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Pituitary hyperplasia induced by ectopic expression of nerve growth factor

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ABSTRACT **Nerve growth factor (NGF) cDNA was fused to the rat prolactin promoter to induce its ectopic expression in pituitary lactotrophs of transgenic mice. High-level expression of both RNA and functional protein was achieved in two pedigrees. Pituitary cells from these animals secreted biologically active NGF that was capable of inducing rapid differentiation of cocultured PC12 pheochromocytoma cells. Despite this robust expression, transgenic pituitaries failed to show any detectable increase in neuronal innervation. Unexpectedly, we observed a dramatic hyperplasia of lactotrophs resulting in pituitaries 10–100 times larger than normal. These results suggest that NGF, in addition to its previously described effects, may act as a new class of mitogen, with a potential role in oncogenesis.**

The discovery of nerve growth factor (NGF) and its actions has served as a model for the study of neuronal development and differentiation (1). Addition of NGF to neural crest-derived neurons in culture blocks cell proliferation, leading rapidly to neurite outgrowth and neurotransmitter synthesis (2). NGF has trophic properties as well, and thus it is essential for the continued survival of these differentiated cells.

The pleiotropic effects of NGF are mediated through the interaction with an NGF receptor present on the membrane of responsive cells (3). The NGF–receptor complex is formed, internalized, and then recycled to the cell surface. The molecular mechanisms underlying the effects of NGF are still unknown. It has been shown that inducers of the protein kinase C signal-transduction pathway, such as the activated p21 ras protein, trigger the same response as NGF in cultured PC12 pheochromocytoma cells (4), leading to the hypothesis that the biological effect of NGF could be mediated by interaction with guanine nucleotide-binding proteins (G proteins) (5, 6). Molecular cloning of the mouse NGF cDNA has allowed a more detailed analysis of its synthesis and distribution (7). Although believed to be important for sympathetic innervation in the peripheral nervous system, NGF has also been detected in tissues in which its presence was not suspected, such as the brain (8), and more surprisingly, in tissues of nonneuronal origin such as the testis and epididymis (9). These findings strongly suggest that NGF is part of a class of growth factors with wide functions, which may not be restricted to neuronal cells.

The ability of NGF to induce and sustain a neuronal phenotype, as well as the increased innervation induced by its overexpression in the pancreas of transgenic mice (10), led us to explore the proposition that its expression may be necessary and sufficient to provoke neuronal innervation. To test this idea, we have genetically engineered transgenic animals to ectopically express NGF in a non-innervated tissue, the anterior pituitary. The rat prolactin (Prl) promoter was used to direct transcription of the mouse NGF cDNA in transgenic mice (11). Previous studies have shown that transgenes fused to the rat prolactin promoter will be expressed selectively in the lactotropes of the anterior lobe of the pituitary gland (11, 12). In this study we show that when overexpressed in a nonneuronal tissue, NGF fails to induce innervation and instead acts as a mitogen to stimulate a profound proliferation of an otherwise nondividing cell type, resulting in a massive pituitary hyperplasia.

METHODS

Gene Construct. A 3033-base-pair (bp) *Bam*HI–*Hin*dIII (−3000/+33) fragment of the rat Prl promoter (11) was fused into the *Sma* I site at position −32 with respect to the first ATG of the mouse NGF cDNA (7). The simian virus 40 (SV40) *Mbo* I fragment (nucleotides 4693–4083) providing the intron for the small tumor antigen and a *Bcl* I–*Bam*HI fragment (nucleotides 2753–2516) providing the SV40 polyadenylylation site were added 3′ of the NGF cDNA.

Transgenic Mice. The isolated linear Prl–NGF fragment was injected into fertilized mouse eggs (13). The presence of the construct was tested by Southern blot analysis (14) of DNA extracted from mouse tails, with hybridization to a ^{32}P-labeled SV40 polyadenylylation-site fragment.

Northern Analysis. Total RNAs from tissues of old mice were extracted by the LiCl/urea method (15) fractionated in a 1% agarose/2.2 M formaldehyde gel, blotted to nitrocellulose, and hybridized to a ^{32}P-labeled fragment homologous to the SV40 polyadenylylation site.

Western Analysis. Frozen mice pituitaries were pulverized, then resuspended and homogenized in 10 volumes of STM buffer (0.25 M sucrose/20 mM Tris·HCl/1.1 mM $MgCl_2$) at pH 7.85 at 25°C. After centrifugation at 800 × *g* for 10 min, the supernatant was evaluated for protein content and fractions were electrophoresed in an SDS/12.5% polyacrylamide gel. The separated proteins were blotted to nitrocellulose and probed as described (16) with a rabbit anti-mouse NGF antibody (1:100 dilution, from Marston Manthorpe, University of California, San Diego).

