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# Induction of Reproductive Tract Developmental Abnormalities in the Male Rat by Lowering Androgen Production or Action in Combination with a Low Dose of Diethylstilbestrol: Evidence for Importance of the Androgen-Estrogen Balance

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**This study tested the hypothesis that testis/reproductive tract abnormalities induced in the rat by neonatal treatment with diethylstilbestrol (DES) result from disturbance of the androgen-estrogen balance. Male rats were treated neonatally with a dose of DES (0.1 μg) that induced either no or small effects on its own or with a dose (10 μg) that induced major reproductive tract abnormalities. To allow quantification, the abnormalities chosen for study were distension of the rete testis and efferent ducts and reduction in epithelial cell height in the efferent ducts and vas deferens. To alter the androgen-estrogen balance, other rats were treated with DES (0.1 μg) in combination with a treatment to suppress either androgen production [GnRH antagonist (GnRHa)] or androgen action (flutamide); other rats were treated with GnRHa or flutamide alone. Testosterone levels were measured to verify the effects of treatment. Combined administration of DES (0.1 μg) plus GnRHa or flutamide induced significantly greater distension/overgrowth of the rete testis and efferent ducts (ED) and a reduction in epithelial cell height of the ED than did DES (0.1 μg) administered alone. Neither GnRHa nor flutamide affected rete or ED distension when administered alone, but both significantly reduced ED epithelial cell height. Neonatal treatment with bisphenol-A (100 μg) with or without GnRHa had no significant effect on any of these parameters. In contrast to the ED, a reduction in cell height of the vas deferens was induced to an equal extent by DES (10 μg), DES (0.1 μg) with GnRHa, and GnRHa alone, suggesting greater sensitivity of this tissue to both androgen and estrogen action. The induction of major abnormalities in rats treated with DES (10 μg) was coincident with loss of androgen receptor immunoexpression in affected tissues. Reduced androgen receptor immunoexpression was also induced by combined treatment with DES (0.1 μg) plus GnRHa or flutamide, whereas treatment with any of these compounds alone had no or only minor effects. These findings suggest that reduced androgen action sensitizes the reproductive tract to estrogens, demonstrating that the balance in action between androgens and estrogens, rather than their absolute levels, may be of fundamental importance in determining normal or abnormal development of some regions of the male reproductive tract. (*Endocrinology* 143: 4797–4808, 2002)**

IT IS BECOMING increasingly evident that estrogens play a role in the development and function of the testis and male reproductive tract. This stems from studies that have localized estrogen receptors (ERs) (1–6), studies of ER knockout mice (7–9), and studies involving exposure of the fetal or neonatal male to exogenous estrogens (10–15). Nevertheless, it is apparent that male ER- or aromatase-knockout mice initially develop a grossly normal testis and reproductive tract (7–9, 16, 17), with the probable exception of the efferent ducts (18, 19), which endorses the central role that androgens play in male reproductive tract development. Indeed, because estrogen production is itself dependent on prior androgen production, it is in many respects difficult to divorce androgen from estrogen effects in tissues in which both ERs and androgen receptors (AR) are expressed (20). Several other pieces of recent evidence add to this view. For example, it has been shown that treatment of pregnant rats with the potent estrogen diethylstilbestrol (DES) dose-dependently suppresses testosterone levels in blood and testis of male fetuses (21), whereas similar treatment neonatally can suppress both testosterone levels and AR expression (22–24). Other studies have shown that interactions between the AR and ERs are possible (25, 26), although the physiological implications of such findings remain to be explored.

Our own studies have shown that neonatal treatment of male rats with relatively high doses of DES (10 μg) causes a range of reproductive tract abnormalities, including overgrowth/distension of the rete testis and reduced epithelial cell height in the efferent ducts, epididymis, vas deferens, seminal vesicles, and prostate coincident with relative overgrowth of stromal tissue at many of these sites (14, 15, 23, 24, 27). All of these DES-induced changes are associated with a loss of expression of AR in the affected tissues as well as in Sertoli cells in the testis (23, 24). Neonatal treatment with a 100-fold lower dose of DES (0.1 μg) or with environmental estrogens such as bisphenol-A or octylphenol do not induce these changes (23, 24, 27). These findings raised the possibility that the effects of the high dose (10 μg) of DES resulted

Abbreviations: AR, Androgen receptor; DES, diethylstilbestrol; ED, efferent ducts; ER, estrogen receptor; ERKO, ERα knockout; GnRHa, GnRH antagonist; TBS, Tris-buffered saline.

not from its interaction with ERs, but instead from the parallel suppression of androgen production/action that this treatment also induced (23, 24). However, this conclusion could not be supported, as we were able to show that blockade of androgen production [GnRH antagonist (GnRHa)] or action (flutamide) by neonatal treatment could not reproduce the reproductive tract abnormalities that were induced by 10 μg DES (23, 24).

These findings led us to hypothesize that the changes induced by DES might stem from disturbance of the androgen-estrogen balance. To test this possibility, we coadministered testosterone esters (200 μg) with DES (10 μg) and showed that this combined treatment prevented DES induction of virtually all of the reproductive tract abnormalities as well as prevented the loss of AR expression (24). This was interpreted as evidence in support of the importance of the androgen-estrogen balance. However, a potential criticism of this experimental design was that the dose of testosterone esters administered resulted in supranormal levels of testosterone *in vivo* that would have accelerated maturational development of the reproductive tract. Theoretically, this change might protect the reproductive tract from the adverse effects of the high estrogen levels resulting from the 10-μg DES treatment. It was therefore reasoned that a more rigorous test of the androgen-estrogen balance hypothesis was to establish whether reproductive tract abnormalities could be induced by lowering androgen production or action in combination with administration of a dose of DES (0.1 μg) that was itself incapable of inducing the reproductive tract abnormalities. This was the aim of the present studies. The results obtained were compared with the major abnormalities induced by treatment with a 100-fold higher dose of DES (10 μg) on its own. To enable accurate comparison, abnormality end points were chosen that could be quantified and that included both anterior (rete, efferent ducts) and more distal (vas deferens) parts of the tract. Finally, as the present findings have implications for the study of endocrine disruptors, we also assessed whether substitution of 0.1 μg DES by the environmental estrogen bisphenol-A in combination with treatment to lower androgen production (GnRHa) was able to induce any effects.

## Materials and Methods

### *Animals, treatments, and sample collection and processing*

Wistar rats, bred in our own animal house, were maintained under standard conditions and were maintained on a soy-free diet (rat and mouse soya-free breeding diet, SDS, Dundee, Scotland, UK). The purpose of the treatment regimens shown below was as follows. Previous studies have established that neonatal treatment with 10 μg DES, but not with GnRHa or flutamide, will induce a range of reproductive tract abnormalities (23, 24). Lowering of the dose of DES to 0.1 μg/injection results in minimization/absence of these abnormalities (23, 24, 27). The latter dose was therefore combined with either GnRHa or flutamide to determine whether the abnormalities could then be induced. All-male litters of 8–12 pups were generated by cross-fostering pups on d 1 (the day of birth). Rats were subjected to 1 of the following treatments administered by sc injection: 1) DES (Sigma, Poole, UK) at a dose of 10 or 0.1 μg in 20 μl corn oil on d 2, 4, 6, 8, 10, and 12; 2) 10 mg/kg of a long-acting GnRHa (Antarelix, Europeptides, Argenteuil, France) in 20 μl corn oil on d 2 and 5 alone [this treatment regimen has been shown previously (14, 24, 28) to effectively suppress gonadotropin secretion until postnatal d 15–20 and to induce a reduction in testis weight on d 18–25 similar to that induced by treatment with 10 μg DES as in treatment 1]; 3) 50 mg/kg of the AR antagonist flutamide (Sigma) in 20 μl corn oil on d 2, 4, 6, 8, 10, and 12 [this dose was chosen based on studies by Imperato-McGinley *et al.* (29) that showed it caused major reproductive tract abnormalities in male offspring when administered to pregnant rats; we have shown that administration of this dose neonatally retards normal development of the reproductive tract (23, 24, 27)]; 4) 100 μg bisphenol A (Aldrich Chemicals Ltd., Dorset, UK) on d 2, 4, 6, 8, 10, and 12; 5) sc injection of 20 μl corn oil alone (control); 6) combined treatment of 0.1 μg DES (as in treatment 1) with GnRHa (as in treatment 2); 7) combined treatment of 0.1 μg DES (as in treatment 1) with flutamide (as in treatment 3); and 8) combined treatment of 100 μg bisphenol A (as in treatment 4) with GnRHa (as in treatment 2).

