Expression of β-Nerve Growth Factor and Its Receptor in Rat Seminiferous Epithelium: Specific Function at the Onset of Meiosis


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Abstract. β-Nerve growth factor (NGF) is expressed in spermatogenic cells and has testosterone-downregulated low-affinity receptors on Sertoli cells suggesting a paracrine role in the regulation of spermatogenesis. An analysis of the stage-specific expression of NGF and its low affinity receptor during the cycle of the seminiferous epithelium in the rat revealed NGF mRNA and protein at all stages of the cycle. Tyrosine kinase receptor (trk) mRNA encoding an essential component of the high-affinity NGF receptor was also present at all stages. In contrast, expression of low affinity NGF receptor mRNA was only found in stages VIIα and VIII of the cycle, the sites of onset of meiosis. The low-affinity NGF receptor protein was present in the plasma membrane of the apical Sertoli cell processes as well as in the basal plasma membrane of these cells at stages VIIα to XI. NGF was shown to stimulate in vitro DNA synthesis of seminiferous tubule segments with preleptotene spermatocytes at the onset of meiosis while other segments remained nonresponsive. We conclude that NGF is a meiotic growth factor that acts through Sertoli cells.
trk proto-oncogene product is a member of a family of at least three structurally related proteins (Martin-Zanca et al., 1989; Klein et al., 1989, 1991; Lamballe et al., 1991). Since brain-derived neurotrophic factor (Rodriguez-Tébar et al., 1990), neurotrophin-3 (Ernfors et al., 1990; Squinto et al., 1991), and neurotrophin-4 (Hallböök et al., 1991) interact with the 75-kD NGF-R with similar binding affinities as NGF, this receptor could be used to mediate the response of all members in the NGF family.

In agreement with a target-derived neurotrophic role of NGF, the levels of NGF mRNA in most peripheral tissues (Heumann et al., 1984; Shelton and Reichardt, 1984) and in the brain (Korsching et al., 1985; Whitemore et al., 1986; Shelton and Reichardt, 1986) have been shown to correlate with the degree of innervation by NGF-sensitive fibers. However, in male mouse submandibular gland, snake venom gland (Levi-Montalcini and Angeletti, 1968), guinea pig and rabbit prostate (Harper et al., 1979), and in bull seminal vesicle (Harper et al., 1982), the levels of NGF protein and mRNA do not correlate with the innervation by NGF-sensitive fibers possibly reflecting a non-neurotrophic role of NGF in these tissues. Testis also belongs to such tissues where NGF-like immunoreactivity has been demonstrated in mouse and rat germ cells (Olson et al., 1987; Ayer-LeLièvre et al., 1988). From these initial data, NGF has been suggested to have a role in sperm maturation and/or motility. In agreement with this possibility, NGF mRNA and protein have been demonstrated in spermatocytes and early spermatids of rat and mouse, and NGF protein has also been detected in the lumen of the epididymis (Ayer-LeLièvre et al., 1988). Furthermore, the gene for the 75-kD LNF-R is expressed in Sertoli cells in the testis under negative control of testosterone (Persson et al., 1990) suggesting that NGF produced by spermatogenic cells mediates an interaction between germ and Sertoli cells. Results of in situ hybridizations (Persson et al., 1990) have suggested that the 75-kD LNF-R is only expressed between stages VI–VIII of the cycle of the seminiferous epithelium (Leblond and Clermont, 1952) providing further evidence for a specific function of NGF and its receptor in spermatogenesis. In this report, we have investigated the functional role of NGF in the testis by accurate localization studies combined with a newly developed in vitro method for studies of stage-specific DNA synthesis during spermatogenesis. Our results show that NGF, presumably by interacting with a stage-specific NGF-R in Sertoli cells, stimulates DNA synthesis at the time of onset of meiosis, suggesting that NGF acts as a meiotic growth factor during spermatogenesis.

