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Role of Neurotropins in Rat Embryonic Testis Morphogenesis (Cord Formation)[1]

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ABSTRACT

The process of seminiferous cord formation is the first morphological event that differentiates a testis from an ovary and indicates male sex determination. Cord formation occurs by embryonic Day 14 (Day 0 = plug date; E14) in the rat. A series of experiments were conducted to determine if neurotropins and their receptors are important for the process of rat embryonic cord formation. The expression of low affinity neurotropin receptor (p75/LNGFR) was determined by immunohistochemistry on sections of both testis and ovary from E13 through birth (Day 0, P0) with an antibody to p75/LNGFR. The staining for p75/LNGFR was present in the mesonephros of E13 gonads and in a sex-specific manner appeared around developing cords at E14 in the embryonic testis. At birth, staining for p75/LNGFR was localized to a single layer of cells (i.e., peritubular cells) that surrounded the seminiferous cords. The genes for both neurotropin 3 (NT3) and for corresponding high affinity neurotropin trkC receptor were found to be expressed in the E14 rat testis, as well as other neurotropins and receptors. Immunocytochemical analysis of E14 rat testis demonstrated that NT3 was localized to the Sertoli cells and trkC was present in individual cells of the interstitium at E16 and in selected preperitubular cells at E18. Previously, the peritubular cells adjacent to the cords were demonstrated to be derived from migrating mesonephros cells around the time of cord formation. To determine if neurotropins were involved in cord formation, the actions of neurotropins were inhibited. A high affinity neurotropin receptor (trk)-specific kinase inhibitor, K252a, was used to treat organ cultures of testes from E13 rats prior to cord formation. Treatment of E13 testis organ cultures with K252a completely inhibited cord formation. K252a-treated organ cultures of E14 testis that contained cords did not alter cord morphology. A second experiment to inhibit neurotropin actions utilized a specific antagonist trk-IgG chimeric fusion protein and E13 testis organ cultures. The trk-IgG molecules dimerize with endogenous trk receptors and inhibit receptor signaling and activation of ligand function. Forty percent of E13 testis organ cultures treated with trkC-IgG had significantly reduced cord formation. TrkA-IgG had no effect on initiation of cords; however, in fifty percent of the treated organs, a "swollen" appearance of the cord structures was observed. Experiments using trkB-IgG chimeric protein on E13 organ cultures had no effect on cord formation or cord morphology. The testes from trkC and NT3 knockout mice were examined to determine if there were any morphological differences in the testis. NT3 knockouts appeared to have normal cord morphology in E15 and E17 testis. TrkC knockout mice also had normal cord morphology in E14 and P0 testis. Both NT3 and trkC knockout-mice testis had less interstitial area than wild-type controls. In addition, the trkC knockout mice have an increased number of cells expressing p75LNGFR within the cords when compared to controls or NT3 knockout mice. Combined observations suggest compensation between the different neurotropin ligands, receptors, and/or possibly different growth factors for this critical biological process. In summary, results suggest a novel nonneuronal role for neurotropins in the process of cord formation during embryonic rat testis development. The hypothesis developed is that neurotropins are involved in the progression of male sex differentiation and are critical for the induction of embryonic testis cord formation.

INTRODUCTION

A critical event in mammalian male sex determination is the development of the testis. The differentiation of the indifferent gonad into a testis is required for male sex determination. The process of cord formation is the critical morphogenetic event in embryonic testis development. Identification of specific factors involved in the induction of cord formation is necessary for an understanding of how genetic sex, derived in part through SRY and SOX expression [1, 2], is translated into testis differentiation. Little is known about the specific factors involved in testis morphogenesis. Fibroblast growth factor has been suggested to be involved in testis development [3]. Recently, analysis of transforming growth factor β2 expression has suggested that it may play a role in the embryonic testis [4]. The functions of these factors remain to be examined. Further elucidation of such factors is needed to provide insight into male sex differentiation and the embryonic testis morphogenic process.

The mammalian testis develops from the genital ridge along the medial surface of the mesonephros. Initially there is no sex-specific differentiation of the gonad, and it is referred to as indifferent or bipotential. The first sign of male development is the differentiation of Sertoli cells, which then aggregate with germ cells and undergo a transition from mesenchyme to epithelium to form cords [5–7]. There is no apparent structure in the ovary at this time [8]. The testis cords remain solid until the lumen forms at puberty to convert them into tubules. The seminiferous tubules are the site of spermatogenesis, and there spermatogonia develop into spermatozoa in close interaction with the Sertoli cells that help form the tubule [9–12]. Surrounding the Sertoli cells is a layer of peritubular myoid cells that are responsible for contraction of the tubule [13–16]. In the interstitial space between the tubules are Leydig cells, which are responsible for testosterone production and for stromal fibroblasts, vascular and lymphatic endothelial cells, and macrophages [17–19].

Organ culture of embryonic testis has revealed a requirement for the mesonephros in testis cord formation. Labeled

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cells from the mesonephros migrate into the testis during the culture period and become peritubular, endothelial, and interstitial cells but not Sertoli, Leydig, or germ cells [20,21]. These observations suggest that the migration of cells from the mesonephros may be critical for cord formation by Sertoli cells. Given the close association between Sertoli and peritubular cells, it appears likely that the pre-peritubular cells may be involved in cord formation. Hind limb buds can substitute for the mesonephros in allowing cord formation in organ culture of embryonic testis. In these cocultures, hind limb stromal cells migrate into the testis and are required for cord formation [22]. This implies that the embryonic testis may induce the migration of mesenchymal cells. The fate of the hind limb cells after migration has not been determined, so it is not known whether they contribute to a particular cell lineage such as peritubular cells. More recently, an organ culture system was used with the mesonephros separated from the testis by an embryonic ovary [23]. Observations confirmed a requirement for mesonephros cells to migrate into the testis through the ovary and suggest the potential presence of a chemotactic factor in the differentiating testis.

The neurotropins are a family of growth factors that are involved in tissue morphogenesis. The neurotropin family includes nerve growth factor (NGF), neurotropin 3 (NT3), neurotropin 4/5 (NT-4/5), and brain-derived neurotrophic factor (BDNF) [24]. The factors interact with the high affinity specific receptors trkA (for NGF), trkC (for NT3), and trkB (for NT-4/5 and BDNF), and the common low affinity receptor p75/LNGFR [25–28]. The trk receptors appear to be capable of mediating most of the identified responses to neurotropins including neurite outgrowth, neuronal survival, and proliferation of several different cells [29,30]. p75/LNGFR can modulate the response of the trks to the neurotropins [26]. In addition p75/LNGFR can signal independently of the trk receptors using pathways involving sphingomyelin hydrolysis to generate the second messenger ceramide [31,32].

Neurotropins are critical in mediating the differentiation, migration, proliferation, and survival of neurons in the developing brain and peripheral nervous system [33–37]. In addition, there is accumulating evidence for nonneuronal roles of the neurotropins, particularly in mesenchymal-epithelial interactions [38–45]. The neurotropins have been implicated in mediating local cell-cell interactions during morphogenesis in several tissues including the dermatome, tooth, kidney, and ovary [38–41]. Neurotropins have the potential to mediate similar processes in the embryonic testis.