Rats from the various treatment groups described above were killed on d 15 or d 18, the age range during which DES-induced reproductive tract abnormalities are at their most prominent (15, 23, 28, 30). Animals were anesthetized with flurothane, and the right testis was dissected out, weighed, and fixed for about 5 h in Bouins' fixative. The left testis was removed with the epididymis and proximal vas deferens still attached and similarly fixed.

After fixation, tissue was transferred into 70% ethanol before being processed for 17.5 h in an automated TP1050 processor (Leica Corp., Deerfield, IL) and embedded in paraffin wax. Sections of 5-μm thickness were cut, floated onto slides coated with 2% 3-aminopropyltriethoxysilane (Sigma), and dried at 50 C overnight before being used for immunohistochemistry and/or image analysis as described below. All of the studies of the rete testis and reproductive tract described below used tissue sections of the left testis with the epididymis attached in order that minimal artifactual distortion was caused to the excurrent duct system.

### *Antibodies used for immunohistochemistry*

Immunolocalisation of AR used a rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) raised against an epitope at the N terminus of human AR and was used at a dilution of 1:200. ERα was immunolocalized using a mouse monoclonal antibody raised against a full-length human ERα recombinant protein (Novocastra, Newcastle upon Tyne, UK) and used at a dilution of 1:20. The specificity of the AR and ERα antibodies has been detailed in our previous studies (6, 24, 27).

### *Immunohistochemistry*

Unless otherwise stated, all incubations were performed at room temperature. Sections were deparaffinized in Histoclear (National Diagnostics, Hull, UK), rehydrated in graded ethanols, and washed in water. At this stage, sections were subjected to a temperature-induced antigen retrieval step (31) in 0.01 M citrate buffer, pH 6.0 (for AR and ERα). After pressure cooking for 5 min at full pressure, sections were left to stand, undisturbed, for 20 min, then were cooled under running tap water before being washed twice (5 min each) in Tris-buffered saline (TBS: 0.05 M Tris-HCl, pH 7.4; and 0.85% NaCl). Endogenous peroxidase activity was blocked by immersing all sections in 3% (vol/vol) $H_2O_2$ in methanol (both from BDH Laboratory Supplies, Poole, UK) for 30 min, followed by two 5-min washes in TBS. To block nonspecific binding sites, sections were incubated for 30 min with the appropriate normal serum diluted 1:5 in TBS containing 5% BSA (Sigma). For AR, normal swine serum was used, and for ERα, normal rabbit serum was used (both from Scottish Antibody Production Unit, Carluke, Scotland, UK). Primary antibodies were added to the sections at the appropriate dilution in either normal swine serum/TBS/BSA (for AR) or normal rabbit serum/TBS/BSA (for ERα) and incubated overnight at 4 C in a humidified chamber. After two 5-min washes in TBS, sections were incubated with a secondary antibody, namely a 1:500 dilution in the appropriate blocking serum of biotinylated swine antirabbit IgG (DAKO Corp., High Wycombe, UK) in the case of AR or biotinylated rabbit antimouse IgG (DAKO Corp.) for ER. After two additional 5-min washes in TBS, all sections were incubated for 30 min with avidin-biotin conjugated to horseradish peroxidase (DAKO Corp.) diluted in 0.05 M Tris-HCl, pH 7.4, according to the manufacturer's instructions. Sections were washed twice (5 min each time) in TBS, and immunostaining was developed using 0.05% 3,3′-diaminobenzidine (Sigma) in 0.05 M Tris-HCl, pH 7.4, containing 0.01% (vol/vol) $H_2O_2$ until staining in positive control tissues

was optimal, when the reaction was stopped by immersing all sections in distilled water. All sections were then lightly counterstained with hematoxylin, dehydrated in graded ethanols, cleared in xylene, and coverslipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK).

To ensure the reproducibility of findings, tissue sections from a minimum of three to six animals in each treatment group were evaluated; this was performed on at least two separate occasions, and similar results were obtained. Further confirmation was obtained by undertaking immunohistochemistry with tissue sections from control and treated animals on the same slide. The specificity of immunostaining was checked for each antibody using previously established procedures. This involved demonstrating that incubation of the primary antibody with either 10× (wt/wt) of the peptide immunogen (AR; peptide sc-816P, Santa Cruz Biotechnology, Inc.) or with the respective recombinant protein (ERα) (6, 24, 27) overnight at 4 C was able to block immunostaining.

Immunostained sections were examined and photographed using a Provis microscope (Olympus Corp., London, UK) fitted with a Kodak DCS330 camera (Eastman Kodak Co., Rochester, NY). Captured images were stored on a G4 computer (MacIntosh, Apple Computers, Cupertino, CA) and compiled using Photoshop 5.0 before being printed using an Epson Stylus 750 color printer (Seiko Epson Corp., Nagano, Japan).

### *Measurement of rete testis lumen area (rete testis distension)*

Rete testis lumen area was quantified as a measure of rete testis distension/overgrowth. Sections immunostained for ERα as described above were used together with an Olympus Corp. BH2 microscope fitted with a ×4 plan Achromat objective and a ×3.3 phototube (Olympus Corp.). The image was captured using a SonyxC77CE video camera (Sony, Tokyo, Japan) linked to a personal computer with frame grabber and Image Pro image analysis software (Media Cybernetics, Silver Spring, MD). To ensure consistency regarding the cross-section of the rete testis that was measured, sections were chosen in which the region of the rete testis draining into the efferent ducts could be viewed in the plane of section. Using the count/size tool, the area was measured by drawing around the edges of the rete lumens as shown in Fig. 1. The total rete area in the plane of sections was determined in at least three to six animals in each treatment group.

### *Measurement of efferent duct lumen area (efferent duct distension)*

Efferent duct lumen area was measured as an index of efferent duct distension (24, 30). Sections immunostained for ERα as described above were used, and the quantification method used was similar to that described for rete testis lumen area, except that a ×20 plan Achromat objective was used. Only round, symmetrical, efferent ductule cross-sections were selected for measurement to avoid errors due to plane of sectioning of individual ductules. Using the count/size tool, the edges of the lumen of individual ductules were drawn around, and the area was measured as shown in Fig. 1. For each animal, 10 cross-sections were measured, and a mean value per animal then calculated.

### *Measurement of epithelial cell height*

To determine whether neonatal treatment altered the height of epithelial cells in the efferent ducts and vas deferens, cross-sections from 3–15 rats from control and treated cohorts were evaluated using image analysis at each time point. Sections immunostained for ERα as described above were used. The height of the epithelial cells within the efferent ducts and vas deferens was measured using a ×40 plan Achromat and ×20 objective for the efferent ducts and vas deferens, respectively. Only round or oval cross-sections were selected for measurement. Using the length tool, the height of the epithelium was measured by drawing a line at right angles to the base of the cell adjacent from the basement membrane to the lumenal surface of the cell. After measuring the length, the angle of the line was measured to ensure that it was at 90°. For each animal, at least 24 cells were measured, with sampling from a number of different ductules, and the mean value then calculated for each animal.

