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BIOLOGY OF REPRODUCTION **63,** 1617–1628 (2000)

Expression and Action of Neurotropin-3 and Nerve Growth Factor in Embryonic and Early Postnatal Rat Testis Development

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ABSTRACT

The current study examines the expression and potential actions of neurotropin-3 (NT3), nerve growth factor (NGF), and their receptors during morphological sex determination (seminiferous cord formation) and perinatal rat testis development. The expression of neurotropins and their receptors was analyzed with immunohistochemistry. Cellular localization of neurotropin ligand and receptor proteins changed during embryonic testis development. Neurotropin-3 was localized to Sertoli cells at Embryonic Day 14 (E14), was present in gonocytes at Postnatal Day 0 (P0), and after birth became localized to the interstitium and Sertoli cells (P3–P5). The expression of trk C (the high affinity receptor for NT3) was localized to mesonephric ducts and cells surrounding the cords (E14–E18). In addition, Sertoli cells and preperitubular cells surrounding the cords at E14 also stained for trk C. Neurotropin-3 was expressed in gonocytes and Sertoli cells at P0–P5. Nerve growth factor was detected in Sertoli cells at E14, was clearly in Sertoli and interstitial cells at E16 and E18, and in Sertoli, germ, and interstitial cells from P0–P5. The expression of trk A (the high affinity receptor for NGF) was located in Sertoli and interstitial cells at E16–P5. To determine the actions of neurotropins during embryonic and perinatal testis development, experiments were conducted on E13 and P0 testis. Antisense oligonucleotide experiments with NT3 were used on E13 testis organ cultures to determine effects on seminiferous cord formation. Cord formation was inhibited in 40% of the organ cultures treated with the antisense NT3 oligonucleotides, while no inhibition was observed with sense oligonucleotides. In P0 testis cultures, both NT3 and NGF alone and in combination stimulated thymidine incorporation into DNA. Therefore, the neurotropins are involved in embryonic morphological events (cord formation; NT3) and in growth of the perinatal testis (P0; NT3 and NGF). To define further the growth effects of neurotropins on testis development, expression of transforming growth factor alpha and beta (TGFα and TGFβ) were examined in response to neurotropins. The P0 testis cultures were treated with neurotropins, and expression of mRNA for TGFα and TGFβ was analyzed utilizing a quantitative reverse transcription-polymerase chain reaction assay. Nerve growth factor and NT3 alone or in combination inhibited expression of mRNA for TGFα while NT3 increased mRNA expression of epidermal growth factor receptor. The combination treatment of neurotropins inhibited expression of TGFβ1 and increased expression of TGFβ3. In summary, observations suggest that NT3, NGF, trk A, and trk C are localized to cells critical to seminiferous cord formation and appear to be important regulators of morphological sex determination. In addition to these morphological effects, both NT3 and NGF stimulate P0 testis growth and may elicit their action through altering the expression of locally produced growth factors such as TGFα and TGFβ. Taken together these results suggest that neurotropins are regulators of paracrine cell-cell interactions that result in morphological sex determination and perinatal testis growth.

developmental biology, growth factors, Sertoli cells, testes

INTRODUCTION

Seminiferous cord formation is an important event in testis development and is the first morphological indicator of sex determination. In the rat, seminiferous cord formation occurs at Embryonic Day 13.5 (E13.5, E0 = plug date) of gestation and is postulated to be in part a result of SRY expression. It is known that both aggregation of pre-Sertoli and germ cells [1, 2] along with cellular migration of preperitubular cells from the adjoining mesonephros [3, 4] are two important events that result in seminiferous cord formation. Several factors, including integrin subunit alpha 6 [5] and lectins [6], have been demonstrated to be involved in the early steps of cell aggregation leading to cord formation. Superphysiological levels of retinoic acid can also disrupt the process of seminiferous cord formation in E13 organ cultures [7, 8] through the apparent disruption of laminin production [7]. Regulation of the events associated with cellular aggregation and migration during seminiferous cord formation occurs in the absence of gonadotropin stimulation [9]. Therefore, the process of seminiferous cord formation requires paracrine cell-cell interactions and locally produced growth factors to orchestrate this important event.

A second critical event in testis development is growth and proliferation of somatic and germ cells that occur after seminiferous cord formation. Testis growth, like seminiferous cord formation, is a sex-dependent event that also appears to be gonadotropin independent [10]. Cellular growth and proliferation is necessary to allow for adequate numbers of somatic cells to support adult germ cell development [11]. Paracrine growth factors such as transforming growth factor (TGF)α [12] and TGFβ [13, 14] have been demonstrated to effect growth of the embryonic testis. Therefore, these and other growth factors must contribute to the growth of the somatic and germ cells during late embryonic and early testis development. The present study investigates neurotropins as potential paracrine regulators of morphogenesis and growth during testis development.

A role for neurotropins and their receptors has been implicated in many non-neuronal systems at sites of mesenchymal-epithelial cell interactions. In the testis, neurotropin-3 (NT3) and its high affinity receptor trk C are expressed in the mouse genital ridge prior to seminiferous cord formation [15]. The low affinity receptor for neurotropins, p75/LNGFR, has been demonstrated to be in preperitubular cells of the rat at E14 [16]. Recently, p75/LNGFR expression was determined to be present in a sex-specific manner in the testis at the time of seminiferous cord formation [17]. Expression of p75/LNGFR was present surrounding cords dur-

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Received: 2 May 2000.
First decision: 31 May 2000.
Accepted: 12 July 2000.
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ISSN: 0006-3363. http://www.biolreprod.org