Neurotropins are expressed in the postnatal testis where they appear to be involved in interactions between Sertoli and germ cells during spermatogenesis [46–49]. Less is known about their expression and possible function in the embryonic testis. The p75/LNGFR has been shown to be expressed by the mesenchyme surrounding testis cords [44,50]. This expression suggests that the neurotropins may be involved in testis development. The objective of the current study was to determine if neurotropins are necessary for morphological sex differentiation (i.e., cord formation).

MATERIALS AND METHODS

Organ Cultures

Timed pregnant Sprague-Dawley rats were obtained from Charles River (Wilmington, MA). Plug date was considered to be Day 0. For embryonic Day 13 (E13) dissections, gonads were dissected out with the mesonephros; for later-stage dissections, testes alone were dissected. The organs were cultured in drops of medium on Millicell CM filters (Millipore, Bedford, MA) floating on the surface of 0.4–1 ml of CMRL 1066 media (Gibco BRL, Gaithersburg, MD) supplemented with penicillin-streptomycin, insulin (10 μg/ml), and transferrin (10 μg/ml). Antibodies and factors were added directly to the culture medium. The medium was changed every 1 or 2 days. E13 gonad + mesonephros cultures were typically kept for 3 days, by which point cords are well developed. E14 testis cultures were typically kept for 4 days.

Genomic DNA Isolation and Polymerase Chain Reaction (PCR) for SRY

To determine the sex of E13 gonads, PCR for SRY was performed. Embryonic tails were collected to make 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 digested 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 (25:24:1), and once with chloroform:isoamyl alcohol. The DNA was then precipitated by adding 1/10 volume 7.5 M NH_4Ac and 3 volumes cold ethanol, and incubating at −80°C for an hour before centrifugation at 4°C for 30 min. Pellets were dried and resuspended in 10 μl dH_2O. PCR was performed using 1 μl of genomic DNA with primers to SRY. The sequences of the SRY primers are: 5′ C G G G A T C C A T G T C A A G C G C C C C A T G - AATGCATTTATG 3′ and 5′ GCGGAATTCACTTTAG-CCCTCCGATGAGGCTGATAT 3′. PCR was performed using an annealing temperature of 55°C for 30 cycles to yield a product of 240 base pairs (bp).

RNA Isolation and Reverse Transcription (RT)-PCR

RNA for RT-PCR was obtained by freezing samples on dry ice and then using the TRI reagent (Sigma, St. Louis, MO) for RNA isolation. RT was performed using MMLV-RT under standard conditions [51]. RT-PCR was performed at 60°C annealing temperature for 30 cycles. All RT-PCR reactions were conducted three times on the same RNA preparation, and three different RNA preparations were used for this experiment. A 3′ RT reaction was used, and the primers spanned an intron. The primer sequences are as follows (PCR product size in parentheses): trkA 5′GACGTGCTGGGCAGAGAA3′ and 5′CTTCCGC-ATTTGTTGAGCA3′ (520 bp); trkB 5′GAGCATCTCTC-GGTCTATGC3′ and 5′GGTCATCCCAATGATGAC-AGC3′ (230 bp); trkC 5′CATGGTTCCA-GCTCTCTAACACAG3′ and 5′ACCAGTCACCAC-TAGCCAAGAATG3′ (223 bp); NGF 5′CTTCAACAGGACTCACAGGA3′ and 5′TCTCT-ACAGGATTTGGGGCT3′ (224 bp); NT3 5′ATGCA-GAGCATAAGAGTCAC3′ and 5′GCCTACGAGTTT-GTTGTTTTC3′ (294 bp); BDNF 5′GAAAGTCCCGG-TATCAAAAG3′ and 5′CCAGCCAATTCTCTTTTTGC3′ (181 bp); NT4/5 5′CTGCGTCAGTACTTCTTCG3′ and 5′ACGCCCGCACATAGGACTG3′ (145 bp). Primers for the constitutively expressed gene cyclophilin were used to confirm the integrity of the RNA and efficiency of the PCR reaction as previously described [51]. The identity of all PCR products were confirmed using restriction di-