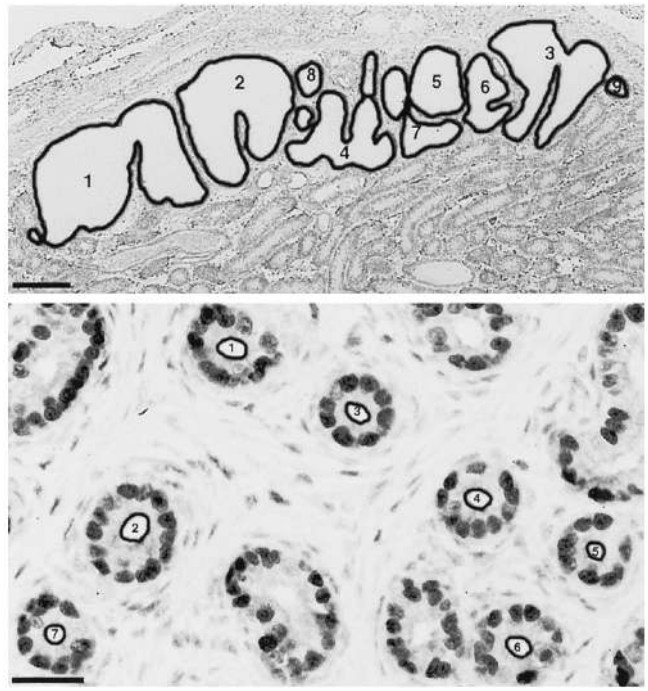

FIG. 1. Illustration of the image analysis method used to measure lumenal area of the rete testis (*top*) and efferent ducts (*bottom*) in control and treated animals. Note that sections have been immunostained for ERα, hence the dark staining of nuclei of the epithelial cells in the efferent ducts. *Scale bar*, 200 μm (*top*) or 100 μm (*bottom*).

### *Measurement of plasma testosterone levels*

Plasma testosterone levels were measured using an ELISA adapted from an earlier RIA method (32). Plasma, to which was added trace amounts of [$^3$H]testosterone (Amersham International, Little Chalfont, UK), was extracted twice with 10 vol hexane-ether (4:1, vol/vol), and the organic phase was dried down under $N_2$ at 55 C. The efficiency of extraction averaged 75%. The second antibody was immobilized to an ELISA plate by addition of 100 μl acid purified donkey antigoat/sheep IgG (250–350 mg/ml) diluted in 0.1 M sodium carbonate buffer, pH 9.6. The plate was sealed and incubated overnight at 4 C. The wells were then washed twice with 0.1% Tween 20 and incubated for 10 min at room temperature with 0.2 ml of the same solution to block nonspecific binding sites. Samples in duplicate (50 μl) were assayed after dilution in 0.1 M PBS, pH 7.4, containing 0.1% gelatin (Sigma) and incubated overnight at 4 C with 50 μl sheep antitestosterone-3-carboxymethyloxide-BSA diluted 1:100,000 plus 50 μl testosterone-3-carboxymethyloxide labeled with 1:20,000 diluted horseradish peroxide (Amdex, Amersham Pharmacia Biotech, Uppsala, Sweden). The plate was then washed several times with 0.1% Tween 20 before addition of 0.2 ml substrate (5 mM *O*-phenylenediamine; Sigma) and 0.03% hydrogen peroxide diluted in 0.1 M citrate-phosphate, pH 5.0, to each well. The plate was incubated in the dark for 10–30 min until the color reaction was optimal. The reaction was stopped by the addition of 50 μl 2 M sulfuric acid to each well, and the OD was read at 492 nm in a plate reader. The limit of detection was 12 pg/ml, and the intraassay coefficient of variation was 8.3%. All samples were assayed together in one run.

### *Statistics*

Comparison of the different parameters for the various treatment groups was made using ANOVA after logarithmic transformation of the data to obtain a normal distribution. Where significant differences between groups were indicated, subgroup comparisons also used ANOVA, but used the variance from the experiment as a whole (for that parameter) as the measure of error.

## Results

### *Rete testis lumenal area in relation to plasma testosterone levels*

*Day 15.* Representative photomicrographs of the effects of the different treatment regimens on rete testis lumenal size and quantification of this parameter on d 15 are shown in Figs. 2 and 3, respectively. As reported previously (23, 24, 30), neonatal treatment with a high dose (10 μg) of DES induced massive distension/overgrowth of the rete testis coincident with 73% suppression of testosterone levels. No change in rete testis area was evident in animals treated with GnRHa or 0.1 μg DES alone, although both treatments, in particular GnRHa, reduced testosterone levels (0.1 μg DES, 35% reduction; GnRHa, 73% reduction); the magnitude of reduction in testosterone levels in GnRHa-treated rats was comparable to that in rats treated with 10 μg DES (Fig. 3). However, combined treatment with 0.1 μg DES and GnRHa, which reduced testosterone levels to a similar extent (77%) as GnRHa treatment alone, induced prominent rete distension/overgrowth (Fig. 2) that was significantly different from the effects of either of the two treatments when administered singly (Fig. 3). Treatment with bisphenol-A, either alone or in combination with GnRHa, did not induce any significant change in rete area relative to control and GnRHa treatment alone, respectively, despite the fact that testosterone levels were grossly lowered (84%) when GnRHa was coadministered (Fig. 3).

*Day 18.* In animals assessed on d 18, the rete testis of animals treated with 10 μg DES exhibited comparable overgrowth/distension as that observed on d 15 (Figs. 2 and 4). Neonatal treatment with 0.1 μg DES had only a minor effect on rete area, and neither flutamide nor GnRHa treatment had any significant effect on rete lumenal area when administered alone. However, combined treatment with 0.1 μg DES plus either GnRHa or flutamide produced significant enlargement of the rete compared with the effect of either treatment alone (Figs. 2 and 4). The changes in testosterone levels on d 18 (Fig. 4) were comparable to those on d 15, with similar

