Glial cell-line-derived neurotrophic factor and its receptors are expressed by germinal and somatic cells of the rat testis

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Abstract

Glial cell-line-derived neurotrophic factor (GDNF) and its receptors glial cell-line-derived neurotrophic factor α (GFR1 α) and rearranged during transformation (RET) have been localized in the rat testis during postnatal development. The three mRNAs, and GDNF and GFR1 α proteins were detected in testis extracts from 1- to 90-day-old rats by reverse transcriptase PCR and Western blotting respectively. The three mRNAs were present in Sertoli cells from 20- and 55-day-old rats, pachytene spermatocytes (PS), and round spermatids (RS). The GDNF and GFR1 α proteins were detected in PS, RS, and Sertoli cells. GDNF and GFR1 α were also detected using flow cytometry in spermatogonia and preleptotene spermatocytes, and in secondary spermatocytes. The localization of GDNF and GFR1 α in germ and Sertoli cells was confirmed by immunocytochemistry. The hypothesis

that GDNF may control DNA synthesis of Sertoli cells and/or spermatogonia in the immature rat was addressed using cultures of seminiferous tubules from 7- to 8-day-old rats. Addition of GDNF for 48 h resulted in a twofold decrease in the percentage of spermatogonia able to duplicate DNA, whereas Sertoli cells were not affected. These results are consistent with a role of GDNF in inhibiting the S-phase entrance of a large subset of differentiated type A spermatogonia, together with an enhancing effect of the factor on a small population of undifferentiated (stem cells) spermatogonia. Moreover, the wide temporal and spatial expression of GDNF and its receptors in the rat testis suggest that it might act at several stages of spermatogenesis.

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Introduction

Spermatogenesis is under the dual control of pituitary gonadotropins and local factors. Follicle-stimulating hormone (FSH) controls the Sertoli cells, which provide structural and nutritional support for the germ cells, and luteinizing hormone controls the production of testosterone by the Leydig cells (Russell *et al.* 1987). Local regulation involves intercellular communications between germ and Sertoli cells, and between Sertoli, myoid, and interstitial Leydig cells through growth factors, including neurotrophic factors such as glial cell-line-derived neurotrophic factor (GDNF; for review, see Parvinen & Ventela 1999).

GDNF and neurturin, artemin and persephin are transforming growth factor β -related neurotrophic factors acting via the same high-affinity receptor, the rearranged during transformation (RET) receptor tyrosine kinase. The signaling receptor complex also includes glycosylphosphatidylinositol-linked co-receptors, the GDNF family receptor α s: glial cell-line-derived neurotrophic factor receptor α (GFR α) 1–4. GFR1 α specifically binds to GDNF and mediates activation of the RET receptor (Jing *et al.* 1996). GDNF has been observed in a number of different cell types and structures of the central

nervous system (Du & Dreyfus 2002), where it stimulates neuronal survival and growth (Heuckeroth *et al.* 1988, Wang *et al.* 2002) and modulates synaptic plasticity (Ribchester *et al.* 1998). GDNF transcripts are also present in non-central nervous system and peripheral organs including the kidneys, lungs, blood, and testes (Suter-Crazzolara & Unsicker 1994, Trupp *et al.* 1995, Suvanto *et al.* 1996).

Although fertile, gene-targeted mice with one GDNF-null allele show depletion of the reserve of stem cells, whereas mice overexpressing GDNF in the testis are infertile and accumulate undifferentiated spermatogonia (Meng *et al.* 2000). These results indicate that GDNF should contribute to the paracrine regulation of spermatogonial self-renewal, differentiation, and/or survival in the mouse. Moreover, since GDNF-overexpressing mice develop testicular tumors on aging, GDNF and its receptors GFR1 α /RET might have a role in the etiology of some testicular cancers (Meng *et al.* 2001). However, transgenesis experiments provide little information about the effect of GDNF on differentiated spermatogonia and no information on the possible involvement of this factor on later steps of spermatogenesis.

Because of the potential importance of the GDNF/RET pathway in male reproductive physiopathology, we determined the localization of GDNF and its two receptors, GFR1 α and RET, during postnatal development of the rat testis. We determined mRNA and protein levels in isolated Sertoli and spermatogenic cells and also used cultures of seminiferous tubules from 7- to 8-day-old rats to examine the effect of GDNF on DNA synthesis by Sertoli cells and type A spermatogonia.