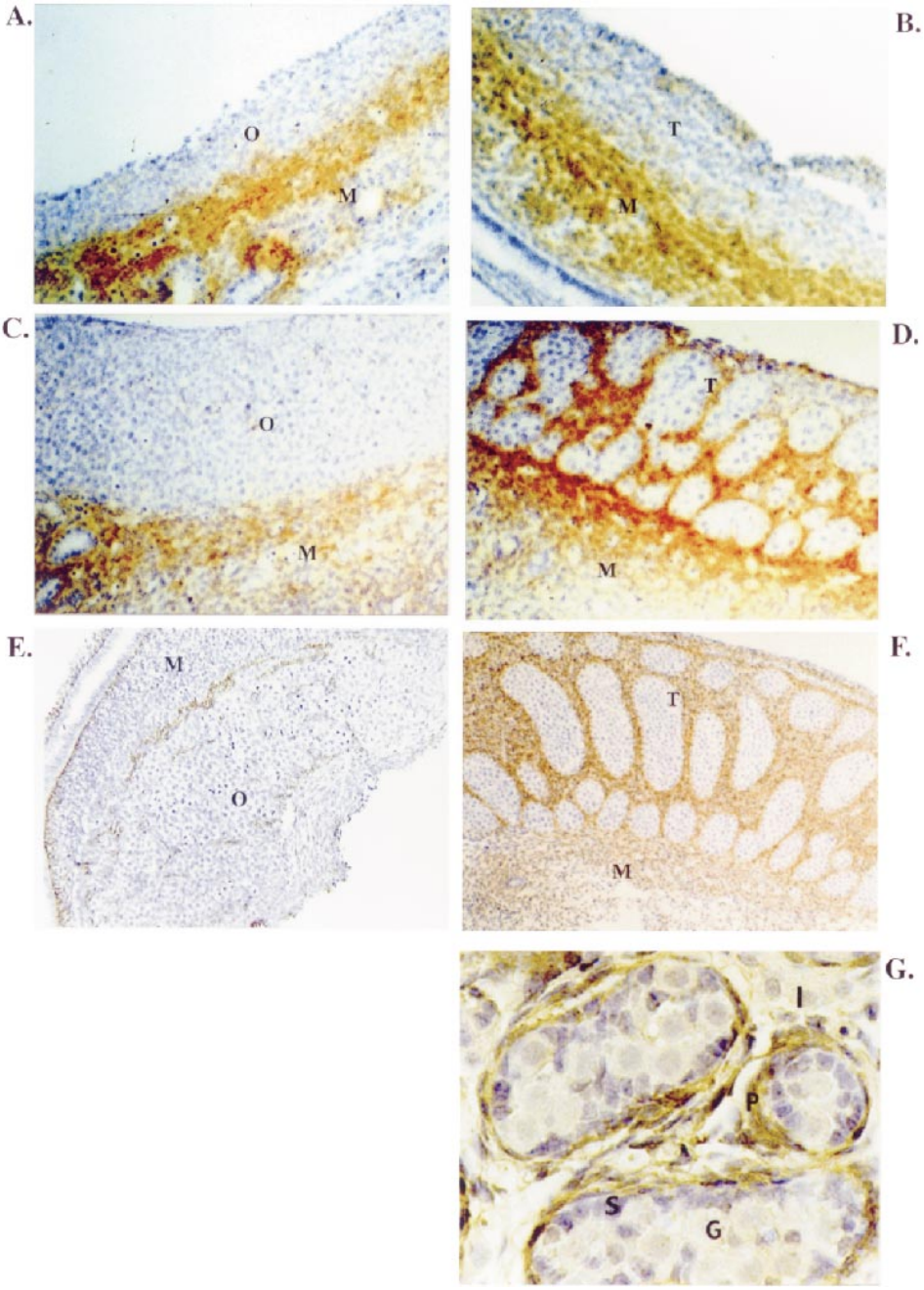

FIG. 1. Immunohistochemistry for p75/LNGFR in the developing urogenital ridge. **A**) E13 ovary + mesonephros; **B**) E13 testis + mesonephros; **C**) E14 ovary + mesonephros; **D**) E14 testis + mesonephros; **E**) E16 ovary + mesonephros; **F**) E16 testis + mesonephros. Ovary (O), testis (T), and mesonephros (M) are labeled. **G**) 0 day testis (P0). S, Sertoli; G, germ cell; P, peritubular; I, interstitial cell. Cells positive for p75/LNGFR are brown, while negative cells are blue/purple. ×200 except **G,** ×400 (published at 80%). Representative of at least three experiments.

gest analysis, and PCR products were subcloned and sequenced.

Embedding, Histology, and Immunohistochemistry

Tissues were fixed in Histochoice (Amresco, Solon, OH) and embedded in paraffin or in JB4 as previously described [52,53]. Sections were stained with hematoxylin and eosin (paraffin sections) or with toluidine blue (JB4 sections) as previously described [52,53]. Immunocytochemistry for p75/LNGFR was performed as previously described [52,53]. Briefly, 7-μm sections were deparaffinized and rehydrated, permeabilized in 0.1% Triton X-100, quenched in 20% methanol/3% hydrogen peroxide, and blocked in 2.5% BSA/10% serum for several hours at room temperature before incubation with primary antibody anti-p75 (REX) (rabbit anti-rat) (generously provided by Lou Reichardt, University of California, San Francisco) at 1:4000 dilution overnight at 4°C. Secondary antibody (biotinylated donkey anti-rabbit from Amersham) was detected using the Vectastain kit (Vector Laboratories, Burlingame, CA) and diaminobenzadine (DAB). Slides were counterstained lightly with hematoxylin to visualize the tissue. Immunohistochemistry for NT3 and trkC was performed as described previously according to standard procedures [52,53]. Briefly 3- to 7-μm sections were deparaffinized and rehydrated, microwaved (10 min on high), and blocked in 10% goat serum for one hour at room temperature. The NT3 primary antibody was an anti-NT3 peptide antibody (Santa Cruz Biotechnology [SCB], Santa Cruz, CA) raised against amino acids 139–158 of human NT3 (which is identical to mouse). The trkC primary antibody was an anti-trkC peptide antibody (SCB) raised against amino acids 798–812 of porcine trkC (identical to corresponding sequences in rat and mouse). The NT3 antibody was diluted 1:1000 in 10%

goat serum. The trkC antibody was diluted 1:600 in 10% goat serum. The biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) was diluted 1:300. As a negative control, serial sections were put through the same procedure without any primary antibody.

Materials

The antibody against p75/LNGFR (anti-p75 rat [REX]) was generously provided by Lou Reichardt, and sections from NT3 knockout mice were generously provided by Isabel Farinas and Lou Reichardt (UCSF, San Francisco, CA). The trkC knockout mouse testes were provided by Dr. Lino Tessarollo, NCI, Frederick Cancer Center, Rockville, MD. The trk-IgG chimeras were generously provided by Regeneron (Tarrytown, NY); the trk-IgG chimera proteins have cross-reactivity on both human and rat tissue. K252a and calphostin C were obtained from Calbiochem (La Jolla, CA). Images of whole organs were obtained using a Lumina digital scanning camera (Leaf Systems, Southborough, MA). NT3 and trkC primary antibodies were obtained from Santa Cruz Biotechnology.

RESULTS

Expression of p75/LNGFR Protein by Immunohistochemistry in the Developing Rat Urogenital Ridge

Previous experiments have demonstrated the presence of p75/LNGFR mRNA and protein in the embryonic rat testis [44,50]. Expression was confined to the mesenchyme surrounding the developing testis cords. At later ages, the p75/LNGFR protein expression became restricted to the peritubular cell layer surrounding the cords [50]. In the current study, the expression of p75/LNGFR was investigated by immunohistochemistry in more detail around the critical period of cord formation. Expression was examined before the formation of cords (E13) as well as after (E14). Expression was also examined in female gonads and in the entire urogenital ridge including the mesonephros.

Expression was first examined in gonads and mesonephros at E13, just before cord formation, when the testis and ovary are indistinguishable (Fig. 1, A and B). At this stage, expression of p75/LNGFR was detectable only in the mesonephros in both the male and female. The expression was particularly high in the region of the mesonephros adjoining the gonad. After cords formed at E14, the p75/LNGFR was found in the testis in the mesenchyme surrounding the cords. There was no p75/LNGFR protein expressed in the ovary at this stage (Fig. 1, C and D). Later in embryonic development there was slight staining in the ovary and extensive staining throughout the testis interstitium (Fig. 1, E and F). Expression in P0 testis was performed to confirm the fate of p75/LNGFR positive cells, and, as expected, the expression was mostly peritubular (Fig. 1G). This data revealed a sex-specific pattern of p75/LNGFR expression at the time of initiation of the male sexual differentiation pathway.

Expression of trk Receptors and Neurotropin Ligands

The observation that the common receptor p75/LNGFR is present in such an interesting temporal and sex-specific pattern suggests that some of the neurotropin ligands and high affinity trk receptors may be present as well. To investigate the presence of specific ligands and receptors in the early urogenital ridge, RT-PCR was performed on RNA isolated from E14 testis (Fig. 2). PCR for a constitutively