Cell Culture. PC12 rat pheochromocytoma cells (4) seeded at low density in 24-well Costar plates were grown as described (17). Primary cultures of pituitary cells were prepared as described (18). The cells so obtained were plated in Transwell plates (Costar) in Dulbecco's modified Eagle's medium with 2% fetal bovine serum. To quantitate the amount of NGF in the pituitary gland, an automated calorimetric assay was used (19).

Immunohistochemistry. Mice were anesthetized with chloral hydrate and perfused transcardially with neutral buffered 10% formalin (Richard Allen) as described (20). Pituitaries were postfixed overnight at 4°C in the same fixative containing 10% sucrose. Frozen sections (20 μm) were cut on a

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Abbreviations: NGF, nerve growth factor; Prl, prolactin; SV40, simian virus 40.

FIG. 1. Prl–NGF hybrid gene. The 3033-bp *Bam*HI–*Hin*dIII fragment of the rat Prl promoter was inserted into the *Sma* I site at position −32 with respect to the first ATG of the mouse NGF cDNA. A fragment from SV40 supplying the intron of the small tumor-antigen gene and the polyadenylylation (pA) site was present 3′ of the NGF cDNA. Arrow indicates the transcriptional start site.

sliding microtome. The antisera used for the immunohistological analysis were rabbit anti-mouse NGF (1:100 dilution, from Marston Manthorpe, University of California, San Diego), monkey anti-mouse growth hormone (1:80,000 dilution, from Y. Sinha, Whittier Institute, La Jolla, CA), and rabbit anti-human Prl (1:1000; Cambridge Medical Diagnostics, Billerica, MA). The secondary antisera were fluorescein- or rhodamine-labeled goat anti-monkey IgG (1:100; Cappel Laboratories) or anti-rabbit IgG (1:200; Tags, Burlingame, CA).

RESULTS

Tissue-Specific Expression of the Prl–NGF Hybrid Gene. To examine the effects of NGF on pituitary development, a 3-kbp fragment of the rat Prl promoter was fused to the mouse NGF cDNA (7) (Fig. 1). This fusion gene was injected into fertilized mouse eggs, and two transgenic pedigrees expressing high levels of the transgene were obtained. To analyze the pattern of expression of the transgene, RNA extracted from a variety of tissues was size-fractionated, transferred to a nylon filter, and hybridized with a DNA sequence specific for the transgene (SV40 polyadenylylation site). The distribution of transgene mRNA was specifically restricted to the pituitary gland (Fig. 2).

NGF Expression in the Pituitary of Transgenic Mice. To determine the effect of NGF expression, 6-month-old transgenic and nontransgenic mice were prepared for immunohistological analysis (20). At a gross level, pituitaries from transgenic mice of this age were normal in appearance and size (data not shown). Immunohistochemical staining of sections with antisera against markers for cell types in the anterior (Prl, growth hormone, corticotropin) and intermediate (corticotropin) lobes and for fiber types in the posterior lobe (oxytocin, vasopressin) failed to reveal any differences between transgenics and controls (data not shown). Antiserum to tyrosine hydroxylase was used as a marker to look for altered patterns of neuronal innervation. This approach also failed to reveal any overt differences, suggesting that in transgenics the ectopic expression of NGF may not affect the normal physiology of the gland.

To verify whether the presence of the NGF mRNA corresponded to the synthesis of the active peptide, an *in vitro* assay was developed to demonstrate the synthesis and secretion of functional NGF from the transgene mRNA. Pituitary glands from 6-month-old transgenic and nontransgenic mice were dissociated and cultured on a semipermeable membrane that allows diffusion of secreted molecules and metabolites. In a Transwell system (Costar), this membrane was suspended above a monolayer of cultured PC12 cells. Induction of neurite outgrowth was observed after 48 hr of coculture only with pituitary cells from transgenic mice, showing that biologically active NGF was synthesized and secreted into the medium (compare Fig. 3 *A* and *B*). The same phenotype was obtained under similar conditions by treatment of PC12 cells with NGF (50 ng/ml) (data not shown). To quantitate this effect, serial dilutions of conditioned media

