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Immunohistochemical localization of nerve growth factor in the rat pineal gland

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Sympathetic nerve fibers arising from the superior cervical ganglia are the main innervation of the rat pineal gland. Since most organs innervated by these ganglia contain nerve growth factor (NGF), the hypothetical existence of NGF in the pineal gland was investigated. The peroxidase anti-peroxidase technique was applied for the immunohistochemical demonstration of NGF using a polyclonal antiserum on Bouin-fixed, paraffin-embedded pineal glands from adult, young and 6-hydroxydopamine (6-OHDA)-treated rats. Few immunopositive cells were observed in the adult pineal gland. A more conspicuous population of immunoreactive cells was noted in young animals (20–45 days old), especially in those chemically denervated with 6-OHDA. NGF immunoreactive cells displayed a stellate shape resembling the interstitial or glial cells previously described in the rat pineal gland. Since NGF plays a trophic effect on sympathetic neurons during development and adulthood, we postulate that its presence in the pineal gland may exert a trophic role on its sympathetic innervation.

INTRODUCTION

Nerve growth factor (NGF) exerts a trophic effect eliciting the growth of the processes of sympathetic and sensitive ganglionic neurons[24,25,37]. NGF has been demonstrated in the most of the target organs of the superior cervical ganglia, i.e. the iris and the submaxillary gland[20].

The rat pineal gland has a double innervation: postganglionic sympathetic fibers that arise in the neurons of the superior cervical ganglia (SCG) and play a major physiological role[10,17,31] and central nerve fibers which reach the pineal gland through the pineal stalk[8,18,19,30]. The biochemical identification of NGF in the rat pineal gland has been described recently[39].

NGF levels differ depending on several factors including the innervation status and age. Thus, higher amounts were found in younger animals[21] and after denervation[3,11,21,39].

It has been previously reported that the neonatal administration of 6-hydroxydopamine (6-OHDA) produces a selective and irreversible destruction of the sympathetic nerve endings[2,4,16,36,38].

In the present study, we have investigated the immunohistochemical localization of NGF in the pineal gland of young and adult rats and after the 6-OHDA induced chemical sympathetic denervation.

MATERIALS AND METHODS

Forty-six pineal glands from Wistar rats were studied. Animals were kept under normal lighting conditions (14 h light/10 h dark) with free access to water and food. Ten pineal glands were obtained from 5-month-old rats of both sexes. For the study of the postnatal period, 6 pups of the same offspring were killed at 10, 20, 30, 45, 60 and 90 days after delivery. In each stage, 3 animals were maintained in standard conditions, while another 3 animals were treated with 6-OHDA during the first 5 postnatal days using the method previously reported by Angeletti[1]. Briefly, a dose of 50 mg/kg of body weight of 6-OHDA hydrochloride (Fluka) diluted in 0.05 ml of a solution of 0.9 g/l NaCl and supplemented with 0.5 mg of ascorbic acid per ml of solution was injected subcutaneously.

Animals were anesthetized with ether inhalation and killed by decapitation at 18.00 h between April and May. The glands were fixed by immersion in Bouin solution for 24 h at room temperature and embedded in paraffin. Serial sections of 7 μm were cut and mounted on glass slides for immunolabelling. The peroxidase anti-peroxidase (PAP) technique was applied to tissue sections according to Taylor[35]. A polyclonal antiserum raised in rabbits against mouse β-NGF (Sigma) was used at 1:300 dilution. Sections were incubated with the antibody for 36 h at 4°C. The immune reaction was developed with diaminobenzidine and enhanced with 1% osmium tetrox-