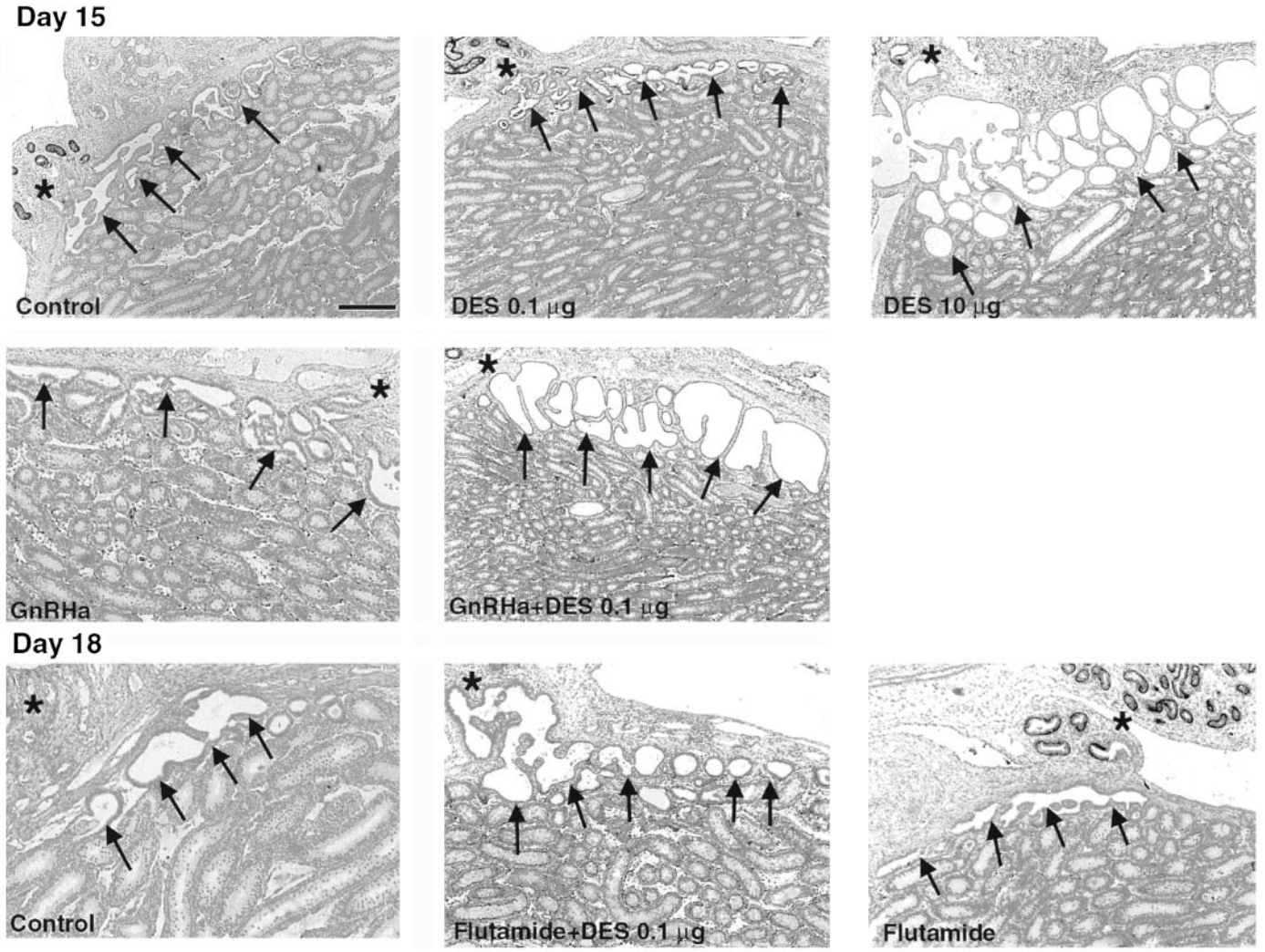


FIG. 2. Effect of neonatal treatment with vehicle (control), 10 μg DES, 0.1 μg DES, GnRHa, or 0.1 μg DES plus GnRHa at 15 d of age (*top two rows*) or with flutamide alone or 0.1 μg DES plus flutamide at 18 d of age (*bottom row*) on the size of the rete testis (*arrows*). Note that all sections were obtained in the region in which the efferent ducts (*asterisks*) emerged from the rete. Sections were immunostained for ERα, hence the dark staining of nuclei of epithelial cells in the efferent ducts. *Scale bar*, 400 μm.

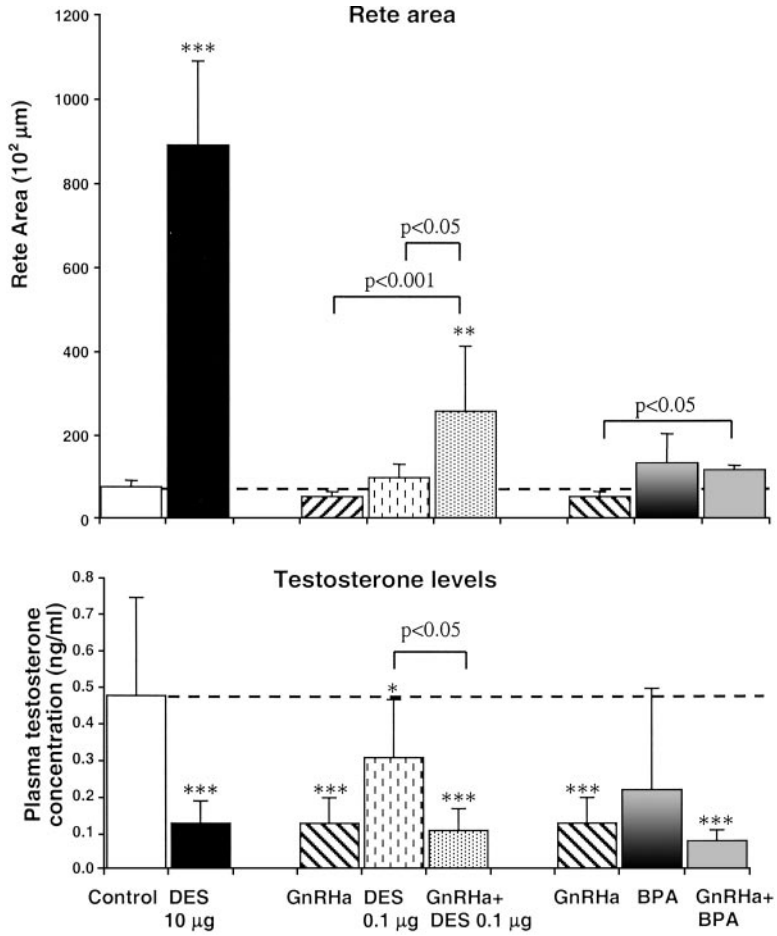


FIG. 3. Effect of neonatal treatment with vehicle (control), 10 µg DES, 0.1 µg DES, GnRHa, 0.1 µg DES plus GnRHa, bisphenol-A (BPA), or BPA plus GnRHa on rete testis lumen area (*top*) and on plasma testosterone levels (*bottom*) at 15 d of age. The *dashed line* shows the mean control value. The data shown are mean ± SD for 3–10 animals/group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with the respective control value).

major reductions being caused by treatment with 10 µg DES (71% reduction) or GnRHa alone (68% reduction) or by any of the combined treatments that included GnRHa (60% reduction for 0.1 µg DES plus GnRHa; 53% reduction for flutamide plus GnRHa). In contrast, treatment with flutamide alone significantly elevated testosterone levels (Fig. 4), presumably because it blocked testosterone negative feedback at the hypothalamus-pituitary.

*Efferent duct lumenal area*

*Day 15.* Neonatal treatment with 10 µg DES induced major distension of the efferent duct lumen compared with controls on d 15 (Figs. 5 and 6). Treatment with 0.1 µg DES alone caused an approximately 5-fold smaller, but highly significant, increase (Fig. 6), although this was not obvious from gross inspection of photomicrographs (Fig. 5). Treatment with GnRHa alone caused a significant reduction in efferent duct lumenal area (Figs. 5 and 6), but combination of this treatment with 0.1 µg DES induced significantly greater ($P < 0.001$) distension of the efferent duct lumen than either treatment alone (Figs. 5 and 6). Neonatal treatment with either bisphenol-A alone or bisphenol-A plus GnRHa did not cause any significant change in lumenal area (Figs. 5 and 6).

*Day 18.* As occurred on d 15, efferent duct lumenal area was increased massively on d 18 in animals treated neonatally with 10 µg DES and to a much smaller, but significant, extent by 0.1 µg DES (Figs. 5 and 6). Neither GnRHa nor flutamide treatment alone induced any significant change relative to controls on d 18. In contrast, combined treatment with 0.1 µg DES plus GnRHa or flutamide caused a major increase in efferent duct lumenal area, and this was significantly greater ($P < 0.01$) than the effect any of these three treatments alone (Figs. 5 and 6).

*Efferent duct epithelial cell height*

*Day 15.* All treatments, with the exception of bisphenol-A, caused a significant reduction in epithelial cell height (Figs. 5 and 7). The biggest reduction (56%) was caused by treatment with 10 µg DES. However, combined treatment with 0.1 µg DES and GnRHa induced a reduction in epithelial cell height of similar magnitude (52%) to that caused by 10 µg DES, and this was significantly greater ($P < 0.01$) than when either 0.1 µg DES (32% reduction) or GnRHa (32% reduction) was administered alone (Figs. 5 and 7). In contrast, combined treatment with bisphenol-A and GnRHa (32% reduction) had no greater effect than did GnRHa administered alone (32% reduction; Fig. 7).

*Day 18.* All treatments caused a significant reduction in epithelial cell height (Figs. 5 and 7). The biggest reduction was caused by treatment with 10 µg DES (51% reduction), comparable to that observed on d 15 (Fig. 7). Although treatment with GnRHa or 0.1 µg DES alone caused a significant reduction in epithelial cell height (27% and 16% reductions,

