Gonadotropins Regulate Rat Testicular Tight Junctions *in Vivo*

Mark J. McCabe, Gerard A. Tarulli, Sarah J. Meachem, David M. Robertson, Peter M. Smooker, and Peter G. Stanton

Prince Henry’s Institute (M.J.M., G.A.T., S.J.M., D.M.R., P.G.S.), Monash Medical Centre, Clayton, Victoria 3168, Australia; School of Applied Sciences (M.J.M., P.M.S.), Royal Melbourne Institute of Technology University, Bundoora, Victoria 3083, Australia; and Department of Biochemistry and Molecular Biology (P.G.S.), Monash University, Clayton, Victoria 3800, Australia

Sertoli cell tight junctions (TJs) are an essential component of the blood-testis barrier required for spermatogenesis; however, the role of gonadotropins in their maintenance is unknown. This study aimed to investigate the effect of gonadotropin suppression and short-term replacement on TJ function and TJ protein (occludin and claudin-11) expression and localization, in an adult rat model *in vivo*. Rats (n = 10/group) received the GnRH antagonist, acyline, for 7 wk to suppress gonadotropins. Three groups then received for 7 d: 1) human recombinant FSH, 2) human chorionic gonadotropin (hCG) and rat FSH antibody (to study testicular androgen stimulation alone), and 3) hCG alone (to study testicular androgen and pituitary FSH production). TJ proteins were assessed by real-time PCR, Western blot analysis, and immunohistochemistry, whereas TJ function was assessed with a biotin permeation tracer. Acyline treatment significantly reduced testis weights, serum androgens, LH and FSH, and adluminal germ cells (pachytene spermatocyte, round and elongating spermatids). In contrast to controls, acyline induced seminiferous tubule permeability to biotin, loss of tubule lumens, and loss of occludin, but redistribution of claudin-11, immunostaining. Short-term hormone replacement stimulated significant recoveries in adluminal germ cell numbers. In hCG ± FSH antibody-treated rats, occludin and claudin-11 protein relocalized at the TJ, but such relocalization was minimal with FSH alone. Tubule lumens also reappeared, but most tubules remained permeable to biotin tracer, despite the presence of occludin. It is concluded that gonadotropins maintain Sertoli cell TJs in the adult rat via a mechanism that includes the localization of occludin and claudin-11 at functional TJs. (*Endocrinology* 151: 2911–2922, 2010)

The blood-testis barrier (BTB) is a permeability barrier, which functions to sequester germ cells undergoing meiotic and postmeiotic differentiation from the vascular environment. Endothelial, peritubular myoid, and Sertoli cells contribute to the barrier (1), but it is recognized that tight junctions (TJs) found basally between Sertoli cells are the major component in the adult testis (1). Sertoli cell TJs are required for fertility, and it is well established that their disruption leads to germ cell atresia and cessation of spermatogenesis (2). Changes in BTB permeability and constituent proteins have been noted in men with infertility (3).

Formation of the BTB at puberty in animal models (2, 4) and humans (5) requires the gonadotropins FSH and LH (6, 7), a requirement that is also supported by *in vitro* models (8–12). Once formed, Sertoli cell TJs undergo a cyclic remodelling during spermatogenesis, as early meiotic preleptotene spermatocytes translocate to the adluminal compartment where they complete meiosis and spermiogenesis (2). An expanding list of cytokines and growth factors, including TGF-β, TNF-α, growth differentiation factor-9, activins, inhibins, and interleukins, produced by both Sertoli and germ cells (10, 13–16; for Discussion, see *Endocrinology* 151: 2911–2922, 2010).
review, see Ref. 17) mediate the local cross talk required for this translocation step.

Sertoli cell TJ s contain three main transmembrane protein groups, the claudins, particularly claudin-11 (18, 19) and claudin-3 (6), occludin (20, 21), and the junctional adhesion molecule (JAM) family (22). Both androgens and FSH can regulate these proteins during TJ formation in vivo (6, 22) and in vitro (9–11). However, the extent to which gonadotropins contribute to the maintenance of the established BTB in the normal adult remains unknown. Animal studies that have previously ablated gonadotropin action on the testis via hypophysectomy (23–25), GnRH antagonism (26, 27), or GnRH immunization (28, 29) have either not examined BTB function or are inconclusive on this point. Regulation of the BTB has potential importance in understanding the mechanism(s) of action of male hormonal contraception, which suppresses spermatogenesis via suppression of circulating gonadotropins (for review, see Ref. 30).

Precedents exist for a regulatory role for gonadotropins on Sertoli cell TJ s in adult animals. In the seasonal breeding, adult Djungarian hamster, circulating gonadotropins, and spermatogenesis are both suppressed during the non-breeding winter months when the BTB is nonfunctional (31). We recently demonstrated that claudin-3 and claudin-11, occludin, and JAM-A were no longer present at organized Sertoli cell TJ s in these animals and that administration of FSH restored BTB functionality and localization of TJ proteins at Sertoli cell TJ s (7, 32). It remains to be seen whether a similar gonadotropin dependence of TJ s occurs in animal models that do not undergo seasonal changes.

The aim of this study was to profoundly suppress circulating gonadotropins and spermatogenesis in adult male rats with the GnRH antagonist acyline (33, 34) and then selectively replace testicular androgens and/or FSH via short-term replacement regimens (29). Changes in germ cell numbers were quantified by stereology, whereas TJ function was assessed using a qualitative biotin permeation tracer (6). Changes in TJ proteins were examined using immunohistochemistry, real-time RT-PCR, and Western blot analysis.