Materials and Methods

Seminiferous Tubule Microdissection and Culture

Testes of adult (3-5 mo) Sprague-Dawley rats (ALAB, Sollentuna, Sweden) were decapsulated and seminiferous tubules teased free by fine forceps under transilluminating stereomicroscope in a petri dish containing PBS (Modified Dulbecco's formula, without calcium and magnesium) (Flow Laboratories, Inc., Irvine, Scotland). The stages were recognized according to light absorption criteria (Parvinen and Vainio-Perttula, 1972). For Northern blot analysis, pools of stages I, II-III, IV-V, VI, VIIa-V, VIIb-VII, IX-XI, XII, and XIII-XIV, each containing a total of 10 cm of seminiferous tubule segments were collected as described (Parvinen and Ruokonen, 1982). Since LNGFR is physiologically downregulated by testosterone (Persson et al., 1990), single subcutaneous injection of 75 mg/kg of ethane dimethane sulphonate (EDS) (dissolved in DMSO/water 1:3) was given specifically to destroy the Leydig cells (Jackson et al., 1986). This results in very low levels of intratesticular testosterone for 3-10 d and a marked increase of NGF receptor and its mRNA (Persson et al., 1991). The same effect can be reached by hypophysectomy. For studies of DNA synthesis in vitro, stages I, V, VII, and VIII-IX were prepared from normal adult rat testes (Parvinen et al., 1991). They contain cells at representative phases of mitotic and meiotic DNA synthesis: Stage I, type A4 spermatogonia in A phase; stage V, type B spermatogonia in S phase; VIIa, preleptotene spermatocytes outside S phase; and VIII-IX, leptotene spermatocytes in S phase (Clermont, 1972). For studies of NGF receptor mRNA expression during in vitro differentiation, quadruplicate pools (2.5 cm each) of stages VIa and VIIc–VII were isolated from normal rats for immediate freezing and RNA isolation, or cultured for 48 h at 37 and 34°C in a medium described below before RNA isolation. For immunohistochemical studies, whole testes or ten pools of microdissected 20 × 2 mm seminiferous tubular segments from stages I, II-III, IV-V, VI, VIIa-V, VIIb-VIIa, VIII, IX-XI, XII, and XIII-XIV were fixed from normal, hypophysectomized (3 and 10 d), and rats given a single dose of EDS 7 d previously.

RNA Blot-Hybridization Analysis

The frozen seminiferous tubule segments were disrupted in 4 M guanidine isothiocyanate, 0.025 M sodium citrate, pH 7.0, and 0.1 M β-mercaptoethanol followed by centrifugation through a cushion of 5.7 M CsCl2 in 0.025 M sodium acetate, pH 5.5. After phenol-chloroform extraction, the samples containing 40 μg of RNA were electrophoresed in a 1% agarose gel containing 0.7% formaldehyde. The gel was blotted onto nitrocellulose and hybridized to a 3.2-kb-long insert derived from a rat LNGF-R cDNA clone (Radeke et al., 1987). After washing and exposure to x-ray film the same filter was boiled for 5 min in 1% glycerol and hybridized to a 456-bp-long polymerase chain reaction fragment from the extracellular region of rat trk. The filters were labeled with α-32P-dCTP by nick-translation to a specific activity of >106 cpm/μg. The filters were washed at high stringency (0.1 × SSC/0.1% NaDodSO4 at 54°C) and exposed to x-ray films at −80°C with an intensifying screen (DuPont Co., Wilmington, DE).

Immunohistochemical Analyses of NGF

Staged 2-mm tubule segments were micro dissected from a normal rat testis. They were fixed in a 4% solution of paraformaldehyde containing 0.2% picric acid for 2 h and cryoprotected with 10% sucrose in PBS. 10 μm cryostat sections were cut with a cryostat (model HM-500, Micron Instruments, Inc., Los Angeles, CA) and thawed onto gelatin-coated glass slides. The sections were incubated for 12–18 h with a rabbit NGF antibody (K 17, Olson et al., 1987) diluted 1:50 in PBS containing 1% BSA and 0.3% Triton X-100. Subsequently, the sections were incubated with FITC-conjugated goat-anti-rabbit IgG (Boehringer Mannheim, GmbH, Mannheim, Germany) diluted 1:10 for 30 min. The sections were mounted in glycerol/PBS containing 0.1% phenylmedamine and examined with a fluorescence microscope (Nikon FX; Nikon Inc., Melville, NY). The controls included omission of the primary antiserum, replacement of the primary antiserum with normal rabbit serum, and preabsorption of the NGF antiserum with 1 μM mouse β-NGF before the immunocytochemical procedure.

