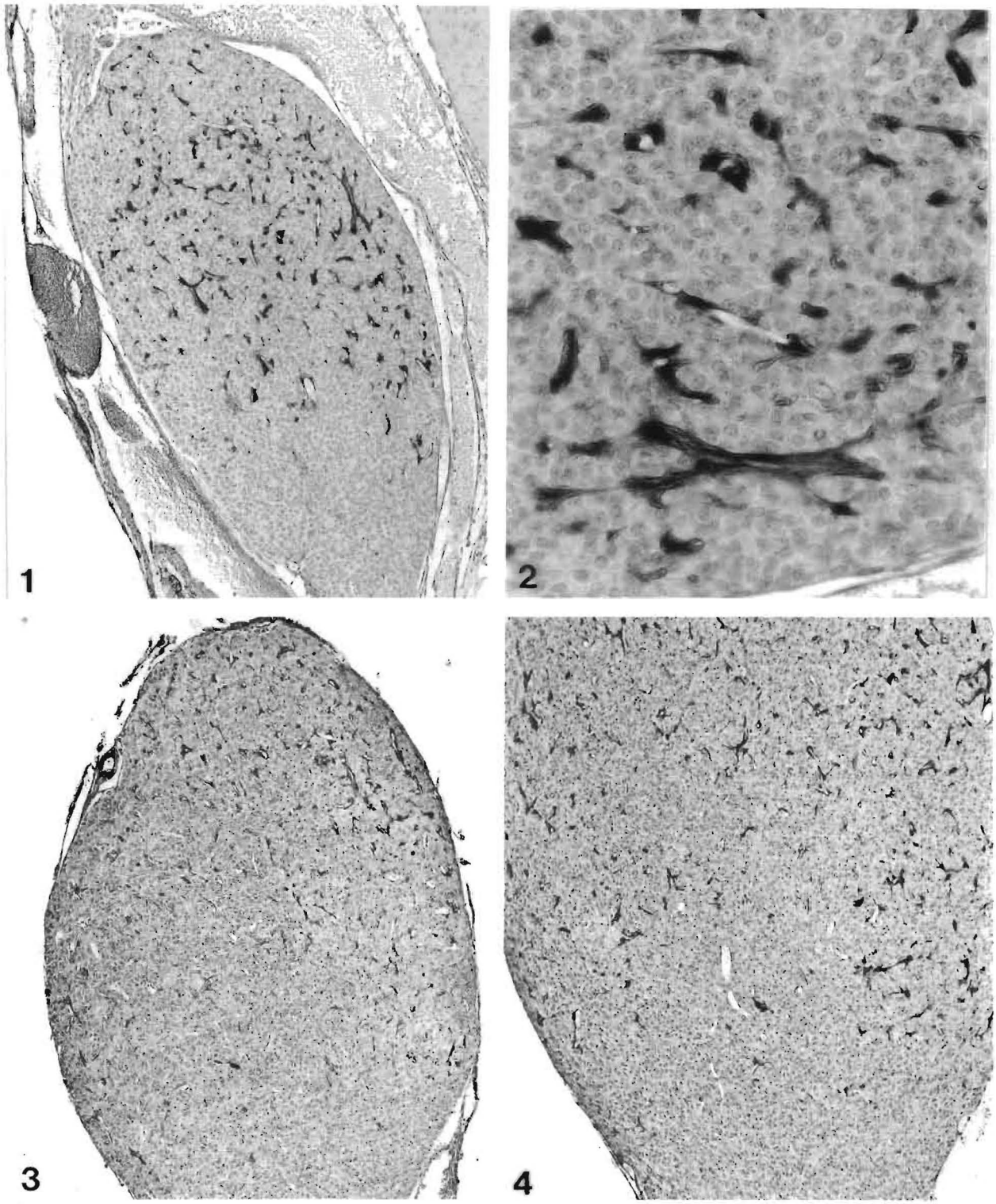


*Fig. 1.* Pineal gland from a rat at 45 days of age (control group). Basic FGF immuno-positive vasculo-connective septa are distributed throughout the gland. (×80).
*Fig. 2.* Higher magnification of Figure 1. Endothelial cells and the subendothelial perivascular spaces are strongly immunostained. Note the complete absence of immunostained pineal parenchymal cells (×350).
*Fig. 3.* Pineal gland from a rat at 3 months of age (control group). Basic FGF immuno-positive blood vessels are mainly located in the distal portion of the gland. Note immuno-negative capillaries in the proximal area of the gland. (×80).
*Fig. 4.* Pineal gland from a 60-day-old, DSP-4 denervated rat. No striking differences in the immunostaining pattern are observed. Note the complete absence of immunostained pineal parenchymal cells. (×90).

bauer et al., 1988] would suggest that pineal b-FGF could participate in the regulation of melatonin secretion. However, further studies are needed to clarify this possibility.

Several in vitro studies have shown that b-FGF has neutrophic effects on neurons in culture, stimulating the growth of the neurite and the differentiation and proliferation of several glial cell types [Unsicker et al., 1993]. At this time the hypothetical role of b-FGF on the nervous tissue in vivo is not well known. In a previous study we have demonstrated that chemical denervation of the rat pineal gland elicits an increase in the expression of nerve growth factor in type II pinealocytes [García-Mauriño et al., 1992]. On the contrary, we have observed no effect either in the time of appearance (20 days) or the localization and intensity of b-FGF immunoreactivity in DSP-4 prenatally-denervated pineal glands when compared with the control group.