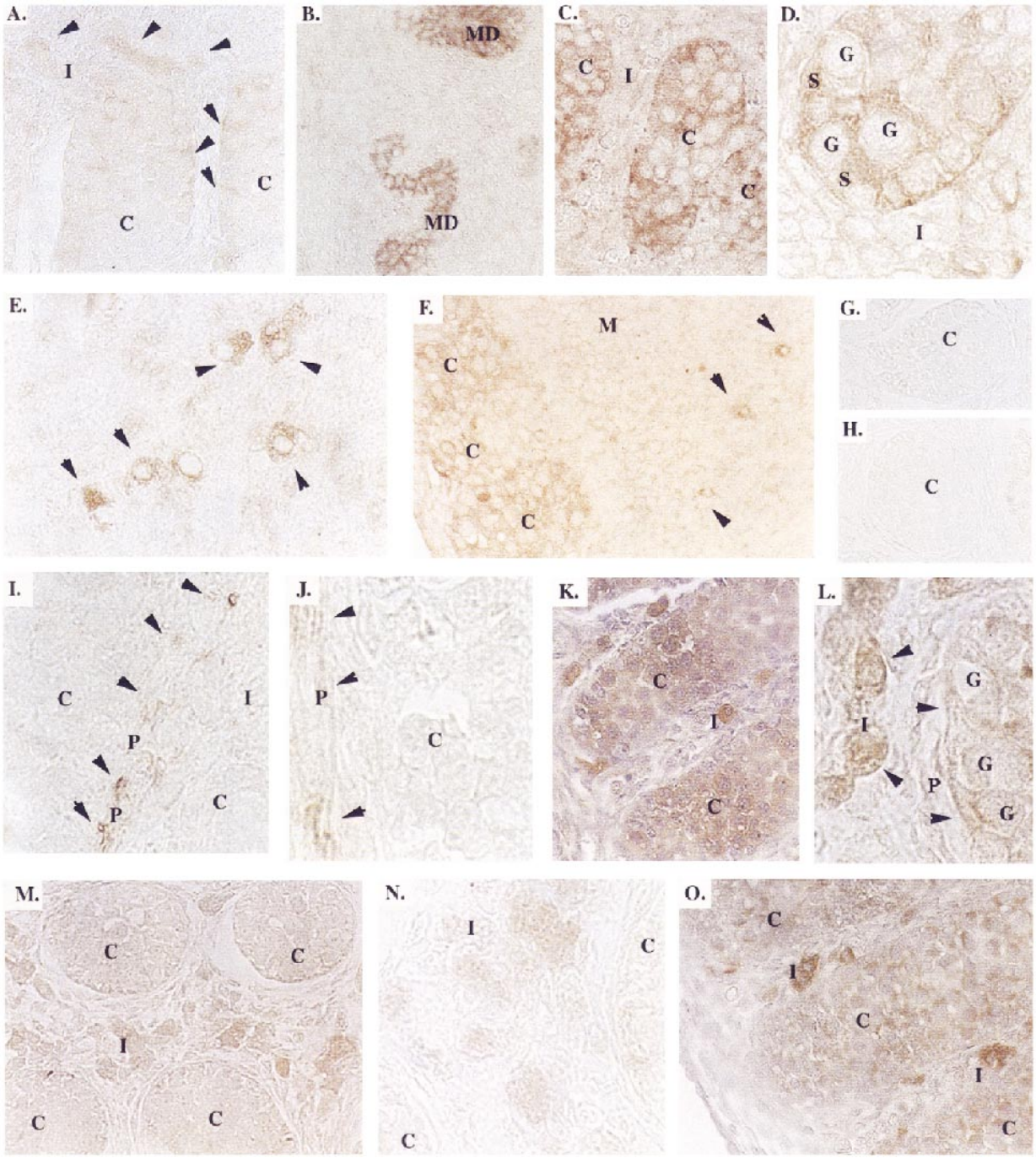

FIG. 1. Immunohistochemistry for trk C in E14 testis (**A**); E14 mesonephros (**B**); NT3 at E14 (**C** and **D**); trk A in mesonephros at E14 (**E**); and NGF in both testis and mesonephros at E14 (**F**). Nonimmune controls are represented for rat testes at E14 (**G**) and E16 (**H**) stages of development. Immunohistochemistry for trk C at E16 is depicted in **I** and **J**; NT3 at E16 (**K**) and (**L**); trk A at E16 (**M** and **N**); and NGF at E16 (**O**). Arrows point to reddish-brown positive staining for neurotropin ligand or receptors. C, Seminiferous cord; MD, mesonephric duct; I, interstitium; S, Sertoli cell; G, gonocyte; P, peritubular cell. **K** and **O** are slightly counterstained with hemotoxylin. Magnification ×400 for **B**, **C**, **F**, **G**, **H**, **K**, **M**, and **O**. Magnification ×600 for **A**. Magnification ×1000 for **D**, **E**, **I**, **J**, **L**, and **N**. These pictures are representative of five different experiments.

ing seminiferous cord formation but was not present in the ovary until E16–17. In addition, previous studies have also demonstrated an inhibitory effect of tyrosine kinase inhibitor K252a and a more specific trk C-IgG chimeric fusion protein on seminiferous cord formation [17]. Therefore, it appears that neurotropins may in part orchestrate seminiferous cord formation in the embryonic rat testis.

In the current study the expression pattern and actions of NT3, nerve growth factor (NGF), and their receptors, trk C and trk A, were examined to elucidate their potential roles in embryonic testis morphogenesis and growth. The hypothesis examined in the current study was that neurotropins are essential for testis morphogenesis leading to gonadal differentiation and growth of the perinatal testis, which is necessary for adult testis function.

MATERIALS AND METHODS

Organ Cultures

Timed pregnant Sprague-Dawley rats were obtained from Charles River (Wilmington, MA). Plug date was con-

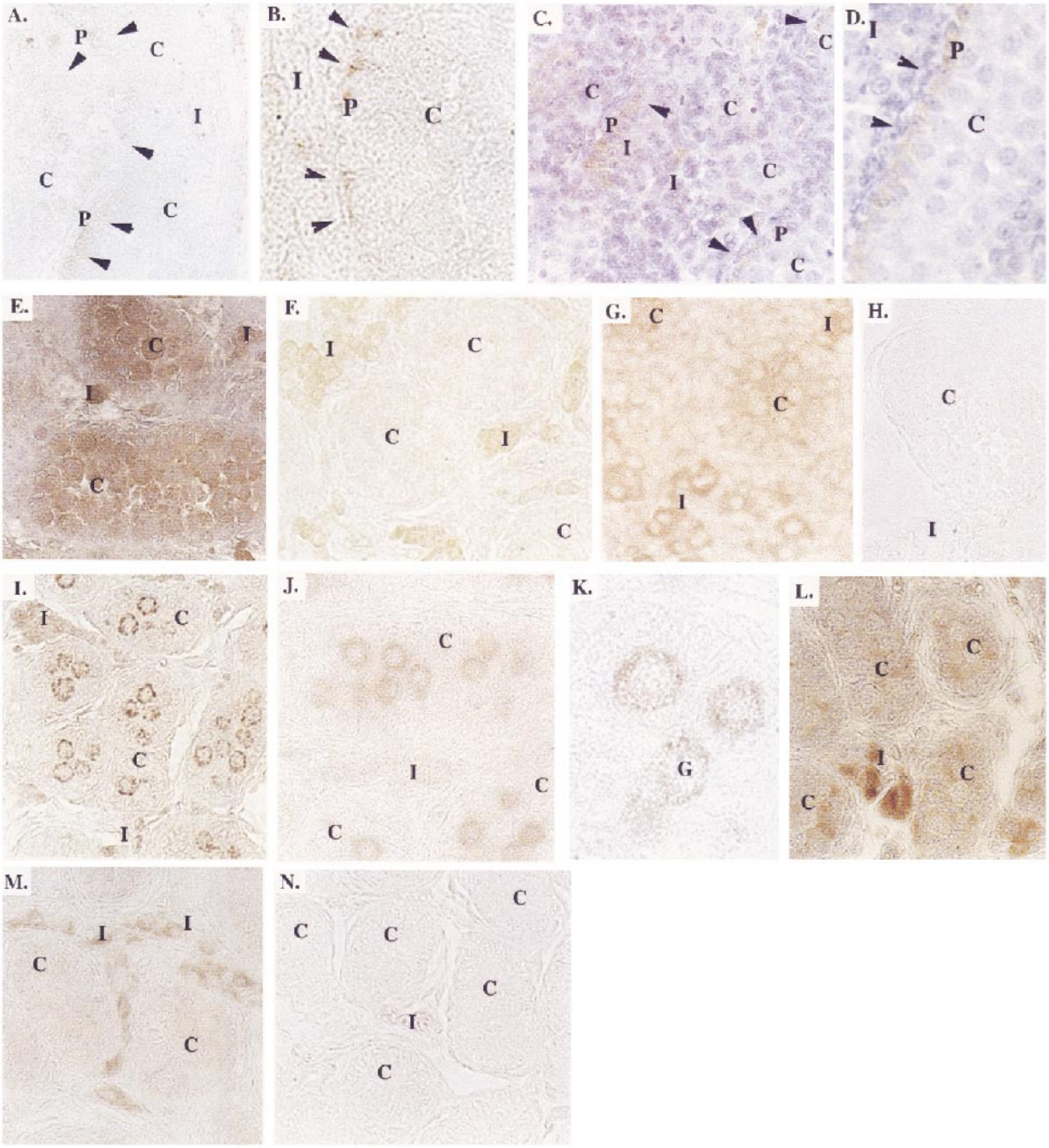

FIG. 2. Immunohistochemistry in E18 testis for trk C (**A**–**D**), NT3 (**E**), trk A (**F**), NGF (**G**), and nonimmune control (**H**). Immunohistochemistry in P0 testis for trk C (**I**), NT3 (**J** and **K**), trk A (**L**), NGF (**M**), and nonimmune control (**N**). Magnification ×400 (**A**, **C**, **E**–**J**, **L**, and **N**). Magnification ×1000 (**B**, **K**). Positive staining is depicted by reddish-brown stain. Arrows point to positive staining for neurotropin ligands and receptors. C, Seminiferous cord; P, peritubular cells; I, interstitium; and G, gonocytes. These pictures are representative of five different experiments. **C**–**E** and **L** are lightly counterstained with hematoxylin.

sidered to be E0. Embryonic Day 13 gonads were dissected out with the mesonephros. The organs were cultured in drops of medium on Millicell CM filters (Millipore, Bedford, MA) floating on the surface of 0.4 ml of CMRL 1066 media (Gibco BRL, Gaithersburg, MD) supplemented with penicillin-streptomycin, insulin (10 μg/ml), and transferrin (10 μg/ml). Antisense or sense oligonucleotides at 10 μg/ml were added directly to the culture medium every 10–12 h. The oligonucleotide sequence for each ligand or receptor is as follows: NT3 antisense: 1) CATCACCTTGTTCAC; 2) GCCACGGAGATAAGC; NT3 sense: GTGAACAAGGTGATG; NGF antisense: 1) CATGTTCACTAGGAG, 2) CTGCCTTGAGGCACA; NGF sense: CTC CTAGTGAACATG; trk A antisense: CCACATCATTCTCTGCCC; trk A sense: GGGCAGAGAATGATGTGG [18, 19]. Where two oligonucleotides are listed, a mixture of both sequences were utilized. The medium was changed every day. The E13 gonad + mesonephros were typically kept for 3 days, by which point cords were developed in the paired controls [14]. The E14 testis organ cultures were cultured without mesonephros and were treated with 100 nM K252a each day for a total of 4 days. Otherwise all other procedures were similar to those stated for the antisense oligonucleotides. Images of whole organs

were obtained by an image analysis system. Areas of testis treated with K253a or those that served as the paired control were measured by use of the NIH image analysis program. Ratios of each testis pair were averaged to determine the average percentage reduction in testis size by K252a treatment. Similar measurements have been previously reported for treatment with TGFβ [14], TGFα antibodies, and epidermal growth factor receptor (EGFR) blocking agents [12] on E13 and E14 testis organ cultures.