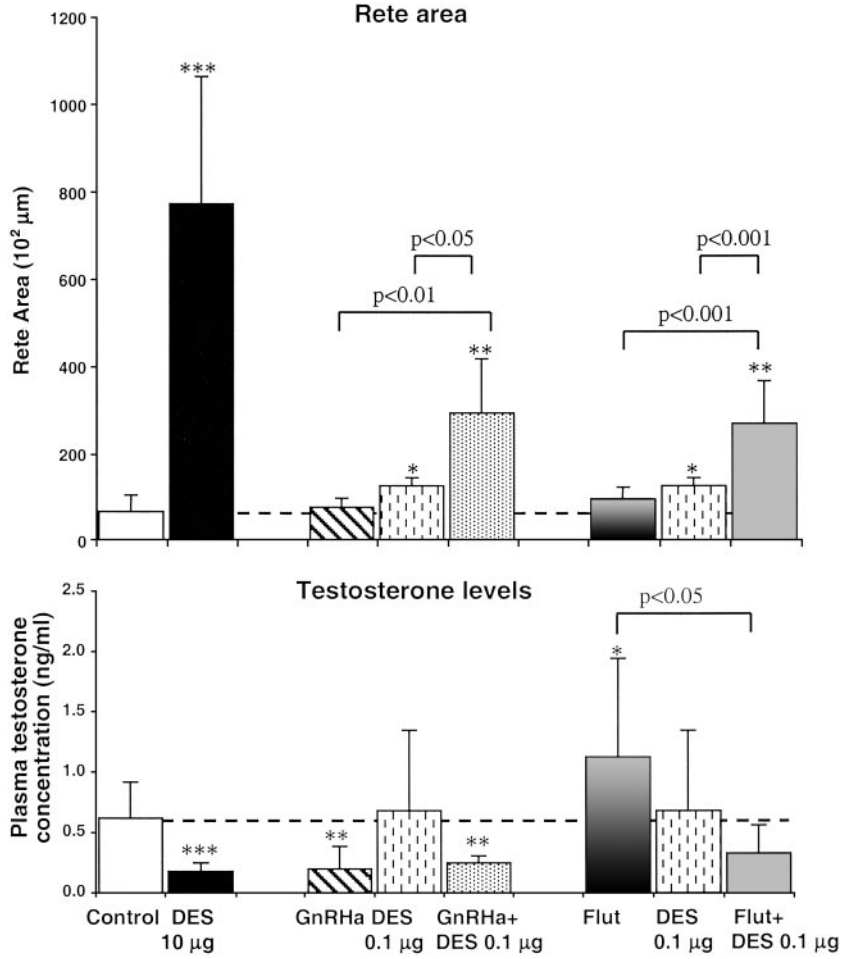


FIG. 4. Effect of neonatal treatment with vehicle (control), 10 µg DES, 0.1 µg DES, GnRHa, 0.1 µg DES plus GnRHa, flutamide (Flut), or 0.1 µg DES plus flutamide on rete testis lumen area (*top*) and plasma testosterone levels (*bottom*) at 18 d of age. The *dashed line* shows the mean control value. The data shown are the mean ± SD for 3–10 animals/group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with the respective control value).

respectively), this was of smaller magnitude than that observed on d 15 after similar treatment. However, combined treatment with 0.1 µg DES and GnRHa (47% reduction) or flutamide (53% reduction) induced a reduction in epithelial cell height similar to that caused by 10 µg DES, and this was significantly greater ($P < 0.005$) than that caused by 0.1 µg DES, GnRHa, or flutamide given alone (Figs. 5 and 7).

*Epithelial cell height in the proximal vas deferens*

*Day 15.* All treatments, with the exception of bisphenol-A, caused a significant and similar reduction in epithelial cell height on d 15 compared with controls (Fig. 8). Combined treatment with 0.1 µg DES plus GnRHa did not cause any greater reduction in epithelial cell height than either treatment given alone. Combined treatment with bisphenol-A and GnRHa had no greater effect than GnRHa administered alone (Fig. 8).

*Day 18.* Similar to results on d 15, all treatments caused a reduction in epithelial cell height on d 18 compared with controls (Fig. 8). Combined treatment with 0.1 µg DES plus GnRHa or flutamide did not cause any greater reduction in epithelial cell height than any of these treatments given alone, although 0.1 µg DES plus GnRHa was marginally different ($P < 0.05$) from 0.1 µg DES alone (Fig. 8).

*Immunoexpression of ERα in the efferent ducts*

As reported previously (15, 33, 34), immunoexpression of ERα was constant and very intense in the efferent ducts in control animals on d 15 and 18, and none of the neonatal treatments had any detectable effect on immunoexpression of ERα (Fig. 5).

*Immunoexpression of AR*

In control animals on d 15 and 18, there was intense immunoexpression of AR in the testis (not shown) and in epithelial and stromal cells of the efferent ducts (Fig. 9) and epididymis (not shown). Treatment with 10 µg DES almost completely abolished AR immunoexpression at all of these sites (*e.g.* Fig. 9). In contrast to these effects, blockage of androgen production (GnRHa) or action (flutamide) was without effect on AR immunoexpression in these tissues, except that the intensity of AR immunostaining in the testes of flutamide-treated animals was consistently slightly weaker than that in controls (not shown) (24). Neonatal treatment with 0.1 µg DES was without obvious effect on AR immunoexpression (*e.g.* Fig. 9). In contrast, combined treatment with 0.1 µg DES and GnRHa or flutamide greatly reduced the intensity of AR immunoexpression in the efferent ducts (Fig. 9), epididymis, and vas deferens (not shown)

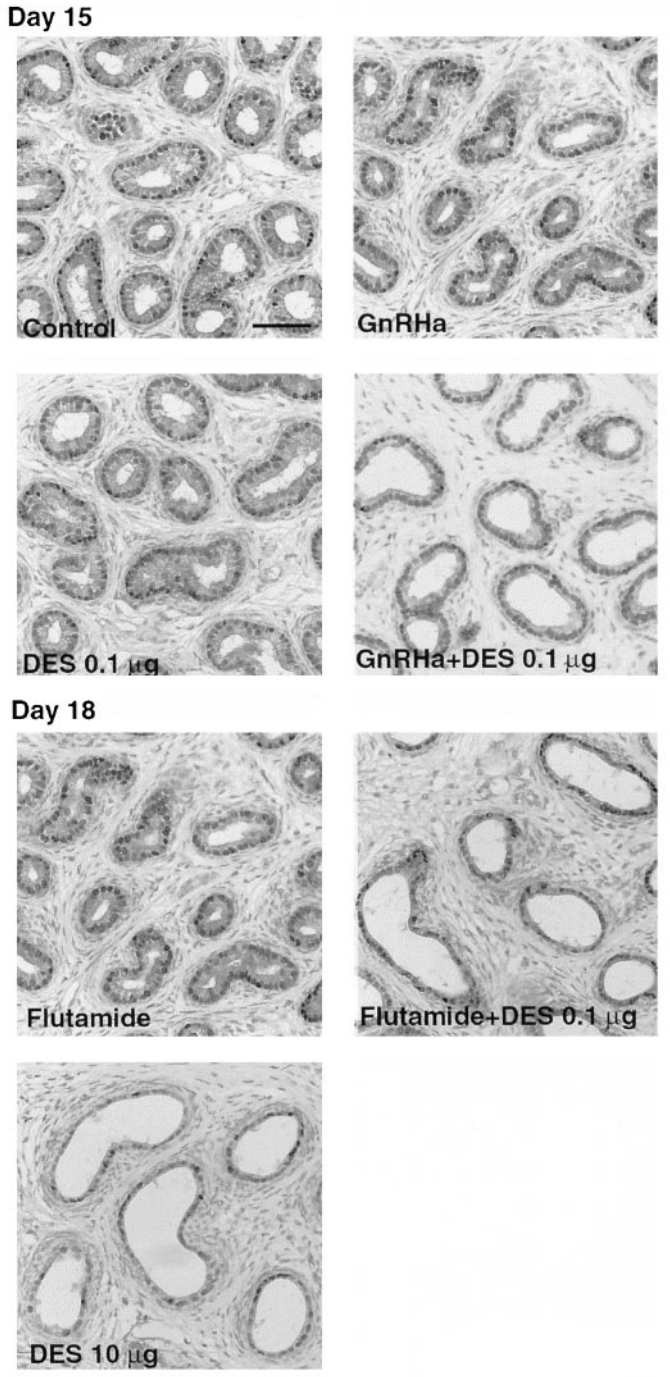


FIG. 5. Effect of neonatal treatment with vehicle (control), 10 $\mu$g DES, 0.1 $\mu$g DES, GnRHa, 0.1 $\mu$g DES plus GnRHa, flutamide, or 0.1 $\mu$g DES plus flutamide on the gross morphology of the efferent ducts and immunoexpression of ER$\alpha$ in the nuclei of epithelial cells. Note that lumenal distension and reduction in height of the epithelium are confined to animals treated with 10 $\mu$g DES or those treated with 0.1 $\mu$g DES plus GnRHa or 0.1 $\mu$g DES plus flutamide, whereas treatment with 0.1 $\mu$g DES, GnRHa, or flutamide alone has no obvious effect. *Scale bar*, 50 $\mu$m.

on d 15 and 18. The reduction in intensity of AR immunoexpression in these combined treatment groups was not as severe as that induced by treatment with 10 $\mu$g DES (*e.g.* Fig. 9).