Immunohistochemical Analyses of the 75-kD LNGF-Receptor

Adult control and hypophysectomized (3 and 10 d) male rats were perfused transcardially with the same fixative as above. The testes were then excised and immersion fixed for 2 h. Staged 2 mm tubule segments from EDS-treated rats (7 d after a single dose) were treated as described above. The sections were incubated overnight with mouse monoclonal rat 75-kD LNGF-R antibody (192-lgG, Taniiuchi and Johnson, 1985) diluted 1:500 or 1:1,000 in PBS containing 1% BSA, followed by incubation with biotinylated sheep-anti-mouse IgG and ABC-complex, 30 min each. In control preparations, the specific antiserum was either omitted or replaced with normal mouse serum (1:500). Diaminobenzidine was used as a chromogen.

Electron Microscopic Immunohistochemical Analysis of the 75-kD LNGF-R

Adult normal or 10-d hypophysectomized male rats were perfused with a fixative containing 4% paraformaldehyde and 0.2% glutaraldehyde with or without 0.2% picric acid in 0.1 M phosphate buffer, pH 7.3. The testes were
removed and immersion fixed for 6-12 h. The tissues were cryoprotected with 50% sucrose in PBS for several days and frozen in liquid nitrogen. Thick sections (~500 μm) were cut with a razor blade and allowed to thaw in PBS. The tissues were then incubated with mouse mAb to the 75-kD LINGF-R diluted 1:500 for 72 h. After several washes, the tissues were incubated in biotinylated goat-anti-mouse IgG and ABC-complex for 2 h each. Diamobenzidine was used as chromogen to reveal ABC-complexes. Subsequently, the tissues were postfixed with 2.5% glutaraldehyde, 1% osmium tetroxide, and 1% uranyl acetate for 30 min each. The tissues were dehydrated and flat embedded in Epon. Sections exhibiting immunolabeling were processed for EM and the ultrathin sections were examined with a microscope (model 1200EX; JEOL USA, Inc., Peabody, MA) without counterstaining.

**Analysis of DNA-Synthesis**

The staged 2-mm tubular segments from normal rats were individually transferred in 10 μl of PBS into 96-well tissue culture plates (Falcon 3027 Microtest III; Becton Dickinson Microbiology Sys., Cockeysville, MD) and incubated for 24, 48, or 72 h in 100 μl alpha modification of MEM (α-MEM) with Earl’s salts (12-312-54; Flow Laboratories, Inc.) supplemented with l-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and α-thioglycerol (7.5 × 10−5 M) (Sigma Chemical Co., St. Louis, MO). During the last 4 h, 20 kBq of methyl-[3H]thymidine (TRA.120, 120, 185 GBq/mmol, Amersham International, Amersham, UK) was added.

**Effects of β-NGF on [3H]Thymidine Incorporation**

After microdissection of 2-mm segments of stages I, V, VII, and VIII-IX, they were transferred to tissue culture wells as described above and incubated for 24, 48, or 72 h in the presence of control (0), 40, 100, and 250 ng/ml of β-NGF purified from the male mouse submandibular gland (Eiben-dal et al., 1984). Cultures were maintained in α-MEM supplemented as described above at a temperature of 37 or 34°C (physiological) in a humidified atmosphere of 5% CO₂ in air. Each preparation was performed, each with six replicate samples.

**NGF Preparation and Purification**

β-NGF was purified from male mouse (NMRI, body weight >30 g) submandibular glands as previously described (Eibendal et al., 1984). Elution from the second column of carboxymethyl-Sepharose was by a linear gradient of sodium chloride yielding a peak of highly purified β-NGF (Eibendal et al., 1984; Olson et al., 1991). The concentration of NGF was determined from specific absorbance at 280 nm (1.6 at 1 mg/ml in a 1-cm cuvette). Identity and purification was further verified by electrophoresis in sodium dodecylsulphate on a gradient polyacrylamide gel, and by immunoblotting of similar gels (Olson et al., 1991).

**Bioassay of NGF Activity**

The biological activity of NGF was measured in a nerve fiber outgrowth assay with explanted sympathetic ganglia from the 9-d-old chick embryo. The dissected ganglia were explanted into a collagen matrix (Eibendal et al., 1984). To determine activity, the purified mouse NGF was added in a series of twofold dilutions with final concentrations ranging from 0 to 20 ng/ml. The cultures were incubated at 37°C using 5% CO₂ and 92% relative humidity. After 1-2 d the cultures were examined in an inverted microscope equipped for phase contrast and dark field illumination. Activity test of the NGF preparation showed that 5 ng NGF/ml evoked a dense circular fiber halo, regarded as corresponding to an activity of 1 biological unit.

