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Cellular Localization of NGF and its Receptors trkA and p75LNGFR in Male Reproductive Organs of the Japanese Monkey, *Macaca fuscata fuscata*

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The actions of neurotropins are not restricted to the nervous system. Immunohistochemical methods were used in the present study to clarify distribution of nerve growth factor (NGF) and its receptors TrkA and p75LNGFR in excurrent ducts of the adult male Japanese monkey (*Macaca fuscata fuscata*). NGF was found in the seminal vesicle, epididymis, and testis, and has been thought to affect male reproductive functions. Leydig cells, Sertoli cells, and spermatogonia at various stages were positively stained for NGF, as well as for TrkA and p75LNGFR. Signals for these proteins were also found in epithelial cells and stromal tissues of the caudal epididymidis, as well as in the seminal vesicle. In the prostate, smooth muscle cells and basal cells were positively stained for NGF, TrkA, and p75 LNGFR. The results were comparatively discussed.

Key Words: NGF; Japanese monkey; testis; epididymis; prostate; seminal vesicle.

Introduction

The neurotropins, which include nerve growth factor (NGF), brain-derived neurotropic factor (BDNF), NT-3, NT-4, and NT-5, are a family of related growth factors that are of major importance in the regulation of neuronal survival and differentiation *(1–3)*. The biological effects of neurotropins are mediated by tyrosine kinase receptors encoded by the *trk* protooncogene family, known as TrkA, TrkB, and TrkC *(4)*. One of these receptors, TrkA, is known as a high-affinity receptor for NGF *(4)*. In addition, all neurotropins are recognized by a more widely expressed low-affinity receptor known as p75 nerve growth factor receptor (p75NGFR), which is a member of the tumor necrosis factor receptor superfamily *(4)*.

In the male mouse, the submandibular gland is a major source of NGF *(1)*. Relatively high levels of NGF protein were also found in the seminal vesicle *(5)* and prostate *(6)*. Because the levels of NGF protein and mRNA do not correlate with the innervations by NGF-sensitive fibers, the physiological roles of NGF outside of the nervous system have received great attention. Several studies have suggested that NGF can have effects outside the nervous system, such as in the prostate *(6)*, ovary *(7)*, testis *(8–12)*, and epididymis *(8)*. In particular, there is considerable evidence for a crucial function of NGF within the male reproductive system. Previous studies have indicated that development of the testis and sex determination are, at least in part, dependent on NGF *(9,13)*. In rat and mouse testes, positive immunoreactions for NGF were observed in germ cells at all stages, from primary spermatocytes to mature spermatids *(8)*. NGF acts as a meiotic growth factor during spermatogenesis by affecting Sertoli cells *(14)*. On the other hand, Persson et al. have demonstrated that p75LNGFR is present in Sertoli cells and is downregulated by testosterone *(12)*. By contrast, the high-affinity receptor TrkA mRNA was upregulated by human chorionic gonadotropin (hCG) *(15)*, suggesting that TrkA and p75LNGFR show reciprocal changes in the testis. Despite these findings, however, the physiological roles of NGF in the human male reproductive system remain unclear. Because a non-human primate would be a good model for studying physiological roles of NGF in the human male reproductive system, we preliminarily examined immunohistochemical localization of NGF and its receptors with high affinity (TrkA) and low affinity (p75LNGFR) in male reproductive organs, including the testis, epididymis, seminal vesicle, and prostate of the Japanese monkey, *Macaca fuscata fuscata.*

Results

Immunohistochemistry revealed that NGF (Fig. 1B) as well as TrkA (Fig. 1C) and p75LNGFR (Fig. 1D) were

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localized in Sertoli cells, Leydig cells, and germ cells at various stages.

Positive immunostainings for NGF (Fig. 2B), TrkA (Fig. 2C), and p75NGFR (Fig. 2D) were observed in both epithelia and smooth muscle of the caudal epididymidis. Strong signals for NGF (Fig. 2B), TrkA (Fig. 2C), and p75NGFR (Fig. 2D) were observed in the basal epithelial cells and smooth muscle in the connective tissue of the caudal epididymidis. Similar staining patterns were also observed in the caput and corpus epididymidis (data not shown).