Experimental design

Circulating FSH and LH/testosterone (T) were suppressed by weekly subcutaneous (sc) injections of the GnRH antagonist, acyline (donated by Richard Blye; National Institutes of Health, Bethesda, MD) for 7 wk into adult rats at 1.5 mg/kg in 5% mannitol/sterile MilliQ water (34), administered as single injections to the hind flank or shoulder (400 µl/site). Control rats received a sc injection of the vehicle alone (n = 10).

After 7 wk of gonadotropin suppression, rats (n = 10/group) in the eighth week received an additional dose of acyline in conjunction with short-term hormone replacement (daily, for 7 d) by sc injections with one of the following: 1) human recombinant (hrec) FSH (2.5 IU/kg) (Pregnyl; Organon, Oss, The Netherlands), 2) human chorionic gonadotropin (hCG) (2.5 IU/kg) (Pregnyl; Organon) + FSH antibody (FSH Ab) (sheep antiserum, 2 mg/kg) (29) to study testicular production of androgens alone, and 3) hCG (2.5 IU/kg) + normal sheep immunoglobulins (2 mg/kg) to study the production of androgens and endogenous rat FSH (29). Three more groups of rats (n = 10/group) continued to receive 1) acyline vehicle, 2) acyline + FSH Ab (daily, sheep antiserum, 2 mg/kg) to suppress residual FSH, and 3) acyline + normal sheep immunoglobulins (daily, 2 mg/kg) for the eighth week. Doses and treatment intervals were as described elsewhere (29, 35). Previous studies demonstrate that endogenous rat FSH levels rise under the influence of T stimulation (28, 36, 37).

At the end of the experiment, rats were killed by CO₂ asphyxiation, and testes were excised and weighed immediately. Five of the left testes per treatment group were snap-frozen in dry ice for RT-PCR and Western blot analyses. The remaining five testes from each group were injected with either the qualitative TJ functional tracer biotin (10 mg/ml, EZ-Link Sulfo-NHS-LC-Biotin; Pierce, Rockford, IL) in a volume of 10% testis weight, or the biotin vehicle, PBS (pH 7.4), and 1 mM CaCl₂ (n = 1). Testes were then incubated at room temperature for 30 min before immersion fixation in Bouin’s fixative for 5 h and were then randomly sampled by a systematic uniform approach (29, 38) for stereological or immunohistochemical assessment.

Shortly after killing rats, blood was taken by cardiac puncture and stored overnight at 4°C before being spun down in a centrifuge. Serum was then collected by centrifugation and snap-frozen before storage at −80°C for hormone analysis.

Immunohistochemistry

Tissues were embedded in paraffin wax, and 5-µm sections were mounted onto Superfrost-Plus slides (HD Scientific, Melbourne, Australia). Antigen retrieval was performed by heating the sections for 10 min in 600 ml 1 mM EDTA-NaOH (pH 8.0) (39) in an 800-W microwave. Primary antibodies [rabbit antiooccludin (no. 71-1500, 2.5 µg/ml, 30 min; Zymed), rabbit anti claudin-11 (no. 36-4500, 2 µg/ml, 1 h; Zymed), rabbit anti zona occludens (ZO)-1 (no. 61-7300, 1.25 µg/ml, 1 h; Zymed), and rabbit anti JAM-A (no. 36-1700, 0.63 µg/ml, overnight; Zymed)] were diluted in 10% normal goat serum (Chemicon International, Temecula, CA) in PBS and then applied to the sections for the times specified at 25°C. Negative controls used an equivalent concentration of nonspecific IgG or normal serum in place of the primary antibody. Detection was with either rabbit antigoat Alexa-488 or Alexa-546 (Molecular Probes, Eugene, OR) at dilutions of 1:400 for claudin-11 and JAM-A or 1:800 for occludin and ZO-1. In some cases, cells

Materials and Methods

Animals

Male outbred Sprague Dawley rats at 75–90 d of age obtained from Monash University Animal Services were maintained at 20°C in a fixed 12-h light, 12-h dark cycle with free access to food and water. All animal experimentation was approved by the Monash Medical Centre Animal Ethics Committee.
Biosciences, Lincoln, NE). in infrared imaging system (Odyssey infrared imaging scanner; Li-Cor
Gilbertsville, PA), and membranes were scanned using a near- antibody (1:5000; Rocklands Immunochemicals, Inc., detection was with IRDye 800-conjugated goat anti-rabbit secur.

Negative controls substituted primary antibody with an equiv-
ductin (no. A-2066, 1:1000; Sigma Chemical Co., St. Louis, MO).

2913

Endocrinology, June 2010, 151(6):2911–2922 endo.endojournals.org

real-time PCR

Qualitative TJ functional analysis

The localization of the biotin tracer in tissue sections was
analyzed by fluorescence microscopy using streptavidin Alexa-546 (Molecular Probes). For colocalization studies with
occcludin, the probe was diluted 1:100 in PBS along with the goat antirabbit Alexa-488 secondary antisera.