**Cell Harvesting and Radioactivity Measurement**

After [3H]thymidine labeling, the tissue cultures were harvested by a Titer-tex Cell Harvester 550 (Flow Laboratories, Inc.) for 1 min/row using distilled water and Titer-tex glass-fiber filter paper (Skatron, Inc., Lier, Norway). The filter disks were transferred into minivials by a punching apparatus (Skatron, Inc.) for liquid scintillation counting by a spectrometer utilizing Ready Safe (Model LS500CE, Beckman Instruments, Inc., Fullerton, CA) scintillation cocktail.

**Autoradiography**

To identify the cell types incorporating the radioactivity derived from [3H]thymidine, four parallel stage VIIa tubule segments (2 mm) were cultured for 48 h at 34°C in the presence and absence of 250 ng/ml of β-NGF and labeled for 4 h with 5 and 20 kBq of [3H]thymidine. After labeling, the tubule segments were carefully squared between coverslips and albumin-coated microscope slides to produce a monolayer of slightly flattened cells (Kangasniemi et al., 1990). The preparations were then frozen with liquid nitrogen, the coverslips removed, and the frozen slides fixed in 99% ethanol/glacial acetic acid 3:1 (vol/vol) for 30 min and air dried. The autoradiography was performed by dipping the slides in Kodak NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, NY) and exposed for 24 or 48 h. By adjusting the temperature of the developer between 5 and 20°C, the optimal grain density for cell identification was found. The preparations were then slightly counterstained with Harris' hematoxylin.

**Data Processing and Statistics**

A Sigma-Plot 3.1 microcomputer program (Jandel Scientific, Corte Madera, CA) was used for data processing and to produce graphs. Statistical analyses were performed by paired Student's t test fitted to a microcomputer program (Nycomed Pharma, Oslo, Norway). In experiments at 34°C, the two largest and the two smallest values were deleted from group of stage VIIa tubule segments cultured for 48 h resulting in n = 14.

**Results**

**Expression of NGF mRNA and Protein during Spermatogenesis**

The cellular distribution of NGF mRNA and protein during spermatogenesis was first studied using a transillumination-assisted microdissection technique (Parvinen and Vanha-Pertula, 1972), where 10 pools of various stages of the seminiferous epithelial cycle were isolated (Parvinen and Ruokonen, 1982). Immunohistochemical staining of sections from such samples revealed a NGF immunoreactivity in spermatogenic cells at all stages of the cycle (Fig. 1 A): Stages I-V, mainly round spermatids; stages VI-VIII, round spermatids and pachytene spermatocytes; stages IX-XIV, mainly pachytene, diakinetik, and dividing spermatocytes. A declining immunoreactivity was seen in acrosome spermatids (steps 9-14). Spermatogonia, early spermatocytes (leptotene through mid-pachytene) and maturation phase spermatids (steps 15-19) were negative (see Fig. 11). Preabsorption of the NGF-antibody with mouse NGF completely abolished all specific staining (Fig. 1 B); other controls did not reveal specific staining either. Northern blot analysis showed equal levels of a 1.3-kb NGF mRNA at all stages of the cycle (data not shown).

**Expression of Low Affinity 75-kD NGF Receptor mRNA and Protein**

Northern blot analysis of different stages of the seminiferous epithelial cycle 4 d after treatment with EDS showed a strictly stage-specific expression of the mRNA for the 75-kD LINGF-R in tubular segments prepared from stages VIIa and VIII of the cycle (Fig. 2). A weak signal was found at stages VIIa but all other stages were negative. Hybridization of the same filters with a probe specific for the tk mRNA revealed an expression of 3.2 kb transcript at all stages of the cycle. Somewhat higher levels were found at stages VIIa and VIII than in other stages (Fig. 3). The expression of tk mRNA in the testis did not change as a result of low testosterone concentration caused by hypophysectomy (Persson,
H., and J.-P. Merlio, unpublished results). In control animals not treated with EDS, the 75-kD LNGF-R mRNA expression at stages VIIa–VIII was much lower, but a clear difference to stages VIIb could be demonstrated (Fig. 4). When seminiferous tubular segments from stages VIIa and VIIc–

VIII were cultured in chemically defined serum-free medium for 48 h, low levels of 75-kD LNGF-R mRNA expression appeared in tubular segments which were at stages VIIa at the onset of culture suggesting that a differentiation occurred in vitro. This differentiation only occurred at the physiological temperature of 34°C; no 75-kD LNGF-R mRNA signal was detected when the seminiferous tubular segments were cultured at 37°C (Fig. 4).