## Discussion

The primary aim of the present studies was to test the hypothesis that testis/reproductive tract abnormalities, which are induced in the neonatal rat by treatment with relatively high doses (10 $\mu$g) of DES, result from disturbance of the androgen-estrogen balance rather than from the estrogenic effect of DES alone (23, 24). This was tested by treating male rats neonatally with a dose of DES (0.1 $\mu$g) that induced either no or small effects on its own and combining this with a treatment that suppressed either androgen production (GnRHa) or androgen action (the androgen receptor antagonist flutamide). The results show unequivocally that either of these combined treatments was able to significantly induce greater abnormalities of the rete testis and efferent ducts than did 0.1 $\mu$g DES administered alone. Neither GnRHa nor flutamide induced any of the abnormalities when administered alone, whereas treatment with a high dose of DES (10 $\mu$g) induced major abnormalities coincident with loss of immunoexpression of the AR in affected tissues. Reduced immunoexpression of AR was also induced by combined treatment with 0.1 $\mu$g DES and GnRHa or flutamide, whereas treatment with any of the compounds alone had no or only minor effects on AR immunoexpression. These results together with our previous demonstration that induction of reproductive abnormalities by neonatal treatment with 10 $\mu$g DES can be blocked by cotreatment with testosterone (24) provide support for the suggestion that these abnormalities result from a disturbance of the normal androgen-estrogen balance rather than from a lowering of androgen action or an elevation of estrogen action *per se*. A slightly alternative view is that the level of androgen exposure determines the sensitivity of the developing male reproductive tract to estrogens, such that low androgen levels increase sensitivity to estrogens and *vice versa*. Either interpretation implies that normal or abnormal development of the male reproductive system may be governed by the relative levels (balance) of androgens and estrogens rather than by the absolute level of either hormone (23, 24). This has several implications, as discussed below.

The finding of key importance from the present study is the demonstration that combined treatment with a relatively low dose (0.1 $\mu$g) of DES plus treatments to either lower androgen production (GnRHa) or block androgen action (flutamide) was able to induce significantly greater distension of the rete testis or efferent ducts, as measured by lumenal area, than any of the treatments administered alone; of the latter, only 0.1 $\mu$g DES had any (minor) effect on its own. Although these contrasting effects were clear-cut, the use of another end point, epithelial cell height in the efferent ducts or proximal vas deferens, resulted in a more complex picture, as all single treatments other than bisphenol-A exerted significant adverse effects. In the efferent ducts it was still clear that combined treatment with 0.1 $\mu$g DES plus either GnRHa or flutamide was able to reduce epithelial cell height to a significantly greater extent than any of these treatments individually; indeed, the combined treatments caused an effect of similar magnitude to that induced by treatment with 10 $\mu$g DES alone. In contrast, several of the individual treatments (10 or 0.1 $\mu$g DES plus GnRHa) in-

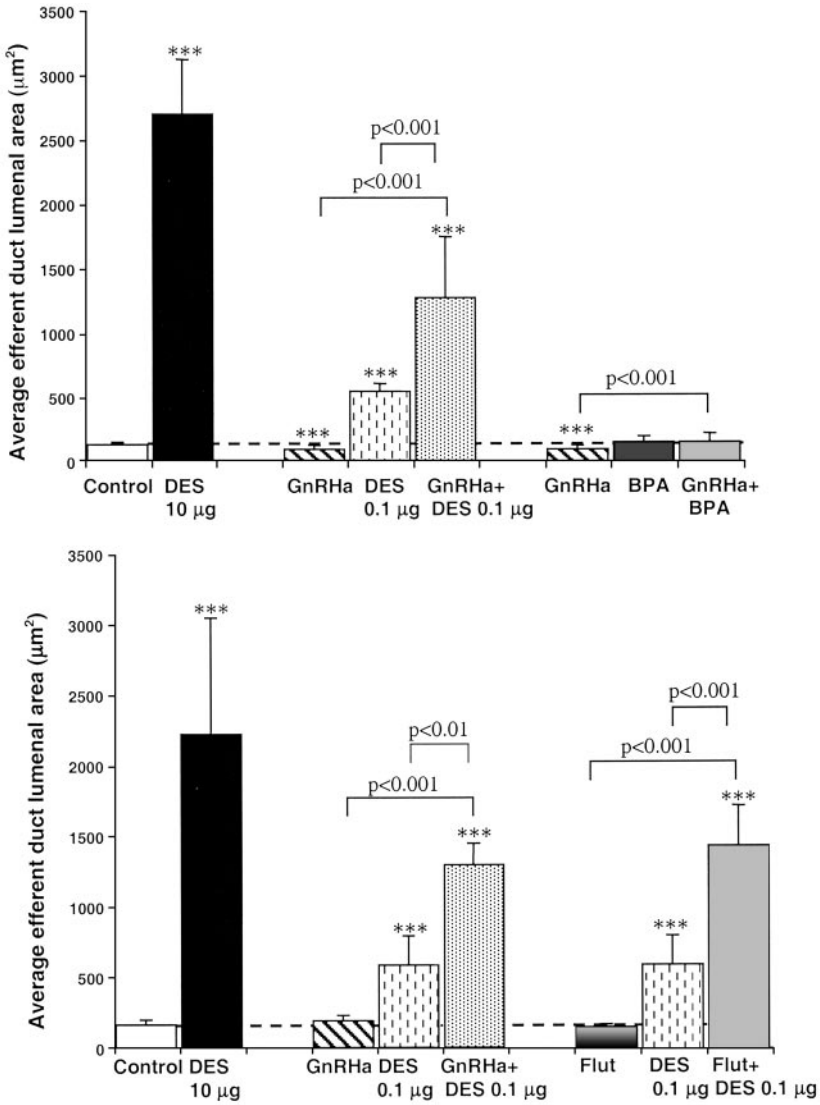


FIG. 6. Effect of neonatal treatment with vehicle (control), 10 $\mu$g DES, 0.1 $\mu$g DES, GnRHa, 0.1 $\mu$g DES plus GnRHa, flutamide (Flut), 0.1 $\mu$g DES plus flutamide, bisphenol-A (BPA), or BPA plus GnRHa on the average lumenal area of an efferent duct at 15 d (*top*) and/or 18 d (*bottom*) of age. The *dashed line* shows the mean control value. The data shown are the mean ± SD for 3–10 animals/group. ***, $P < 0.001$ (compared with the respective control value).

duced very similar effects on epithelial cell height in the proximal vas deferens, and combined treatment with 0.1 $\mu$g DES and either GnRHa or flutamide was unable to exacerbate this effect compared with administration of 0.1 $\mu$g DES or GnRHa alone. This suggests that more posterior parts of the reproductive tract, such as the vas deferens, may be more sensitive to androgens and estrogens than the more anterior parts (rete and efferent ducts) such that all single treatments, other than flutamide and bisphenol-A, induced maximal effects. This makes it fundamentally difficult to test whether the androgen-estrogen balance is as important in the vas as it appears to be in the more anterior regions of the tract.

Although the different responses of epithelial cells from the efferent ducts and vas deferens to altered androgens and estrogens remain to be explained, the fact that normal development of the rete and efferent ducts appear particularly dependent on a normal androgen-estrogen balance may have relevance to findings in ER$\alpha$ knockout (ERKO) mice. The latter exhibit distension of both the rete and efferent ducts (8, 18, 19, 35) in the face of supranormal testosterone levels (7, 9) and the absence of normal expression of ER$\alpha$ in the epithelium of both the rete (30, 35) and efferent ducts (33–35). This raises the possibility that the abnormalities reported in ERKO males do not result only from the loss of ER$\alpha$-mediated estrogen action, but might also be affected by disturbance of the androgen-estrogen balance, although in the opposite direction (supranormal androgen plus subnormal estrogen action) to that induced in the present studies. However, this would fail to explain why aromatase knockout mice, which also have elevated testosterone levels, do not show the same structural abnormalities as ERKO mice (16, 17, 36). Nevertheless, the present findings caution that whenever androgen and/or estrogen levels are altered substantially from normal in the developing male, changes resulting from disruption of the androgen-estrogen balance should be kept in mind.