Western blot analysis

Protein was extracted from frozen rat testes using 1% sodium dodecyl sulfate (MP Biomedicals, Solon, OH) (16) and quantified using the bicinchoninic acid protein assay (Pierce). Protein (40 μg for occludin, 120 μg for claudin-11) was boiled in Laemmli sample buffer, separated by SDS-PAGE on 4–20% precast gels (Bio-Rad, Hercules, CA), and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA) for Western blot analysis. Membranes were blocked in 5% skim milk/PBS, 4°C overnight. Primary antibodies [rabbit anti-occludin (no. 71-1500, 1:125; Zymed) or rabbit anticaludin-11 (no. 7474, 1:200; Abcam, Cambridge, UK)] were applied to the membranes for 2 h with the latter caitained with rabbit antih-β- actin (no. A-2066, 1:1000; Sigma Chemical Co., St. Louis, MO). Negative controls substituted primary antibody with an equivalent concentration of nonspecific IgG or normal serum. Band detection was with IRDye 800-conjugated goat antirabbit secondary antibody (1:5000; Rocklands Immunoochemicals, Inc., Gilbertsville, PA), and membranes were scanned using a near-infrared imaging system (Oydessy infrared imaging scanner; Li-Cor Biosciences, Lincoln, NE).

Total RNA extraction, RT, and quantitative real-time PCR

Total RNA was extracted from frozen rat testes using the RNA Isolation kit (QIAGEN, Hildern, Germany), and contaminating DNA was removed with a deoxyribonuclease-free kit (Ambion, Austin, TX), following the manufacturers’ instructions. RT used avian myeloblastosis virus-reverse transcriptase (Roche, Basel, Switzerland) and random hexamers as described elsewhere (11).

mRNA expression was quantified using the Roche Light Cycler 380 (Roche) and the FastStart DNA Master Sybr Green 1 systems (Roche). Oligonucleotide primer pairs were obtained from published sources or were designed using the Primer3 program (available at http://frodo.wi.mit.edu/primer3/) and were ordered from Sigma Genosys (Castle Hill, Australia). Primer details and PCR conditions, including anneal temperature and Mg²⁺ concentration, are presented in Table 1. Primers produced a single band on DNA agarose gels, which corresponded to the target protein as shown by DNA sequencing. Standard curves for PCR analyses were generated using dilutions of an adult rat testicular cDNA preparation of arbitrary unitage. Unless otherwise noted, PCR of all samples was performed using triplicate reactions for 38 cycles, after which a melting curve analysis was performed to monitor PCR product purity (see Table 1).

Serum hormone assays

Serum androgens, rat FSH, and rat LH concentrations were determined as described previously (29, 35, 40, 41). The cross-reactivities of hCG in the FSH and LH immunofluorometric assays were approximately 1 and 4%, respectively (42, 43), and assay sensitivities were 0.013 ng/ml (serum androgens), 0.015 ng/ml (rat FSH), and 0.012 ng/ml (rat LH).

Stereological assessment of germ cell type and number

The total number of cells per testis was calculated using the optical dissector method as previously described (29, 38). Germ cells were identified using the criteria of Russell et al. (44) as published previously (45), and approximately 100 germ cells of each type were counted per animal. Germ cells were counted in the following subgroups: type A/intermediate spermatogonia, type B/preleptotene spermatocytes, leptotene/zygotene spermatocytes, pachytene spermatocytes (associated with stages I–VIII and IX–XIV), round spermatids (steps 1–8), elongating spermatids (steps 9–14), and elongated spermatids (steps 15–19).

were counterstained with the fluorescent nuclear marker 4-,6- diamidino-2-phenylindole (Molecular Probes) (100 nM in PBS) for 5 min after the secondary antibody incubation. Sections were then washed in PBS, mounted with FluorSave (Calbiochem, La Jolla, CA), and visualized with a conventional fluorescent microscope (Olympus BX-50; Olympus, Mt. Waverley, Australia).

TABLE 1. Primer-specific conditions used for quantitative PCR analysis

<table>
<thead>
<tr>
<th>Gene (source)</th>
<th>Species</th>
<th>accession no.</th>
<th>Primer sequence (5’-3’)</th>
<th>Size (bp)</th>
<th>Mg²⁺ (μM)</th>
<th>Anneal temperature (C)</th>
<th>Read temperature (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin-11 (70)</td>
<td>Rat</td>
<td>NM.053457</td>
<td>F TTAGAATGGGCACCTCAGG</td>
<td>624</td>
<td>2.5</td>
<td>68</td>
<td>85</td>
</tr>
<tr>
<td>Occludin (13)</td>
<td>Rat</td>
<td>NM.031329</td>
<td>R ATGATGTGGCCACTGCTCG</td>
<td>294</td>
<td>2.5</td>
<td>64</td>
<td>75</td>
</tr>
<tr>
<td>JAM-A (Primer 3)</td>
<td>Mouse</td>
<td>NM.172647</td>
<td>G TGCAGCCACACCACTTAATTA</td>
<td>425</td>
<td>2.5</td>
<td>64</td>
<td>72</td>
</tr>
<tr>
<td>ZO-1 (Primer 3)</td>
<td>Rat</td>
<td>NM.009386</td>
<td>F AGAGAGGAAGAGCGAATGTCTA</td>
<td>248</td>
<td>3.0</td>
<td>60</td>
<td>72</td>
</tr>
<tr>
<td>INSL-3 (Primer 3)</td>
<td>Rat</td>
<td>NM.053680</td>
<td>C TCTCTCTACAGCGGCTTC</td>
<td>241</td>
<td>2.0</td>
<td>58</td>
<td>72</td>
</tr>
<tr>
<td>β-actin (Primer3)</td>
<td>Rat</td>
<td>NM.031144</td>
<td>R CCGTAAACCACTTCACTGCA</td>
<td>103</td>
<td>2.0</td>
<td>67</td>
<td>72</td>
</tr>
<tr>
<td>GAPDH (71)</td>
<td>Mouse</td>
<td>NM.008084</td>
<td>G ACCCCCCCCTATGACCTCAAC</td>
<td>560</td>
<td>2.5</td>
<td>60</td>
<td>72</td>
</tr>
</tbody>
</table>

F, Forward; R, reverse.