In immunohistochemical analysis of staged seminiferous tubule segments from EDS-treated rats using a mAb specific for the 75-kD LNGF-R, some of the tubules at stages VIIa showed a reaction in the apical parts of the Sertoli cells. In contrast, all the tubules at stages VIIa and VIII showed 75-kD LNGF-R immunoreactivity mainly at the apical portions of the Sertoli cells (Fig. 5). Some reaction was also

Figure 1. (A) Fluorescent immunocytochemical analysis of the expression of NGF protein during spermatogenesis (stages of the cycle are indicated at lower left corner of each panel). The spermatogenic cells in all stages of the cycle showed immunoreactivity. In stages I-V, mainly round spermatids were labeled; in stages VI–VIII both round spermatids and pachytene spermatocytes; and in stages IX–XIV mainly pachytene, diakinetic, and dividing spermatocytes were labeled. During stages IX–XIV, a declining immunoreactivity was seen in acrosome phase spermatids (steps 9–14). Spermatogonia and early spermatocytes for up to mid-pachytene as well as maturation phase spermatids (steps 15–19) were negative. (B) A stage IV–V control tubule incubated with an antiserum preabsorbed with NGF. No specific NGF immunostaining is seen in the layers of spermatogonia (G), spermatocytes (C), round spermatids (R), or elongated spermatids (E). The finding was similar in all stages of the cycle. Bars, 25 μm.

Figure 2. Northern blot analysis of 75-kD LNGF-R mRNA in seminiferous tubule segments microdissected from defined stages of the epithelial cycle 4 d after a single dose of EDS. Total cellular RNA (40 μg/slot) was electrophoresed in a formaldehyde containing agarose gel, blotted onto a nitrocellulose filter, and hybridized to a cDNA probe for the 75-kD low-affinity NGF-R. The filter was washed at high stringency and exposed to x-ray film.

Figure 3. Northern blot analysis of rat trk mRNA in the same filter as in Fig. 2.
progressed at a slightly accelerated rate as compared with the physiological temperature of the testis. At 37°C, spermatogenesis that observed in vivo (Clermont and Harvey, 1965). After 48 h at 37°C, the cryptorchid temperature, and at 34°C, the physiological temperature of the testis, spermatogenesis showed clear signs of degeneration, many tubules were intensively positive. At this time, all parts of the Sertoli cells were strongly labeled (Fig. 6).

Localization of the 75-kD LNGF-R by Immunoelectron Microscopy

After hypophysectomy (10 d), the 75-kD LNGF-R immunoreactivity was found in the apical Sertoli cell processes while the adjacent germ cells were negative (Fig. 7 A). The reactivity was associated with plasma membranes and it was particularly strong in the narrow and long apical extensions of the Sertoli cells (Fig. 7, B and C). Some 75-kD LNGF-R immunoreactivity was also seen in the basal plasma membrane of the Sertoli cell (Fig. 7 D).

Effects of NGF on Spermatogenic Stage-specific DNA Synthesis

To study the effect of NGF on DNA synthesis in spermatogenic cells, 2-mm segments of seminiferous tubules from stages I, V, VII, and VIII–IX of the cycle were micropipette and cultured in vitro. The cultures were maintained at 37°C, the cryptorchid temperature, and at 34°C, the physiological temperature of the testis. At 37°C, spermatogenesis progressed at a slightly accelerated rate as compared with that observed in vivo (Clermont and Harvey, 1965). After 24 h in culture, the highest basal DNA synthesis was observed in tubular segments from stages VIII–IX of the cycle that contain preleptotene spermatocytes at the onset of meiosis (Fig. 8 A). NGF had a dose-dependent significant effect on this DNA synthesis, whereas no effect was found in other stages. After 48 h, resting preleptotene spermatocytes of stage VII, were activated in DNA synthesis but NGF did not further stimulate them (Fig. 8 B). However, a significant dose-dependent effect on the same stages was found after 72 h at 37°C (Fig. 8 C). At this time, the meiotic DNA synthesis had started also in what was originally stage V tubules, but NGF did not have an effect on this. At 34°C, in the in vitro differentiation of the seminiferous tubules progressed at the same rate as in vivo (Clermont and Harvey, 1965). Significant stimulation of meiotic DNA synthesis of stages VIII–IX was demonstrated after 24 h in culture by the highest concentration of NGF (Fig. 9 A). A significant dose-dependent stimulation of premeiotic DNA synthesis was found after a 48-h culture. All concentrations of NGF had a stimulatory effect on what was originally stage VII, tubular segments at their onset of meiotic DNA synthesis (Fig. 9 B). The autoradiographic analysis revealed that the cells incorporating radioactivity in these conditions were preleptotene spermatocytes at the onset of meiosis (Fig. 10). The other stages showed no response. At 72 h, the meiotic DNA synthesis at stage VII, was fully activated, and the stimulatory or maintaining effects of NGF were no longer seen (Fig. 9 C).