Positive signals for NGF (Fig. 3B), TrkA (Fig. 3C), and p75LNGFR (Fig. 3D) were observed in the epithelial cell and smooth muscle of the seminal vesicle.

In the prostate, strong signals for NGF (Fig. 4B), TrkA (Fig. 4C), and p75LNGFR (Fig. 4D) were observed in the smooth muscle. Basal cells of the prostate were also positively stained with the antibodies.

Throughout Figs. 1–4, negative control sections did not show any immunostaining (Figs. 1A, 2A, 3A, and 4A).

Discussion

This study reports the cellular localization of NGF and its receptors TrkA and p75LNGFR in the reproductive organs of a non-human primate. Positive signals for NGF were observed in various types of cells in the testis. Our data seem in agreement with immunohistochemical localization of NGF in the testis of the mouse *(8, 11)*, rat *(8)*, and human *(16, 17)*. The previous study has also shown localization of NGF mRNA in germ cells and expression of NGF receptor mRNA in the testis *(8)*. Expression of NGF and its receptors in the round spermatids in the rat was demonstrated by Chen et al. *(18)*. Studies on expression sites of its receptor demonstrated that TrkA was localized in Leydig cells and relatively weak immunoreactivity was also observed in Sertoli cells in the rat and human adult testes *(19)*, although TrkA was expressed in rat Sertoli and interstitial cells at embryonic and early postnatal stages *(9)*. These studies suggest that the cellular localization of TrkA in the mammalian testis is probably age-dependent. Study in adult human testes has shown strong immunoreactions for TrkA in the Leydig cells, one of the major sites followed by Sertoli cells and cellular elements of the germinative epithelium *(10)*. Our staining in the adult Japanese monkey is consistent with previous human localization. With respect to the p75LNGFR, it showed wide distribution in the testis, which included peritubular cells of the semniferous tubules, Sertoli cells, Leydig cells, and germ cells at various stages. The wide distribution in the Japanese monkey is proved by several previous studies in the rat and human. In the rat, Parvinen et al. reported that p75LNGFR was localized in the seminiferous tubules, most probably in Sertoli cells *(14)* as confirmed in study by Western blotting analysis *(20)*. In the human testis, expression of p75LNGFR was shown by immunohistochemistry *(10)*. Northern blot hybridization demonstrated the presence of p75LNGFR mRNA in pachytene spermatocytes and round spermatids *(21)*. Only the spermatids at stages VII–IX of the seminiferous epithelium cycle express p75LNGFR. The roles of NGF in mammalian testes are not altogether clear. However, tropic effects of NGF in the maintenance of Sertoli cell viability have been demonstrated *(18)*. NGF has been shown to stimulate DNA synthesis at the onset of meiosis in the rat testis *(14)*. Both NGF and round spermatids stimulate expression of transcription factors in a mouse Sertoli cell line, indicating that spermatid-derived NGF may be involved in Sertoli cell function in a paracrine fashion *(22)*. In addition, administration of Chinese cobra NGF to male rats increases sperm motility and pregnancy rate *(23)*. Our preliminary study using the hamster also supports stimulatory effects of NGF on sperm motility (unpublished data). NGF has been shown to stimulate angiogenesis *(24)*. Because dynamic vascularization occurs in postnatal hamster testes *(25)*, testicular NGF may be involved in this process. Testicular NGF may also be involved in the regulation of testicular function through the effects on nerve system because catecholamines affects testicular steroidogenesis *(26)*. NGF is secreted by various types of cells in different tissues, and may act as a paracrine or autocrine factor regulating spermatogenesis and other testicular functions.

The present study determined a strong signal for NGF, TrkA, and p75LNGFR in the epithelial cells of the excurrent ductules. The ductal system is physiologically important, particularly in the reabsorption of fluid and other substances and for the maintenance of proper sperm concentrations in the lumen of the epididymis *(27)*. These ductules form a series of small tubules that transport sperm from the testis to the epididymis. Ductules in the rat reabsorb nearly 90% of the rete testis fluid to concentrate sperm as they enter the epididymis *(28)*. Previous data and our present observations lead us to hypothesize that the NGF signaling pathway may participate in the regulation of fluid reabsorption in the male reproductive tract. Ayer-LeLievre et al. showed NGF mRNA-containing cells in the epithelium of convoluted ducts in the mouse corpus epididymidis *(8)*.