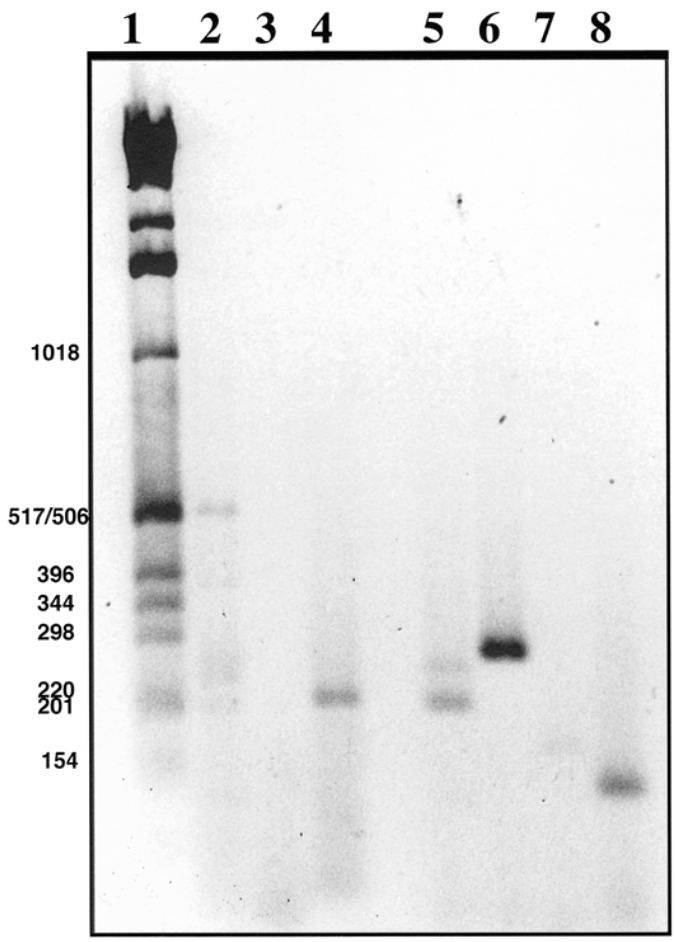

FIG. 2. Expression of trk receptors and neurotropin ligands by RT-PCR in E14 testis. The lanes are labeled as follows: 1, molecular weight standards; 2, trkA; 3, trkB; 4, trkC; 5, NGF; 6, NT3; 7, BDNF; 8, NT4/5. The sizes of the PCR products are as follows: trkA 520 bp, trkB 230 bp, trkC 223 bp, NGF 224 bp, NT3 294 bp, BDNF 181 bp, and NT4/5 145 bp. Product identity was confirmed with restriction analysis and sequencing. Representative of at least 3 experiments for each.

expressed gene cyclophilin was done to confirm the integrity of the RNA for each sample (data not shown). PCR for SRY was done to confirm the male genotype of the gonad analyzed (data not shown). A brain RNA sample was used as a positive control, and all the neurotropins and receptors were detected with the RT-PCR analysis (data not shown). The RT-PCR was done on the same RNA sample, and the experiment was repeated three times with three different RNA samples. The identity of the individual PCR products were confirmed with a restriction digest and sequence analysis (data not shown). Of the three known high affinity receptors, trkC and trkA were both present while trkB was not. The neurotropin ligands NGF, NT3, BDNF, and NT4/5 all yielded detectable PCR products, although NT3 gave the strongest signal (Fig. 2). Therefore, the genes for the neurotropins and receptors are expressed in the E14 embryonic testis.

To complement the RT-PCR analysis, an immunocytochemical procedure was used to investigate the cellular localization of NT3 and trkC in the embryonic rat testis (Fig. 3). NT3 was primarily localized within the Sertoli cells at E14. Interestingly, the trkC was primarily localized to individual cells of the interstitium at E16 and to the basal surface of the cords in the peritubular cell region at E18 (Fig. 3). These data confirm the expression of the NT3 and

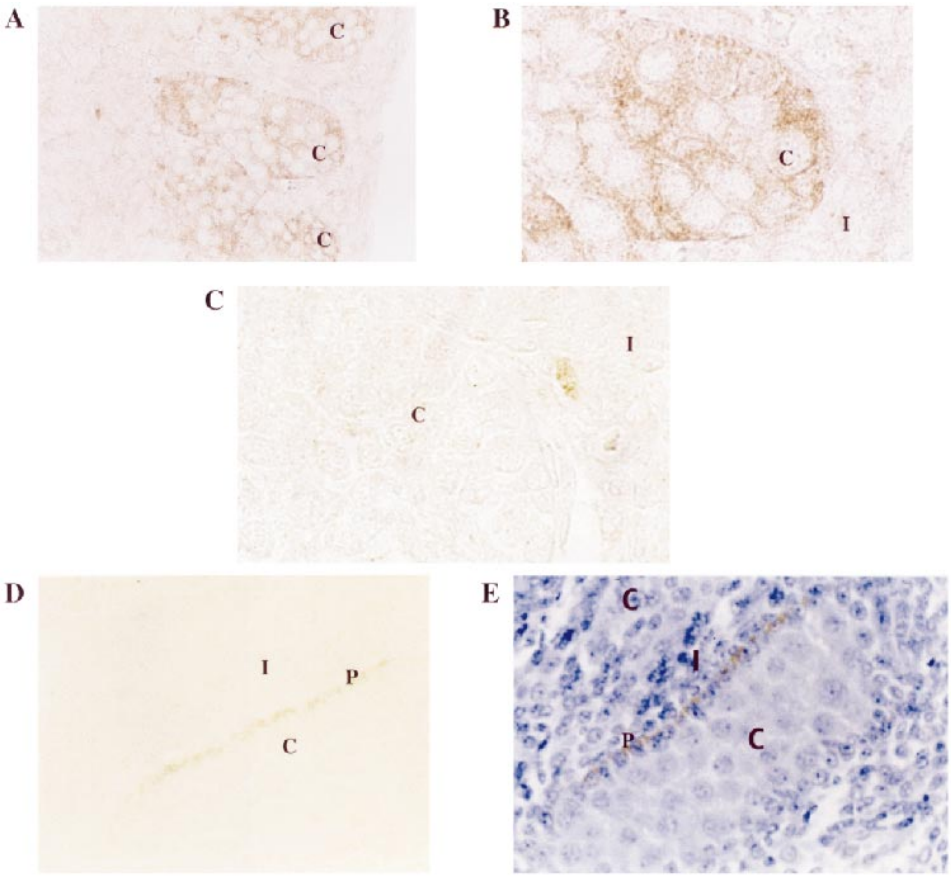

FIG. 3. Immunohistochemistry for **A**) NT3 at E14 (×400), **B**) NT3 at E14 (×1000), **C**) trkC at E16 (×1000), **D**) trkC at E18 (×600) no counterstain, and **E**) trkC at E18 (×600) counterstained with hemotoxylin to show morphology (published at 68%). Staining for NT3 is red and trkC is brown. C, Cords; I, interstitium; P, peritubular cells. Representative of at least three experiments.