Genomic DNA Isolation and Polymerase Chain Reaction for SRY

To determine the sex of E13 embryos polymerase chain reaction (PCR) for SRY was conducted on each embryo. Embryonic tails were collected to isolate genomic DNA by standard procedures. Briefly, the tissue was homogenized through a 25-gauge needle in digestion buffer (100 mM NaCl, 10 mM Tris, pH 8, 25 mM EDTA, 0.5% SDS), and treated with proteinase K (0.15 mg/ml) for at least 4 h at 60°C. The samples were then extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1), and once with chloroform:isoamyl alcohol. The DNA was then precipitated by adding 1/10 volume 7.5 M ammonium acetate and three volumes cold ethanol and collected by centrifugation at 4°C for 30 min after an hour incubation at −20°C. Pellets were dried and resuspended in 10 μl distilled H_2O. The PCR was performed using 1 μl of genomic DNA with primers to SRY. The sequences of the SRY primers are: 5′-CGGGATCCATGTCAAGCGCCCCATGAATGCATTTATG-3′ and 5′-GCGGAATTCACTTTAGCCCTCCGATGAGGCTGATAT-3′. The PCR was performed using an annealing temperature of 55°C for 30 cycles to yield a product of 240 base pairs (bp) [20].

Testicular Cell Culture and Growth Assay

To generate a testicular culture from postnatal Day 0 (P0) testis, the tunica was removed and the testis digested with 0.125% trypsin, 0.1% EDTA, and 0.02 mg/ml DNase in Hanks balanced salt solution (HBSS), for 15 min at 37°C. The trypsin was inactivated with 10% calf serum. The samples were triturated with a pipette tip and washed twice in 1 ml HBSS. The pellet was resuspended and either used in growth assays immediately or placed in 10-mm plates in F12 media supplemented with 10% bovine calf serum until confluent (approximately 2 days). For growth assays cells were plated at a 25% confluence in 24-well plates and allowed to settle overnight in Dulbecco modified Eagle medium without thymidine. Medium was replaced the next day, and cells were treated for 24 h with different hormones or growth factors. Medium was removed after the 24-h treatment period, and media containing tritiated thymidine (10 μCi/ml) were placed on cells for 5–6 h. After 5–6 h, media were discarded and cells were either frozen or processed using the tritiated thymidine assay. Briefly, a solution of 0.5 M NaH_2PO_4 (pH 7.3; 500 μl) was added to each well, and cells were sonicated. Half of the sonicated cells were placed on DE-81 filters on a manifold, and a vacuum was applied. After three washes with the NaH_2PO_4 buffer the filters were dried, placed in counting vials with 5 ml of scintillation fluid, and counted. The remaining sonicate was used for DNA assays to normalize number of cells per well [14].

RNA Isolation and Quantitative Reverse Transcription-PCR

Total RNA was obtained using Tri Reagent (Sigma, St. Louis, MO). Briefly, tissue or cells were lysed in Tri Reagent (1 ml/50–100 mg tissue or 1 ml/100 mm of a culture plate). After adding 0.2 ml chloroform/ml Tri Reagent, the mixture was centrifuged at 12 000 × *g* for 15 min at 4°C, the colorless upper aqueous phase was transferred to a fresh tube, and 0.5 ml isopropanol/ml Tri Reagent was added to pellet the RNA. Reverse transcription (RT) was performed using Moloney murine leukemia virus reverse transcriptase under standard conditions [21].

Quantitative RT-PCR (QRT-PCR) procedures were performed as previously published [14, 21]. Briefly, total RNA (1 μg) was reverse transcribed using the specific 3′ primers. Plasmid DNA containing subclones of interest were used to generate standard curves from 1 ng/μl to 10 pg/μl, each containing 10 ng/μl Bluescript carrier DNA. Identical 10-μl aliquots of each sample were used for PCR amplification. At least 0.25 μCi of ^{32}P-labeled dCTP was included in each sample during amplification. Specific PCR products were quantitated on 4–5% polyacrylamide gels. The gels were exposed to a phosphor screen for 8–24 h, followed by quantification of specific bands on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and analyzed with Image Quant. Equivalent steady-state mRNA levels for each gene were determined by comparing each sample to the appropriate standard curve. All gene expression data were normalized for 1B15 (cyclophilin) mRNA. Optimal cycle number for amplification was determined for each assay in order to achieve maximum sensitivity while maintaining linearity. The sensitivity of each quantitative PCR assay is below 1 fg with intra-assay variability of 6–15%. Primers utilized for the QRT-PCR were as follows: TGFβ1, 5 prime, 5′-TCG ATT TTG AC TCA CTG GAG TTG T-3′ and 3 prime, 5′-GGG GTG GCC ATG AGG AGC AGG-3′; TGFβ2, 5 prime, 5′-CCG CCC ACT TTC TAC GAG CCC-3′ and 3 prime, 5′-GCG CTG GGT GGG AGA TGT TAA-3′; TGFβ3, 5 prime, 5′-TGC CCA ACC CGA GCT CTA AGC G-3′ and 3 prime, 5′-CCT TTG AAT TTG ATC TCC A-3′; cyclophilin, 5 prime, 5′-ACA CGC CAT AAT GGC ACT GG-3′ and 3 prime 5′-ATT TGC CAT GGA CAA GAT GCC-3′; TGFα, 5 prime, 5′-TTGCTG TCC TCA TTA TCA CC-3′ and 3 prime, 5′-CAG AGT GGC AGC AGG CAG TC-3′; EGFR, 5 prime, 5′-CTG CTG GGG AAG AGG AGA GGA GAA C-3′ and 3 prime, 5′-GAG TGG TGG GCA GGT GTC TT-3′ [21]. The sizes of the PCR products generated are as follows: TGFα, 138 bp; EGFR, 208 bp; TGFβ1, 200 bp; TGFβ2, 194 bp; TGFβ3, 288 bp; cyclophilin, 105 bp.

Embedding, Histology, and Immunohistochemistry

Tissues were fixed in Histochoice (Amresco, Solon, OH) and embedded in paraffin according to standard procedures [21]. The tissue sections (3–5 μm) were deparaffinized, rehydrated, and microwaved in 0.01 M sodium citrate to boil for 5 min. The sections then were blocked with 10% goat serum for 30 min at room temperature. Immunohistochemistry was performed as described previously [21]. The NT3 antibody was an anti-NT3 peptide antibody (Santa Cruz Biotechnology [SCB], Santa Cruz, CA; cat. no. sc-547) raised against amino acids 139–158 to the carboxy terminus of human NT3 (which is also identical to the mouse sequence). The NGF antibody was an anti-NGF peptide antibody (SCB; cat. no. sc-549) raised against amino acids 1–

20 of mouse NGF. Both the NGF and NT3 antibodies (200 μg/ml) were diluted 1:300 in 10% goat serum. The trk A antibody was an anti-trk A peptide antibody (SCB; cat. no. sc-118) raised against amino acids 763–777 of human trk A. The trk C antibody was an anti-trk C peptide antibody (SCB; cat. no. sc-117) raised against amino acids 798–812 of porcine trk C. Both the trk A and trk C antibodies (200 μg/ml) were diluted 1:50 in 10% goat serum. As a negative control, serial sections were put through the same procedure without any primary antibody. Additional negative control sections were incubated with 50–100× excess of synthetic blocking peptide (SCB) for each ligand and receptor with the respective antibody (anti-NGF, anti-NT3, or anti-trk A or anti-trk C antibody). All sections utilized for negative controls (without specific primary antibody or with excess synthetic blocking peptide) had no positive staining. This demonstrated that all antibodies for the neurotropin receptors and ligands were not due to nonspecific staining or due to artifacts of tissue fixation and processing. The biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlington, CA) was diluted 1:300. The secondary antibody was detected by using the histo stain-SP kit (Zymed Laboratories, South San Francisco, CA), and immunohistochemical images were digitized with a slide scanner (Sprint Scan). Five different experiments were conducted for each neurotropin ligand and receptor. In each experiment three serial sections of four to five testes for each developmental age were analyzed. One serial section was utilized for the nonimmune control for each time period. Figures 1 through 3 represent the data from all five experiments. There was uniform and reproducible staining at each developmental age for the respective neurotropin ligands and receptors in all five experiments.