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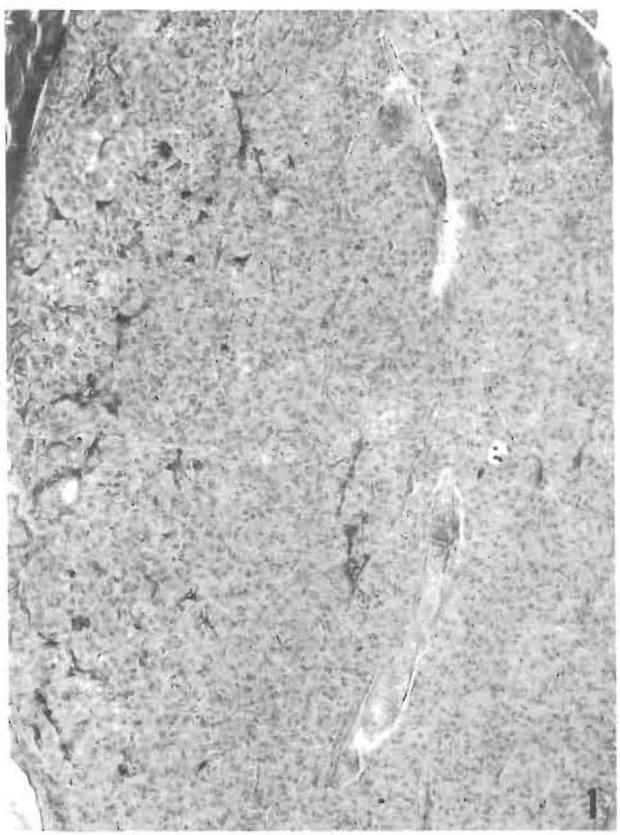
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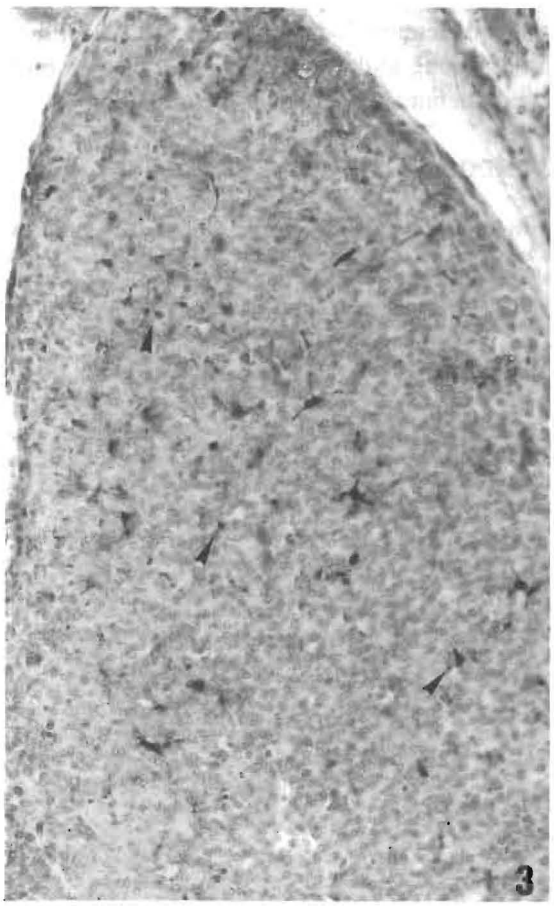
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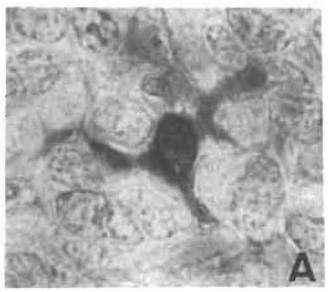
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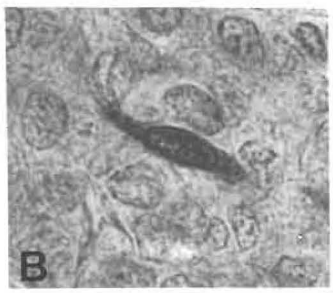
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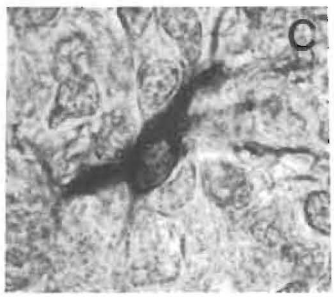
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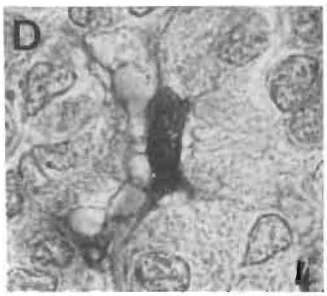
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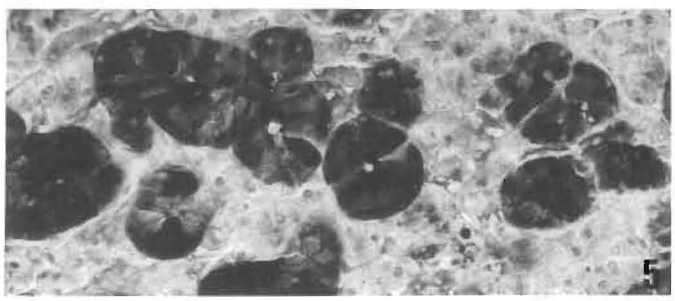
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ide. A light hematoxylin nuclear counterstaining was applied. Negative controls included sections immunostained with anti-β-NGF pre-absorbed with purified mouse β-NGF (Chemicon) and sections in which non-immune rabbit serum was used in lieu on the primary antibody. In the absorption experiments, the antibody was pre-incubated overnight with an equal volume of β-NGF at a dilution of 500 μg/ml in PBS. As a control for positive immunostaining, sections from male mouse submaxillary glands were used.

RESULTS

Few immunopositive cells were observed in the pineal gland of adult rats. These immunoreactive cells were mainly located in the apical region and along the dorsal zone (Fig. 1). In the postnatal period, especially between 20 and 45 days, the extent and intensity of the immunoreaction was more notable than in adult animals (Fig. 2). NGF immunostaining was increased in the pineal glands of those animals chemically denervated with 6-OHDA. In these pineal glands, immunoreactive cells were also detected in the apical region (Fig. 3), as was observed in adults.