Materials and Methods

Reagents and chemicals

Trizol reagent, restriction enzymes, and Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) were purchased from Invitrogen SARL, pGEM-T Vector System from Promega, pd(N)₆ random hexamer from Amersham Biosciences Europe Group, DNA polymerase from Eurobio (Les Ulis, France), Qiagen Midi kit from Qiagen SA, polyacrylamide and other electrophoresis reagents were from BioRad Life Sciences Group, and nitrocellulose membrane from Schleicher and Schuell (Mantes-la-Ville, France). BCA kit was from Pierce (Perbio Science France, Bezons, France). ECL Plus Western Blotting Detection System was from Amersham Biosciences Europe Group. Collagenase was from Serva (Paris, France) and DMEM/NUT MIX F12 culture medium from Invitrogen SARL. Recombinant GDNF was purchased from PeproTech, Inc. (Tebu-bio, Le Perray-en-Yvelines, France). DakoCytomation SA (Trappes, France) provided anti-vimentin monoclonal and anti-bromodeoxyuridine (BrdU) monoclonal and secondary antibodies, normal serum, and the streptavidine-horseradish peroxidase (HRP) detection system. Anti-GDNF goat polyclonal antibody was from R&D Systems Europe (Lille, France), anti-GFR1 α monoclonal antibody from BD Biosciences (Le Pont-de-Claix, France) and anti-RET antibody (rabbit serum) was produced and characterized by Pelet *et al.* (1998). All other products were purchased from Sigma-Aldrich.

Animals

Male Sprague-Dawley rats ranging from 1 to 90 days (adult) of age were used. All procedures were approved by the Scientific Research Agency (Approval No. 69306) and conducted in accordance with the guidelines for care and use of laboratory animals.

Preparation of Sertoli and germinal cell fractions

Sertoli cells were obtained by collagenase digestion using 20- or 55-day-old rat testes (Weiss *et al.* 1997). The number of contaminating germ cells was reduced to a very low level (about 10%) by several cycles of fragmentation of the cell

clusters through a 19-gauge syringe needle, followed by sedimentation. Vimentin immunoreactivity was used to determine the percentage of germ cells (vimentin negative) contaminating the (vimentin positive) Sertoli cell preparations (Franke *et al.* 1979, Suter *et al.* 1997, Kopecky *et al.* 2005). An aliquot of each Sertoli cell preparation was cytopun onto aminoalkylsilanized slides. The cells were fixed in Bouin's solution, and rinsed twice with ethanol (70%), then once with NaCl 9%. Cell membranes were permeabilized with 0.03% Triton X-100. An anti-vimentin specific monoclonal antibody was used at a dilution of 1:1000. Vimentin immunoreactivity was revealed by a biotin-coupled goat anti-mouse antibody incubated with streptavidine-coupled HRP giving a brown coloration to the Sertoli cells after reaction with diaminobenzidine (DAB). The cell nuclei were then stained with hematoxylin. The percentage of vimentin-negative cells in the Sertoli cell preparations was (mean $\pm$ S.E.M.) 11 ± 1 and $8 \pm 1\%$ in 20- and 55-day-old rats respectively ($n=3$). Pachytene spermatocytes (PS) and round spermatids (RS) were obtained by centrifugal elutriation as previously described (Onoda *et al.* 1991, Weiss *et al.* 1997). Early and middle to late PS were recovered from 90-day-old rat testes, whereas early and middle PS were recovered from 22-day-old rat testes (Godet *et al.* 2000). The purity of the PS and RS fractions was assessed by flow cytometry (see below); $94 \pm 3\%$ of the cells in the PS fractions were 4C cells, $3 \pm 2\%$ were 2C cells and $1 \pm 0.5\%$ were 1C cells ($n=5$). In the RS fractions, $81 \pm 2\%$ of cells were 1C cells, $5 \pm 1\%$ were 2C cells, and $10 \pm 2\%$ were 4C cells ($n=3$). Cell fractions were frozen until processed.

Culture of seminiferous tubule segments from 7- to 8-day-old rats and BrdU incorporation

At 7–8 days of age, the only germ cells are type A spermatogonia, since the first B spermatogonia do not develop until 11 days of age (Boitani *et al.* 1993, Dym *et al.* 1995, Jahnukainen *et al.* 2004). Culture of seminiferous tubule segments was performed as previously described for 21-day-old rat seminiferous tubules (Hue *et al.* 1998, Staub *et al.* 2000, Perrard *et al.* 2003), but omitting testosterone in the culture medium. Indeed, Boitani and co-workers (1993) have shown that, as opposed to FSH, testosterone has no effect on the preservation of the cellular morphology of testicular explants from immature rats. Ovine National Institute of Health FSH-20 was obtained through the National Hormone and Peptide Programme, the National Institute of Diabetes and Digestive and Kidney Diseases, and Dr A F Parlow. Between 20 and 25 animals were used for each culture. Culture was started at day 0 in the absence or presence of GDNF (2.5, 10, or 50 ng/ml) (Linnarsson *et al.* 2001, Powers *et al.* 2001). In order to focus on the S-phase of spermatogonial mitosis (not S-phase of meiosis), we limited the time of culture to 48 h and added BrdU (1 μ M) to the culture medium during the last 20 h. On day 2, the cells were detached from the culture dishes by trypsinization. Cell viability was assessed by Trypan

Blue exclusion. Sertoli (vimentin positive) and germ cells (vimentin negative) were sorted by flow cytometry as described below, collected on slides, and BrdU was revealed by immunodetection (see below).