Hofmann and Unsicker revealed that relatively high levels of NGF protein were present in the seminal vesicle *(5)*. They purified seminal vesicle–derived neuronotrophic factor (SVNF), which is present in seminal vesicle extracts (SVEs), and used several experimental approaches to demonstrated that SVNF could not be distinguished from NGF on the basis of biological activity. Consistent with this, in our study, we observed positive signals for NGF and its receptors co-localized in the both epithelial cell and smooth muscle of the seminal vesicle.

Stromal–epithelial interactions mediated by paracrine or autocrine factors such as fibroblast growth factor-2 and NGF play a fundamental role in androgen-induced development of normal and neoplastic prostate *(29–31)*. Indeed, the prostate is relatively abundant in NGF in several species

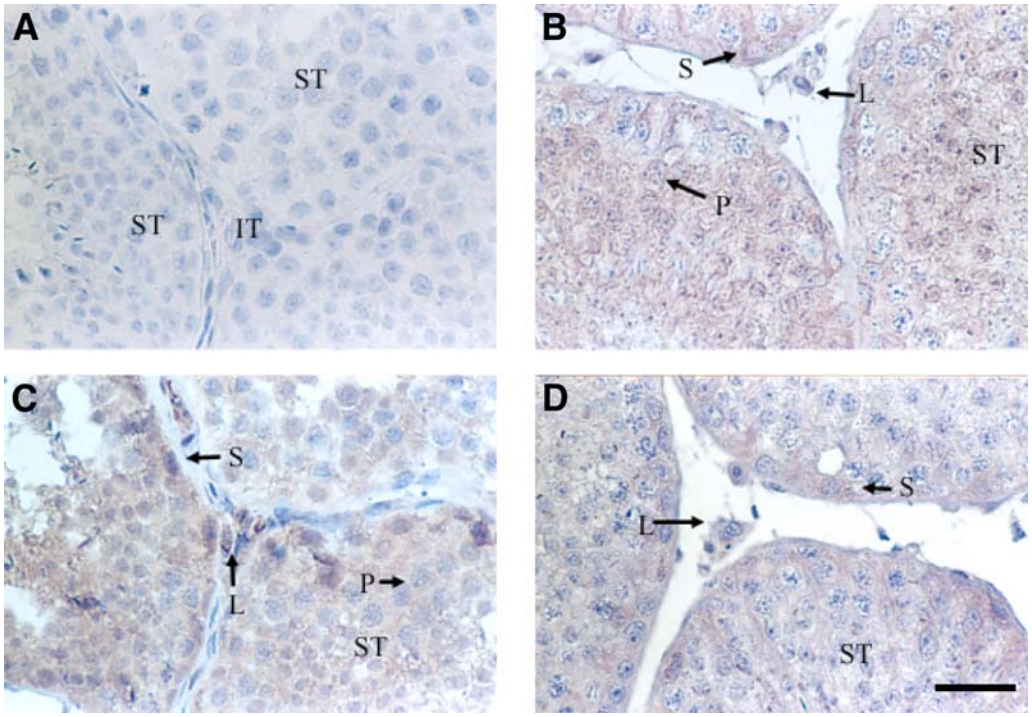

Fig. 1. Localization of NGF (**B**), TrkA (**C**), and p75LNGFR (**D**) in the testis of the Japanese monkey. Sertoli cells, Leydig cells, spermatocytes, and elongated spermatids were positively stained for NGF, as well as for TrkA and p75LNGFR. Normal rabbit serum used as a negative control did not show any immunostaining (**A**). Bar = 25 μm. Abbreviations: ST, seminiferous tubules; IT, interstitial tissue; L, Leydig cell; S, Sertolil cell; P, pachytene spermatocytes.