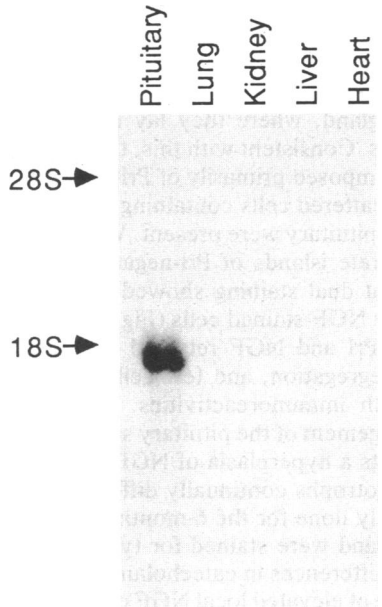

FIG. 2. Northern blot analysis of total RNA extracted from tissues of a 6-month-old transgenic mouse. RNA (20 μg per lane) was electrophoresed in a formaldehyde/agarose gel, transferred to nitrocellulose, and hybridized with a nick-translated DNA fragment homologous to the SV40 polyadenylylation site. The 28S and 18S ribosomal RNAs were used as size markers.

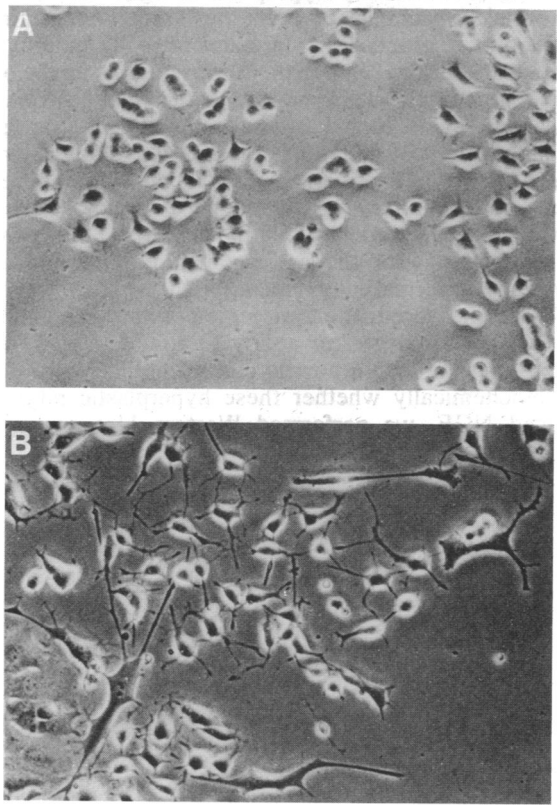

FIG. 3. Biological effect of transgenic NGF on PC12 culture. PC12 cells were grown on polylysine-coated 24-well Costar plates. Twenty-four hours later, permeable Transwells containing dissociated pituitary cultures from either normal (*A*) or transgenic (*B*) mice were laid in the wells in contact with the medium of the underlying cells. Pictures were taken 48 hr later. (×35.)

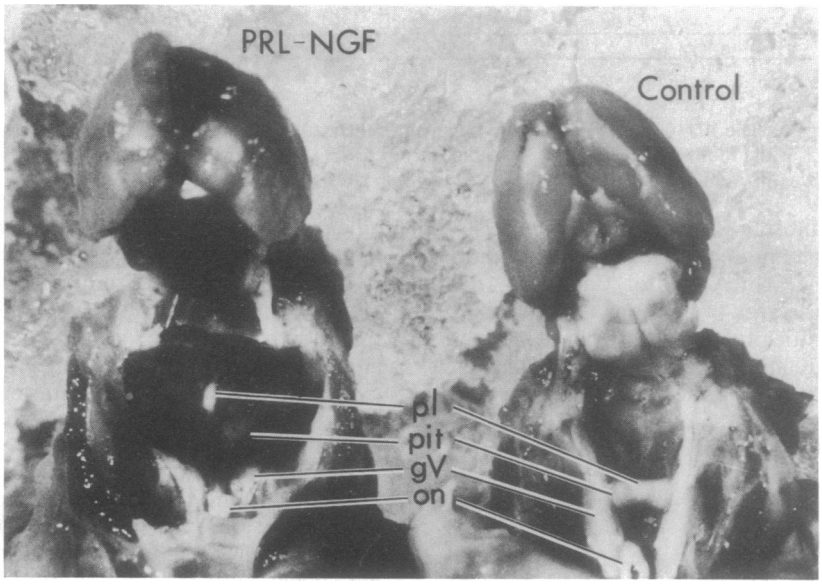

FIG. 4. Hypertrophic effect of NGF on the pituitary of 16-month-old mice. (*Left*) Transgenic mouse pituitary. (*Right*) Control mouse pituitary. For reference, the relative positions of the anterior lobe of the pituitary (pit), the posterior lobe of the pituitary (pl), the trigmenal ganglion (gV), and the optic nerve (on) are indicated.

from cultured pituitary cells were added to primary cultured sensory neurons (19). Survival of these cells was scored and trophic units were measured. Medium from transgenic pituitary cells was 3.5 times more effective than the control in promoting survival of sensory neurons. Addition of anti-NGF antiserum to the culture medium eliminated all trophic effects, indicating that the inducing substance was NGF.