Immunolabeling and flow cytometric analyses

Cultured cells from seminiferous tubules of 7- to 8-day-old rats or cells isolated from freshly prepared seminiferous tubules of 15- or 90-day-old rat testes (ten and four animals

respectively) by collagenase and trypsin treatment (Weiss *et al.* 1997, Godet *et al.* 2000) were fixed in 70% ethanol, then immunolabeled with an anti-vimentin antibody followed by an anti-mouse phycoerythrin (PE) conjugated antibody as previously described in details (Godet *et al.* 2000, 2004).

Cell sorting of cultured Sertoli cells and spermatogonia Hoechst 33342 at a final concentration of 20 µg/ml was added to the immunolabeled cells from cultured tubule

Figure 1 Representative flow cytometry analysis of the cell populations of (A) cultured seminiferous tubule segments of 7–8-day-old rats or of (B–E) seminiferous tubules from pubescent rats and (F–J) GFR1α labeling of these latter germ cell populations. Distribution of vimentin-positive diploid (R1) and tetraploid (R2) Sertoli cells or vimentin-negative diploid (R3), tetraploid (R4), and haploid (R5) populations of germ cells according to their ploidy (A and B). The different germ cell populations of pubescent rats were subsequently characterized according to their forward light scatter (FSC) and side angle light scatter (SSC) (C–E). Isotype control (---) and GR1α labeling (—) (F–J) of the different germ cells populations of pubescent rats. R5, round spermatids; R6, spermatogonia and preleptotene spermatocytes; R7, secondary spermatocytes and doublets of round spermatids; R8, young spermatocytes; R9, middle to late spermatocytes.

segments from 7- to 8-day-old rats to assess their DNA content. The vimentin-positive 4C and 2C somatic cells and the vimentin-negative 4C and 2C germ cells were separated by bivariate analysis (Fig. 1A): DNA content/vimentin, and sorted on slides (500 cells per spot, five spots per slide) for BrdU detection. Analyses were performed using a fluorescence activated Vantage SE cell sorter (FACS; BD Biosciences) equipped with a 50 mW argon laser, tuned to 488 nm, and an Innova 300 ion multilined/UV laser tuned to UV. Emission fluorescence was measured with a BP 275/26 filter for PE and BP 424/44 filter for Hoescht. Cell sorting were performed with Clone Cyt Plus software and data acquisition with CellQuest Pro 3 software, both from Beckton-Dickinson Le Pont-de-Claix, France.

Quantification of GDNF and GFR1 α in freshly isolated Sertoli cells and in germ cells

To detect and quantify GDNF and GFR1 α in Sertoli cells, PS, RS, and in germ cell populations, which are difficult to purify (spermatogonia and preleptotene spermatocytes, young spermatocytes, and secondary spermatocytes), cells from seminiferous tubules of 15- or 90-day-old rats were further incubated overnight at 4 °C with the specific antibody (15 μ g/ml for anti-GDNF antibody and dilution at 1:100 for anti-GFR1 α antibody), then with a fluoresceine isothiocyanate (FITC)-conjugated secondary antibody (anti-goat or anti-mouse for GDNF and GFR1 α respectively). Finally, Hoescht 33342 was added to the immunolabeled cells at a final concentration of 20 μ g/ml. The reactions allowed the quantification of GDNF in germinal and Sertoli cells and of GFR1 α only in germinal cells, as GFR1 α labeling in Sertoli cells was revealed by both PE- and FITC-conjugated antibodies (both anti-vimentin and anti-GFR1 α antibodies being mouse monoclonal). For each experiment (repeated twice), negative controls were performed with non-immune sera to determine background fluorescence for each cell population. Emission fluorescence was measured with a BP 530/30 filter for FITC, a BP 275/26 filter for PE, and a BP 424/44 filter for Hoechst 33342. Results were expressed relative to the FITC fluorescence levels observed in middle/late PS, which were given an arbitrary value of 100. Analyses were performed as described previously (Godet *et al.* 2000, 2004). Five data parameters were acquired; linear forward light scatter (FSC) and linear side angle light scatter (SSC), which roughly represent cell size and cellular granularity respectively, logarithmic PE and FITC to detect immunolabeling, and linear Hoescht to measure the DNA content of the different populations of cells. The vimentin-negative germ cells and vimentin-positive somatic cells were separated by bivariate analysis, DNA content/vimentin. For germ cells, three populations with 4C, 2C, and 1C DNA content were selected; the bivariate FSC/SSC analysis identified five populations as described by Godet *et al.* (2000, 2004) (Fig. 1B–J). Contaminating events such as debris

and clumped cells were eliminated from the analysis. Each acquisition was performed on 50 000 events.

