Testis Developmental Phenotypes in Neurotropin Receptor *trkA* and *trkC* Null Mutations: Role in Formation of Seminiferous Cords and Germ Cell Survival[1]

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ABSTRACT

The objective of the present study was to determine if the neurotropin receptors *trkC* and *trkA* are involved in embryonic testis development. These receptors bind neurotropin 3 and nerve growth factor, respectively. The hypothesis tested was that the absence of *trkC* or *trkA* receptors will have detrimental effects on testis development and morphology. The *trkA* and *trkC* homozygote knockout (KO) mice generally die either at or shortly after birth. Therefore, heterozygote mice were mated to obtain homozygote gene KO mice at Embryonic Day (E) 13, E14, E17, and E19 of gestation, with E0 being the plug date. Gonads from approximately 80 embryos were collected and fixed, and each embryo was genotyped. To determine gonadal characteristics for each genotype, the number of germ cells, number of seminiferous cords, seminiferous cord area, and interstitial area were calculated at each developmental age. Germ cell numbers varied in *trkA* gene KO mice from those of wild-type mice at each age evaluated. In *trkC* gene KO mice, differences were detected in germ cell numbers when compared to wild-type mice at E17 and E19. At E19, germ cell numbers were reduced in both *trkA* and *trkC* gene KO mice when compared to wild-type animals. Apoptosis was evaluated in testes of wild-type, *trkC* gene KO, and *trkA* gene KO mice to determine if the alteration in germ cell numbers at each developmental age was influenced by different patterns of germ cell survival or apoptosis. No differences were found in germ cell apoptosis during embryonic testis development. Interestingly, *trkA* gene KO mice that survived to Postnatal Day 19 had a 10-fold increase in germ cell apoptosis when compared to germ cells in wild-type mice. Evaluation of other morphological testis parameters demonstrated that *trkC* KO testes had reduced interstitial area at E13, reduced number of seminiferous cords at E14, and reduced seminiferous cord area at E19. The *trkA* gene KO testes had a reduction in the number of seminiferous cords at E14. Histology of both *trkA* and *trkC* gene KO testes demonstrated that these gonads appear to be developmentally delayed when compared to their wild-type testis counterparts at E13 during testis development. The current study demonstrates that both *trkA* and *trkC* neurotropin receptors influence germ cell numbers during testis development and events such as seminiferous cord formation.

developmental biology, gametogenesis, growth factors, Sertoli cells, testis

INTRODUCTION

Although the neurotropin family of growth factors was first discovered in the nervous system, neurotropins have been demonstrated to be important in tissue morphogenesis of a large number of organs [1–5] and in germ cell maturation and survival [6]. The neurotropin family contains nerve growth factor (NGF), neurotropin 3 (NT3), neurotropin 4/5 (NT4/5), and brain-derived neurotrophic factor (BDNF) [7]. All four ligands can bind to a low-affinity receptor P75/NTR and have specific high-affinity receptors: *trkA* (for NGF), *trkC* (for NT3), and *trkB* (for NT4/5 and BDNF) [8–11]. P75/NTR is speculated to modulate the response of the trk's to the neurotropins [9], and it may signal independently of the trk receptors using sphingomyelin hydrolysis to generate the second-messenger ceramide [12, 13].

Expression of P75/NTR in the embryonic gonad has demonstrated localization to the testis in a sex-specific manner during seminiferous cord differentiation [14]. NT3 is expressed by Sertoli cells during seminiferous cord formation, with localization of its receptor *trkC* to the preperitubular cells that migrate from the adjacent mesonephros. Both these cell types, Sertoli and preperitubular, are required for the formation of seminiferous cords [14, 15]. Experiments designed to inhibit neurotropins or neurotropin receptor signaling in testis organ cultures have shown an inhibition of seminiferous cord formation [14, 15]. The current hypothesis is that, on the induction of male sex determination, the Sertoli cells produce NT3, which acts as a chemotactic agent for preperitubular cell migration from the mesonephros and, after migration, promotes seminiferous cord formation. Effects of the neurotropins NGF and NT3 have also been examined after seminiferous cord formation and been found to influence embryonic and perinatal testis growth [15] and germ cell survival [16]. Therefore, the present study was conducted to extend these previous observations and to determine the effects of *trkA* or *trkC* gene null mutations on testis growth and development.

Knockout (KO) mice for both the *trkA* and *trkC* genes have been generated [17, 18] and studied in regard to neurological function. These mice are born alive but do not survive more than 1 wk in most cases; their death is caused by severe neurological problems. To our knowledge, the reproductive potential and effects of these null mutations on testis development have not been addressed in previous reports. Therefore, the present study was designed to determine if mice with targeted mutations in either the *trkA* or *trkC* genes have altered embryonic testis development.