Statistical Analysis

Data were analyzed with the JMP 3.1 statistical analysis program (SAS Institute, Cary, NC). All values are expressed as the mean ± SEM. Statistical analysis was performed using one-way ANOVA. Significant differences were determined using the Dunnett test for comparison to controls and using the Tukey-Kramer honestly significant difference tests for multiple comparisons. Statistical difference was confirmed at $P < 0.05$.

RESULTS

Protein Expression and Cellular Localization of NT3, NGF, trk C, and trk A During Embryonic Testis Development

Immunohistochemistry was conducted on testis sections from E14, E16, E18, P0, P3, and P5 rats to determine expression of NT3, NGF, trk C, and trk A (Table 1). Negative controls for each ligand and receptor (excess blocking peptide or no primary antibody) did not have positive staining in any of the experiments in the current study. Thus, the antibodies for the neurotropin ligands and receptors appear to be specific and can be utilized to localize neurotropin ligands and receptors in the rat testis.

Neurotropin-3 was localized to Sertoli cells at E14 (Fig. 1, C and D, and Table 1), was present in interstitial cells with expression in Sertoli cells at E16 and E18 (Figs. 1, K and L, and 2E), and by P0 was localized to the gonocytes (Fig. 2, J and K). From P3 to P5, NT3 protein expression was localized to surrounding germ Sertoli and interstitial cells (Fig. 3, B and G, and Table 1). The expression of trk C (the high affinity receptor for NT3) was faintly detected in E14 testis (Fig. 1A and Table 1) around the seminiferous cords and in Sertoli cells. Expression of trk C was also expressed in mesonephric ducts of the mesonephros (Fig. 1B). At E16, NT3 was localized to specific cells of the interstitium (i.e., preperitubular cells; Fig. 1, I and J, and Table 1) and at E18 trk C appeared to be localized to selective preperitubular cells (Fig. 2, A through D) [17] and at P0–P5 was localized to germ cells (Figs. 2I, 3, A and F, and Table 1).

Nerve growth factor was detected in the cords of the testis and specific cells of the mesonephros at E14 (Fig. 1F). At E16–E18, NGF was localized to the Sertoli cells and interstitial cells (Figs. 1O and 2G) and by P0–P3 NGF was localized to the interstitium and germ cells with low levels of expression in the Sertoli cells (Figs. 2M and 3D). At P5, NGF was localized to germ and interstitial cells (Fig. 3K). The expression of trk A (the high affinity receptor for NGF) was located in specific cells in the mesonephros at E14 (Fig. 1E) and in interstitium at E16 (Fig. 1M) and in interstitial cells with slight staining in Sertoli at E18, P0, and P3 (Figs. 2, G and M and 3D, and Table 1). By P5, trk A was localized mainly to the Sertoli cells with expression also in the interstitium (Fig. 3, I and J).

Effects of Antisense Oligonucleotides to NGF, NT3, and trk A on Seminiferous Cord Formation

Embryonic Day 13 testis organ cultures were utilized to determine the effect of antisense oligonucleotides to NGF, NT3, and trk A on seminiferous cord formation. These experiments expanded previous results where K252a (a tyrosine kinase inhibitor reported to be specific to trk receptors) [22] and trk C-IgG (recombinant chimeric fusion proteins) [23] both caused perturbation of seminiferous cord formation in E13 testis organ cultures [17]. Antisense oligonucleotides to NT3 (10 μM) completely inhibited cord formation in 40% (10 of the 24 testis pairs had inhibition of cord formation in the treated testis when compared to its control) of the E13 testis organ cultures treated (Fig. 4B). In contrast, antisense oligonucleotides to NGF (10 μM) inhibited cord formation in 20% (5 of the 24 testis pairs had inhibition of cord formation in the treated testis when compared to its paired control) of E13 testis organ cultures (data not shown). Furthermore, trk A (10 μM) antisense oligonucleotides had no effect on perturbation of cord formation (data not shown). Only dramatic reductions in cord formation were included as a response in the organ culture data. Histological analysis may have been able to provide a more accurate analysis of the extent of the reduction in cord formation in the testis pairs not as severely affected by the treatments. However, due to the size and manipulation of the organs, accurate histology could not be obtained for all of the experimental organs.

Control testes were treated with sense oligonucleotides given at a 10-μM dose to determine if the concentration of oligonucleotide had any adverse effects on the organ cultures. No effect on seminiferous cord formation was detected in the control organ cultures at the 10-μM dose for any of the sense oligonucleotides (Fig. 4A and data not shown). These data further demonstrate that the neurotropins (NT3 in particular) and their respective receptors may be important regulators of morphological sex determination resulting in seminiferous cord formation.

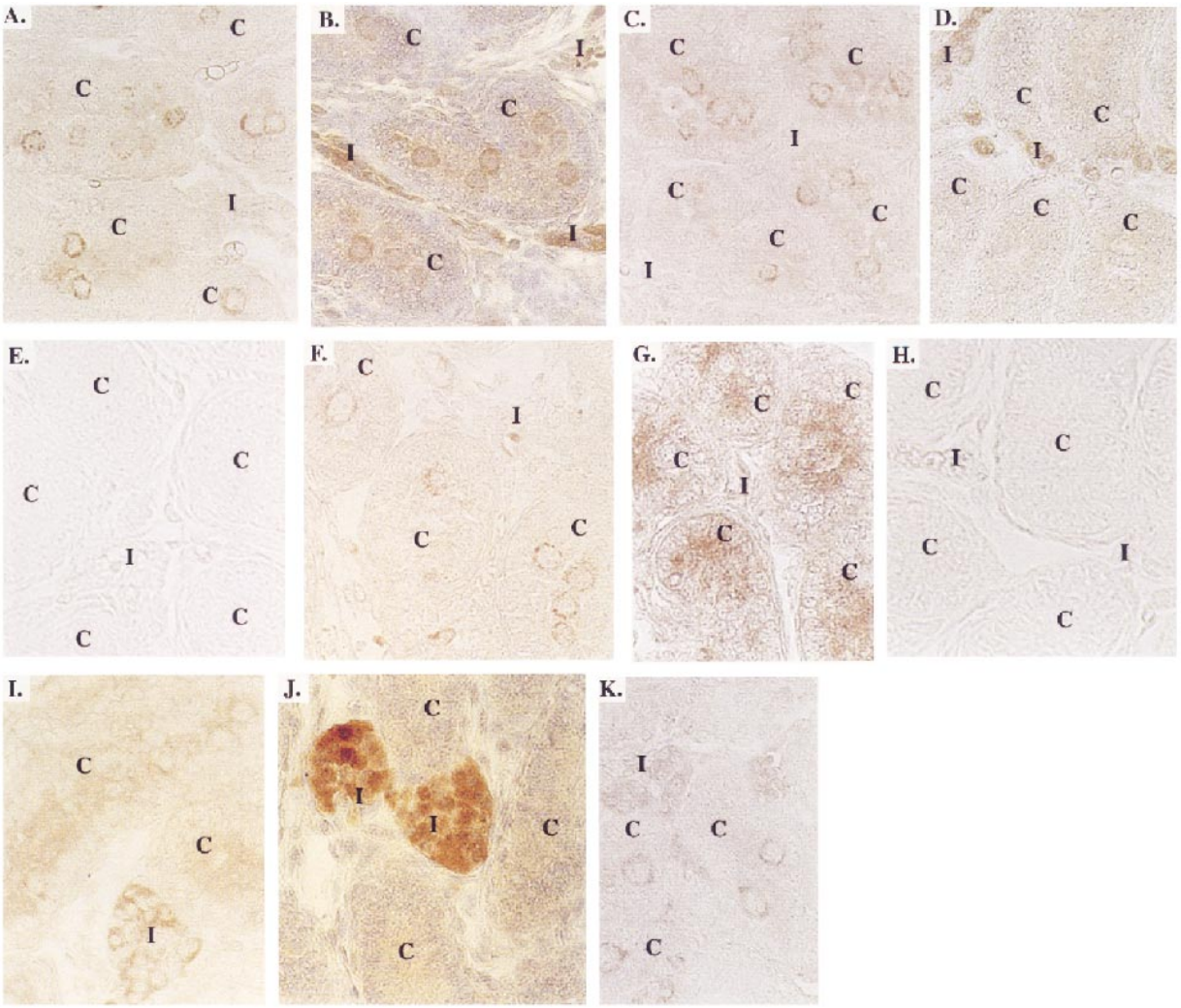

FIG. 3. Immunohistochemistry in P3 testis for trk C (**A**), NT3 (**B**), trk A (**C**), NGF (**D**), and nonimmune control (**E**). Immunohistochemistry in P5 testis for trk C (**F**), NT3 (**G**), nonimmune control (**H**), trk A (**I**, **J**), and NGF (**K**). C, Seminiferous cord; I, interstitium. Reddish-brown stain indicates positive stain for neurotropin ligands and receptors. These pictures are representative of five different experiments. Magnification ×400.