© Temperature at which the fluorescence of the PCR product was quantified during LightCycler analysis.

Gene (source) | Species | accession no. | Primer sequence (5’-3’) | Size (bp) | Mg²⁺ (μM) | Anneal temperature (C) | Read temperature (C) |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin-11 (70)</td>
<td>Rat</td>
<td>NM.053457</td>
<td>F TTAGAATGGGCACCTCAGG</td>
<td>624</td>
<td>2.5</td>
<td>68</td>
<td>85</td>
</tr>
<tr>
<td>Occludin (13)</td>
<td>Rat</td>
<td>NM.031329</td>
<td>R ATGATGTGGCCACTGCTCG</td>
<td>294</td>
<td>2.5</td>
<td>64</td>
<td>75</td>
</tr>
<tr>
<td>JAM-A (Primer 3)</td>
<td>Mouse</td>
<td>NM.172647</td>
<td>G TGCAGCCACACCACTTAATTA</td>
<td>425</td>
<td>2.5</td>
<td>64</td>
<td>72</td>
</tr>
<tr>
<td>ZO-1 (Primer 3)</td>
<td>Rat</td>
<td>NM.009386</td>
<td>F AGAGAGGAAGAGCGAATGTCTA</td>
<td>248</td>
<td>3.0</td>
<td>60</td>
<td>72</td>
</tr>
<tr>
<td>INSL-3 (Primer 3)</td>
<td>Rat</td>
<td>NM.053680</td>
<td>C TCTCTCTACAGCGGCTTC</td>
<td>241</td>
<td>2.0</td>
<td>58</td>
<td>72</td>
</tr>
<tr>
<td>β-actin (Primer3)</td>
<td>Rat</td>
<td>NM.031144</td>
<td>R CCGTAAACCACTTCACTGCA</td>
<td>103</td>
<td>2.0</td>
<td>67</td>
<td>72</td>
</tr>
<tr>
<td>GAPDH (71)</td>
<td>Mouse</td>
<td>NM.008084</td>
<td>G ACCCCCCCCTATGACCTCAAC</td>
<td>560</td>
<td>2.5</td>
<td>60</td>
<td>72</td>
</tr>
</tbody>
</table>
TABLE 2. Effect of GnRH antagonist acyline treatment (8 wk) and short-term (1 wk) hormone replacement on body and testis weights, and serum hormones (androgens, rat LH, and rat FSH)

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Testis weight (mg)</th>
<th>Serum LH (ng/ml)</th>
<th>Serum androgens (ng/ml)</th>
<th>Serum FSH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>439 ± 38 (10)</td>
<td>1755 ± 91g (10)</td>
<td>2.37 ± 1.40a (7)</td>
<td>2.22 ± 1.27a (10)</td>
<td>5.55 ± 1.01a (7)</td>
</tr>
<tr>
<td>Acyline</td>
<td>403 ± 22 (10)</td>
<td>332 ± 44b (10)</td>
<td>0.38 ± 0.21b (8)</td>
<td>0.28 ± 0.10b (10)</td>
<td>0.68 ± 0.23b (8)</td>
</tr>
<tr>
<td>Acyline + FSHAb</td>
<td>402 ± 37 (10)</td>
<td>299 ± 47b (10)</td>
<td>0.12 ± 0.01c (5)</td>
<td>0.33 ± 0.15c (10)</td>
<td>na</td>
</tr>
<tr>
<td>Acyline + hrecFSH</td>
<td>416 ± 40 (10)</td>
<td>426 ± 33b (10)</td>
<td>0.32 ± 0.19b (6)</td>
<td>0.31 ± 0.08b (9)</td>
<td>0.40 ± 0.16b (6)</td>
</tr>
<tr>
<td>Acyline + hCG + FSHAb</td>
<td>413 ± 14 (10)</td>
<td>433 ± 54b (10)</td>
<td>0.020 ± 0.007b (6)</td>
<td>0.78 ± 0.39b (9)</td>
<td>na</td>
</tr>
<tr>
<td>Acyline + hCG</td>
<td>419 ± 20 (10)</td>
<td>482 ± 74b (10)</td>
<td>0.35 ± 0.25b (5)</td>
<td>0.67 ± 0.26b (10)</td>
<td>1.36 ± 0.27c (6)</td>
</tr>
</tbody>
</table>

Data are mean ± sd (n/group). Different letters denote significant differences between groups at P < 0.05 or greater, as detailed in Results. na, Not assayed.

Cell counting was performed with a ×100 objective lens on an Olympus BX-50 microscope (Olympus, Tokyo, Japan), using a systematic uniform random sampling method as described elsewhere (29). A set of unbiased frames was used to count the germ cells, with the number of frames for each germ cell type being determined by their frequency (29). The final screen magnification was ×2708, and no correction for tissue shrinkage was necessary as determined previously (29, 45, 46).

Statistical analysis

Treatments were compared with controls by ANOVA followed by the Student’s Newman-Keuls test, or where data were nonparametric, Kruskal-Wallis test, followed by Newman-Keuls analog (Equal N’s) test. P < 0.05 was used to determine whether results were statistically significant. All statistics were performed using SigmaStat version 3.5 (Systat Software, Inc., San Jose, CA), and data have been expressed as mean ± sd with n = 5–10 rats per group unless otherwise stated.