MATERIALS AND METHODS

trkA *and* trkC *KO Mice*

The *trkA* and *trkC* KO mice were obtained from Dr. Lino Tessarollo (National Cancer Institute, Frederick, MD). The *trkC* and *trkA* mice were
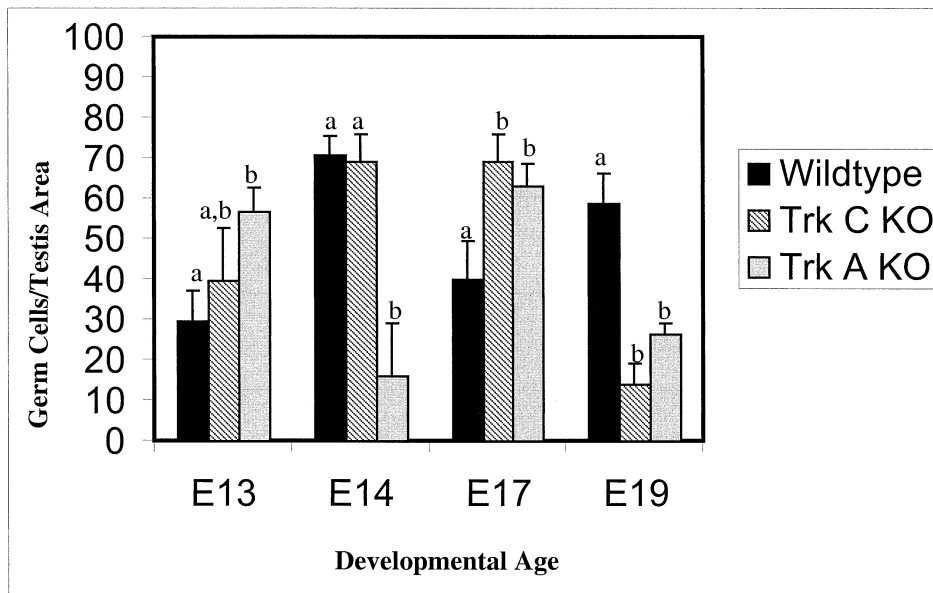
FIG. 1. Number of germ cells per testis area in wild-type (WT), *trkA* gene KO, and *trkC* gene KO at E13, E14, E17, and E19 of testis development. At least 6 different areas were counted for each testis. Number of mice per genotype at each developmental age: E13 = 1 WT, 1 *trkA* KO, and 1 *trkC* KO; E14 = 4 WT, 1 *trkA* KO, and 2 *trkC* KO; E17 = 1 WT, 2 *trkA* KO, and 4 *trkC* KO; and E19 = 1WT, 2 *trkA* KO, and 2 *trkC* KO. A minimum of 2 different testes were analyzed for each genotype at each developmental stage. Data are presented as the mean ± SEM, and different superscript letters over bars represent statistical differences at $P < 0.05$ within a developmental age.

generated as previously reported [17, 18]. Breeding of two *trkC* heterozygote (+/−) mice gave rise to homozygous (−/−) mutant mice. Generation of *trkA* KO mice was conducted in a similar manner [18].

Gonadal Tissue Collection

Heterozygote gene KO mice for *trkA* and *trkC* (−/+, −/+), *trkA* (−/+, +/+), or *trkC* (+/+, −/+) were mated, and embryos were collected from timed pregnant mice at Embryonic Day (E) 13, E14, E17, and E19 of gestation (E0 = plug date). Tails from embryos were collected for genotyping, and embryos were fixed with Histochoice (Amresco, Solon, OH) overnight at 4°C. Later, gonads were dissected and processed for immunohistochemistry, histology, or analysis of apoptosis. All protocols utilized in these experiments were approved by the Animal Care and Use Committee at Washington State University. Approximately 80 embryos were utilized in the present study.

Genomic DNA Isolation and Southern Blot Analysis for Genotyping

To determine the genotype of mice, tail fragments were collected and genomic DNA extracted for Southern blot analysis. Briefly, the tails were treated with proteinase K (0.15 mg/ml) overnight at 55°C. Genomic DNA was separated with saturated NaCl, precipitated with 100% ethanol, and resuspended in buffer. Genomic DNA was cut with *Hinc*II (*trkC*) or *Bam*HI (*trkA*) and run on a gel for Southern blot analysis to determine the genotype for each neurotropin receptor [17–19].

Histology and Immunohistochemistry

Tissues were fixed in Histochoice and embedded in paraffin according to standard procedures [20–22]. The tissue sections (thickness, 3–5 μm) were deparaffinized, rehydrated, heated in a microwave for 15 min, and blocked in 10% (v/v) goat serum for 30 min at room temperature. The germ cell nuclear antigen (GCNA1) antibody was a monoclonal antipeptide antibody generously provided by Dr. George Enders (University of Kansas, Kansas City, KS). The GCNA1 antibody was diluted 1:50 in 10% goat serum and used to detect germ cells through immunohistochemistry [23]. As a negative control, serial sections were put through the same procedure with nonimmunogenic antibody (Sigma, St. Louis, MO). The biotinylated second antibody (Vector Laboratories, Burlington, CA) was diluted 1:300. The secondary antibody was detected by using the Histostain-SP kit (Zymed Laboratories, South San Francisco, CA), and immunohistochemical images were digitized with a slide scanner. Three different experiments were conducted for GCNA1 antibodies for each developmental time. In each experiment, 3 serial sections of 4–5 testes for each developmental age were analyzed. Uniform and reproducible staining was found at each developmental age for GCNA1 in all 3 experiments.