In summary, b-FGF is present in the endothelial cells and the perivascular spaces in the normal adult pineal gland in rats. Its hypothetical physiological role remains unresolved. However, its absence in early postnatal development of the pineal gland would suggest that it does not play a major role in the cytodifferentiation and neovascularization of the gland. Moreover, its expression does not seem to be influenced by the prenatal sympathetic denervation with DSP-4. In clear contrast with the findings with NGF, we have never observed immuno-positive parenchymal pineal cells.

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evident when compared with the previous stages. In older animals, this decline was still more evident. However, b-FGF immuno-positivity was always present, and it did not disappear even in the oldest animals studied (6 months). It is remarkable that in the oldest animals, the immunostained vessels and perivascular spaces were mainly located in the dorsal zone of the gland (Fig. 3).

The brain parenchyma adjacent to the pineal gland and included in the tissue block, its blood vessels, and the choroid plexus were consistently immuno-negative. On the contrary, positivity was detected in the meninges and in ependymal cells of the lateral ventricles in those cases in which this area was included in the tissue section.

Bouin-fixed tissues exhibited the same immunocytochemical pattern; however, b-FGF immunolabeling was weaker, and unspecific background immunostaining was present. The immunoreaction in these tissues was not enhanced after the enzymatic digestion of the sections.

No immunostaining was observed when the sections were incubated with the primary antiserum pre-adsorbed with excess recombinant bovine b-FGF or after the substitution of the primary antiserum with NSS.

There were no differences in the b-FGF immunostaining pattern in the group of DSP-4 denervated rats. Both the time of appearance of immunopositive elements (20 days) and the development and morphology of the b-FGF immunoreactive structures were indistinguishable from the control group (Fig. 4).

## Discussion

In the present study we have demonstrated the presence and distribution of b-FGF in the pineal gland with immunocytochemical techniques. According to our results, b-FGF is first detected in 20-day-old rats, reaching its maximal expression in animals at 30–45 days old, simultaneously with the maturation of the parenchymal pineal cells [Calvo and Boya, 1983]. In these stages, nearly all the endothelial cells and perivascular spaces presented b-FGF immuno-positivity that was maintained, although less extensively, in adult animals.

Endothelial cells are a well-known source of b-FGF [Schweigerer et al., 1987; Hannan et al., 1988; Rosenbaum et al., 1989; Bikfalvi et al., 1990]. This growth factor, acting by paracrine and autocrine mechanisms, plays an important role in the growth, proliferation, and migration of the endothelial cells during the processes of angiogenesis and tissue repair [Schweigerer et al., 1987; Montesano et al., 1986; Vlodarvsky et al., 1987; Ingbar and Folkman, 1989]. The ubiquitous immunocytochemical localization of b-FGF in the endothelial cells of rat embryos during development [González et al., 1990; Hanneken et al., 1989] could reflect the remodeling processes and rapid growth that immature tissues display. However, the finding of high amounts of b-FGF in the pineal gland of adult animals [Taylor et al., 1988; present work] raises the question of the physiological role of this growth factor in mature tissues, since the proliferation of the endothelial cells is low and the cell turnover may be very prolonged [Denekamp, 1984].

Although the pineal gland receives a very high blood flow per gram of tissue [Goldman and Wurtman, 1964], this fact would not explain the notable presence of b-FGF in this gland, since other highly vascularized organs, such as the liver and kidney, contain very small amounts of b-FGF [Taylor et al., 1988; González et al., 1990; Cordón-Caro et al., 1990].

One possibility is that endothelial cells and the subendothelial extracellular matrix of the pineal gland are singularly abundant deposits of b-FGF that could be stored at this level in an inactive form bound to heparin-like molecules [Bashkin et al., 1989; Folkman et al., 1988], and could be mobilized when needed for angiogenesis and tissue repair, as has been suggested by several authors [Cordón-Caro et al., 1990; Bashkin et al., 1989; Folkman et al., 1988]. According to our findings, this deposit of b-FGF would not be a universal feature in the central nervous system, since the blood vessels of the brain and cerebellum lack b-FGF immunoreactivity. The reason for this heterogeneous distribution of b-FGF remains unclear, but it has to be pointed out that the subendothelial spaces of the rat pineal gland are substantially different from those of the central nervous system. They show a broad perivascular space filled with abundant extracellular matrix, nerve fibers, collagen and oxytalan fibers, pinealocyte cell processes, fibrocytes, etc. [Matsushima and Reiter, 1975; Vollrath, 1981; Boya and Calvo, 1984], which could require b-FGF to be maintained. On the other hand, the characteristic fenestrations of the capillary endothelial cells of the rat pineal gland [Matsushima and Reiter, 1975] are not found in the capillaries of the central nervous system. However, this is unlikely to be the reason for this heterogeneous distribution of b-FGF, since the choroid plexus share this feature with the pineal gland, but do not exhibit b-FGF immunoreactivity.

The hypothetical role of b-FGF in the modulation of hormonal release in several endocrine cells [Baird et al., 1985; Baird and Hsueh, 1986; Ober-

bauer et al., 1988] would suggest that pineal b-FGF could participate in the regulation of melatonin secretion. However, further studies are needed to clarify this possibility.

Several in vitro studies have shown that b-FGF has neutrophic effects on neurons in culture, stimulating the growth of the neurite and the differentiation and proliferation of several glial cell types [Unsicker et al., 1993]. At this time the hypothetical role of b-FGF on the nervous tissue in vivo is not well known. In a previous study we have demonstrated that chemical denervation of the rat pineal gland elicits an increase in the expression of nerve growth factor in type II pinealocytes [García-Mauriño et al., 1992]. On the contrary, we have observed no effect either in the time of appearance (20 days) or the localization and intensity of b-FGF immunoreactivity in DSP-4 prenatally-denervated pineal glands when compared with the control group.

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