Results

Body and testis weights and serum hormone levels

Hormone suppression with acyline for 8 wk had no effect on body weights but induced a significant (P < 0.001) decrease in testis weights (Table 2) to 19% of control. The addition of the FSH Ab in the eighth week caused a further 10% decrease in testis weights compared with acyline alone, but this was no more significant than acyline alone.

Short-term hormone replacement with hrecFSH, hCG + FSH Ab, or hCG + control IgG all gave significant (P < 0.001) recoveries in testis weights to 24–27% of controls (Table 2).

LH

Acyline treatment significantly decreased rat serum LH to 16% (P < 0.05) (Table 2) of control, and addition of the FSH Ab in the eighth week resulted in a further decrease in serum LH to 5% of controls (P < 0.05 compared with acyline alone). Short-term hrecFSH and hCG replacement had no effect on suppressed serum LH levels, whereas short-term replacement with hCG + FSH Ab resulted in a further significant decrease of rat LH (P < 0.05, Table 2).

Androgens

Acyline treatment reduced serum total androgens to 13% (P < 0.001) of control, and the addition of the FSH Ab caused no further decrease (Table 2). Short-term hormone replacement with hrecFSH had no effect on serum androgen levels, but significant (P < 0.01) recoveries were achieved with short-term replacement of hCG (to 30% of control, P < 0.01) and hCG + FSH Ab (to 35% of control, P < 0.001).

FSH

Serum FSH was reduced to 12% (P < 0.01) of controls in response to acyline treatment (Table 2). As the FSH Ab interfered with the assay (data not shown), no measurements were possible in FSH Ab-containing treatment groups. However, the short-term replacement of hrecFSH further decreased serum rat FSH levels to 7% of control (P < 0.05), whereas the addition of hCG induced a significant recovery to 25% of control (P < 0.001).

Changes to germ cell types and numbers

The effects of chronic GnRH antagonist treatment on spermatogenesis in the rat are well understood (26, 47). Because this study used the more recently developed antagonist, acyline (33, 34), for which quantitative data on germ and Sertoli cell numbers is not yet available, we first assessed changes in cell populations in response to the various treatment regimes.

Spermatogonia and preleptotene spermatocytes

Spermatogonia and preleptotene spermatocytes reside outside the BTB in the normal animal. Hormone suppression with acyline for 8 wk significantly (P < 0.001) reduced type A and intermediate spermatogonia to 66% of controls, and addition of the FSH Ab caused a further significant reduction compared with acyline alone (P < 0.05) to 46% of controls (Fig. 1). Type B/preleptotene spermatocytes were similarly suppressed by the acyline...
and FSH Ab treatment (Fig. 1). Short-term FSH replacement was the only treatment that significantly restored germ cell numbers in the type B/preleptotene spermatocyte group to control levels ($P < 0.001$ vs. FSH Ab) (Fig. 1).

**Leptotene/zygotene spermatocytes**

Leptotene/zygotene/pachytene spermatocytes and later spermatids all reside inside the BTB in the normal animal. Acyline reduced leptotene/zygotene spermatocyte numbers to 50% of control ($P < 0.001$), and the addition of the FSH Ab caused a further decrease to 34% of control ($P < 0.001$ vs. control, $P < 0.01$ vs. acyline alone) (Fig. 1). None of the hormone replacement groups resulted in any significant increases compared with acyline alone.

**Pachytene spermatocytes stages I–VIII**

Pachytene spermatocytes in stages I–VIII were suppressed by acyline to 15% or by acyline + FSH Ab to 8% (both $P < 0.001$) of controls (Fig. 1). Short-term replacement produced significant increases in all treatment groups (hrecFSH, hCG + FSH Ab, and hCG, all $P < 0.001$ vs. acyline), although animals receiving FSH (either hrecFSH or via hCG) recovered to a greater extent than the hCG + FSH Ab group (hrecFSH vs. hCG + FSH Ab, $P < 0.001$; hCG vs. hCG + FSH Ab, $P < 0.001$). The increase in pachytene spermatocyte (stages I–VIII) number in the hrecFSH-treated group was significantly ($P < 0.001$) greater than in the hCG-alone group (Fig. 1).

**Pachytene spermatocytes stages IX–XIV**

Acyline treatment reduced pachytene spermatocytes at stages IX–XIV to 3% of control ($P < 0.001$), with a further significant suppression to 2% of control numbers with the FSH Ab treatment ($P < 0.05$ vs. acyline) (Fig. 1, note log scale). As seen for pachytene spermatocytes in stages I–VIII, short-term hormone replacement produced significant 3- to 5-fold increases in all treatment groups for stages IX–XIV pachytene spermatocytes, with the group receiving hrecFSH significantly greater than either hCG + FSH Ab or hCG-alone groups.

**Round spermatids steps 1–8**

Round spermatids at steps 1–8 responded similarly to the various treatments as pachytene spermatocytes, although a greater extent of initial suppression with acyline (0.4% of control numbers) was observed (Fig. 1). Short-term hrecFSH replacement resulted in a partial but significant ($P < 0.05$) recovery of round spermatid numbers to 6.5% of controls (Fig. 1), whereas androgen alone (hCG + FSH Ab group) resulted in a nonsignificant increase to 1.4% of control numbers. However, the combination of endogenous rat FSH and androgen due to hCG treatment significantly increased round spermatids to 3.1% of control, but this was significantly ($P < 0.05$) less than the number obtained with hrecFSH alone (hrecFSH vs. hCG $P < 0.05$) (Fig. 1).