Analysis of Apoptosis

Analysis of apoptotic cells was carried out with the assessment of DNA fragmentation through a TUNEL procedure [24] and utilized the Apoptosis Detection System Fluorescein kit (Promega, Madison, WI). The labeling and detection of fragmented DNA was accomplished with fluorescent probes. Briefly, Histochoice-fixed and paraffin-embedded histological sections [20] were deparaffinized and then fixed in 4% methanol-free formaldehyde solution. They were then treated with a solution of proteinase K (20 μg/ml) for 8 min. Subsequently, they were washed and fixed in 4% methanol-free formaldehyde. The sections were then placed in an equilibrating solution containing a fluorescein tag (fluorescein-12-deoxyuridine triphosphate [fluorescein-12-dUTP]) with nucleotide mix and incubated at 37°C for 1 h in a humidified chamber. This incubation allows the fluorescein to bind and to label fragmented DNA in cells. The reaction was terminated and washed to remove unincorporated fluorescein-12-dUTP. The slides were then processed with a drop of Anti-Fade mounting solution (Molecular Probes, Eugene, OR) and analyzed on a confocal microscope in the Histology Core of the Center for Reproductive Biology at Washington State University. Fluorescently labeled cells were counted per arbitrary fixed area of testis. Positive controls were generated with DNase I instead of proteinase K to determine if enzyme (fluorescein-12-dUTP) was labeling properly, because DNase I causes DNA strand breaks. Negative controls were also generated using no enzyme to determine background fluorescence. At least 3 separate experiments were conducted for each developmental age, with a minimum of 3–6 sections for each experiment. The number of different testis analyzed is stated in *Results*.

Morphological Analysis

Testis sections from genotyped animals at each developmental age were randomly analyzed at 200× magnification for number of germ cells, number of seminiferous cords, area of seminiferous cords, and area of interstitium. The NIH Image program (a public domain image-analysis program from the National Institute of Health, Bethesda, MD) was utilized to determine the area of seminiferous cords and interstitium. Two independent measurements were made for each section included in the analysis. Seminiferous cords were circled within a section to calculate the total number of pixels involved in seminiferous cords. The seminiferous cord area was then subtracted from the total area represented to determine the interstitial area of each section. The data for each averaged area (for a particular genotype) are depicted as the number of pixels per designated testis area. Because the sections were relatively small, serial sections on each slide were utilized to obtain these measurements. Therefore, for each genotype represented, at least 6 sections were utilized to obtain the number of germ cells, cord area, interstitial area, and number of seminiferous cords. The number of testis analyzed is stated in *Results*.

FIG. 2. Histology of fluorescently labeled apoptotic cells in testes from two *trkA* P19 KO mice (**A–D**) and wild-type littermates (**E** and **F**). Magnification is noted in the bottom-left corner in microns. The TUNEL assay was conducted in triplicate on 3 serial sections from the 2 *trkA* gene KO littermates and wild-type littermate controls.
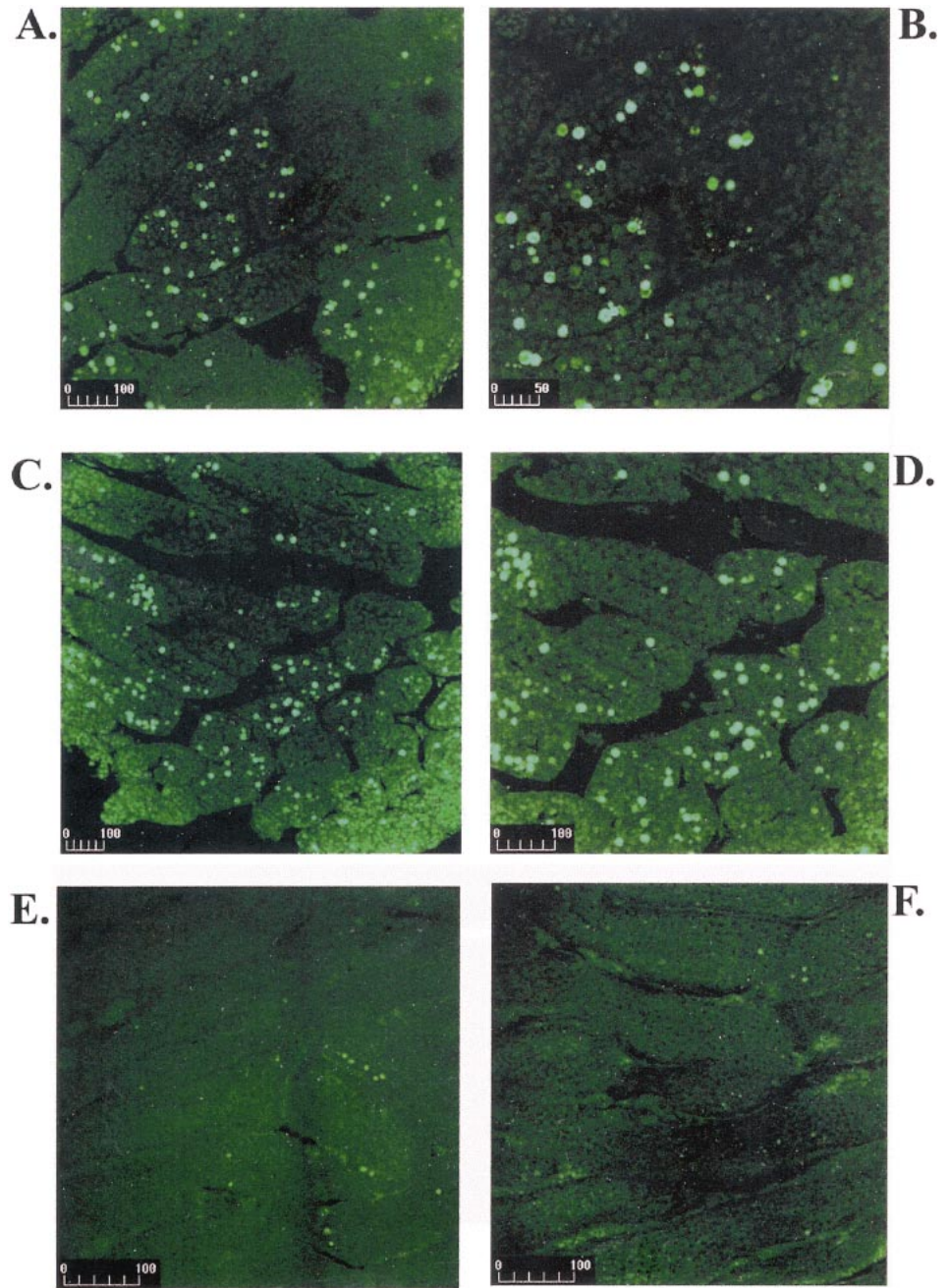
Statistical Analysis