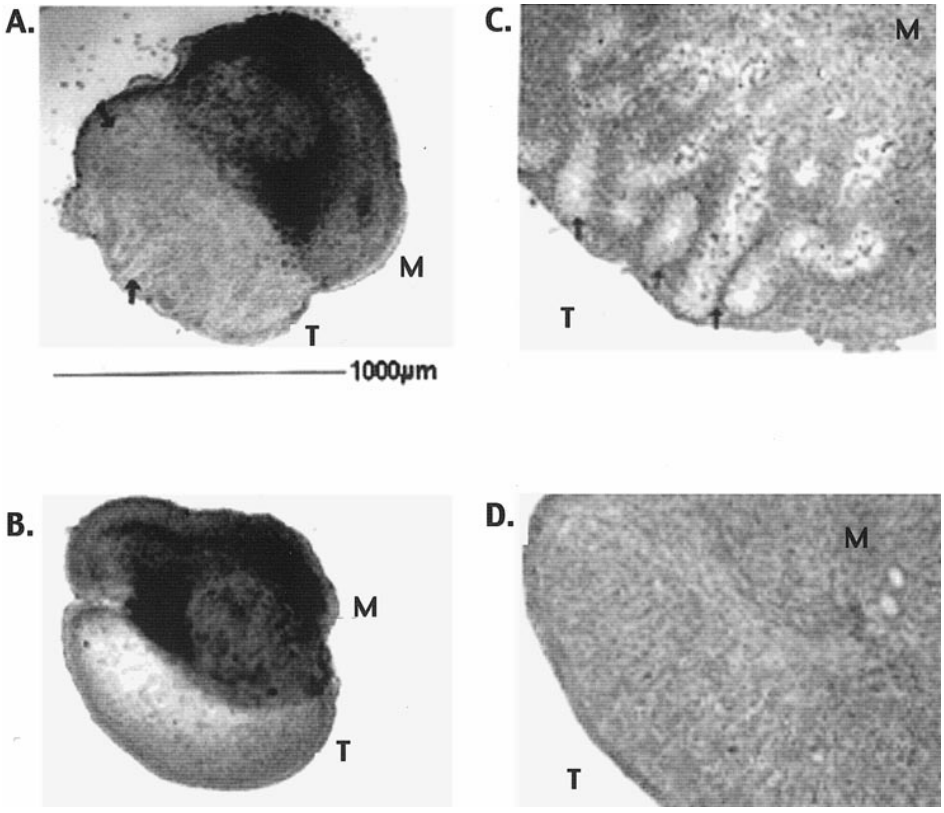

FIG. 4. Effects of trk inhibitor K252a on organ cultures of E13 testis + mesonephros in culture for three days. Images of whole organs treated with **A**) control solvent DMSO or **B**) K252a (100 nM, representative of three experiments with approximately six organ testis culture pairs per experiment; one testis serves as a control, the other is treated). JB4 sections of organ cultures were stained with toluidine blue for **C**) control solvent DMSO or **D**) K252a (100 nM). Testis (T) and mesonephros (M) are labeled. **C,D**) Representative cords are marked by arrows.

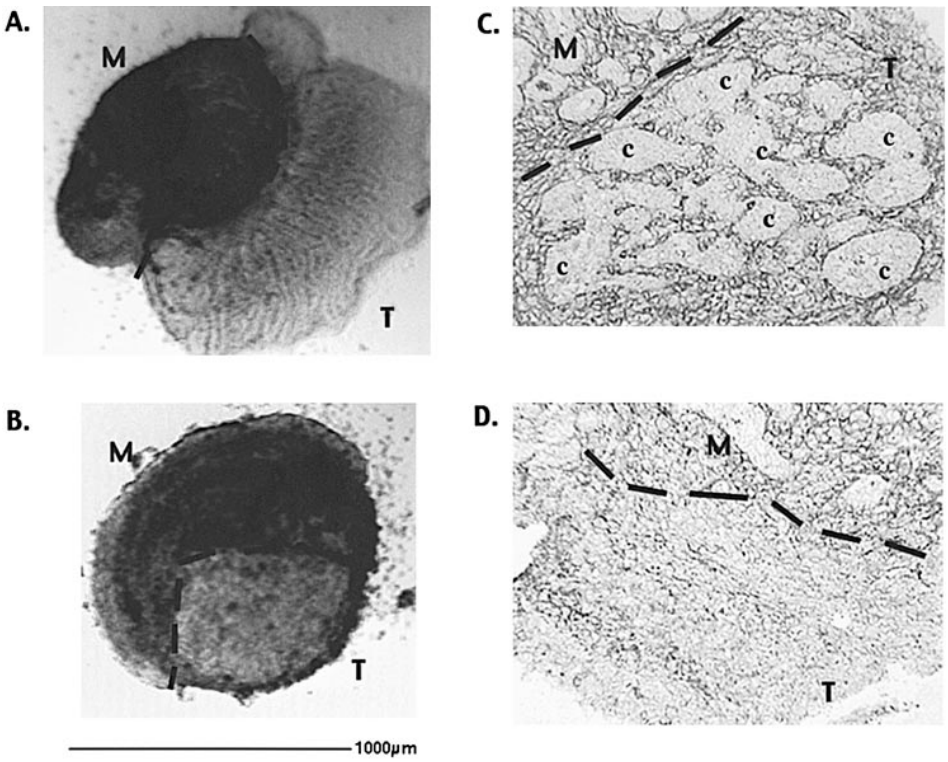

FIG. 5. Effects of trkC-IgG chimera on organ cultures of E13 testis + mesonephros in culture for three days. **A**) Control, **B**) trkC-IgG (7.5 μg/ml). Testis (T) and mesonephros (M) are labeled. Representative of four experiments with approximately six organ culture pairs per experiment. p75LNGFR expression in E13 organ cultures treated with TrkC-IgG (7.5 μg/ml). Dark gray stain denotes p75LNGFR expression in organ cultures. In 60% of organ cultures, cord formation occurred (**C**), while in 40% of organ cultures no cord (**D**) formation occurred. P75LNGFR does not stain cords and therefore is a good marker to detect cord formation. M, Mesonephros; T, testis; C, cord. Dashed lines separate testis from mesonephros.

trkC proteins. The localization suggests a potential interaction between NT3 produced in the cords with the peritubular/adjacent mesenchymal surrounding the cords. As previously discussed [20,21], the peritubular/adjacent mesenchyme to the cords appears to be derived in part from the migrating mesonephros cells.

Perturbation of Neurotropin Action in Rat Testis Organ Cultures

The presence of neurotropin ligands and receptors in the rat embryonic testis, as well as the sex-specific expression of p75/LNGFR just at the time of cord formation, suggest that the neurotropins may be functioning in the morphogenesis of the early testis. To test this hypothesis, neurotropin signaling was inhibited with the trk kinase inhibitor K252a in embryonic testis organ cultures. K252a has been shown to be specific for the trk receptors over that of any other receptor-linked tyrosine kinases tested. However, at high concentrations it can inhibit serine-threonine kinases such as protein kinase C (PKC) [54–56]. Organ cultures of the testis and the mesonephros were established at E13 before the development of cords. Over three days in culture, cords formed in control organs. However, in cultures treated with 100 nM K252a, cord formation was completely inhibited (Fig. 4, B and D). Organ cultures of E13 testis and mesonephros were also treated with a more specific highly potent PKC inhibitor, calphostin C, as a control to ensure that inhibition of PKC by K252a was not responsible for these effects. Treatment with calphostin C did not inhibit either cord formation or size of the embryonic testis (data not shown). This implies that inhibition of PKC was not responsible for the effects of K252a on the testis. Observations suggest that the inhibition of testis cords by K252a is likely due to an inhibition of neurotropin actions.