**Elongating spermatids steps**

Elongating spermatids at steps 9–14 were suppressed by acyline to 0.2% of control ($P < 0.001$), with no further reduction obtained with the FSH Ab. None of the hormone replacements gave any recovery in these germ cell numbers (Table 3). Elongated spermatids at steps 15–19 were not detected in any of the treatment groups (Table 3).
TABLE 3. Stereological data for elongating and elongated spermatids (steps 9–14 and 15–19) and Sertoli cell numbers

<table>
<thead>
<tr>
<th></th>
<th>Elongating spermatids, steps 9–14 (cells/testis)</th>
<th>Elongated spermatids, steps 15–19 (cells/testis)</th>
<th>Sertoli cells (cells/testis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccontrol</td>
<td>$9.45 \times 10^7 \pm 0.84 \times 10^7$</td>
<td>$3.63 \times 10^8 \pm 0.59 \times 10^8$</td>
<td>$4.11 \times 10^7 \pm 0.31 \times 10^7$</td>
</tr>
<tr>
<td>Acyline</td>
<td>$1.42 \times 10^8 \pm 1.93 \times 10^8$</td>
<td>$&lt;0.01 \times 10^9$</td>
<td>$4.44 \times 10^7 \pm 0.88 \times 10^7$</td>
</tr>
<tr>
<td>Acyline + FSHAb</td>
<td>$0.24 \times 10^8 \pm 0.35 \times 10^8$</td>
<td>$&lt;0.01 \times 10^9$</td>
<td>$4.71 \times 10^7 \pm 1.51 \times 10^7$</td>
</tr>
<tr>
<td>Acyline + hrecFSH</td>
<td>$3.69 \times 10^8 \pm 4.10 \times 10^8$</td>
<td>$&lt;0.01 \times 10^9$</td>
<td>$5.18 \times 10^7 \pm 1.17 \times 10^7$</td>
</tr>
<tr>
<td>Acyline + hCG + FSHAb</td>
<td>$1.22 \times 10^8 \pm 2.42 \times 10^8$</td>
<td>$&lt;0.01 \times 10^9$</td>
<td>$5.54 \times 10^7 \pm 1.64 \times 10^7$</td>
</tr>
<tr>
<td>Acyline + hCG</td>
<td>$3.32 \times 10^8 \pm 4.04 \times 10^8$</td>
<td>$&lt;0.01 \times 10^9$</td>
<td>$5.18 \times 10^7 \pm 1.15 \times 10^7$</td>
</tr>
</tbody>
</table>

Quantification was conducted as per the earlier germ cell types presented in Fig. 2. Data are mean ± SD, n = 5 rats/group. Different letters denote significant differences between groups at $P < 0.05$ or greater, as detailed in Results.

Sertoli cell numbers

Sertoli cell numbers were unchanged with hormone suppression or short-term replacement compared with the control (Table 3).

Effect of hormone suppression and short-term replacement on TJ structure/localization and function

In normal adult rats where the BTB is intact, permeation of the biotin tracer was restricted to the interstitial space in the testis (Fig. 2A, arrowheads) and only migrated into the tubules as far as the intact TJs, as visualized by occludin immunostaining (Fig. 2A, inset) in conjunction with the nuclear counterstain 4′,6-diamidino-2-phenylindole (blue). A similar phenotype was observed for animals that received acyline plus the FSH Ab (Fig. 3C).

Acyline treatment markedly altered seminiferous tubule morphology, because tubule diameters were suppressed and tubule lumens were almost absent (compare Fig. 3, A and B). In addition, occludin immunostaining was absent in acyline-treated animals, and tubules were permeable to the biotin tracer that surrounded all remaining germ cell types (Fig. 3B). Based on stereological analysis, these cells were spermatogonia, leptotene/zygotene spermatocytes, and some (3–15%) pachytene spermatocytes (Fig. 1). A similar phenotype was observed for animals that received acyline plus the FSH Ab (Fig. 3C).

Short-term hormone replacement for 7 d with either hrecFSH (Fig. 3D), hCG + FSH Ab (Fig. 3E), or hCG alone (Fig. 3F) resulted in increased tubule and lumen diameters with some occludin staining at the TJ in hCG ± FSH Ab-treated animals but minimal staining in the hrecFSH group (Fig. 3, D–F, insets). The biotin tracer generally permeated throughout the tubules (Fig. 3, D–F), but in a low proportion (<5% of tubules/cross-sections observed), the tracer was predominantly restricted to the interstitium, and occludin immunostaining was more readily apparent (Fig. 3, G–I). This latter phenotype (Fig. 3, G–I) was not observed in the acyline ± FSH Ab hormone-suppressed groups.

The localization of the TJ proteins claudin-11 and JAM-A, and the cytoplasmic plaque protein ZO-1, was...
also altered after hormone suppression (Fig. 4). In control animals, staining of claudin-11, JAM-A, and ZO-1 was predominantly basal, consistent with the localization of intact TJs (Fig. 4, arrows). Unlike occludin, immunostaining for all three proteins remained observable in acyline-treated and acyline + FSH Ab-treated animals but was predominantly observed both at membranes adjacent to germ cells (Fig. 4, asterisks) and as a diffuse pattern in Sertoli cell cytoplasm, indicative of a change in distribution of the protein. There did not appear to be any major change in claudin-11 immunolocalization in the hrecFSH-treated group (Fig. 4), but staining for claudin-11, JAM-A, and ZO-1 was concentrated more basally in hCG-treated animals ± FSH Ab (Fig. 4, arrows).