Immunocytochemistry of BrdU on sorted cells and counting

For BrdU detection, the cells were first treated with 0.03% Triton X-100, then with 3% hydrogen peroxide and with 0.07 M NaOH in 50% ethanol for 5 min (DNA denaturation). Incubation was then performed with an anti-BrdU antibody diluted at 1:100 for one night at 4 °C. The staining reaction was performed using 3-amino-9-ethylcarbazole or DAB. Cells were counterstained with hematoxylin. BrdU-positive and BrdU-negative cells were counted under microscopic examination.

Electrophoresis and Western blotting

Proteins were extracted by homogenizing tissues (two pools of testes, at least three animals per age) in 50 mM Tris-HCl (pH 7.4), 0.5 mM EDTA and 2% v/v of a protease inhibitor cocktail. Elutriated PS and RS and Sertoli cell fractions were lysed by sonication in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1 mM Na₃VO₄, 10 mM NaF, 10 mM β -glycerophosphate, 2 mM EDTA and 2% v/v of a protease inhibitor cocktail. Protein concentrations were determined with BCA kit from Pierce. SDS-PAGE separation was carried out according to the method of Laemmli (1970) on polyacrylamide gels (9% for RET detection, 12% for other immunodetections). One hundred and fifty micrograms protein per well were loaded for RET and 50 μ g for other determinations. After electrotransfer, nitrocellulose membranes were stained with Ponceau Red S to verify that the same amount of protein was loaded for each well. Reactions were carried out in 1 $\times$ PBS–0.05% Tween. The membranes were incubated with the specific antibodies diluted as follows: anti-vimentin antibody 1:3000; anti-GFR1 α antibody 1:5000; anti-RET antibody 1:2500; anti-GDNF antibody was diluted to 0.1 μ g/ml. After incubation with appropriate secondary HRP-conjugated antibodies, detection was performed by chemiluminescence (ECL Plus Western Blotting Detection System; Amersham).

Detection of GFR1 α and GDNF by fluorescence confocal microscopy in isolated testicular cells

Freshly prepared germ cell and Sertoli cell fractions (Weiss *et al.* 1997, Godet *et al.* 2004) were immunolabeled as for flow cytometry analysis except (1) for vimentin revelation, the LSAB kit (DakoCytomation SA) and DAB or a FITC-conjugated secondary antibody were used; (2) GDNF and GFR1 α were revealed by PE- or cyanin 3-conjugated secondary antibodies. Cells were mounted in Gel/Mount (Biomedica Corp., Foster City, CA, USA). Scanning fluorescence images were acquired using a confocal laser unit

(Leica TCSSP₂) coupled to a microscope equipped with a $\times 63$ oil immersion objective. In control reactions, the first antiserum was replaced by normal (non-immune) IgG used at the same concentration as the first antiserum.

mRNA extraction and RT-PCR

Total RNA was prepared from frozen tissues (testes and pituitary) or cell pellets using Trizol reagent. RT was performed on at least two different pools of testes or cell pellets by M-MLV RT following the manufacturer's instructions. PCR (denaturation 94 °C, annealing 62 °C, elongation 72 °C, 30 cycles) was performed in an Eppendorf thermocycler using the pairs of forward (F) and reverse (R) primers previously used by Urbano *et al.* (2000) to detect GDNF, GFR1 α , and RET cDNA in anterior pituitary gland tissue (used as a positive control in our experiments): *GDNF*: (F): 5' ATG AAG TTA TGG GAT GTC GTG GCT 3' (exon 1); (R): 5' GGG TCA GAT ACA TCC ACA CCG (exon 2). *GFR1 α* : (F): 5' GCA CAG CTA CGG GAT GCT CTT CTG 3'; (R): 5' GTA GTT GGG AGT CAT GAC TGT GCC AAT C 3'. *RET*: (F): 5' CGG CAC ACC TCT GCT CTA TG 3' (exon 2); (R): 5' CTG GAG GAA GAC GGT GAG CA 3' (exon 3).

These pairs of primers produced specific bands of 640, 286, and 235 bp for GDNF cDNA, GFR1 α , and RET respectively. The cDNAs were ligated in pGEM-T vector and plasmids were amplified (Qiagen Midi kit) and sequenced (Biofidal, Vaulx-en-Velin, France).

Statistical analysis

ANOVA was used to compare data from more than two groups. Paired *t*-test was used to assess statistical differences between treated cells and their corresponding control cells.

Results

GDNF, GFR1 α , and RET proteins and mRNAs during postnatal development of the rat testis

The specificity of the commercial anti-GDNF and anti-GFR1 α antibodies was tested by Western blotting: only specific bands at the expected size were observed and no non-specific bands (see below and data not shown).