Several more specific reagents were used in order to disrupt signaling through neurotropins and the trk receptors. Chimeric fusion proteins that contain a trk extracellular domain fused to human IgG have been developed that inhibit receptor dimerization and bind to their corresponding ligand to block biological activity [57]. The chimeric proteins used have cross-reactivity with human and rat tissues. A trkC-IgG chimera was used to treat embryonic testis and mesonephros organ cultures. Experiments were conducted using 7.5 μg/ml (four experiments with approximately six testis organ-culture pairs per experiment) and 50 μg/ml (four experiments with approximately six testis organ-culture pairs per experiment) of trkC-IgG on E13 testis organ cultures. Testis pairs were divided with one testis being treated while the other served as the control. SRY PCR was used to confirm the male genotype of the gonads (data not shown). In 10 of 24 (i.e., approximately 40%) of testis organ cultures treated with trkC-IgG, there was either inhibition or dramatic reduction of cord formation (Fig. 5, A and B). There did not appear to be a difference in cord reduction between the doses of trkC-IgG used (data not shown). Additionally, the trkC-IgG did not have an effect on relative expression of p75LNGFR, but the staining with p75LNGFR does demonstrate the reduction in cord structures present in the trkC-IgG chimera-treated testis organ cultures (data not shown).

Experiments were conducted with a trkA-IgG chimera (five experiments with approximately six testis organ-culture pairs per experiment) to determine what effect this fusion protein receptor antagonist may have on cord formation in E13 treated testis organ cultures. TrkA-IgG did not effect cord formation, but in 8 of 15 (53%) of the organ cultures (Fig. 6), there was an increase in cord diameter or swollen appearance in the cords. This swollen appearance was determined to be an increase in size of some cells in the cords and potential hypertrophy of the cells.

Experiments were conducted using a combination of trkA-IgG and trkC-IgG (50 μg/ml; four experiments with six testis organ-culture pairs per experiment) to determine the effect on cord formation. In 12 of 24 (50%) of E13

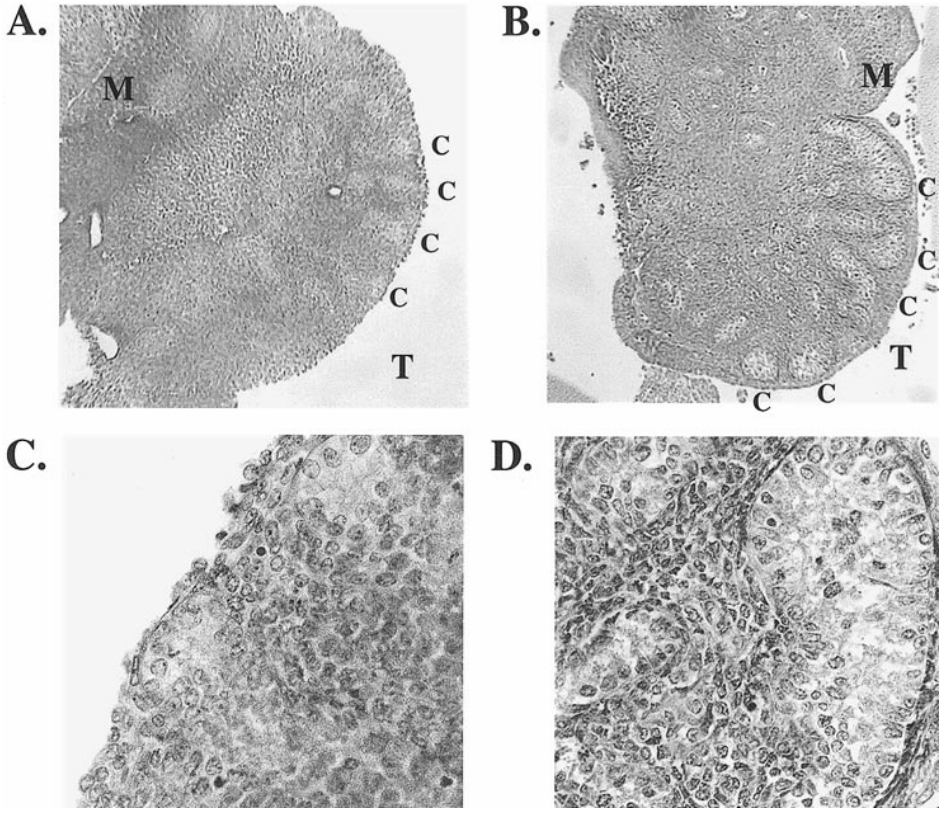

FIG. 6. Effects of trkA-IgG (50 μg/ml) on of E13 testis organ cultures. H and E sections of control testis organ cultures **A**) at ×80 or **C**) ×1000 magnification (published at 68%). Organ cultures treated with trkA-IgG that had ''swollen cord effect'' at the same magnifications of **A** and **C,** respectively. P75LNGFR staining is dark grey. M, Mesonephros; T, testis; C, cords. These are representatives of six experiments with approximately 6 organ testis pairs per experiment.

testis organ cultures treated, there appeared to be a reduction or absence of cords, and in approximately 20% there were swollen cords (data not shown). This reduction is not significantly different from trkC-IgG treatment alone. Treatment of E13 testis organ cultures with trkB-IgG had no effect on cord formation or cord morphology (data not shown). This corresponds to the lack of trkB being detected by PCR and provides a negative control protein for the trk-IgG experiments.

To support the previous experiments, an additional experiment was conducted with trkC antisense oligonucleotide to determine what effect it might have on seminiferous cord formation (data not shown). In fifty percent of organ cultures treated (17 of 36 testis organ pairs) with a trkC antisense oligonucleotide, seminiferous cord formation was perturbed. Therefore, these experiments provide additional support for a role for trkC in seminiferous cord formation.

Examination of NT3 and trkC Knockout Mouse Testis

Mice lacking NT3 have severe sensory and sympathetic defects, and most die within 24 h of birth [58]. The testes from NT3 knockout mice and their wild-type litter mates were examined at E15 and E17. The testes from mice lacking NT3 appear normal and contain histologically normal cords (Fig. 7). Similar results were observed in a minimum of 5 regions for each of 3 different sections (data not shown). Interestingly, a reduced interstitium was observed in the NT3 knockout mice (Fig. 7B), which remains to be quantified.

Mice lacking trkC generally live until birth and die between Days 3 and 21 postnatally. They are completely deficient in muscle afferents that innervate muscle spindles in the periphery and project to the spinal cord. Thus, these mice have difficulty in movement and localization of limbs in space [59]. While the testes from trkC knockout mice have normal formation of cords at E14 and P0, there appears to be less interstitium with the seminiferous cords in closer apposition to each other (Fig. 8B). Staining with an antibody for LNGFR demonstrated an increased population of cells within the cords expressing p75/LNGFR in trkC knockout mice when compared to heterozygous knockout mice (Fig. 8B). This is an additional phenotype of the knockout animals when compared to the heterozygous or wild-type animals (Fig. 5). Therefore, the expression of different neurotropin receptors (e.g., LNGFR) may be altered in the knockout animals.

DISCUSSION

The results presented suggest that neurotropins play a role in the morphogenesis of the developing testis. This adds to the growing body of evidence for nonneuronal actions of the neurotropins in development. Prior analysis of the expression patterns of NGF and p75/LNGFR suggested that neurotropins were potentially involved in many developmental processes at sites of mesenchymal-epithelial cell interaction [44]. The identified sites of function so far associate with morphogenetic processes involving inductive interactions leading to transitions from mesenchymal to epithelial cells (i.e., kidney and testis) or from epithelial to mesenchymal cells (i.e., dermatome).