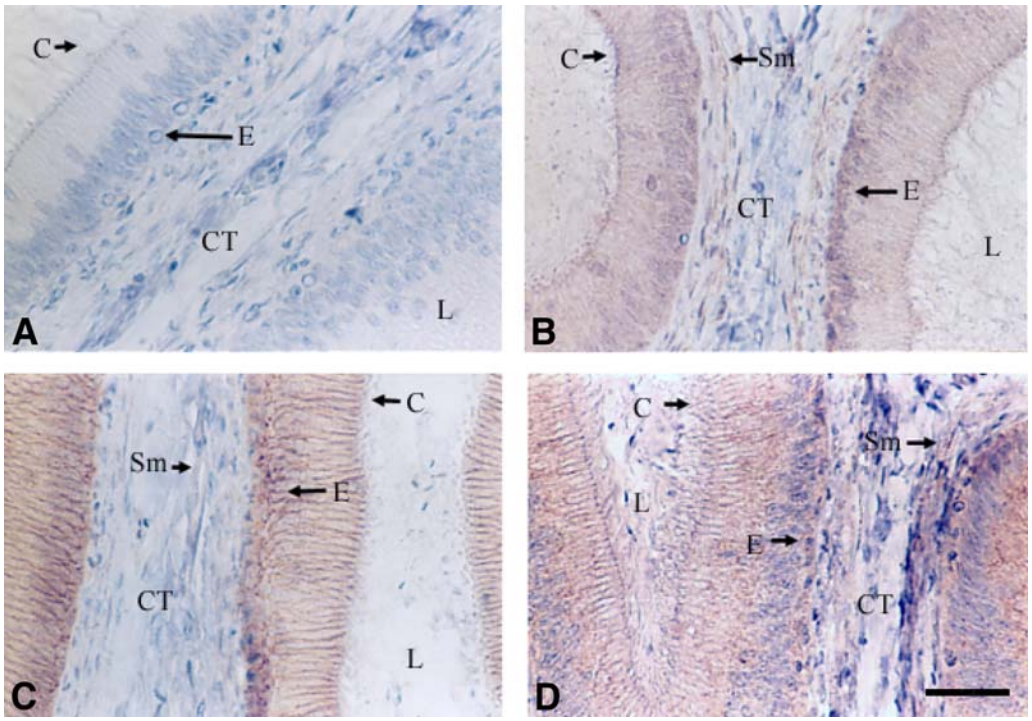

Fig. 2. Localization of NGF (**B**), TrkA (**C**), and p75LNGFR (**D**) in the caudal epididymidis of the Japanese monkey. Positive signals for NGF were observed in the epithelial cell and smooth muscle, as well as for TrkA and p75LNGFR. Normal rabbit serum used as a negative control did not show any immunostaining (**A**). Bar = 25 μm. Abbreviations: Sm, smooth muscle; CT, connective tissue; E, epithelial cell; L, lumen; C, ciliated cell.