NGF immunoreactive cells displayed bipolar (Fig. 4B) or stellate shapes (Fig. 4A, C, D). Nuclei were elongated and showed dense and homogeneous chromatin. The immunoreaction product was detected both in the soma and the cell processes. Immunopositive cells appeared frequently in close relationship with the connective tissue septa. Because of their shape, NGF immunopositive cells resembled interstitial or glial pineal cells.

Selective and specific immunostaining of the secretory tubules in the mouse submaxillary glands was observed (Fig. 5), as previously described by Schwab et al.[33]. No immunostaining was obtained following incubation with antibody pre-absorbed to excess antigen or with substitution of the primary antiserum with non-immune rabbit serum.

DISCUSSION

In the present immunohistochemical study we demonstrated the existence of NGF immunopositive cells in the pineal gland for the first time. In a biochemical study using ELISA, Weskamp and Otten[39] described the presence of NGF in several brain regions, showing great amounts in the pineal gland. Although a close structural relationship between NGF and brain-derived neurotrophic factor and neurotrophin-3 has been described[15,23], the absence of immunostaining observed in the pre-absorption tests makes it unlikely that a cross-reaction with these neurotrophic factors has occurred. Furthermore, the presence of these proteins has not been described in the pineal gland.

NGF exerts trophic effects on sympathetic neurons, increasing their size and promoting the sprout and growth of cell processes[24,26,37], On the other hand, the presence of NGF in organs innervated by the SCG, i.e. the submaxillary gland and the iris has been clearly demonstrated[20,21]. These observations led us to consider the hypothetical existence of NGF in the pineal gland since its innervation is mainly sympathetic and also arises from the SCG.

Biochemical studies have demonstrated that the amount of NGF in organs innervated by the sympathetic system varies depending on the age[21] and after denervation[3,11,12,20,26,39]. Thus, maximal levels were found at the 20th postnatal day in several organs in the mouse[21], but no data have been reported in the pineal gland. According to our results, NGF immunopositive cells were more in evidence between the 20th and the 45th postnatal days. These findings were even more noteworthy in those animals of the same age treated with 6-OHDA, a substance that produces a very specific and irreversible sympathetic denervation[36]. This increased immunopositivity may be the result of the synergistic effect of the sympathetic denervation on young animals.

NGF immunoreactive cells displayed a stellate shape resembling the so-called interstitial cells or type II pinealocytes[7]. These pineal cells in the rat were considered glial in nature because they express markers including glial fibrillary acidic protein (GFAP), S-100 protein and vimentin, which are markers for astrocytes[9,29,32]. Several in vitro[13,14,34,41] and in vivo[26] studies have suggested that astrocytes in the central nervous system synthesize and release NGF. Recently Lu et al.[28] detected mRNA-NGF in this cell type.

In a previous study on the postnatal development of the rat pineal gland, Calvo and Boya[6] reported a consistent relationship between glial cells and nerve

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Figs. 1–5. Figs. 1 and 2: pineal glands from two rats of 5 months and 45 days, respectively. NGF immunopositive cells are mainly located in the apical and dorsal zones. Notice a stronger immunopositivity in the pineal gland of a 45-day-old rat. Magnification ×128. Fig. 3: pineal gland from a 20-day-old rat, denervated with 6-OHDA. Stellate-shaped immunopositive cells are observed in the distal portion. Transverse sections of immunopositive cell processes (arrowheads) are frequently seen. Magnification ×200. Fig. 4: stellate (A, C, D) and bipolar (B) immunopositive cells. Observe the lack of immunostaining of the remaining parenchymal cells. A and B are from a 20-day-old rat pineal gland treated with 6-OHDA, C and D are from a 30-day-old rat pineal gland. Magnification ×1,225. Fig. 5: strong and specific NGF immunoreactivity in the secretory ducts of the submaxilary gland of an adult male mouse. Magnification ×225.

fibers. Furthermore, the occurrence of sympathetic fibers in the rat pineal gland takes place during the first postnatal days, simultaneously with the differentiation of glial cells. It is conceivable that NGF in glial pineal cells elicits tropic and trophic effects in the sympathetic fibers during the development and in the adult rat. Similar findings have been reported in organs innervated by sympathetic fibers[21,24,25,37] and in the central nervous system[22,27,40].

NGF immunoreactive cells in the rat pineal gland were unevenly distributed showing a preferential location in the apical region of the gland. Calvo et al.[9] demonstrated that glial cells in the rat pineal gland expressed different glial markers according to their situation within the gland. Thus, vimentin immunopositive cells were diffusely distributed throughout the gland, while both GFAP and S–100 immunoreactive cells were predominantly found near the pineal stalk. The different location of NGF immunoreactive cells may support the hypothesis that diverse populations of glial cells are present in the rat pineal gland.

Kappers[17] and Bowers et al.[5] have described that sympathetic fibers reach the rat pineal gland through the apical and dorsal regions, where NGF immunopositive cells are mainly located. This topographic relationship may support the role of these cells on the maintenance of the sympathetic innervation of the gland.

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