GDNF protein and mRNA (Fig. 2A–C), GFR1 α protein and mRNA (Fig. 2D and E) and RET mRNA (Fig. 2F) were detected in the rat testis from 1 to 90 days postnatally. The GDNF protein had an apparent molecular weight (MW) of 15 kDa under reducing conditions (Fig. 2A and B) and of 30 kDa under non-reducing conditions (Fig. 2B) as in the rat brain (Lin *et al.* 1993). The GFR1 α protein migrated as a doublet with a MW of 55–60 kDa (Fig. 2D). The RET protein was not detected in total testicular protein extracts (not shown). The mRNAs corresponding to these proteins were detected at the expected sizes: 640, 286, and 235 bp for

GDNF (Fig. 2C), GFR1 α (Fig. 2E), and RET (Fig. 2F) respectively at all ages.

Cellular localization of GDNF and GFR1 α proteins and mRNA, and RET mRNA in the rat testis

Localization of GDNF and GFR1 α proteins and mRNA, and RET mRNA in purified fractions of germinal and Sertoli cells PS and RS fractions recovered by elutriation from adult rat testes were free from detectable somatic cell contamination, as shown by the absence of a vimentin signal (Fig. 3A). GDNF (Fig. 3B and C), GFR1 α (Fig. 3D and E) proteins and mRNAs, and RET mRNA (Fig. 3F) were detected in PS (from 22- and 90-day-old rats), and RS (from 90-day-old rats), and in Sertoli cells (from 20- and 55-day-old rats). The GFR1 α protein appeared as a single band with a MW of 55 kDa in PS and SR, whereas two bands with a MW of 55–60 kDa were present in Sertoli cells (Fig. 3D).

Localization and relative amounts of GDNF and GFR1 α proteins in germinal and Sertoli cells assessed by flow cytometry

GDNF (Fig. 4A) and GFR1 α (Fig. 4B) proteins were detected in the five germ cell populations obtained by flow cytometry in the adult rat (see Fig. 1): (1) spermatogonia and preleptotene spermatocytes, (2) young spermatocytes, (3) middle to late PS, (4) secondary spermatocytes and doublets of RS, and (5) round and elongating spermatids. Likewise, at the age of 15 days, both proteins were detected in the populations corresponding to spermatogonia + preleptotene spermatocytes and young spermatocytes, the only germ cells present at this age. GDNF was also detected in Sertoli cells at both ages.

Localization of GDNF and GFR1 α in germinal and Sertoli cells by immunocytochemistry and scanning fluorescence microscopy

As a confirmation of the results presented above, the immunoreactivity of GFR1 α (Fig. 5) and GDNF (Fig. 6) was observed in both Sertoli and germ cells (spermatogonia, PS, and RS). As expected, the labeling of GFR1 α was localized at the cell periphery, while that of GDNF appeared within the cells.

In vitro effect of GDNF on DNA synthesis (labeling index) of Sertoli cells and type A spermatogonia in the immature rat

Seminiferous tubules from 7- to 8-day-old rats were cultured for 2 days in the absence or presence of different concentrations of GDNF, and the numbers of somatic and germ cells in S-phase were determined by BrdU labeling after cell sorting (see Fig. 1A). Cell viability was close to 95% throughout the culture period, irrespective of the absence ($5.1 \pm 0.6\%$ of dead cells) or presence ($4.9 \pm 0.6\%$ of dead

Figure 2 Detection of GDNF, GFR1 α , and RET mRNA and proteins during postnatal development of the rat testis. GDNF (A and B) and GFR1 α (D) proteins were immunodetected in total testicular protein extracts at the ages of 1, 5, 8, 10, 12, 20, 28, and 90 days by Western blotting. For GDNF, recombinant GDNF was used as a positive control (A) and migration was performed under reducing (R) conditions for samples corresponding to ages from 1 to 28 (A) and 90 days (B) and under non-reducing (NR) conditions for the latter sample (B). For GFR1 α (D), migration was performed under reducing conditions. The molecular weights are indicated on the left. mRNAs coding for GDNF (C), GFR1 α (E) and RET (F) were detected by RT-PCR using total RNA from rat testes at the ages of 1, 5, 8, 15, 20, 45, and 90 days. Negative controls (no reverse transcriptase, no RT) were performed with RNA from testes at the ages of 8 and 90 days, and without sample (H₂O). cDNA length is indicated on the left in bp. At least two RNA preparations and three protein preparations were tested with similar results.