Effect of Neurotropins on Embryonic and Early Testis Growth

To determine the effects of NGF and NT3 on embryonic growth, E14 testis organ cultures were treated with 100 nM K252a (a tyrosine kinase inhibitor reported to be specific to trk receptors) [22] for 3 days. An evaluation of the area of testes treated with K252a demonstrated a reduction in testis size by 50% (an average reduction of testis area of 12 K252a treated testes when compared to their paired testis culture control; data not shown). Therefore NGF and NT3 may be important regulators of somatic and germ cell growth during the period after cord formation.

To determine the effects of NGF and NT3 on P0 testis growth, a tritiated thymidine assay was conducted on neurotropin-treated P0 whole testis cultures. In previous experiments FSH, EGF, and 10% calf serum were demonstrated to be positive stimulators of P0 testis culture growth and thymidine incorporation into DNA [8, 14]. In the present study, both NT3 and NGF stimulated thymidine incorporation over controls ($P < 0.05$; 50 ng/ml dose; Fig. 5) in a dose-responsive manner. Nerve growth factor and NT3 given in combination stimulated growth over that of either growth factor alone ($P < 0.001$). Nerve growth factor and NT3 appeared to be as effective as FSH, EGF, and 10% calf serum in stimulating P0 testis thymidine incorporation at the 50-ng/ml dose and more effective at the 100-ng/ml dose. In addition, NGF and NT3 when given in combination increased tritiated thymidine incorporation in a synergistic manner. As a control K252a was found to block NT3 and NGF actions (data not shown). Thus, both NT3 and NGF may be important regulators of embryonic and perinatal testis growth, which is necessary to obtain adequate numbers of somatic and germ cells for normal spermatogenesis in the adult male.

Effect of Neurotropins on Expression of mRNA for TGFα, EGFR, and TGFβs

A hypothesis tested was that NGF and NT3 may be acting to influence P0 testis growth through altering the expression of other growth factors known to increase cell growth, such as TGFα and its receptor EGFR. After 24 h, P0 testis treated with both NGF and NT3 alone or in combination had a reduced mRNA expression for TGFα ($P < 0.05$) when compared to controls (Fig. 6A). Because TGFα [12], trk A, and trk C are all localized to the interstitium

TABLE 1. Expression of NT3, NGF, trk A, and trk C during testis development.

Age	NT3	Trk C	NGF	Trk A
E14	Sertoli	Mesonephric ducts in mesonephros and in testis; Sertoli and specific cells around cords in interstitium	Specific cells in mesonephros and Sertoli cells of seminiferous cords	Specific cells in mesonephros not detected in testis
E16	Sertoli/interstitium	Specific cells around cords in interstitium	Sertoli/interstitium	Interstitium
E18	Interstitium/Sertoli	Preperitubular cells	Sertoli/interstitium	Sertoli (slight staining) interstitium
P0	Surrounding germ cells	Interstitium/surrounding germ cells	Interstitium/Sertoli	Sertoli/interstitium
P3	Interstitium/Sertoli, surrounding germ	Germ	Interstitium/Sertoli	Surrounding germ
P5	Sertoli	Surrounding germ	Interstitium/surrounding germ	Sertoli/interstitium

at P0, the neurotropins may be acting directly on interstitial cells to suppress expression of mRNA for TGFα.

In contrast, NT3 but not NGF increased mRNA expression of EGFR ($P < 0.05$) in P0 testis after 24 h (Fig. 6B). The combination of NGF and NT3 did not affect mRNA expression for EGFR after 24 h of treatment. The receptor for NT3, trk C, was present in germ and interstitial cells in P0 testis while trk A was present in Sertoli and interstitial cells. Nerve growth factor and NT3 may act through different mechanisms to regulate EGFR expression differentially.

An alternative hypothesis to the neurotropins increasing expression of growth stimulators would be that they might inhibit the actions of growth inhibitors such as the TGFβ isoforms. Therefore, expression of TGFβ isoforms were also examined in P0 testis treated with NT3 and NGF. Treatment with NGF for 24 h suppressed the expression of mRNA for TGFβ1 ($P < 0.07$; Fig. 7A). The combination of NGF plus NT3 also suppressed expression of mRNA for TGFβ1 in P0 testis. Treatment with NT3 alone had no effect on TGFβ1 mRNA expression (Fig. 7A). There were no changes in expression of the TGFβ2 isoform after 24 h of neurotropin treatments in P0 testis cultures (Fig. 7B). However, the combination treatment of neurotropins increased expression of mRNA for TGFβ3 after 24 h of treatment ($P < 0.05$; Fig. 7C). Therefore, neurotropins may directly regulate the expression of TGFβ isoforms.

Long-term indirect effects of neurotropins were also evaluated by measuring the effects on mRNA for TGFβ

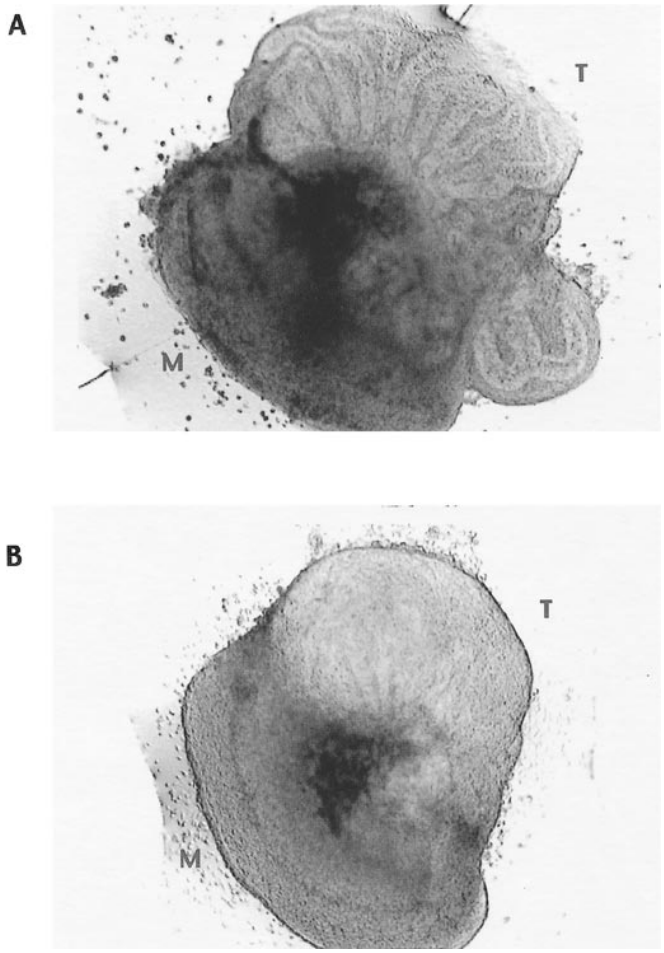

FIG. 4. Embryonic Day 13 testis organ cultures treated with sense oligonucleotide to NT3 (**A**) or antisense oligonucleotide to NT3 (**B**). Organ cultures were treated every 10–12 h with a 10 μM concentration of oligonucleotide. These are representative images from 24 testis pairs (n = 24; 24 treated and 24 were controls). M, Mesonephros; T, testis. Magnification ×100.