NGF Induces Pituitary Hyperplasia in Aged Mice. Despite the normal appearance of pituitaries from younger mice, examination of pituitaries from older mice (12 months old) revealed an abnormal enlargement with weights on the order of 5 mg, compared with 1 mg for age-matched controls. An analysis of an additional 21 male and female transgenic animals of ages 15–18 months revealed even more dramatic changes (Fig. 4). The enlargement was more pronounced in females, with pituitary weight ranging between 40 and 100 mg. Male pituitaries consistently fell toward the lower end of this range. None of the age-matched control pituitaries showed any abnormal enlargement. When the trophic units of NGF were measured, the transgenic pituitary sample demonstrated a 60-fold greater ability to stimulate survival of sensory neurons than a normal control pituitary. To determine biochemically whether these hyperplastic pituitaries produced NGF, we performed Western blot analysis of protein extracted from the pituitary of a 15-month-old transgenic mouse. The analysis revealed a 13-kDa band corresponding to the size of purified NGF, whereas no such bands were observed from the controls (Fig. 5).

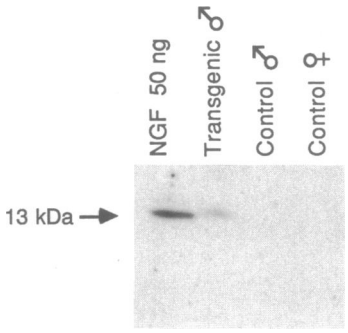

FIG. 5. Western blot analysis of extracts from the pituitary gland of a 15-month-old transgenic male mouse and age-matched control mice. Proteins were separated by SDS/12.5% polyacrylamide gel electrophoresis, blotted to nitrocellulose, and incubated with a polyclonal anti-NGF antibody. The binding was revealed by ^{125}I-labeled protein A. Authentic NGF (50 ng) was run in the lane at left.

We next investigated the morphological aspect of the hyperplastic tissues. In sections, enlarged pituitaries from these older transgenic animals retained a region at the base of the mass where a discrete visual semblance of the basic three-lobed organization was evident, though this was distorted to various degrees by the overlying growth. Immunohistochemical analyses supported this view, and separate and distinct regions in the pituitary could be identified that contained intermixed growth hormone- and corticotropin-positive cells (anterior lobe), a continuous band of corticotropin-positive cells (intermediate lobe), and oxytocin- and vasopressin-immunoreactive terminals (posterior lobe). The sole exception to this otherwise normal pituitary organization was a segregation of Prl-positive cells toward the periphery of the intact gland, where they lay in contiguity with the overlying mass. Consistent with this, the overlying mass was found to be composed primarily of Prl-positive cells (Fig. 6); only widely scattered cells containing the other markers for the "normal" pituitary were present. Within this vast field of Prl cells, discrete islands of Prl-negative cells were found, and concurrent dual staining showed that these comprised predominantly NGF-stained cells (Fig. 6). Within all aspects of the mass, Prl and NGF retained this tendency toward topographic segregation, and few cells were identified that expressed both immunoreactivities. We suggest that the aberrant enlargement of the pituitary seen in older transgenic mice represents a hyperplasia of NGF-producing cells from which the lactotrophs continually differentiate.