Figure 3 Cellular localization of vimentin, GDNF, GFR1 α , and RET in the rat testis. The presence of the proteins vimentin (A), GDNF (B), and GFR1 α (D) was investigated by Western blotting in the following fractions: pachytene spermatocytes (PS) and round spermatids from 90-day (d)-old rats (90d PS and 90d RS respectively) and Sertoli cells from 20-day-old rats (20d S). The presence of GDNF and GFR1 α proteins was also investigated in PS from 22-day-old rats (22d PS) (B and D). The molecular weights are indicated on the left. mRNAs coding for GDNF (C), GFR1 α (E) and RET (F) were detected by RT-PCR using total RNA from testicular tissue from 8-day (d)-old rats (8d T); PS from 22- and 90-day-old rats (90d PS and 22d PS); round spermatids obtained from 90-day-old rats (90d RS); and Sertoli cells from 20- and 55-day-old rats (20d S and 55d S). Pituitary gland (P) was also tested. Negative controls (no RT) were performed with RNA from 8-day (8d T)-old rat testes, adult PS (90d PS), and without sample (H₂O). cDNA length is indicated on the left in bp. At least two RNA preparations and three protein preparations were tested with similar results.

cells) of GDNF. The proportion of somatic cells in S-phase was not changed in the presence of GDNF (Fig. 7A). By contrast, GDNF induced a significant decrease in the proportion of BrdU-labeled spermatogonia (Fig. 7B) when used at 10 ng/ml (0.19 ± 0.04 versus 0.34 ± 0.08), or 50 ng/ml (0.22 ± 0.08), both $P < 0.01$, but not at 2.5 ng/ml. Similar results were observed after 5 days of culture for both somatic and germ cells (data not shown).

Discussion

Expression and localization of GDNF and its receptors in the rat testis during postnatal development

This is the first time, to our knowledge, that the localization of GDNF and its receptors, GFR1 α and RET, has been investigated in different cell types of the rat testis in the same

Figure 4 Determination of relative GDNF and GFR1 α levels in germ and Sertoli cells separated by flow cytometry. Relative GDNF (A) and GFR1 α (B) levels in germ cells, and relative GDNF levels in Sertoli cells were determined from 15- (gray) and 90- (black) day-old rats. Five germ cell populations were obtained from 90-day-old rat testes: (1) spermatogonia (SG) and preleptone spermatocytes (preL), (2) young spermatocytes (yS), (3) middle to late PS (m-l PS), (4) secondary spermatocytes (SII) and doublets of round spermatids (dRS), and (5) round (RS) and elongating spermatids (ES). At the age of 15 days, the only germ cells present were SG+preL and yS. The level of GFR1 α was not determined in Sertoli cells (S) as both anti-vimentin and anti-GFR1 α were monoclonal antibodies. Results shown are the mean of two independent experiments and are expressed relative to the level of fluorescence observed in m-l PS, which were given an arbitrary value of 100.

study throughout postnatal development. Most of our findings were obtained at the protein level by several techniques and were confirmed at the mRNA level. GDNF and GFR1 α proteins and mRNA and RET mRNA were observed from 1 to 90 days of age, and were present in Sertoli cells, PS, and RS. GDNF and GFR1 α proteins were also detected in spermatogonia and preleptone spermatocytes and in secondary spermatocytes.

In the mouse, GDNF mRNA and protein were first localized only in Sertoli cells (Meng *et al.* 2000, Viglietto *et al.* 2000). However, more recently, GDNF mRNA was reported in gene expression profiles of germ cells from spermatogonia to spermatids (Yu *et al.* 2003). In the human testis, GDNF

protein is present in both somatic (Sertoli and Leydig) and germ (spermatocytes, RS) cells (Davidoff *et al.* 2001). In the mouse, GFR1 α was shown to be restricted to a subset of spermatogonia (Meng *et al.* 2001, Dettin *et al.* 2003, Buageaw *et al.* 2005, Hofmann *et al.* 2005), whereas RET was detected in more differentiated germ cells (up to spermatids) (Cao *et al.* 1996, Creemers *et al.* 2002). By contrast, in human, GFR1 α immunoreactivity was reported in Sertoli and Leydig cells, but not in germ cells (Davidoff *et al.* 2001). It might be argued that the cell fractions used in our studies are not 100% pure. However, such an explanation cannot hold for all the differences observed between the mouse and the rat. (1) It is inconceivable that the presence of the protein GFR1 α in PS, RS, or Sertoli cells could be explained by a contamination of these fractions by a sufficient number of GFR1 α expressing spermatogonial stem cells which are in very low number in the testis (Orwig *et al.* 2002, Dettin *et al.* 2003, Ryu *et al.* 2003, 2005, Buageaw *et al.* 2005, Hofmann *et al.* 2005). (2) The molecular forms of GFR1 α are not identical in Sertoli and germ cells (see below). (3) The contamination of the germ cell fractions by Sertoli cells was below the threshold of detection of the intermediate filament vimentin. (4) The results obtained with the methods used to detect GDNF and GFR1 α at a one cell level (FACS analysis and immunocytochemistry) corroborated quite well with the biochemical approaches. Taken together, these results might indicate species variability in the localization of the GDNF pathway in the testis.