The appearance of p75/LNGFR in the testis correlated with the morphogenetic event of cord formation. The sex specificity and the timing of expression suggest that the neurotropins may be involved in the early steps of male sexual differentiation and particularly in the process of cord formation. The parallels in the expression of p75/LNGFR in the developing testis and ovary are instructive in thinking about the potential roles of this receptor. p75/LNGFR is present in the ovary mesenchyme surrounding the developing follicles in the late embryonic and early postnatal

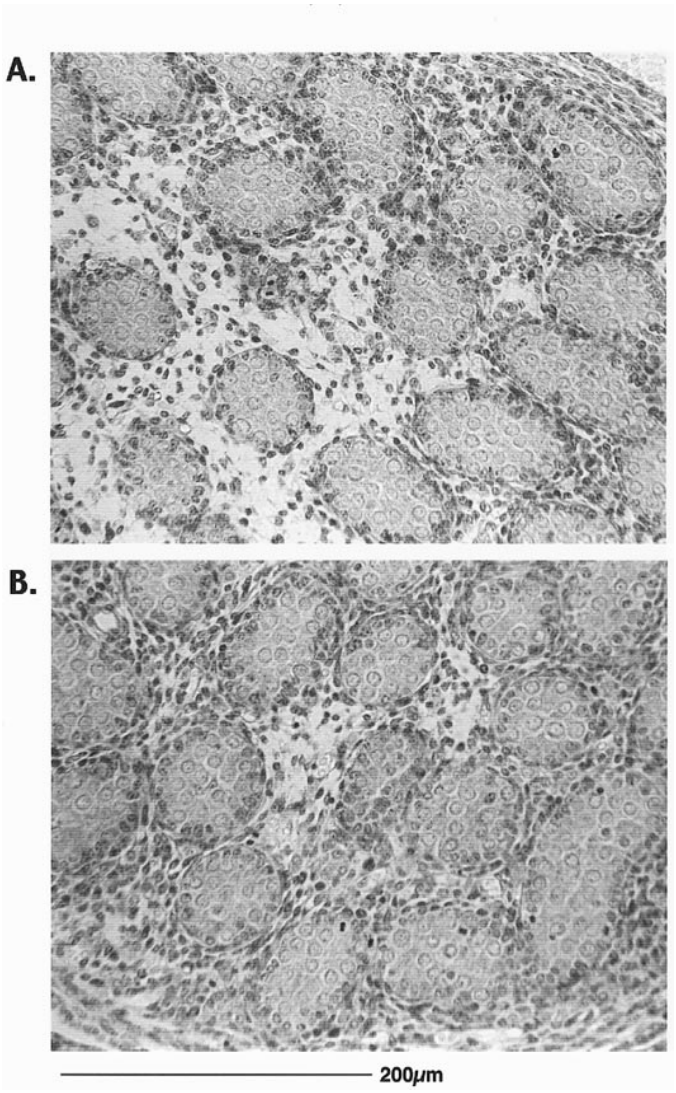

FIG. 7. NT3 knockout E15 testis. **A**) Wild-type, **B**) NT3 knockout. Representative of three different animals.

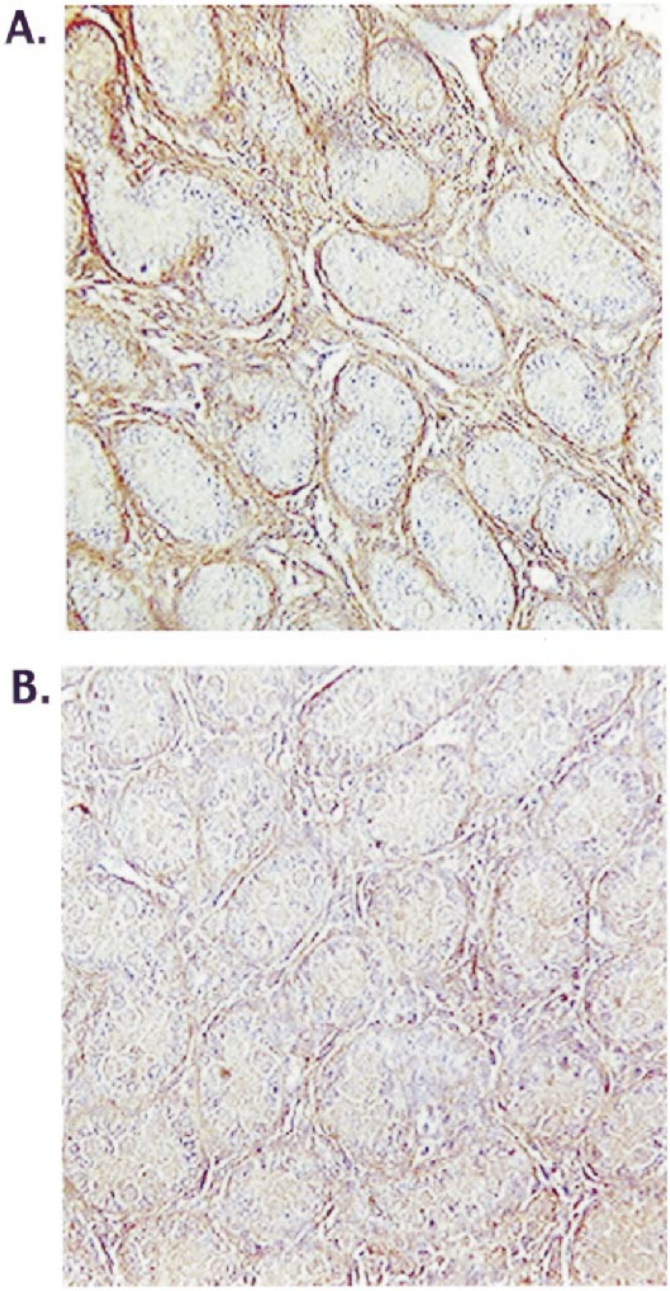

FIG. 8. Immunohistochemistry for p75LNGFR in **A**) heterozygous controls or **B**) homozygous trkC knockout mice in zero day testis. P75LNGFR is dark brown. Sections are lightly counterstained with hemotoxylin. ×200 (published at 91%). Representative of four different animals for each group.

period [39]. In the current experiments we have found that expression is undetectable in the earlier ovary, which remains unstructured during the time of cord formation in the testis. p75/LNGFR protein is first detectable at E16 and increases through E19 in the ovary just before the morphological rearrangements leading to follicle formation. Its expression in both male and female gonads specifically at the time of the initial formation of testis cords and ovarian follicle structures suggests that it may be performing a critical and analogous role in these processes. The presence of trkC in the cells surrounding the cords (presumed to be the peritubular cells) in E18 rat testis also supports the theory that NT3 and trkC are important during the process of morphological cord formation.

Treatment of embryonic testis in organ culture with the tyrosine kinase inhibitor K252a resulted in complete inhibition of cord formation. K252a is widely used as a trk-specific inhibitor since other tyrosine kinases that have been examined are not affected by K252a. K252a was found to have no effect on the responses of the epidermal growth factor receptor, platelet-derived growth factor receptor, fibroblast growth factor receptors, or src [46,48]. However, several serine/threonine kinases can be inhibited by high concentrations of K252a. The most obvious candidate is protein kinase C (PKC), which is known to be a target of K252a at high concentrations. However, PKC inhibition by calphostin C did not interfere with cord formation, suggesting that the effects of K252a were not mediated through inhibition of PKC. The inhibition of cord formation by K252a suggests that a trk receptor and neurotropin signaling may be involved in testis cord formation.