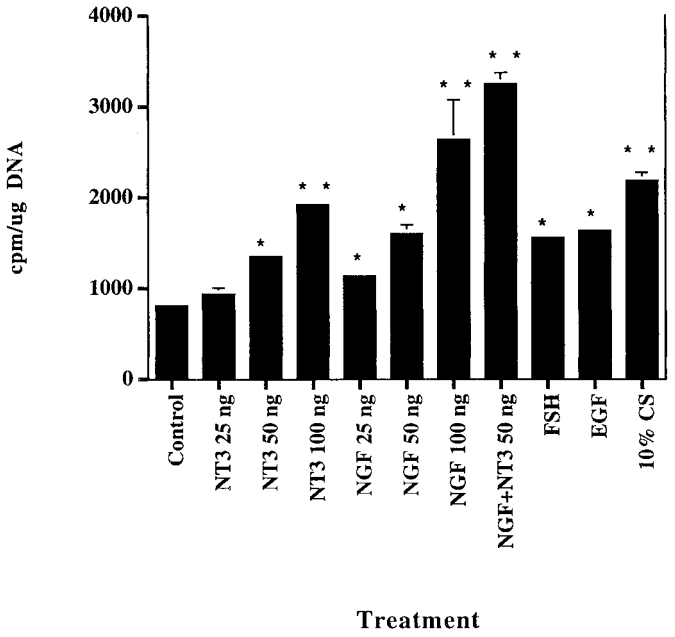

FIG. 5. Effects of NT3 and NGF on P0 testis growth. Results are presented as percentage of control and represent three to four individual experiments in triplicate. Follicle-stimulating hormone and 10% calf serum serve as positive controls. $^{*}P < 0.05$. $^{**}P < 0.01$.

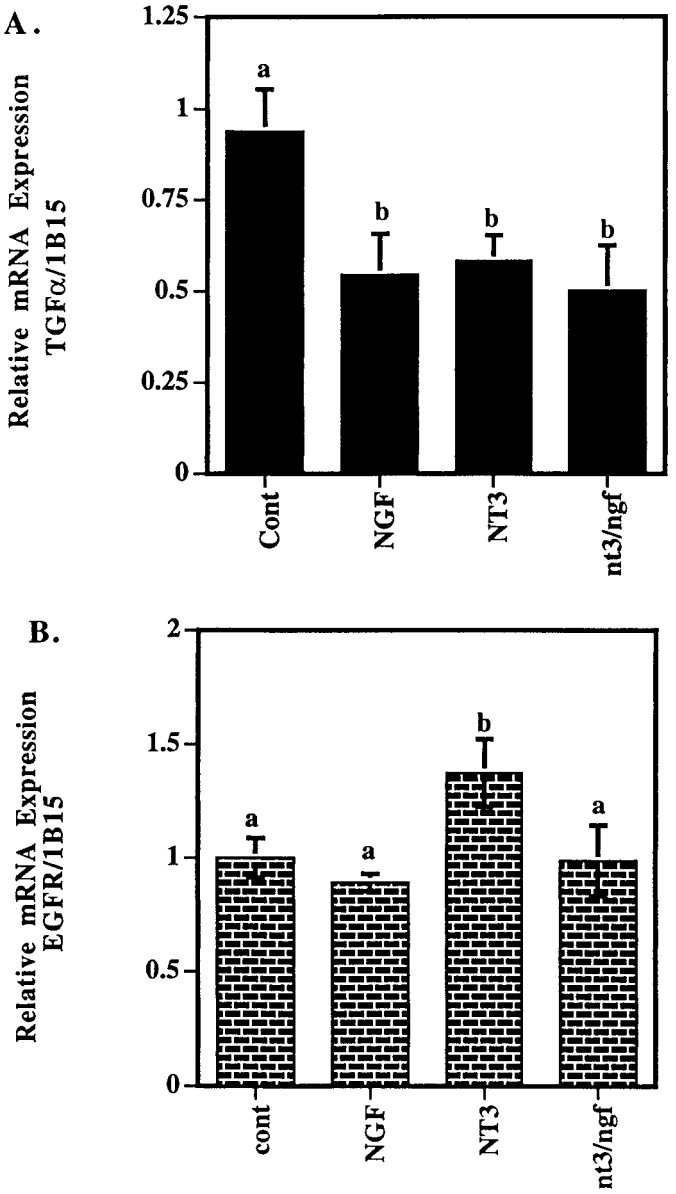

FIG. 6. Effects of neurotropins on relative amounts of mRNA for TGFα (**A**) and EGFR (**B**) after 24 h of treatment. Amounts of mRNA for TGFα were normalized to cyclophilin (1B15) and expressed relative to controls. These data represent three individual experiments assayed in duplicate. Different superscript letters for each mean represent statistical difference at $P < 0.05$.

isoforms after 72 h. Expression of TGFβ1 was increased in P0 testis treated with NGF or the combination of NGF and NT3 ($P < 0.05$; Fig. 8A). This increase in TGFβ1 appeared to be a compensatory rebound effect due to NGF and NGF/NT3 suppression of mRNA for TGFβ1. A similar rebound in expression of TGFβ2 was evaluated 72 h after NGF or NGF/NT3 treatment ($P < 0.05$; Fig. 8B). Expression of TGFβ3, in contrast to the other isoforms, appeared to be affected similarly by neurotropins 24 and 72 h after treatment. Expression of TGFβ3 was increased after 72 h after both NT3 and NGF/NT3 treatments. Therefore, TGFβ isoforms appear to be regulated differentially by NT3 and NGF. These data further support the theory that NGF and NT3 do not appear to have overlapping functions during testis development and may function independently to elicit similar morphological and growth effects.

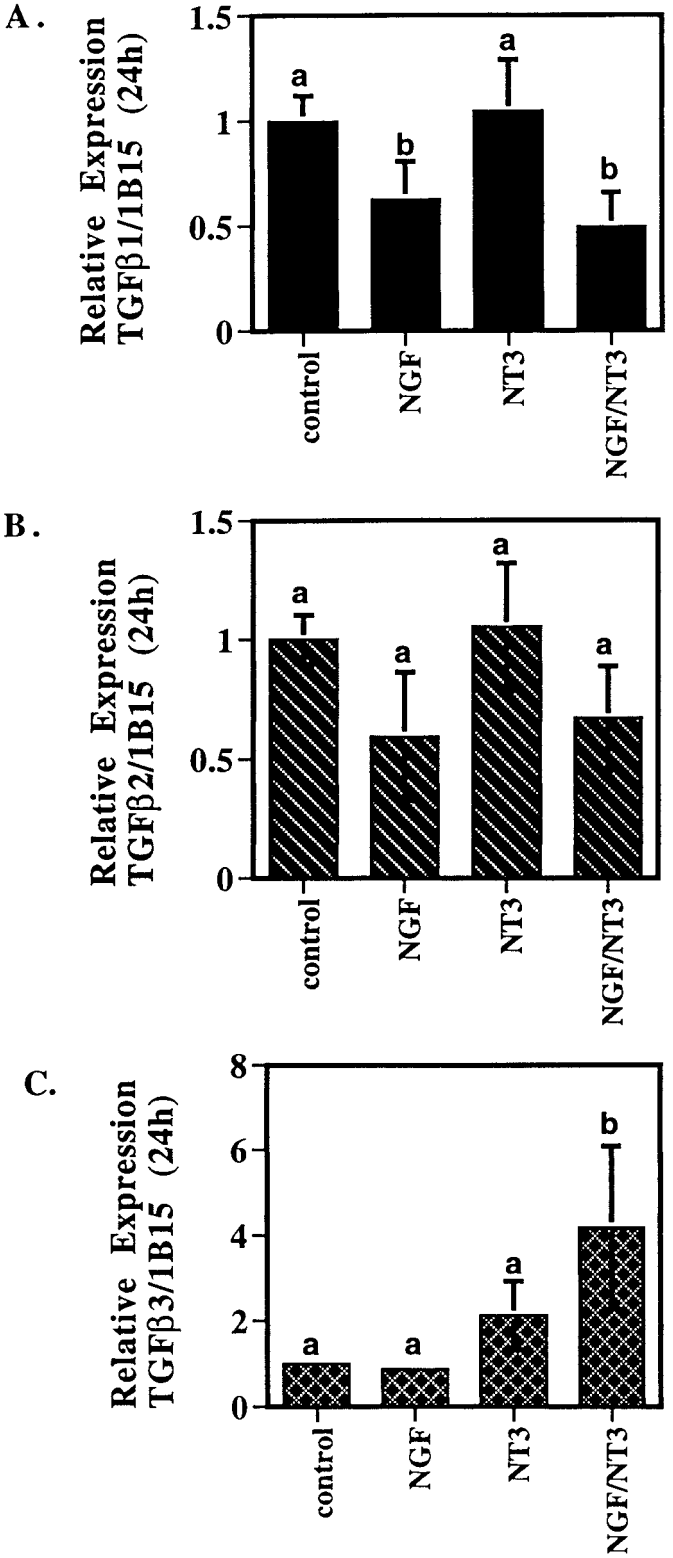

FIG. 7. Effects of neurotropins on relative amounts of mRNA for TGFβ1 (**A**), TGFβ2 (**B**), and TGFβ3 (**C**) after 24 h of treatment. Amounts of mRNA for TGFβ isoforms were normalized to cyclophilin (1B15) and expressed relative to controls. These data represent three individual experiments assayed in duplicate. Different superscript letters for each mean represent a statistical differences at $P < 0.05$.