Data were analyzed with the JMP 3.1 statistical analysis program (SAS Institute, Cary, NC). All values are expressed as the mean ± SEM of the parameter measured. Due to variability between testes, the n value was based on testis number and not on embryo number. Each testis section analysis involved a minimum of 6 different areas. Statistical analysis was performed using one-way ANOVA. Significant differences were determined using the Dunnett test for comparison to controls and the Tukey-Kramer honestly significant difference test for multiple comparisons between different treatment groups. Statistical difference was confirmed at $P < 0.05$. Specific comparisons, analyses, and results are presented in the different figure legends.

RESULTS

Testis germ cell numbers were compared between wild-type mice and homozygous gene KO *trkA* and *trkC* mice at E13, E14, E17, and E19 of gestation (Fig. 1). Germ cells were identified by the use of positive staining for GCNA1, which has been reported to stain germ cells during early testis and ovarian development [23]. Germ cell numbers in wild-type mice fluctuated during testis development, with the lowest numbers of germ cells being detected at E13 and E17. Previous studies have reported reduced germ cell numbers at E13 and E17 and have correlated these changes to alterations in germ cell proliferation and apoptosis [24]. Numbers of germ cells varied the most in *trkA* gene KO testes when compared to wild-type testes at each age evaluated. Greater numbers of germ cells were found in *trkA* gene KO mice testes at E13 and E17, with lower numbers found at E14 and E19, compared to wild-type testes at similar time points. Differences were also detected in *trkC* gene KO mice, with an increase in germ cell numbers from those of wild-type mice at E17 and a dramatic reduction at E19
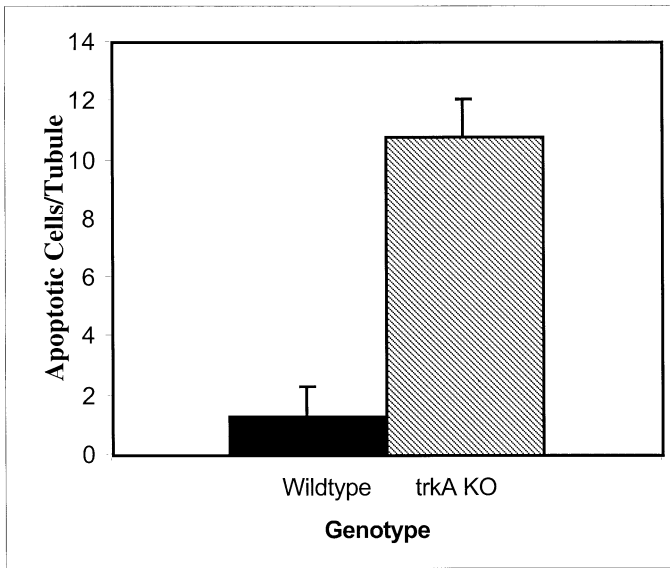
FIG. 3. Quantitative analysis of the number of apoptotic cells/seminiferous tubule for P19 *trkA* gene KO mice compared to wild-type mice at P19 of testis development. At least 25 areas from all 3 triplicate experiments were counted to determine the mean ± SEM, with a significant difference at $P < 0.01$ according to the Dunnett analysis.

(Fig. 1). The alterations observed in germ cell numbers in both the *trkA* and *trkC* gene KO mice testes may be due to changes in the expression pattern of genes that regulate germ cell proliferation, apoptosis, and/or maturation.

To determine if differences in testis cell apoptosis exist during embryonic development, the TUNEL assay [24] was utilized. No differences were detected in the number of cells that had DNA fragmentation between the *trk* gene KO mice and wild-type mice at any of the embryonic time points evaluated (E13, E14, E17, and E19; data not shown). Interestingly, differences were detected in the number of apoptotic cells in testes from two *trkA* gene KO males that survived to Postnatal Day (P) 19 when compared to P19 wild-type controls (Figs. 2 and 3). A 10-fold increase was found in the number of cells that contained DNA fragmentation per seminiferous tubule in the *trkA* P19 mice testes when compared to wild-type mice (Fig. 3). The cells that contained the DNA fragmentation appeared to be spermatocytes (Fig. 2) based on morphology and cellular location. Analysis could not be conducted in postnatal gonads from *trkC* KO mice, because these mice did not survive past P0. Therefore, the *trkA* gene appears to be important in germ cell development, survival, and/or maturation.