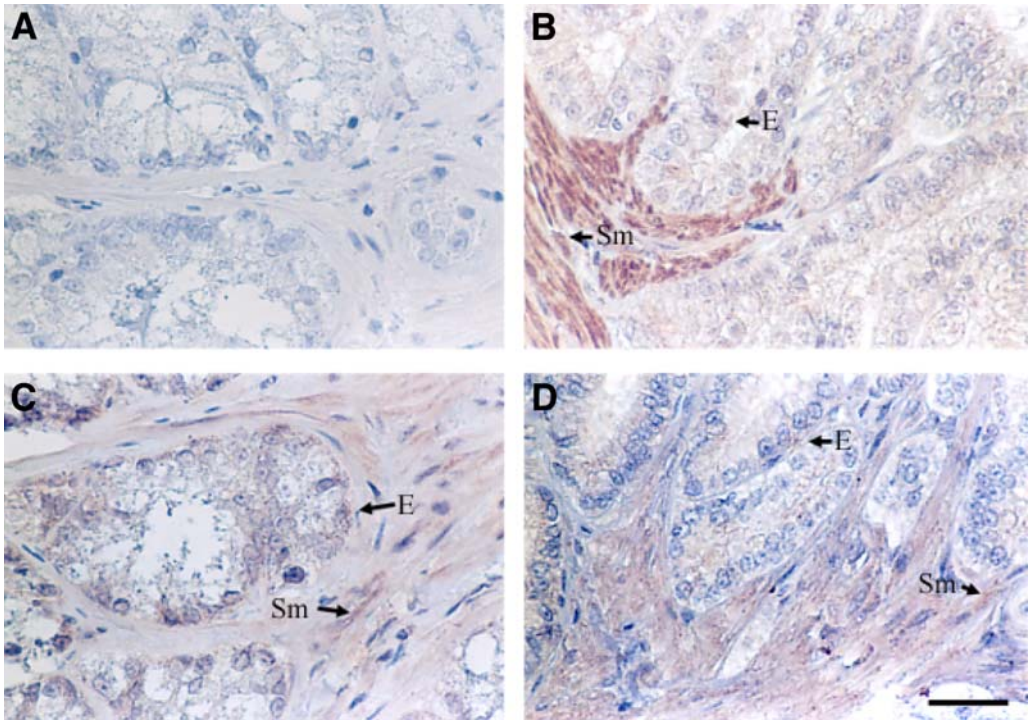

Fig. 3. Immunohistochemical analysis of NGF (**B**) and its receptors TrkA (**C**) and p75LNGFR (**D**) in the seminal vesicle of the Japanese monkey. Positive signals for NGF, TrkA, and p75LNGFR were observed in the epithelial cell and smooth muscle. Normal rabbit serum used as a negative control did not show any immunostaining (**A**); bar = 25 μm. Abbreviations: Sm, smooth muscle; E, epithelial cell; B, basal cell.

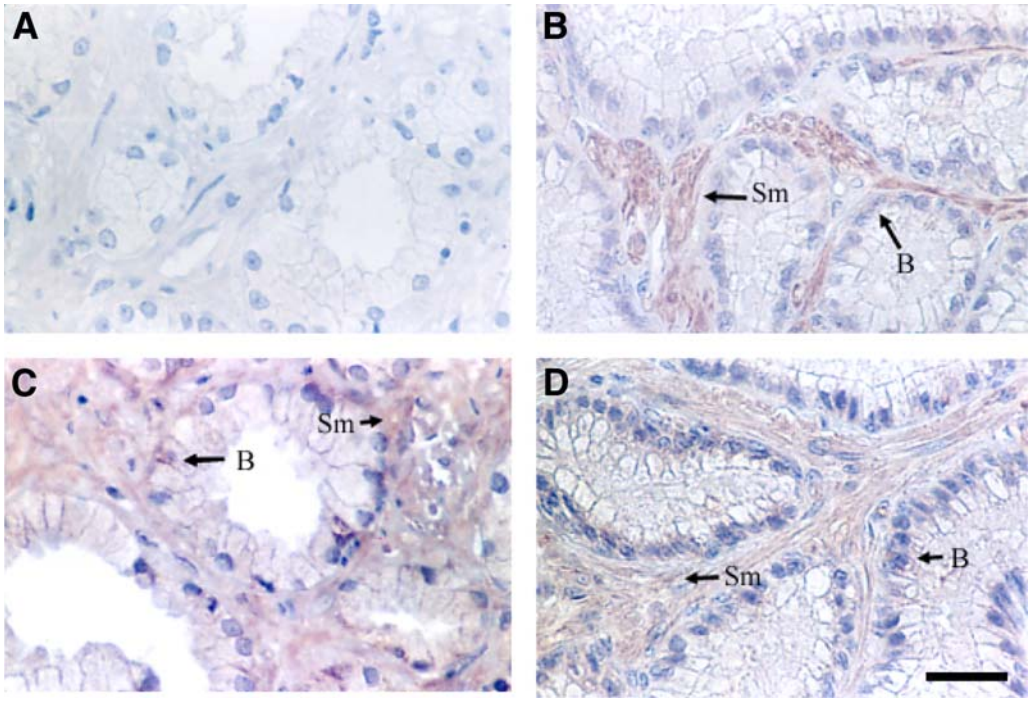

Fig. 4. Immunohistochemical analysis of NGF (**B**) and its receptors TrkA (**C**) and p75LNGFR (**D**) in the prostate of the Japanese monkey. Positive signals for NGF (**B**), TrkA (**C**), and p75LNGFR (**D**) were observed in the basal cell and smooth muscle. Normal rabbit serum used as a negative control did not show any immunostaining (**A**); bar = 25 μm. Abbreviations: Sm, smooth muscle; B, basal cell.