There are examples in the literature indicating a role for the androgen-estrogen balance. For example, gynecomastia in men can be induced either by raising estrogen levels or by lowering androgen levels, such that in either situation the androgen-estrogen balance is altered in favor of estrogens (37–39). Another example is clover disease, in which castrated rams that fed on Mediterranean clover containing

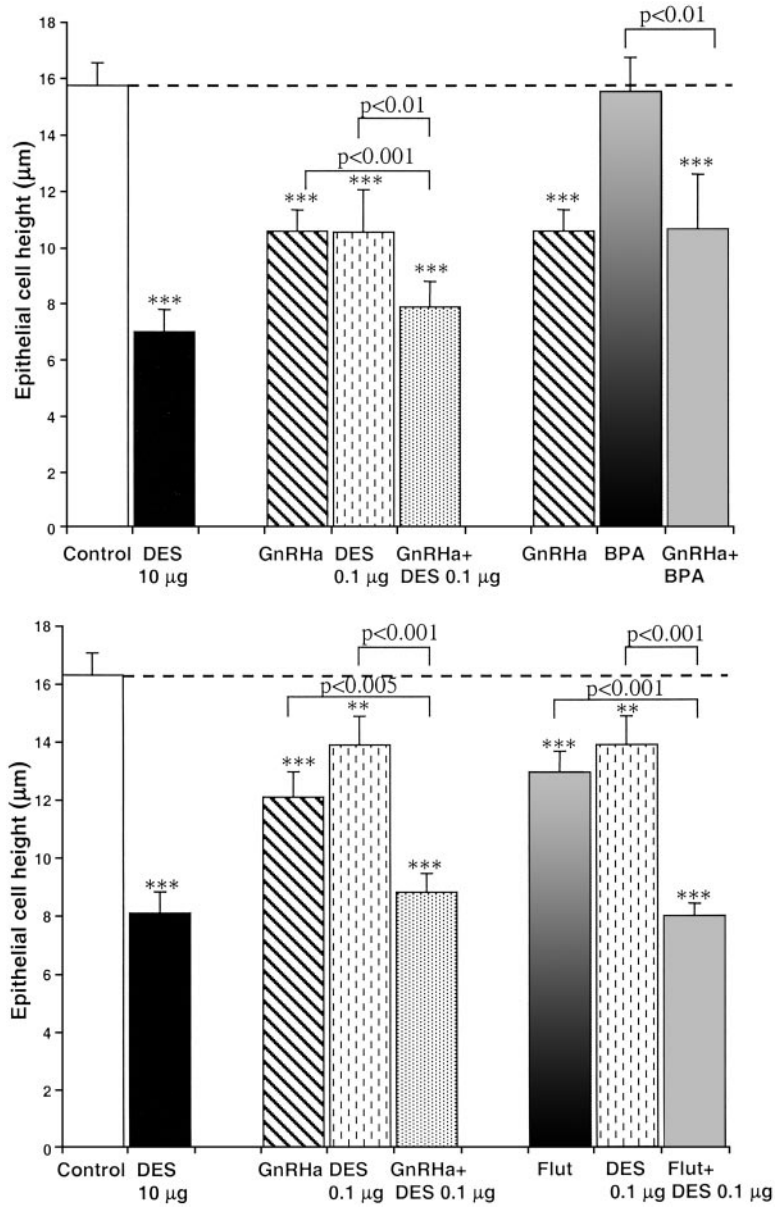


FIG. 7. Effect of neonatal treatment with vehicle (control), 10 μg DES, 0.1 μg DES, GnRHa, 0.1 μg DES plus GnRHa, flutamide (Flut), 0.1 μg DES plus flutamide, bisphenol-A (BPA), or BPA plus GnRHa on epithelial cell height in the efferent ducts at 15 d (*top*) and/or 18 d (*bottom*) of age. The *dashed line* shows the mean control value. The data shown are the mean ± SD for 3–10 animals/group. **, $P < 0.01$; ***, $P < 0.001$ (compared with the respective control value).

weakly estrogenic phytoestrogens died from urinary retention due to overgrowth of the bulbo-urethral glands (40, 41), which are both an estrogen and an androgen target (41, 42). Intact rams or castrated rams treated with androgens and fed on the same clover showed no overgrowth or ill effects. Thus, in castrated rams with low androgen levels, phtyoestrogens induced a catastrophic effect, whereas in intact rams with high androgen levels or in castrated rams supplemented with androgens, no effect occurred despite exposure to the same "estrogen" level. In many respects this example arises from a situation in which hormonal status would be comparable to that induced in the present studies by combined treatment with 0.1 μg DES plus GnRHa. There may be other examples in which a role for altered androgen-estrogen balance could be important. For example, abnormal prostatic structure and growth induced by neonatal estrogen treatment are known to involve both altered androgen and estrogen action (22, 43, 44), and this may apply also to reproductive tissues in the female (45).

If the relative, rather than absolute, levels of androgens and estrogens are important for normal development and/or function of the testis and reproductive tract, as our findings suggest, how would this work at the cellular level, given that the present understanding is that androgens and estrogens act via separate, if related, signaling systems? There are various possibilities based on published data, although none has been shown to operate physiologically. For example, estrogens can *trans*-activate the AR/$ARA_{70}$ complex at high concentrations and thus activate the transcription of androgen-dependent genes, although DES could not exert this effect (26). Interaction between the C-terminal domain of ERα and the AR has been demonstrated using two-hybrid systems, and cotransfection of the two receptors into CV-1 cells has demonstrated a mutual ability of each receptor to antagonize

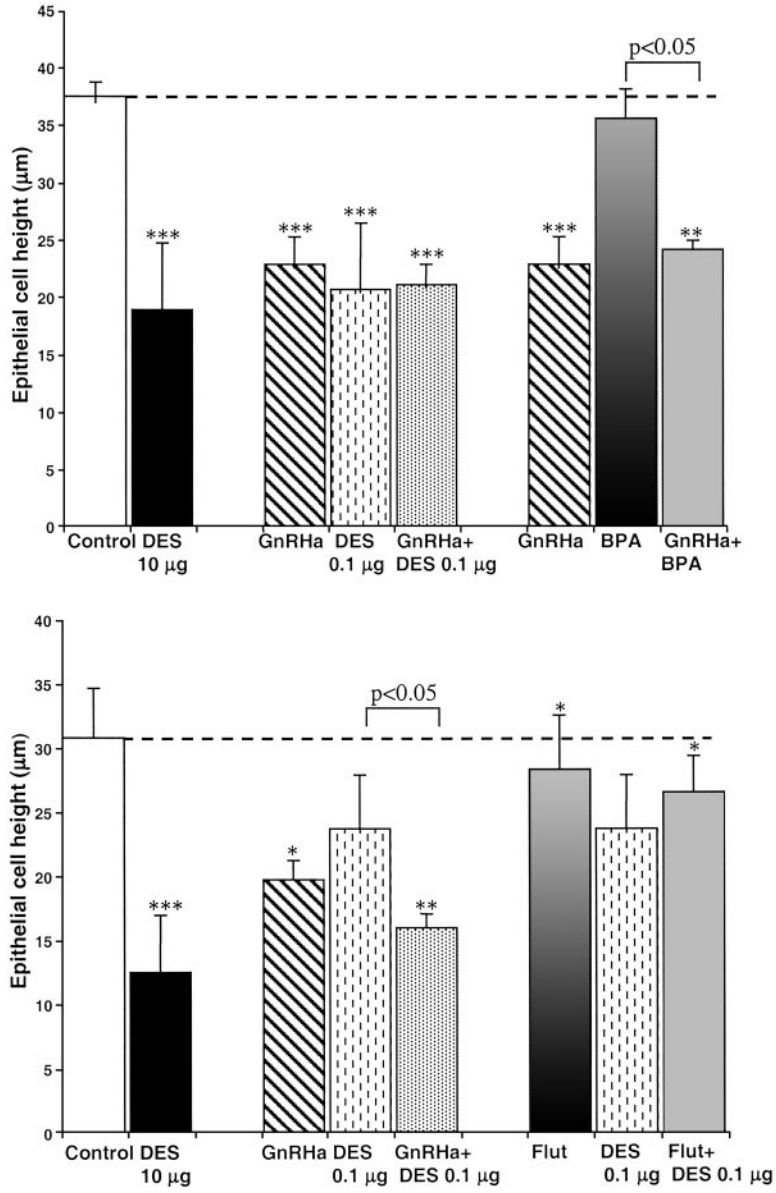


FIG. 8. Effect of neonatal treatment with vehicle (control), 10 $\mu$g DES, 0.1 $\mu$g DES, GnRHa, 0.1 $\mu$g DES plus GnRHa, flutamide, 0.1 $\mu$g DES plus flutamide, bisphenol-A (BPA), or GnRHa plus BPA on epithelial cell height in the proximal vas at 15 and 18 d of age. The *dashed line* shows the mean control value. The data shown are the mean ± SD for 3–10 animals/group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with the respective control value).