Western blot analysis of occludin and claudin-11 expression

Protein extracts from control rat testis displayed bands corresponding to the expected molecular masses of occludin, which appeared as a doublet at approximately 64 kDa (Fig. 5A, lane 1), claudin-11 (∼22 kDa; Fig. 5B, lane 1), and β-actin (43 kDa; Fig. 5B). Fainter bands were also present in each of the gels but were also detectable in the negative controls. Hormone suppression resulted in a
marked decrease in occludin protein expression (Fig. 5A, lanes 2 and 3), and no changes were observed after short-term hormone replacement (Fig. 5A, lanes 4–6). Similarly, claudin-11 protein expression was suppressed to beyond the limit of detection after hormone suppression (Fig. 5B), with no detectable recovery after any of the short-term hormone replacement regimens. β-Actin protein expression did not change with any of the treatments compared with control (Fig. 5B).

**Real-time PCR analysis of TJ mRNA expression**

Hormone suppression with acyline resulted in significant ($P < 0.001$) 3- to 4-fold increases in claudin-11, occludin, and JAM-A mRNA expression compared with controls (Fig. 6, A–C). Short-term hormone replacement with each of the treatments resulted in a significant ($P < 0.001$) decrease in both claudin-11 and occludin mRNA expression levels relative to the acyline group, although there were no differences between groups (FSH alone, hCG + FSH Ab, or hCG). In contrast, JAM-A levels remained high in all treatment groups (Fig. 6C). Hormone suppression and replacement had no effect on ZO-1 mRNA expression compared with controls (Fig. 6D). Expression of the testicular androgen-regulated protein insulin-like factor 3 (INSL-3) was suppressed 12-fold after hormone suppression (Fig. 6E, note log y-axis), and was restored to control levels by androgen treatment groups (hCG and hCG/FSH Ab) but not hrecFSH (Fig. 6E). β-Actin mRNA expression remained unchanged after hormone suppression and replacement compared with control (Fig. 6F).

**Discussion**

This study has established that chronic gonadotropin suppression in adult rats caused the BTB to become permeable to a low molecular weight tracer in association with protein redistribution (claudin-11) or complete loss (occludin) from the basally located Sertoli cell TJ. In contrast, JAM-A levels remained high in all treatment groups (Fig. 6C). These data indicate that the BTB in the adult rat requires ongoing gonadotropic support to remain functional, due...
in part to a regulated localization of TJ proteins to the Sertoli cell TJ's and, therefore, establishes a new site of gonadotropin action in the adult rat testis. In addition, we show that spermatogenic restoration through meiotic and postmeiotic cell stages can occur even when Sertoli cell TJ's remain permeable to a low molecular weight tracer, suggestive of a size-selectivity function of the Sertoli cell TJ.

**Testis weights and serum hormones**

The gonadotropins LH and FSH were chronically suppressed for 8 wk in this study using the GnRH antagonist acyline, which is comparable with other GnRH antagonists in terms of the extent of suppression of LH and T and its effectiveness after multiple weekly doses (33, 34, 48).

Acyline did not alter body weights, as observed elsewhere (34, 49, 50); however, testis weights were decreased to 19% of control, consistent with suppression studies using a GnRH immunogen [19–29% (28, 29, 35)]. Acyline also significantly suppressed serum rat LH, FSH, and androgen levels to 12–16% of control values, mirroring other findings with this antagonist in rats (34, 48, 51–54) and also similar to the effects of active GnRH immunization on LH and androgens (29, 35). Interestingly, acyline treatment in this study brought about a 3- to 4-fold greater suppression in serum FSH than GnRH immunization [12 vs. 34–54% of controls (29, 35)], indicating a greater suppressive action for the antagonist regime. Further possible decreases in serum FSH in treatment groups that received FSH Ab were not able to be analyzed, but significant decreases in germ cell numbers suggested that an additional suppressive effect had occurred.

Short-term hormone replacement had differential effects on the serum hormones studied. Administration of hrecFSH to acyline-treated animals caused a further significant reduction in endogenous rat FSH, presumably via negative feedback or via a GnRH-independent pathway (36, 37). Treatment with hCG increased serum androgens (3-fold), but these remained at 30–35% of control, whereas rat FSH levels increased 2-fold to approximately 25% of control levels. Differences in the effectiveness of hCG in elevating androgens and rat FSH between this and previous GnRH immunization studies (29, 35) are most probably due to the greater level of testicular suppression achieved with acyline. It is noted that all hormone replacement treatments induced similar significant increases in testis weights, consistent with previous studies where this, or similar models, have been employed (28, 35).

**Germ cell numbers and BTB function**

The major changes in germ cell numbers observed after GnRH antagonism occurred in the cell types normally located adluminal to the BTB (leptotene/zygotene spermatocytes, pachytene spermatocytes at stages I–VIII and IX–XIV, and round/elongating spermatids) with only moderate decreases in basally located germ cells (type A/intermediate spermatogonia and type B/preleptotene spermatocytes). The 10- to 50-fold decreases observed for pachytene spermatocytes and round spermatids are con-
sistent with the known requirement for FSH and androgen to maintain spermatocyte survival and spermatid differentiation, respectively (29, 55, 56). All short-term hormone replacements stimulated 3- to 4-fold recoveries in these cell types, with a greater dependency on FSH action noted, as seen elsewhere (56). Importantly, tubules from both the suppressed (acyline ± FSH Ab) and hormone-replaced groups showed a marked disruption in BTB function by biotin tracer permeation, demonstrating that this hormone-induced restoration of germ cell numbers can occur when Sertoli cell TJs are not fully functional. Lack of TJ function is also supported by the almost total absence of tubule lumens in the suppressed groups, which is a qualitative phenotype indicative of disrupted TJ function (1, 7, 31, 57). However, tubule lumens were observable in the short-term hormone replacement groups, indicating that a level of TJ function was restored despite the continued permeability to the low molecular weight biotin tracer. These data suggest that Sertoli cell TJs do not need to be fully functional to allow the early restoration of spermatogenesis, particularly at meiotic and postmeiotic stages, to occur. A similar permeability of seminiferous tubules to a low molecular weight tracer ([51Cr]-EDTA) has been observed in postpubertal 25-d-old rats (58), which contain meiotic and postmeiotic germ cells (46).