The number of seminiferous cords per area of testis had a trend for a reduction in *trkC* KO mice at E13 but was only significantly different at E14 of testis development (Fig. 4). Statistical analysis was only performed within a specific age group and not between the different ages. In addition to that in *trkC* KO mice, a reduction was found in the number of seminiferous cords in *trkA* gene KO mice at
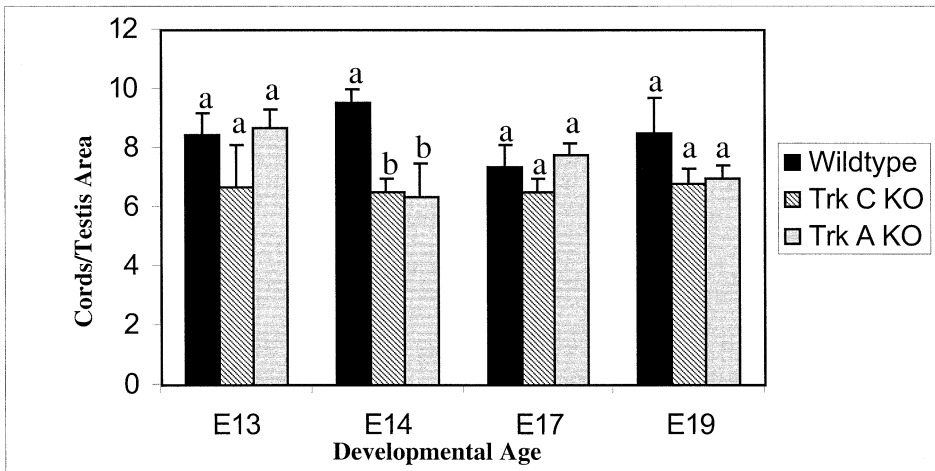

FIG. 4. Number of seminiferous cords per testis area in wild-type, *trkA* gene KO, and *trkC* gene KO mice at E13, E14, E17, and E19 of testes development. At least 6 different areas were utilized for measurements in each testis. A minimum of 2 different testes were analyzed for each genotype at each developmental stage. The mean ± SEM are presented with different letter superscripts over bars representing statistical differences at $P < 0.05$ within a developmental age.
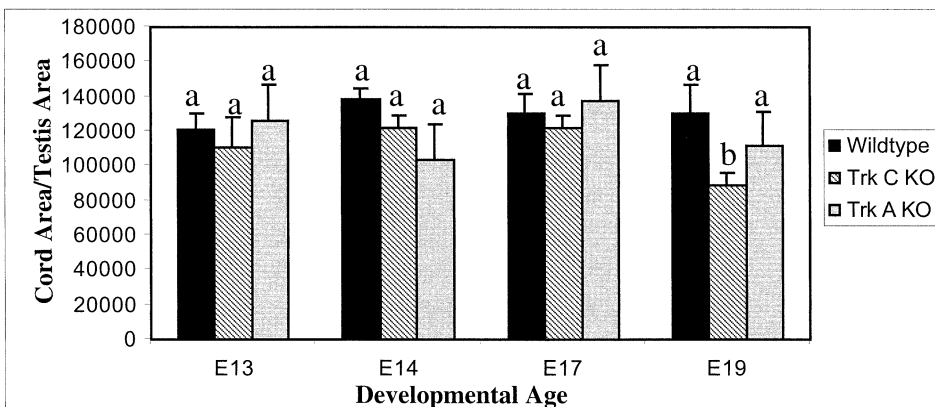

FIG. 5. Area of seminiferous cords per testis area in wild-type, *trkA* gene KO, and *trkC* gene KO mice at E13, E14, E17, and E19 of testes development. At least 6 different areas were utilized for measurements in each testis. A minimum of 2 different testes were analyzed for each genotype at each developmental stage. The mean ± SEM are presented with different letter superscripts over bars representing statistical differences at $P < 0.05$ within a developmental age.

FIG. 6. Area of interstitium per testis area in wild-type, *trkA* gene KO, and *trkC* gene KO mice at E13, E14, E17, and E19 of testes development. At least 6 different areas were utilized for measurements in each testis. A minimum of 2 different testes were analyzed for each genotype at each developmental stage. The mean ± SEM are presented with different letter superscripts over bars representing statistical differences at $P < 0.05$ within a developmental age.
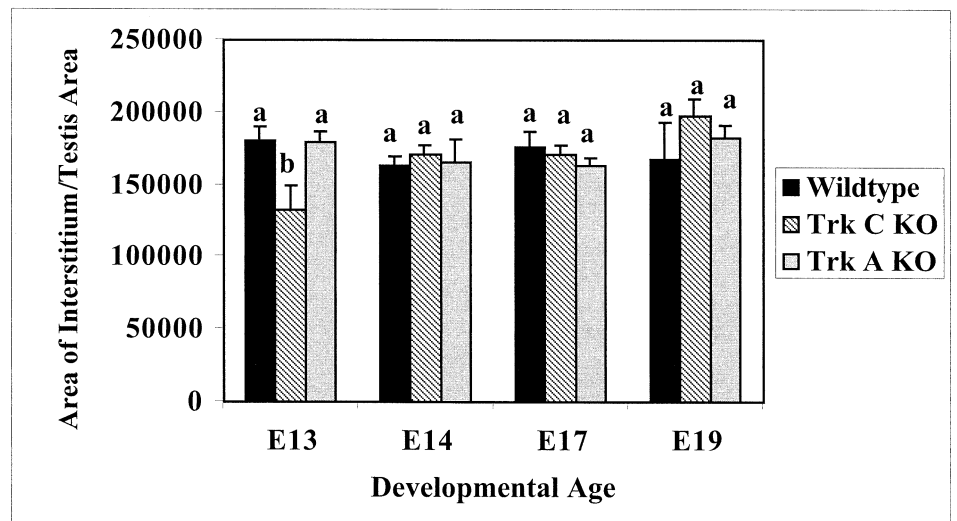