It might be objected that it is not logical to evaluate Sertoli cells from 20-day-old rats and germ cells from 90-day-old rats. However, the results obtained with the Sertoli cells from 55-day-old rats (an age by which all the germ cell populations, including postmeiotic germ cells, are present in the testis) were quite similar to those obtained with Sertoli cells from younger animals (see Fig. 3). Moreover, for those proteins and cell populations that could be studied by cytometry, the results were close at 15 and 90 days (see Fig. 4).

Aside from the localization of the GDNF pathway in the rat testis, two points deserve further discussion. First, GFR1 α , which is a protein of 468 amino acids with an N-terminal hydrophobic domain characteristic of a secretory signal peptide, was detected in testis extracts as a doublet (MW 55–60 kDa) as in the rat brain and pituitary, where the upper band was suspected of being related to the precursor and the lower band to the mature form (without the signal peptide) (Matsuo *et al.* 2000). Surprisingly, the upper band (MW 60 kDa) was present in Sertoli cells but not in germ cells, which exhibited only the lower band (MW 55 kDa). The significance of the presence of the precursor in Sertoli cells is unknown and needs further investigation. Secondly, it should be emphasized that the Western and the Northern blotting methods, as used in the present study to detect GDNF, GFR1 α or RET proteins or mRNAs, are in no sense quantitative methods. Hence, no conclusion about the relative levels of either protein or mRNA between the different samples can be made. This also holds true for

Figure 5 Scanning immunofluorescence immunocytochemical localization of GFR1 α in freshly prepared spermatogonia, PS, RS, and Sertoli cells. GFR1 α was detected in (a) spermatogonia (vimentin negative) and Sertoli cells (vimentin positive). (b) Transmission microscopy of (a) shows the vimentin labeling of the Sertoli cells (revealed with DAB). (c) PS, (d) RS, and (e) corresponding control (reaction with control mouse IgG for RS). All bars = 10 μ m.

a comparison between the relative levels observed by FACS analysis (on a per cell basis) and those obtained by Western or Northern blots (on a protein- or RNA-amount basis). Taken together, the above results support the view that GDNF, GFR1 α , and RET exhibit overlapping patterns of expression

in the rat testis and are constitutively expressed. Such an overlapping pattern for GDNF and its receptors has been found in other tissues such as the retina, where GDNF can stimulate fiber growth from ganglion cells (Karlsson *et al.* 2002). This suggests that GDNF participates in auto and/or

Figure 6 Scanning fluorescence immunocytochemical localization of GDNF in freshly prepared spermatogonia, PS, RS, and Sertoli cells. GDNF was detected in (a) spermatogonia (vimentin negative) and Sertoli cells (vimentin positive). Note that vimentin was revealed with a FITC conjugated secondary antibody giving a yellow staining when both GDNF (red) and vimentin (green) are expressed in the same cells. (b) PS, (c) RS, (d) corresponding control (reaction with control goat IgG for PS). All bars = 10 μ m.

paracrine cellular mechanisms in the testis and may influence both somatic and germ cell functions throughout postnatal development.

Biological effect of GDNF in seminiferous tubules in vitro

In different organs, GDNF may have either stimulatory or inhibitory effects on DNA synthesis and cell proliferation *in vitro* through RET/GFR1 α signaling. For example, it stimulates the proliferation of enteric neurons and glial progenitors (Heuckeroth *et al.* 1988), but inhibits the proliferation of the embryonic carcinoma cell line NT2/D1 (Baldassarre *et al.* 2002). Human adrenal chromaffin cells respond to GDNF by differentiation (Powers *et al.* 1998) or mitogenesis (Powers *et al.* 2001) according to their *in vitro* environment.

In the rat testis, Sertoli cells stop proliferating around 15 days of age (Steinberger & Steinberger 1971, 1977). In our study,

using 7- to 8-day-old rat seminiferous tubule cultures, the labeling index of Sertoli cells was around 4%, similar to the indices reported previously in 3-day cultures of 6- and 9-day-old rats (Boitani *et al.* 1993, 1995, Schlatt *et al.* 1999). We found that GDNF did not affect the percentage of Sertoli cells duplicating their DNA, in contrast to what Hu *et al.* reported in testicular explants of younger rats (6 days old) (1999). This apparent divergence may be related to different experimental conditions or more likely to the different age of the animals. Indeed, the same authors have shown that FSH increases the labeling index of Sertoli cells at the age of 6 days, whereas two previous studies have shown that this index is FSH-independent at the age of 9 days in a similar culture system (Boitani *et al.* 1995, Schlatt *et al.* 1999). Thus, the positive effect of GDNF on the Sertoli cell labeling index observed by Hu *et al.* (1999) may be limited to a very defined period of development. Hence, our results do not suggest a significant action of GDNF on the regulation of Sertoli