Perturbing neurotropin function using more specific reagents used trk-IgG fusion proteins that inhibit the specific trk receptors [57]. Treatment with the inhibitory trkC-IgG antagonist resulted in inhibition of cord formation in 40% of E13 testis organ cultures treated. The ability of trkC-IgG

to prevent cord formation in testis organ cultures suggests a role for trkC in testis cord formation. The variability of the trkC-IgG response is not considered to be due to dose, since the same response in cord reduction was achieved using either 7.5 or 50 μg/ml. In addition, experiments with antisense oligos to trkC had similar results as the trkC-IgG fusion proteins. Several possibilities exist that may explain the variability of these experiments. Three isoforms for the trkC receptor exist with one being a nonfunctional receptor and two containing different portions of the intracellular c-terminus. There is no known function for these trkC isoforms, and the trkC-IgG chimeric protein used in these experiments may be titering out a form of the receptor that is inactive in the cell as a signaling molecule. NT3 may also act at trkA or trkB with less efficiency to elicit its effect [60]. There is also a possibility that a crucial window of time exists in the development of testis where cord formation can be perturbed, and this time could have been partially missed in these experiments. Alternatively, since the process of cord formation is the first morphological event in the process of sex determination, this may be accomplished by more than one factor. There may be several factors that act in a compensatory manner to orchestrate the process of cord formation.

Both trkA and trkC receptors are expressed in the embryonic testis, as well as minimally three ligands NGF, NT3, and NT4/5. Treatment of E13 organ cultures with trkA-IgG had no effect on cord formation but did influence cord morphology. Therefore, trkA may effect embryonic testis development, but not cord formation. A combination of trkA-IgG and trkC-IgG did not significantly inhibit further the reduction of cord formation compared to that of trkC-IgG treatment alone. This suggests that trkC and not trkA appears to be the important neurotropin receptor for cord formation. The absence of detectable trkB by RT-PCR suggests that BDNF and NT4/5 are unlikely to function during embryonic testis development (at least not through trkB). Treatment with trkB-IgG did not have an effect on cord formation in testis organ cultures. Therefore, the trkB-IgG protein can be considered a negative control for the IgG chimera protein experiments.

The morphology of the NT3 and trkC knockout mice was examined to determine if cord formation and testis morphology were effected. In the current study, examination of the mice lacking NT3 revealed apparently normal testis. Slight reductions in interstitial volume were observed and require further investigation. If NT3 is involved in cord formation, the knockout phenotype suggests that other factors are capable of compensating for its absence. While the neurotropin knockouts have dramatic phenotypes, there are many tissues in which there do not appear to be abnormalities. This is despite extensive evidence for specific neurotropin function from expression and in vitro studies ([36,37] and [60–63]). This literature supports the idea that a lack of phenotype in the knockout does not rule out a role for the factor in a developmental process. In several instances, the phenotypes of the trk receptor knockouts were more dramatic than that of the cognate ligands [58,59,61–64]. This suggests that other ligands may compensate for the absence of one, which is reasonable given the ability of multiple ligands to interact with a single receptor.

Knockout mice lacking trkC were also obtained and the testis morphology examined. After observing several testes from these knockouts, a reduction in area of the interstitium was observed. Immunohistochemistry was conducted on sections of trkC knockout mice testis, NT3 knockout testis, and heterozygous controls for expression of p75LNGFR. NT3 knockout mice had a similar pattern of expression of p75LNGFR to controls; however, there appeared to be an increase in the number of stained cells within the cords that expressed p75LNGFR in the trkC knockout mice testis. In controls and NT3 knockout mice, p75LNGFR stained intensely around the cords in the interstitium. However, in the trkC knockout mice there was low level of expression throughout the testis including inside of the cords surrounding germ cells. The significance of this staining pattern remains to be elucidated. The reduction in p75LNGFR positive cells in the interstitium in trkC knockouts may involve reduced migration of cells from the mesonephros that eventually become part of the interstitium. p75LNGFR knockout mice do not appear to have as dramatic a phenotype as NT3, trkC, or trkA knockout mice [65].

There are several possible roles for neurotropins at different steps in the processes leading to cord formation. One possible function is stimulation of the initial migration of peritubular cells from the mesonephros into the testis. This would be consistent with the known abilities of neurotropins in other systems. NGF has been widely studied as a factor responsible for guiding the migration of neurons and is capable of inducing neuronal morphogenesis [66]. NGF also functions as a chemoattractant for melanocytes in the skin and promotes migration by Schwann cells [67,45]. Therefore, neurotropins could be involved in the migration of pre-peritubular cells into the testis. The expression of p75/LNGFR on cells in the mesonephros before the appearance of any p75/LNGFR positive cells in the testis is consistent with this idea. The expression of p75/LNGFR in the mesonephros is particularly pronounced in the region adjacent to the testis, suggesting that those cells may either migrate into the testis or be involved in signalling interactions with the testis. The immunohistochemical analysis of NT3 and trkC in embryonic testis also support this hypothesis. NT3 was localized within the Sertoli cells while trkC was present in the layer of cells adjacent to the cords (i.e., pre-peritubular cells). Previously the mesonephros cells that migrate into the testis have been shown to derive in part the peritubular layer of cells surrounding the cords [20]. Therefore, the possibility exists that the NT3 produced in the cords (i.e., Sertoli-germ cell aggregates) promote the migration of mesonephros cells into the testis to become peritubular cells and help promote cord formation. Another possible role for neurotropins in the testis is in the inductive interactions leading to cord formation by Sertoli cells. Neurotropins might stimulate Sertoli cells to undergo a transition from mesenchyme to epithelia, which promotes aggregation and cord formation. There is evidence for neurotropin function in morphogenetic inductions in other systems. The best functional data on NT3 outside of the nervous system is in the process of dermis formation from the dermatome, involving an epithelial to mesenchymal transition [38].

The complex process of seminiferous cord formation is the result of expression and activation of a series of genes that initiate and maintain Sertoli cell differentiation. Initially, SRY is proposed to trigger the process of Sertoli cell differentiation. In addition, SOX9 has been determined to assist in sex determination and may potentially be regulated or work cooperatively with SRY. Downstream of SRY and SOX9, expression other genes such as TGFβ2 and MIS have been detected in Sertoli cells prior to seminiferous cord formation. Their potential role appears to be in main-

tenance of Sertoli cell differentiation and the creation of an adequate environment to favor testis differentiation. The current results lead to the intriguing possibility that neurotropins may be involved in the process of cord formation by Sertoli cells. The speculation is made that one of the earliest steps in mammalian male sex determination after SRY and SOX9 expression may be the production of neurotropins and their receptors in the testis. Understanding more about this process will provide insight into similar morphogenetic processes in other developing organs. In addition, this data adds to the evidence for crucial nonneuronal functions of the neurotropins in development.

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