DISCUSSION

Embryonic testis development is initiated when cells migrate from the adjacent mesonephros to surround Sertoli-germ cell aggregates to form seminiferous cords [1, 2]. The importance of the mesonephros in the induction of cord formation in the testis has been demonstrated because its removal completely blocks cord formation [3, 4]. Indifferent testes placed in direct apposition to mesonephros from ovarian tissue (up to E16 of age) or hind limb form seminiferous cords [24, 25]. In contrast, indifferent ovaries are not capable of forming seminiferous cords when placed in opposition to mesonephros from indifferent testes [24]. Therefore, the hypothesis proposed was that factors produced by the indifferent testis induce migration of cells from the mesonephros to result in cord formation. The logical cell to induce these migration events is the Sertoli cell. The Sertoli cell is thought to be the first cell type to differentiate in the testis [26–28]. Furthermore, mice lacking germ cells produce testes with normal cord formation, suggesting that germ cells do not direct the events resulting in seminiferous cord formation [29]. The paracrine factors involved in this migration process are unknown; however, recent data have demonstrated sex-specific expression of the low affinity receptor for neurotropins, p75LNGFR [17].

The family of neurotropins is composed of four ligands: NGF, NT3, brain-derived growth factor (BDNF) and neurotropin-4/5 (NT4/5). Each of these ligands bind with low affinity to the p75/LNGFR (p75 low affinity nerve growth factor receptor). In addition to this receptor, each ligand has a high affinity specific receptor called a trk (tyrosine kinase receptor). The trk A receptor binds with high affinity to NGF but also will bind with a lower affinity to NT3. Neurotropin-3 binds to trk C and BDNF and NT4/5 both bind with high affinity to trk B [30, 31]. Neurotropins were initially found to be critical in mediating the differentiation, migration, proliferation, and survival of neurons in the developing brain and peripheral nervous system. In the past decade there has been accumulating evidence for non-neuronal roles of the neurotropins. In these systems neurotropins have been implicated in mediating local cell-cell interactions during morphogenesis in the dermatome, tooth, kidney, ovary, and even more recently, the testis [17, 32–34].

One of the objectives of the current study was to localize expression of neurotropin ligands and receptors to determine their potential regulation of seminiferous cord formation. Previous investigators had demonstrated in the mouse that trk C [15] and p75/LNGFR [16] were localized to the urogenital ridge in cells that were involved in the process of seminiferous cord formation. Sex-specific expression of p75/LNGFR in the rat has also been demonstrated in the testis at the time of seminiferous cord formation and is also suggestive of a potential role for neurotropins at this time [17]. Recently, NT3 was localized in the mouse to interstitial cells at E14.5 to P20 [35]. An E14.5 mouse testis corresponds to an E16 rat testis. Even though we are using the same antibodies in the current study, our method of antigen retrieval (microwaving) may make our tissue more sensitive to antibody binding. In addition, we evaluated testes from an earlier time-point, E14 (cord formation in rat is at E13.5 to E14), while Russo evaluated protein expression of neurotropins 3 days after cord formation [35]. The current study extends this research by looking earlier and localizes the expression of NT3, NGF, and their high affinity receptors trk C and trk A

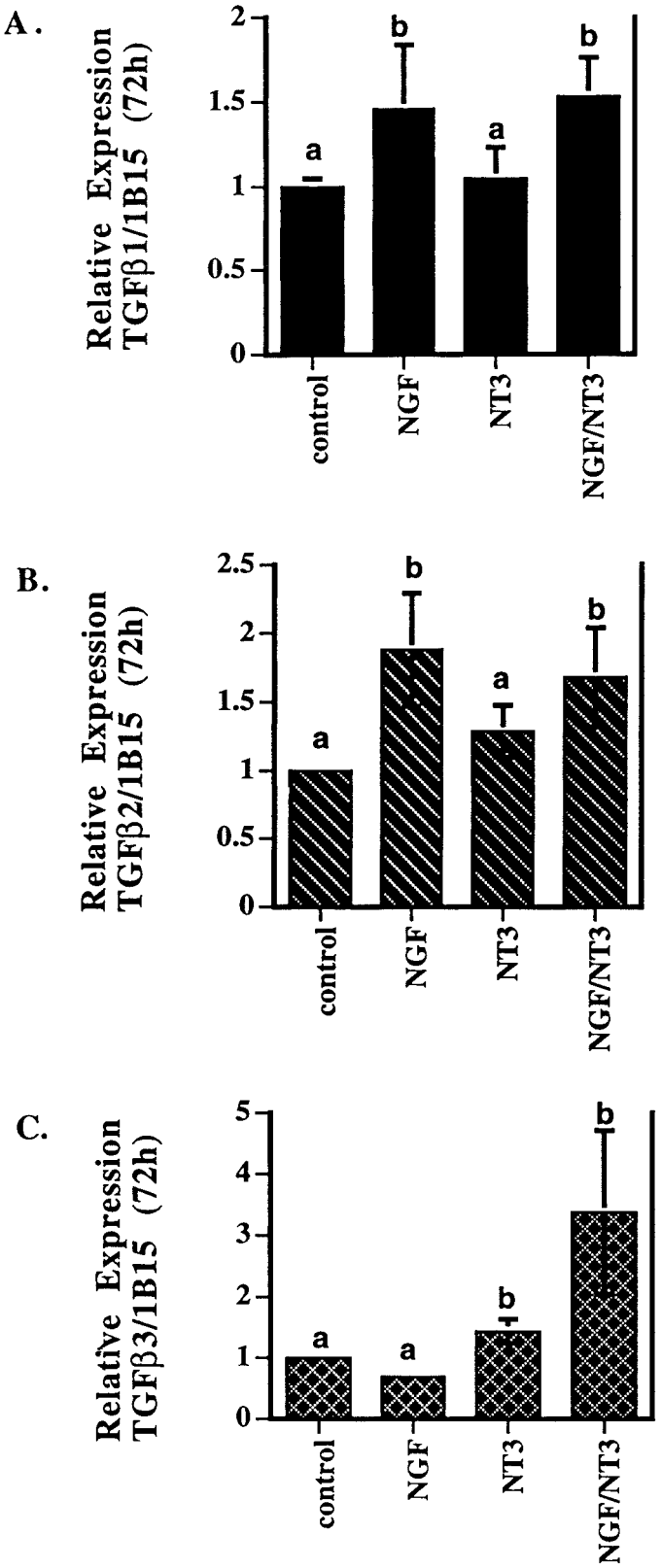

FIG. 8. Effects of neurotropins on relative amounts of mRNA for TGFβ1 (**A**), TGFβ2 (**B**), and TGFβ3 (**C**) after 72 h of treatment. Amounts of mRNA for TGFβ isoforms were normalized to cyclophilin (1B15) and expressed relative to controls. These data represent three individual experiments assayed in duplicate. Different superscript letters for each mean represent statistical differences at $P < 0.05$.

throughout embryonic and early postnatal testis development.

The localization of NT3 and trk C in the current study around the time of seminiferous cord formation suggests possible functions in testis morphogenesis. Neurotropin-3 localized to the Sertoli cells at E14 and may be produced in order to induce migration of preperitubular cells from the mesonephros into the gonad. At E14 and E16 protein localization of trk C was detected in selective cells of the interstitium and at E18 appeared to be localized to preperitubular cells. Preperitubular and endothelial cells are the primary cell types that migrate from the mesonephros into the differentiating testis [4]. Therefore, the localization of trk C is also suggestive of a potential role for NT3 in the induction of the migration of cells from the mesonephros into the differentiating gonad to complete seminiferous cord formation. Immunohistochemistry on earlier time points is necessary to determine if trk C is localized to cells within the mesonephros that eventually migrate into the testis.