E14 (Fig. 4). By E17 and E19 of testis development, no differences were observed in the number of seminiferous cords in either *trk* gene KO mice (Fig. 4). All the *trk* receptors are expressed at this stage of testis development [15]. Therefore, both *trk* genes may be important early in testis development for initiation of seminiferous cord formation.

The area of seminiferous cords had a trend for a slight reduction in *trkC* gene KO mice testes at E13, E14, and E17 of testis development, but a statistically significant re-

FIG. 7. Histology of E13 testes from wild-type (**A**, **E**, and **G**), *trkA* gene KO (**B**, **F**, and **H**), and *trkC* gene KO (**C** and **D**) mice. Sections were stained with hematoxylin (except **C**), and the germ cells were immunostained (brown/reddish) for GCNA1 (except **D**). Testis (T) and mesonephros (M) are shown, with the broken line indicating the separation between the two. Data are representative of a minimum of 3 different experiments. Magnification ×100 (**A–C**), ×200 (**D–F**), and ×400 (**G** and **H**).
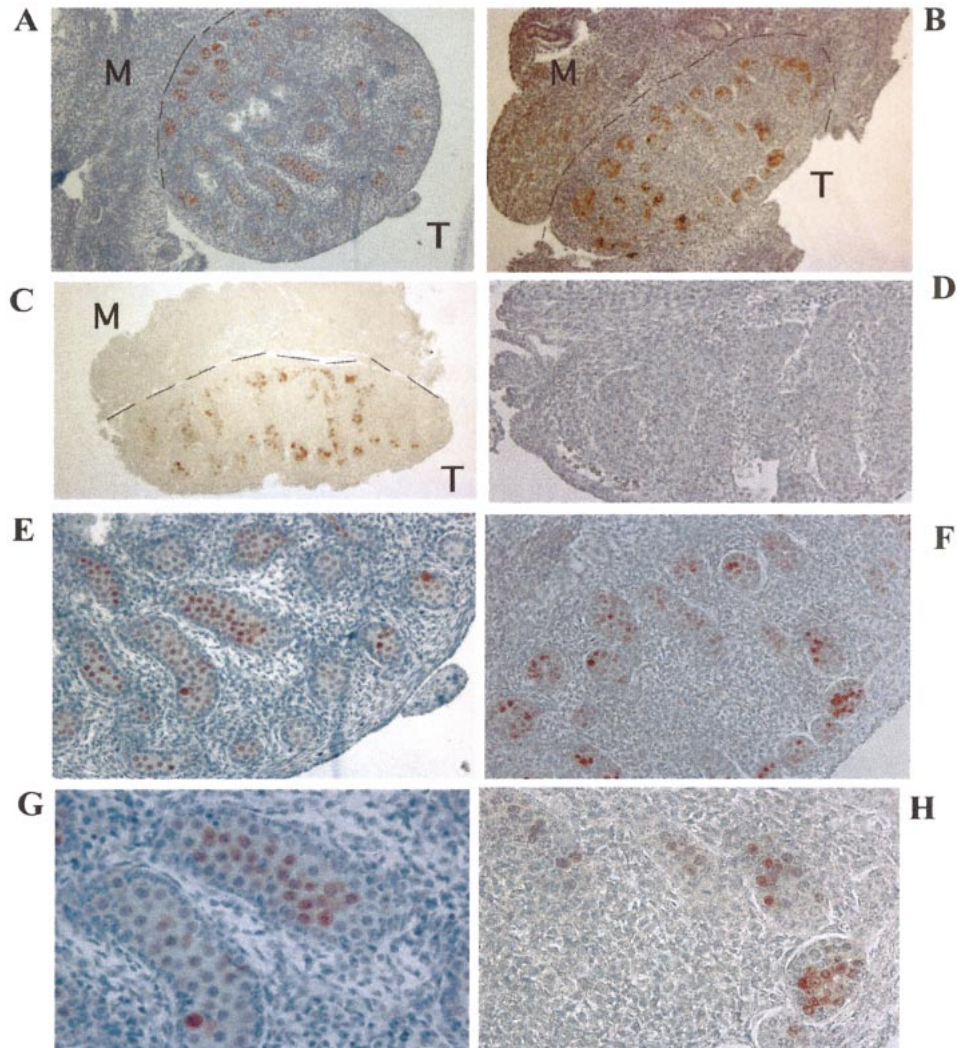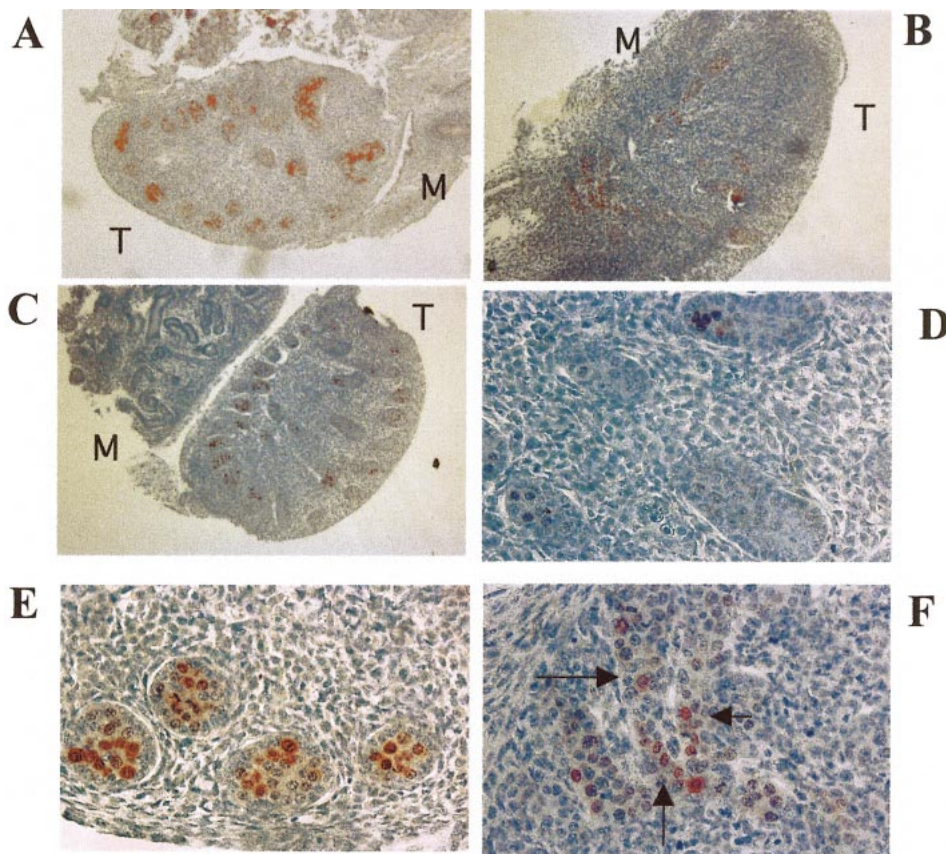
FIG. 8. Histology of E14 testes from wild-type (**A** and **E**), *trkA* gene KO (**B** and **F**), and *trkC* gene KO (**C** and **D**) mice. Sections were stained with hematoxylin, and germ cells were immunostained (brown/reddish) for GCNA. Testis (T) and mesonephros (M) are shown. Arrows indicate a branched cord in **F**, whereas normal cords are shown in **E**. Magnification ×100 (**A–C**) and ×400 (**D–F**).