As previously done for the 6-month-old animals, sections through the gland were stained for tyrosine hydroxylase to evaluate any differences in catecholaminergic innervation as a consequence of elevated local NGF expression. No evident differences were detected between transgenics and controls insofar as the "normal" pituitary was concerned. In the transgenics, however, fascicles of immunoreactive fibers were found around the periphery of the pituitary-associated mass and occasionally penetrated its outer margins in association with vascular elements. However, no extensive ar-

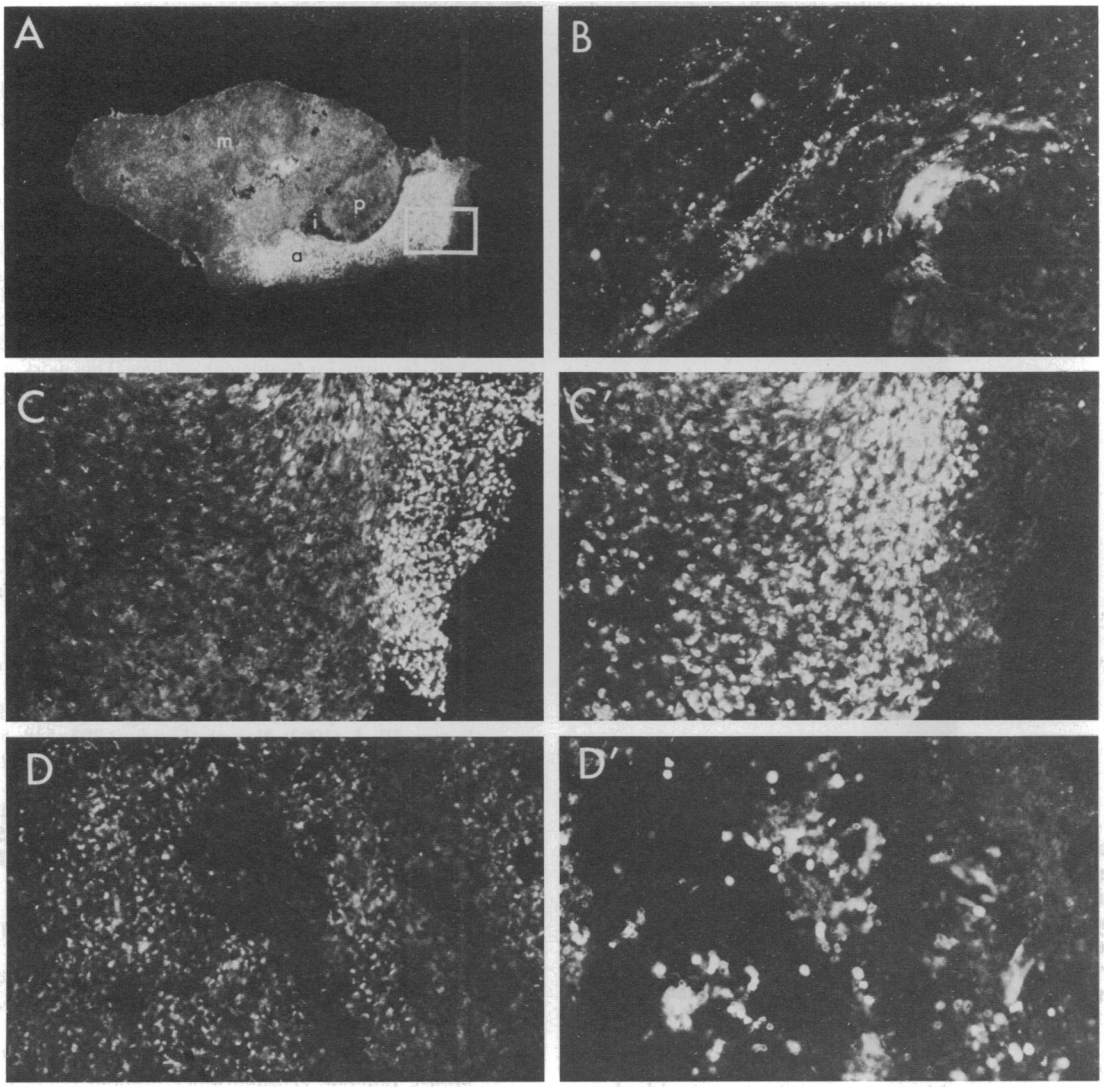

FIG. 6. Histochemical appearance of the pituitary gland in transgenic mice. (*A*) Dark-field photomicrograph of a section through the pituitary of a transgenic male mouse. The anterior (a), intermediate (i), and posterior (p) lobes are discernable and approximate their normal appearance. Imposed upon this is a hyperplastic mass (m) associated with the anterior lobe. (*B*) Fluorescence photomicrograph to show tyrosine hydroxylase-immunoreactive fibers near the periphery of the mass. Despite the expression of NGF, no extensive arborization of catecholaminergic fibers within any aspect of the pituitary was evident. (*C* and *C'*) Fluorescence photomicrographs of a portion of the section shown in *A* (boxed region) stained concurrently for Prl (*C*) and growth hormone (*C'*) immunoreactivities. Prl cells are abnormally sequestered at the margins of the anterior lobe and are contiguous with the overlying mass. (*D* and *D'*) Fluorescence photomicrographs of a section through the hyperplastic mass stained concurrently for Prl (*D*) and NGF (*D'*). More-or-less discrete islands of NGF-positive cells are seen amid a field of Prl-immunoreactive cells. (*A*, ×10; *B–D*, ×120.)

borizations of aminergic fibers were seen within the depth of the mass.