**TJ protein localization and expression**

Immunohistochemical analysis showed that hormone suppression with acyline disrupted the localization of Sertoli cell TJ proteins. In control testes, both occludin and claudin-11 were present at functional TJs, which excluded the biotin tracer from the epithelium. After acyline treatment, occludin protein was difficult to detect by immunofluorescence microscopy and Western blot analysis, whereas claudin-11 protein was suppressed and tended to be redistributed within the Sertoli cell but away from the basal aspect of the tubules. This phenotype was similar to the gonadotropin-deplete testis of the short-day Djungarian hamster (7, 32) and, in both cases, was associated with disrupted TJ function as shown by extensive penetration of biotin tracer throughout the seminiferous epithelium. Several recent in vivo and in vitro studies have demonstrated that TJ proteins, including occludin and JAM-A, can undergo endocytosis from the BTB (59) via a clathrin-dependent pathway (60) in response to changes in the cytokines TGF-β3, TGF-β2, TNFα, and androgen (59, 60). Endocytosed TJ proteins are also seen in other epithelial tissues (6, 7, 60-66), where they can be either recycled back to the cell surface or targeted for intracellular degradation (for reviews, see Refs. 67 and 68). Although not investigated in this study, it is speculated that the altered localization of Sertoli cell TJ proteins (claudin-11 and JAM-A) observed after gonadotropin antagonism represents similar recycling and/or degradation processes.

Short-term hormone replacement with FSH ± androgen for 7d led to a partial relocation of occludin and claudin-11 to the TJ, although this was not complete and remained below the sensitivity of the Western blot analysis, due to the small testis sizes and reduced amount of total TJ protein available. In occasional tubules, complete occludin localization to the TJs with marked biotin exclusion was also seen, indicative of either a more rapid recovery of tubular function under hormonal stimuli, or an incomplete suppression in all tubules by the acyline treatment. As no evidence for this latter point was observed, it is concluded that tubule recovery was heterogeneous. On the basis of other studies, which show that testicular TJs in the suppressed hamster testis remain permeable to biotin tracer after 2 d of exogenous FSH treatment but are impermeable after 10 d (7), it is suggested that longer periods (>7d) of hormonal stimulation in this acyline-treatment model would result in a greater restoration of TJ protein and function.

In contrast to the protein responses, acyline treatment increased occludin and claudin-11 mRNA levels, whereas short-term gonadotropin replacement decreased expression toward control levels. Similar in vivo responses for TJ mRNA expression have been reported elsewhere (7, 69), suggesting that gonadotropin regulation of TJ protein localization is more indicative of TJ function than mRNA expression.

In conclusion, this study demonstrates that maintenance of the adult rat Sertoli cell TJ is regulated by gonadotropins in vivo. Chronic gonadotropin suppression resulted in decreased TJ function, as shown by permeability to tracer and loss of tubule lumens, in conjunction with altered or lost TJ protein localization and a loss of adluminal germ cells. Short-term hormone replacement with FSH and/or hCG partially reversed this phenotype, as shown by a reappearance of tubule lumens, some relocation of TJ proteins to basally located TJs, and a renewed proliferation of adluminal germ cell types. However, the TJ remained permeable to a low molecular weight tracer, demonstrating that TJs need not be fully functional for early spermatogenic restoration to occur. Collectively, these data establish a new site of gonadotropin action at Sertoli cell TJs in the adult rat testis.

**Acknowledgments**

We thank the excellent technical contributions made by Fiona McLean (androgen assay), G. A. Balourdos (stereology), and Enid Pruysers (gonadotropin assays) and the donation of acyline from Dr. Richard P. Blye (Contraception and Reproductive Health Branch, National Institute of Child Health and Human Development/National Institutes of Health).
Address all correspondence and requests for reprints to: Peter G. Stanton, Ph.D., Prince Henry’s Institute of Medical Research, P.O. Box 5152, Clayton 3168, Victoria, Australia. E-mail: peter.stanton@princehenrys.org.

This work was supported by National Health and Medical Research Council (Australia) Program Grants 241000 and 494802 (to D.M.R. and P.G.S.) and by the Victoria Government’s Operational Infrastructure Support Program.

Disclosure Summary: The authors have nothing to disclose.

References

32. Tarulli GA, Stanton PG, Lercil A, Meachem SJ 2006 Adult sertoli cells are not terminally differentiated in the Djungarian hamster: effect of FSH on proliferation and junction protein organization. Biol Reprod 74:798–806
35. Pratis K, O’Donnell L, Ooi GT, Stanton PG, McLachlan RI, Robertson DM 2003 Differential regulation of rat testicular 5α-
reductase type 1 and 2 isoforms by testosterone and FSH. J Endocrinol 176:393–403