Previous studies have also implicated NT3 and trk C in participating in the process of seminiferous cord formation [17]. In these experiments a trk-specific tyrosine kinase inhibitor, K252a, inhibited seminiferous cord formation. To extend these experiments, the present study utilized antisense oligonucleotides to NT3, NGF, and trk A each at a 10 μM concentration that has been demonstrated to be adequate to inhibit the actions of these ligands and receptor [18, 19]. Antisense oligonucleotides to NT3 completely inhibited seminiferous cord formation in 40% of the organ cultures treated while NGF inhibited 20% of the organ cultures. The trk A antisense treatment had no effect on seminiferous cord formation in these experiments. The control sense oligonucleotides had no influence on cord formation. These results are similar to previous experiments where a specific trk C-IgG chimeric fusion protein inhibited cord formation in 40% of the embryonic testis organ cultures treated. Additionally, a specific trk A-IgG chimeric fusion protein inhibited cord formation in 20% of the testis organ cultures treated [17]. Therefore, these data confirm previous experiments and further demonstrate that neurotropins are potential regulators of seminiferous cord formation. The variability of the specific antisense oligonucleotides and trk-IgG antagonist may suggest a compensatory mechanism that occurs among the neurotropin family of ligands and receptors to allow the critical process of seminiferous cord formation to occur in the absence of one ligand or receptor.

The expression of NGF at E14 was localized to the seminiferous cords surrounding germ cells and also in the interstitium. However, there was no detectable trk A staining in the testis only in specific cells of the mesonephros. There may be trk A protein present that was undetectable with our assay. Further experiments are necessary to determine whether trk A is present in the testis during cord formation (using our current data), the localization of trk A and NGF does not suggest a major role for NGF in migration of mesonephric cells during testis morphogenesis. Embryonic testis organ cultures using NGF antibodies, specific trk A-IgG chimeric fusion proteins [17], and antisense oligonucleotides (present study) have demonstrated some alteration in cord morphology and formation. However, these experiments have not resulted in the extent of perturbation of seminiferous cord formation that has been demonstrated with NT3 and trk C antagonists. Neurotropin-3 has been shown in other systems to bind to trk A with low affinity, and NGF has been demonstrated to bind to trk C. Therefore, mesonephric cells containing both trk A or trk C may migrate in response to NT3 or NGF establishing compensatory mechanisms for seminiferous cord formation.

After the process of seminiferous cord formation occurs, the embryonic testis undergoes dramatic growth in somatic and germ cell populations. Both NT3 and NGF receptors are present in crucial cells that are rapidly proliferating at this time [17, 34]. The protein trk C is present in preperitubular interstitial cells and germ cells, while trk A is present in Sertoli interstitial and germ cells from E16 through P5. Neurotropin-3 and trk C may be important in the differentiation of the preperitubular cells to form the single cell layer around the seminiferous cords. Nerve growth factor and trk A may be important in the differentiation of the Leydig or germ cells and the initiation of steroidogenic functions or germ cell maturation.

The localization of the neurotropin ligands and receptors was different during the late embryonic and early postnatal period when compared to the early embryonic testis development. Most of the receptors and ligands appeared to have similar cellular localization after birth and retained this pattern until P5 of testis development. Neurotropin-3 was the exception with localization to germ cells at P0 and P3, while localization prior to and after P0 was to the Sertoli cells and/or interstitium. Therefore, the neurotropin ligands and receptors may be important for differentiation and migration events that occur early in testis development. For example, NT3 produced by Sertoli cells may initiate the migration of trk C-bearing cells from the mesonephros into the gonad. After migration occurs, NT3 and trk C may be required further to allow for preperitubular cell differentiation into the single layer of cells that surround the cords by E18.

At P0, NT3 and trk C appear to be involved in germ cell survival and/or maturation because both the ligand and receptor are localized to the germ cell population. It is possible that NT3 is involved in an autocrine function to sustain germ cell populations at this time in development. After birth (P3–P5) NT3 was localized to Sertoli cells, germ cells, and interstitial cells, while trk C was present in the germ cells. Therefore, NT3 may be an important autocrine factor involved in germ cell maturation.

Nerve growth factor localization during testis development was in the Sertoli cells early and then became localized to Sertoli, germ, and interstitial cells after birth. The trk A was primarily localized in Sertoli and interstitial cells during testis development. Therefore, the major function of NGF may be to allow for and maintain Sertoli cell differentiated functions. At P0, NGF stimulated thymidine incorporation in a dose-responsive manner, suggesting that NGF may be a crucial paracrine factor at this time of testis development.

Some of these data are in contrast to immunohistochemistry reported by Russo et al. [35] in the mouse. Russo and coworkers reported expression of neurotropin ligands and receptors mainly in the interstitium and did not report expression in the seminiferous cords in their results. The experiments in the present paper were conducted at earlier time points and also were conducted in 2-day periods during development. In addition, the more extensive time points in the present study demonstrate the dynamic change in expression of neurotropin ligands and receptors. The dynamic nature of the neurotropins may have been missed in the Russo results, because fewer time points were evaluated. A second explanation may be species difference, because Russo and coworkers evaluated the mouse, and the current study examined neurotropin ligand and receptor lo-

calization in the rat. Furthermore, the differences in tissue handling between the two laboratories, and the differences in antigen retrieval also may have added to the differences in localization of the neurotropin ligands. In the current study, a more sensitive method of antigen retrieval was utilized that may have exposed more epitope sites and allowed for an increase in localization of neurotropin ligands within the seminiferous cords of the testis.

To determine how neurotropins may be promoting growth of P0 testis, effects of neurotropins on known paracrine growth stimulators (TGFα and its receptor EGFR) and growth inhibitors (TGFβ isoforms) previously shown to influence testis growth were evaluated. Treatment of P0 testis with NGF and NT3 altered the expression of TGFα, EGFR, TGFβ1, TGFβ2, and TGFβ3. Therefore, NT3 and NGF appear to promote a cascade of locally produced growth regulators to influence testis growth. Further studies are needed to elucidate how the individual cell types interact and the role of specific growth factors. However, it is clear the neurotropins will have an important role in the regulation of early postnatal testis growth.

After birth, NGF is expressed in Sertoli cells and interstitium with more expression on P5 in germ cells. Primarily, expression of trk A at this time is in Sertoli cells. This pattern of expression of NGF and trk A also suggests germ cell-Sertoli cell paracrine interactions during adult testis development. These results are supportive of other data where NGF has been proposed to be an important germ cell paracrine factor for Sertoli cells [36]. Nerve growth factor was demonstrated to stimulate DNA synthesis in seminiferous tubules [37], stimulate androgen-binding protein secretion from Sertoli cells [38], maintain morphology of seminiferous epithelium [39], and increase survival of Sertoli cells in culture [40]. Thus, NGF has been proposed to be an important regulator of Sertoli cell differentiated functions.

Less research has been conducted on NT3 and its role during postnatal testis development. By the expression data collected in the present experiment, NT3 secreted by the Sertoli cells acts on specific germ cell populations containing trk C. Further studies are needed to delineate the role of NT3 during postnatal testis development, but the current data implicate an important role for NT3 in potential paracrine and autocrine maturation or survival of germ cells. Thus, NGF appears to be important in Sertoli cell survival, and NT3 may be important in germ cell maturation in the postnatal testis.

The current study demonstrates dramatic changes in localization of NGF and NT3 during embryonic and early postnatal testis development. The localization of both NGF and NT3 are indicative of potential roles in seminiferous cord formation in the early testis and critical germ and Sertoli cell interactions after birth. The current observations support a previous study with embryonic testis organ cultures [17] that suggest that NT3 and trk C may orchestrate the events resulting in seminiferous cord formation. In addition to these early roles in testis morphogenesis, NT3 and NGF also stimulate testis growth during perinatal testis development. These proliferative events may be in part through novel interactions to suppress or increase mRNA for paracrine growth factors (TGFα and EGFR) or growth inhibitors (TGFβ isoforms). In summary, neurotropins appear to be important paracrine regulators that may regulate crucial morphological and growth processes resulting in sex-specific testis development.

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