Complementary Prl and NGF mRNA analyses of pituitaries from 15- to 18-month-old control and male and female transgenic mice were carried out. In line with the histological results, both mRNA species were found to be increased, especially in females (Fig. 7). The same RNA samples were examined in parallel for expression of the NGF receptor by using ^{32}P-labeled cDNA as a hybridization probe (21). No hybridization was observed (data not shown), suggesting that the receptor was either not present or expressed at extremely low levels.

DISCUSSION

Although NGF is referred to as a growth factor, its typical effects are to induce cellular differentiation and to maintain the viability of differentiated cells. These properties led us to explore the question of whether NGF expression alone, in a non-innervated tissue, could be sufficient to provoke innervation from surrounding pioneer neurons. The clear result from this study is that, at least in the case of the anterior pituitary, no significant innervation could be provoked. This indicates that NGF cannot act on its own to promote stable innervation and that it does not serve a chemotactic function in this particular tissue.

Unexpectedly, NGF stimulated a dramatic and specific hyperplasia of the pituitary cell type in which it was being overexpressed; thus, NGF can function as a growth factor. In previous studies we have shown that overexpression of other gene products, such as herpes thymidine kinase, does not lead to any noticeable change in cell phenotype or function (22). Hence, we conclude that the hyperplasia is a specific consequence of the ectopic expression of NGF. We propose that NGF triggers the expansion of a rare or normally transient cell population that only slowly differentiates into postmitotic lactotrophs. Preliminary studies have failed to detect NGF receptor mRNA in the pituitary of either normal or transgenic animals. Therefore, the mechanisms by which the hyperplasia is provoked are not clear. Either the observed

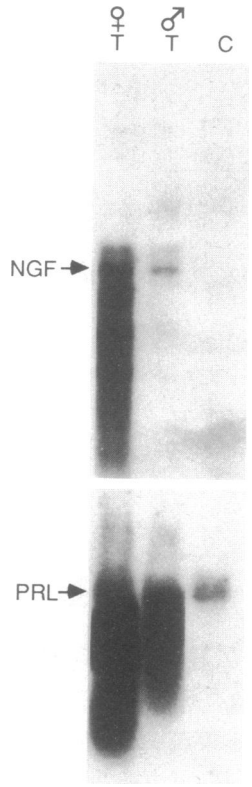

FIG. 7. Northern blot analysis of hyperplastic pituitaries. Total RNA extracted from pituitary glands of 15-month-old female and male transgenic (T) mice and control (C) female mouse (the control male lane is not shown because it was identical to the female) was electrophoresed in a formaldehyde/agarose gel, blotted to nitrocellulose, and hybridized to nick-translated probes to detect either the Prl–NGF transgene or the endogenous Prl, as indicated by the arrows.

effects are caused through the NGF receptor, which is present in low amounts in this tissue, or it is conceivable that high-level expression of NGF might lead to inappropriate activation of another class of pituitary receptor through a low-affinity interaction. It has also to be taken into account that NGF might function as a starting signal to induce the postmitotic lactotroph population (22) to proliferate, in which case the overproduction of Prl could be responsible for the observed hyperplasia. It has been shown that transgenic mice secreting high levels of growth hormone present a similar phenotype (23). Consequently, it is conceivable that some pituitary hormones could act as cell growth-stimulating factors. One puzzling question is why the hyperplasia is observed only in old animals. A delayed onset of expression, which has been postulated to depend on the site of transgene integration in the genome, has been observed for other transgenes (ref. 10 and references therein). This does not appear to be the case in our study, as both pedigrees show the same phenotype and express detectable amounts of NGF from the first months of life. We propose that some regulatory mechanisms are either removed or replaced in older animals. Alternatively, the continuous supply of NGF may disrupt an intracellular signal-transduction system.

Whatever the mechanistic basis for the observed hyperplasia is, these results suggest that NGF may serve roles other than triggering neuronal differentiation and cell survival. Thus, in addition to its previously described effects, NGF may act as a new class of mitogen, with a potential role in oncogenesis.

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