Discussion

Fig. 11 summarizes the findings of the present work and relates them to the previously described stage-specific DNA synthesis during spermatogenesis (Parvinen et al., 1991). LNGF protein is expressed in spermatogenic cells from late meiosis to the mid-spermiogenesis, but its receptor is only expressed in Sertoli cells at stages VII, and VIII where preleptotene spermatocytes activate in the meiotic DNA synthesis. NGF appears to be the first growth factor described that specifically affects meiosis by mediating an interaction between germ cells and Sertoli cells. Although NGF is known to have important functions in the development and maintenance of sensory and sympathetic neurons in the mammalian nervous system (Levi-Montalcini, 1987), its function in the testis has remained obscure. However, the strictly limited expression of 75-kD LNGF receptor mRNA to Sertoli cells at stages VII, and VIII of the seminiferous epithelial cycle suggests a specific paracrine function. In contrast, LNGF mRNA and protein did not show such a stage-specific localization and were found in primary spermatocytes and in spermatids at all stages. The expression of trk mRNA did not show a great variation during the cycle suggesting that the trk proto-oncogene protein is present in the testis although its expression is not regulated by the same strictly stage-specific manner as that of the 75-kD LNGF-R. Provided that the 75-kD LNGF-R can mediate a response of NGF in the testis, perhaps as a heterodimer between the 75-kD LNGF-R and trk (Hempstead et al., 1991), events at stages VII, and VIII of the cycle may be targets for NGF-mediated actions.

Stages VII and VIII of the cycle of the seminiferous epithelium are preferentially regulated by androgens (Parvinen, 1982), and synthesis of specific proteins in these stages is influenced by testosterone (Sharpe et al., 1992) and by germ cells (McKinnell and Sharpe, 1992). Surprisingly, the ex-
Figure 5. Immunohistochemical analysis of 75-kD LNGF-R protein during the cycle of rat seminiferous epithelium in a rat treated with EDS 7 d previously (stages are indicated at lower left corner of each panel). Some of the tubules at stage VIIa showed a reaction in the apical parts of the Sertoli cells, whereas all the tubules at stages VIIb and VIII showed immunoreactivity mainly at the apical portions of the Sertoli cells. An increasing reactivity was found at the basal part of Sertoli cells during stages VIIc–XI (arrows). In stage XII, the reactivity was disappearing. Stages XIII–XVI were negative. Bar, 50 μm.
pression of 75-kD LNGF-R mRNA and protein is regulated in a negative fashion by testosterone at these stages. This further supports the central role of androgens in the regulation of this part of the seminiferous epithelium. In terms of DNA synthesis, the same stages belong to the most active ones during the cycle of the seminiferous epithelium. The A₁-spermatogonia located most basally in the seminiferous epithelium enter S phase during stages VII₈-VIII and undergo the first mitotic division that starts the entire spermatogenesis (Clermont, 1972). However, the A₁-spermatogonia are too few to significantly contribute to the total DNA synthesis of stages VII₈-VIII. The preleptotene spermatocytes that enter DNA synthesis to start meiosis at the same stage of the cycle are far more abundant in number (Wing and Christensen, 1982). Without doubt they contribute the bulk of [3H]thymidine incorporation considered as meiotic DNA synthesis, also revealed by autoradiography (Monesi, 1965; Söderström and Parvinen, 1976). Finally, the most mature step 19 spermatids become released from the seminiferous epithelium at stage VIII of the epithelial cycle (Leblond and Clermont, 1952). The present data do not allow us to evaluate the role of NGF in the regulation of all of these processes. However, a hypothesis to explain the existence of the constant cell associations in the seminiferous epithelium is that different generations of spermatogenic cells require similar metabolic activation of the Sertoli cells. This may be the case for DNA synthesis in A₁-spermatogonia and preleptotene spermatocytes at the same stage of the seminiferous epithelial cycle.