duction was found only at E19 (Fig. 5). At E19, *trkC* gene KO mice also had reductions in seminiferous cord area when compared to wild-type mice. In contrast, there appeared to be no differences in the area of seminiferous cords in the *trkA* gene KO mice when compared to wild-type mice at any of the ages evaluated. Trends of a slight reduction at E14 and E19 were found in *trkA* KO compared to wild-type testes, but this reduction was not statistically different (Fig. 5). Therefore, the *trkC* receptor appears to be important in the proliferation of cells within the seminiferous cords that, when absent, result in reductions in seminiferous cord area at E19.

Significant reductions in the area of interstitium were found in the *trkC* gene KO mice testes when compared to wild-type mice testes at E13, but not at other ages, during testis development (Fig. 6). The *trkA* gene KO mice testes did not have a significant influence on the interstitium at any stage of development examined (Fig. 6).

In addition to the quantitative differences in testis morphology described above, qualitative differences in *trk* gene KO and wild-type mice testis morphology were observed. At E13 of gestation, the testes from wild-type mice appeared to be at a later stage of development when compared to testes from either *trk* gene KO testes (Fig. 7, B and C). The *trkA* and *trkC* gene KO testes were still tubular in shape, and the mesonephros had not started to differentiate into other portions of the male reproductive tract, such as the epididymis. In contrast, the testes from the wild-type littermate had become rounded, and the mesonephros had started to differentiate into an epididymis (Fig. 7A). Cord morphology was not grossly different (Fig. 7, D–H). In addition to these morphological differences at E13, there appeared to be increased branching of seminiferous cords in

the *trkA* gene KO mice testes at E14 (Fig. 8F). Because tail somites were counted to define the specific age of the embryos, it does appear that the observed delay in development of *trkA*- and *trkC*-deficient testes is attributable to a specific gene deficiency rather than to developmental variations within litters. Increased branching of seminiferous tubules was also demonstrated in the *trkA* gene KO mice that survived to P19 of development (data not shown). Therefore, the *trkA* or *trkC* receptors may be important in the early stages of testes differentiation, and absence of the *trkA* or *trkC* gene may result in delayed testis development and increased seminiferous cord abnormalities.