(32) including humans *(16,33)*. Our present results in the Japanese monkey suggest possible involvement of prostatic NGF in the regulation of prostate function.

In conclusion, the present study demonstrates the presence and the site of expression of NGF and its receptors in the testis, epididymis, seminal vesicle, and prostate in the

adult male Japanese monkey. Thus, involvement of NGF signaling in the regulation of male reproduction could be quite probable. Studies are in progress to define the possible involvement of NGF signaling in the regulation of male reproduction in this species.

Material and Methods

Animals

Six male Japanese monkeys (*Macaca fuscata fuscata*) at least 6 yr old and weighing 7–9 kg were used. They were housed individually in an air-conditioned room with controlled temperature (20 ± 5°C) and lighting (lights-on: 06:00 to 18:00 h) at the Primate Research Institute, Kyoto University, Inuyama, Aichi, Japan. They were fed standard monkey pellet foods with sweet potatoes or fruits daily, and were allowed free access to water. Animal care and the experimental protocol were in accordance with the Guide for the Care and Use of Laboratory Primates issued by the Primate Research Institute, Kyoto University, Japan (2002).

Tissue Preparation

Animals were sacrificed by bloodletting from the artery under deep anesthesia with ketamine hydrochloride (10 mg/kg, im) and sodium pentobarbital (25 mg/kg, iv), following the euthanasia guidelines approved by the Primate Research Institute, Kyoto University (2002). The male reproductive organs were immediately fixed in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA) in 0.05 *M* phosphate-buffered saline, pH 7.4, and embedded in paraffin. The paraffin-embedded testicular tissues were serially sectioned at 6-μm thickness and placed on poly-L-lysine (Sigma Diagnostics. INC., St. Louis, MO, USA)–coated slide glasses (Dako Japan Co., Kyoto, Japan) for use in immunohistochemistry.

Immunohistochemistry for NGF, TrkA and LNGFR p75

After deparaffinization, the tissue sections were subjected to antigen retrieval by autoclaving in 0.01 *M* sodium citrate buffer (pH 6.0) at 121°C for 15 min. The sections were then treated with 6% H_2O_2 in methanol at room temperature for 1 h, followed by blocking with 0.5% casein–Tris saline (0.05 *M* Tris-HCl with 0.15 *M* NaCl, pH 7.6; CTS) at 37°C for 1 h. Then, they were incubated at 37°C for 16–18 h with primary antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibodies used were a rabbit polyclonal antibody to NGF (M-20), a rabbit polyclonal antibody to TrkA (763), or a rabbit polyclonal antibody to p75LNGFR (H-92). The antibodies to NGF and TrkA were diluted to 200 ng/mL with CTS. The antibody to p75LNGFR was diluted to 20 μg/mL for the testis or 200 ng/mL for the other tissues. For negative control sections, normal rabbit serum was used instead of the primary antibodies. After incubation with the primary antibodies, sections were treated with 0.25% (v/v) biotinylated goat anti-rabbit secondary antibody (Elite ABC kit; Vector Lab. Burlingame, CA, USA) in CTS for 1 h at 37°C. These sections were subsequently incubated with 2% (v/v) avidin–biotin complex (Elite ABC kit) in CTS for 30 min at 37°C. The reaction products were visualized by treating with 0.025% (w/v) 3.3'-diaminobenzidine tetrachloride (DAB, Sigma) in 100 m*M* Tris-buffered saline containing 0.01% H_2O_2 for 1–30 min. The sections were examined with Olympus BX50 microscope.

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