The new method for quantitative analysis of DNA synthesis during rat spermatogenesis (Parvinen et al., 1991) is sometimes influenced by unexpected modulations of the seminiferous epithelial wave (Perey et al., 1961), particularly in rats that were more than 5-mo-old. Therefore, only rats between 60 and 90 d of age gave results with a variability small enough to reveal the subtle effects of NGF. In all positive experimental conditions (four out of six), the highest concentration of NGF significantly stimulated the premei-
Figure 7. Electron microscopic immunohistochemical analysis of 75-kD LNGF-R protein 10 d after hypophysectomy. (A) The main reactivity was found in the apical Sertoli cell processes approximately at stages VIIa–VIII of the cycle (arrow); the adjacent germ cells were negative (A). (B) The immunoreactivity was associated with plasma membranes and it was particularly strong in the narrow and long extensions of the Sertoli cells (arrows). (C) 75-kD LNGF-R immunoreactivity was also seen in the basal plasma membrane of the Sertoli cell (arrows) around the typical indented nucleus (n). (D) Some Sertoli cells showed immunoreactivities only at limited areas of the plasma membrane (arrows); m, mitochondrion typical for Sertoli cell. Bars, 500 nm.
Figure 8. In vitro DNA synthesis (cpm incorporated from [H]thymidine during the last 4 h of culture) of staged 2-mm seminiferous tubule segments after 24 (A), 48 (B), and 72 (C) h at 37°C (means ± SEM, n = 18). The approximate progression of stages V, VIIa, VIII-IX, and I of the seminiferous epithelial cycle is indicated by arrows at the x-axis of each panel. After 24 h, the preleptotene–leptotene spermatocytes at stage X are still active in DNA synthesis, and NGF has a dose-dependent maintaining effect on this. After 48 h, the resting preleptotene spermatocytes at original stage VIIa have spontaneously activated in meiotic DNA synthesis (B), but NGF had no effect. However, after 72 h, when stage VIIa has differentiated to IX, preleptotene spermatocytes are activated in meiotic DNA synthesis and NGF has a dose-dependent effect (C). Premetiotic DNA synthesis was not stimulated or maintained by NGF in any conditions examined. *, P < 0.05; **, P < 0.01 compared with controls.

The absence of NGF effect in two conditions (48 h/37°C and 72 h/34°C) is puzzling and may be because of an interaction of growth promoters and thus far poorly investigated inhibitors of DNA synthesis.

An interesting finding was that at 37°C, NGF seemed primarily to be a maintaining factor for meiotic DNA synthesis. Apparently some LNGF-Rs survive to support this effect although the mRNA was shown to disappear. The physiological temperature of the testis (34°C) seems to be important and NGF clearly had a promoting effect on the premeiotic DNA synthesis. It is possible that in adverse conditions such as cryptorchidism, NGF is particularly important in maintaining and supporting the testicular functions. In agreement with this, a stabilizing effect of NGF has been demonstrated...
in cultures of human seminiferous tubules (Seidl and Holstein, 1990a,b).

The absence of any effect on DNA synthesis at stages I and V containing spermatogonia in S phase, but a dose-dependent stimulation at stages VII, and VIII–IX shows that NGF in vitro stimulates DNA synthesis at the onset of meiosis but not during mitotic proliferation of spermatogonia. Our data suggest that NGF synthesized in male germ cells stimulates meiosis through interaction with testosterone regulated stage-specific receptor on Sertoli cells. Other
growth factors may have functions in regulating S phases of type A2-4, intermediate and type B-spermatogonia. Interleukin 1c is an example of a testicular growth factor that has effects on both spermatogonial (mitotic) and meiotic DNA synthesis (Parvinen et al., 1991), and there is evidence that IGF-1 is not mitotic but only a spermatogonial growth factor (Söder et al. 1991). It appears that a very complex interaction of several growth factors together with follicle stimulating hormone and testosterone ultimately leads to a proper regulation of spermatogenesis.

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