DISCUSSION

The objective of analyzing gonads from the *trkA* and *trkC* gene KO mice was to determine the effects of the absence of these neurotropin receptor genes on testis differentiation and growth. Previous data from our laboratory in the rat have described a potential role for the neurotropins in testis morphogenesis and growth [14, 15]. Specifically, NT3 and its receptor *trkC* have been implicated in the process of seminiferous cord formation in the rat testis. Observations suggest that, on sex determination, Sertoli cells produce NT3, which acts as a chemotactic agent to promote preperitubular cell migration from the mesonephros to induce cord formation [14, 15]. In addition to NT3 binding to the *trkC* receptor, additional experiments in the nervous system have shown that NT3 can bind to and elicit effects through the *trkA* receptor in neurons [25, 26]. Therefore, the present study examined both *trkC* and *trkA* gene KO mice and their potential effects on testis development. Although the number of animals was low in several of the

developmental age groups, multiple testes were analyzed, and the phenotypes were consistent. To control for variation between testes, the testis number was used to determine the n value. The low numbers of animals at some embryonic ages is a limitation that needs to be considered in data interpretation.

The most dramatic effect of either *trkA* or *trkC* gene KO mice was the alteration observed in germ cell numbers during embryonic development of the testis. The rebound of the germ cell number during later stages of embryonic development is speculated to involve, in part, the onset of other compensatory growth regulators. The differences in germ cell numbers between *trkA* and *trkC* gene KO mice suggest that the pattern of germ cell proliferation and/or apoptosis may be altered from that of wild-type mice. Subsequent experiments to measure differences in cellular apoptosis during embryonic development did not reveal differences in *trk* KO and wild-type mice. However, dramatic differences were demonstrated in cells of *trkA* P19 gene KO testes when compared to wild-type testes. The increased cell apoptosis identified appeared to be primarily in spermatocytes. Therefore, *trkA* may be important in the maturation of these spermatocytes during spermatogenesis or be a survival factor in these cells to reduce apoptosis.

Another explanation for the differences in germ cell numbers during embryonic development may be due to a reduced cellular proliferation or migration due, in turn, to a delay in testis differentiation. This apparent delay in development was noted in the E13 *trkA* and *trkC* gene KO testes (Fig. 7) and in the E14 *trkA* gene KO testes (Fig. 8) when compared to the littermate wild-type testis at the same developmental age (Figs. 7 and 8). The delay in morphological differentiation from a tubular into a round testis and the differences detected in the mesonephros stage of differentiation suggest that the *trk* gene KO mice testes are not at the same stage of testis development. Therefore, the pattern of germ cell proliferation and apoptosis may be shifted several days in the *trk* KO mice, which may explain the differences in germ cell numbers at the developmental time points evaluated. Tail somites were counted both within and between litters to determine the specific age of the embryos; therefore, it does appear that these *trkA* and *trkC* gene KO testes are developmentally delayed when compared to their littermate, wild-type counterparts.

Nerve growth factor has been depicted as a survival factor for both neuronal cells [5, 25, 26] as well as germ cells [6] of the testis. Therefore, it is not surprising that absence of the NGF receptor may affect germ cell growth and survival. Recent studies analyzing ovaries of mice carrying null mutations of both NT4 and BDNF demonstrated that these neurotropins are required for primordial follicle growth, whereas NGF-deficient mice have decreased formation of both primary and secondary preantral follicles [27]. Thus, NGF and *trkA* appear to be critical regulators of germ cell development in both gonads. Observations in the present study suggest a similar role for NT3 in germ cell maturation and survival. Further experiments need to be conducted to determine how NT3 and NGF may interact to influence germ cell development.

In *trkC* gene KO mice, several morphological effects were observed in the embryonic testis. At E13, a reduction was seen in the area of interstitium. This reduction may be the result of inefficient mesonephros cell migration. The *trkC* receptor is present on cells migrating from the mesonephros [15], suggesting that a reduced migration of these cells may have occurred that, in turn, resulted in dispro-

portional numbers of interstitial cells being present at E13. Because no other differences were observed later during gestation, some compensation presumably occurred in the differentiation or migration of these cells at later stages of development.

At E14, a reduction was seen in the number of seminiferous cords that were present in the testis of both *trkC* and *trkA* gene KO testes. A slight reduction was also noted at E13 for both *trkC* KO genotypes. These reductions in the number of seminiferous cords may be the result of a delay in the initiation or formation of cords. Because NT3 can act through both *trkA* and *trkC* [25, 26], both receptors may be important in the process of seminiferous cord formation and compensate for each other. The effect of a double KO of both *trkA* and *trkC* is under investigation. The in vitro observation with organ cultures and the use of *trk* inhibitors also has demonstrated a reduction in cord formation [14], supporting the results of the present study.

The combined observations of the present study demonstrate a role for *trkA* in regulating the number of germ cells within the early stages of testis development and for both *trkA* and *trkC* in the later stages of testis development. Presumably, this regulation is through increasing survival or allowing for critical maturation of germ cells during development. The *trkC* appears to be important for morphogenic events in the testis and potentially may regulate the migration of mesonephric cells and, indirectly, the interstitial area of the testis during early development. In addition, both *trkA* and *trkC* may be important regulators of the number of seminiferous cords. The hypothesis that the neurotropin receptors *trkA* and *trkC* influence somatic and germ cell function during embryonic testis development was supported by the present study. Further studies are necessary to determine the potential interactions of these neurotropin receptors on testis morphology.

ACKNOWLEDGMENTS

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