Figure 7 Effect of GDNF on DNA synthesis of Sertoli cells (A) and spermatogonia (B). Seminiferous tubule segments from 7- to 8-day-old rats were cultured for 2 days in the absence or presence (2.5, 10, and 50 ng/ml) of GDNF. One micromole BrdU was added to the medium for the last 20 h of culture. The proportion of BrdU-labeled Sertoli cells (2C vimentin-positive cells) and spermatogonia (2C vimentin-negative cells) was determined by immunodetection on slides, after cells were sorted by flow cytometry. Values are the means $\pm$ S.E.M. of five independent experiments for 2.5 and 10 ng/ml, and three for 50 ng/ml. * $P < 0.01$ versus control (0).

cell mitosis in 7- to 8-day-old rats. Besides, the number of Sertoli cells is not affected in GDNF overexpressing mouse testes (Meng *et al.* 2000).

In control wells, the labeling index of spermatogonia found in our study was very close (around 35%) to that reported by Boitani *et al.* using similar conditions (age of rats and culture medium) (Boitani *et al.* 1993, 1995). The proportion of BrdU-labeled spermatogonia was reduced by GDNF by around twofold. In 7- to 8-day-old rat testes, only type A spermatogonia are present (Boitani *et al.* 1993, Dym *et al.* 1995, Jahnukainen *et al.* 2004). Hence, these results suggest that GDNF inhibits the S-phase entrance of a large subset of type A spermatogonia under our culture conditions.

In the mouse, and more recently in the rat, the *in vitro* studies reported in the literature have shown a positive effect of GDNF on the number of spermatogonial stem cells which are a small subset of type A spermatogonia (Kamatsu-Shinohara *et al.* 2003, Nagano *et al.* 2003, Kubota *et al.* 2004a, 2004b,

Hofmann *et al.* 2005, Ryu *et al.* 2005). It has been proposed that this effect is due to self-renewing of stem cells encouraged by suppression of the differentiation pathway (Nagano *et al.* 2003). This hypothesis fits quite well with the results of the *in vivo* studies. Although fertile, knockout mice with one GDNF-null allele show a depletion of undifferentiated spermatogonia (Meng *et al.* 2000). Moreover, undifferentiated spermatogonia self-renew in mice overexpressing GDNF in the testis, but do not differentiate (Meng *et al.* 2000, Creemers *et al.* 2002, Yomogida *et al.* 2003). Likewise, in heterozygous mutants for the *dominant white spotting* locus and *steel* locus encoding the c-kit receptor and the c-kit ligand (stem-cell factor) respectively high levels of GDNF are observed in the testis, in which undifferentiated spermatogonia actively proliferate, but are decreased in number and do not differentiate (Tadokoro *et al.* 2002). In an extensive study, quantifying type A spermatogonia in the adult rat seminiferous epithelium, Huckins (1971) calculated the proportion of undifferentiated type Ais and Apr spermatogonia (stem cells) to be 11% of total type A spermatogonia. Moreover, Orwig *et al.* (2002) and Ryu *et al.* (2003) demonstrated only moderate differences in stem-cell concentration between adult and neonate rat testes. Taken together, these results indicate that the population of type A spermatogonia studied in our work was most likely composed of at least 80% of differentiated type A spermatogonia. Hence, it seems reasonable to suggest that the overall effect of GDNF observed in the present study is the sum of an inhibitory effect of GDNF on the large population of differentiated spermatogonia together with an enhancing effect of the factor on a small population of undifferentiated (stem cells) spermatogonia. This assumption appears substantiated by the results showing that even at maximal concentration, GDNF did not induce a decrease of the labeling index of spermatogonia higher than 50% (see Fig. 7).

Presently, the effects and means of action of GDNF on spermatogonia remain not completely understood in all studied species. For instance, the number of bovine spermatogonial stem cells arising from cells cultured with GDNF was lower than in controls after 1 week of culture, but higher than controls after 2 weeks (Oatley *et al.* 2004). Although some mouse spermatogonial stem cells can survive for more than 3 months in culture (Nagano *et al.* 1998), about 50 and 90% are lost after 3 and 7 days of culture respectively (Nagano *et al.* 2001). Spermatogonial stem cells derived from DBA/2J strain mice can be cultured in serum-free medium supplemented with GDNF alone, whereas spermatogonia from other strains cannot, suggesting an inherent genetic difference between strains (Kubota *et al.* 2004b). Besides, Buageaw *et al.* (2005) have shown that the stem-cell pool of immature mouse testes is heterogeneous with respect to the level of GFR1 α expression.

In conclusion, our *in vitro* results indicate that GDNF inhibits the S-phase entrance of differentiating A spermatogonia. Finally, the wide temporal and spatial expression of GDNF and its two receptors in the rat testis suggest that GDNF might act at several stages of spermatogenesis by either a direct effect on

the different types of germ cells and/or by an indirect action through Sertoli cells. Further investigations using similar *in vitro* approaches are in progress to answer these questions.

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