*trans*-activation mediated by ligand binding to the other receptor (25). An alternative explanation might be activation of the Src-Raf1/Shc-Erk2 pathway, in which androgens and estrogens may induce assembly of a novel ternary complex comprising the AR, ER (either ER$\alpha$ or ER$\beta$), and Src (46, 47). This complex triggers activation of the protein kinase domain of Src and downstream effects, such as cell proliferation (46) or inhibition of apoptosis (47). The androgen-AR and estrogen-ER complexes bind to separate domains on the Src protein, and antagonists of either the AR or ER can block activation of this pathway by either androgens or estrogens (46). Our recent findings that expression of classical androgen-regulated genes in the prostate can also be regulated by estrogens and that antiestrogens can block androgen activation of these genes *in vivo* (48) are consistent with the activation of such a pathway.

Regardless of the pathway involved, the present findings have implications for issues such as endocrine disruptors, in which considerations of risk are focused largely on the absolute dose/level of exposure as opposed to the relative levels of androgen and estrogens. The present study has shown that combined treatment with a weak environmental estrogen, bisphenol-A, plus a GnRHa was unable to induce any of the abnormalities induced by 0.1 $\mu$g DES plus GnRHa, suggesting that in this situation the estrogenicity of the bisphenol-A, when injected in moderately high amounts (100 $\mu$g/injection), was still insufficient to perturb the androgen-estrogen balance. Whether this balance can be disturbed by higher doses of this or other environmental estrogens or in combination with environmental antiandrogens are obvious questions that need to be addressed.

In summary, the present findings add to the growing evidence of a close interrelationship between the actions of androgens and estrogens in regulating normal and abnormal development of the male reproductive system. Our findings suggest that the balance in action between androgens and

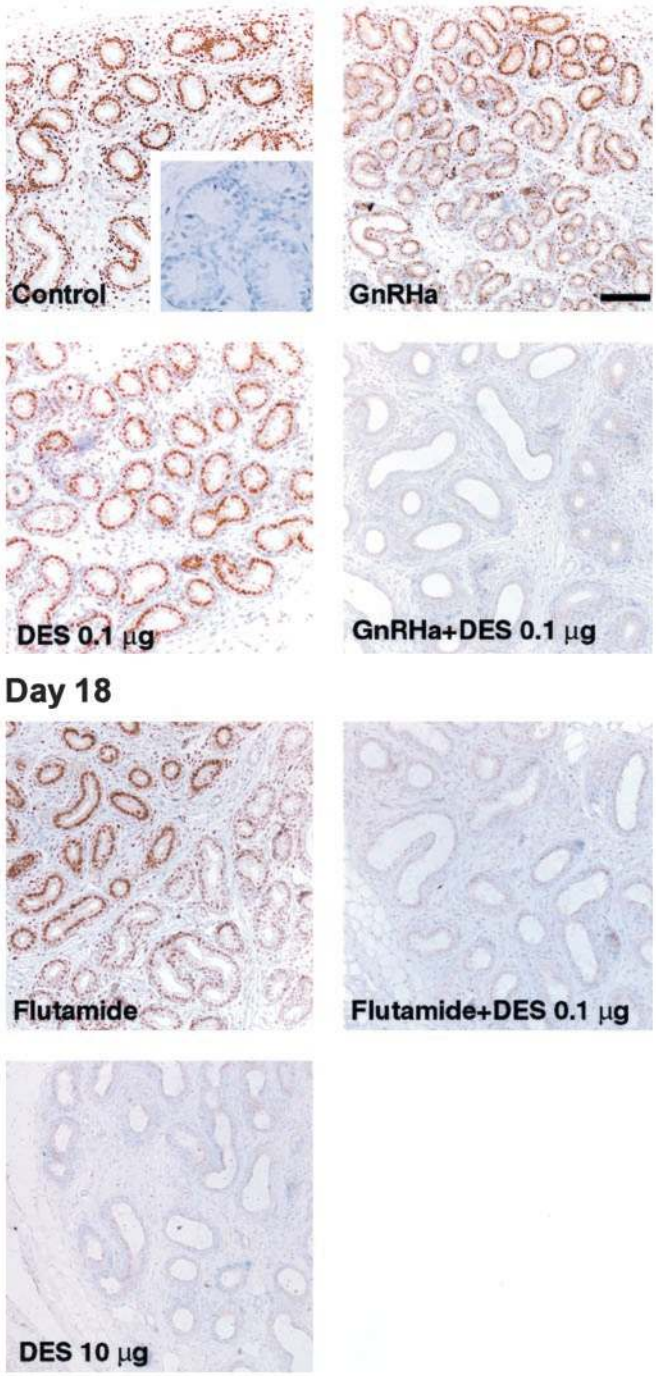


FIG. 9. Effect of neonatal treatment with vehicle (control), 10 $\mu$g DES, 0.1 $\mu$g DES, GnRHa alone, 0.1 $\mu$g DES plus GnRHa, flutamide, or 0.1 $\mu$g DES plus flutamide on AR immunoexpression in the efferent ducts on d 18. Note that reduction in immunoexpression of AR (and lumenal distension) is confined to animals treated with 10 $\mu$g DES, 0.1 $\mu$g DES plus GnRHa, or 0.1 $\mu$g DES plus flutamide, whereas treatment with 0.1 $\mu$g DES, GnRHa, or flutamide alone had no obvious effect. The *inset* in the control panel shows immunoexpression of AR after preabsorption of the primary antibody with the peptide immunogen. *Scale bar*, 50 $\mu$m.

estrogens, rather than the absolute levels of either hormone, may be of fundamental importance at least for some regions of the reproductive tract. From a physiological perspective, local regulation of relative levels of androgens and estrogens, for example by differential expression of aromatase or 5$\alpha$-reductase, may be critical factors that ensure an appropriate steroid milieu for specific regions of the reproductive system.

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## References

1. **Saunders PTK, Maguire SM, Gaughan J, Millar MR** 1997 Expression of estrogen receptor $\beta$ (ER$\beta$) in multiple rat tissues. J Endocrinol 154:R13–R16
2. **Couse JF, Lindzey J, Grandeien K, Gustafsson JA, Korach KS** 1997 Tissue distribution and quantitative analysis of estrogen receptor-$\alpha$ (ER$\alpha$) and estrogen receptor-$\beta$ (ER$\beta$) messenger ribonucleic acid in the wild-type and ER$\alpha$-knockout mouse. Endocrinology 138:4613–4621
3. **Saunders PTK, Fisher JS, Sharpe RM, Millar MR** 1998 Expression of oestrogen receptor $\beta$ (ER$\beta$) occurs in multiple cell types, including some germ cells, in the rat testis. J Endocrinol 156:R13–R17
4. **van Pelt AM, de Rooij DG, van der Burg B, van der Saag PT, Gustafsson JA, Kuiper GG** 1999 Ontogeny of oestrogen receptor-$\beta$ expression in rat testis. Endocrinology 140:478–483
5. **Jefferson WN, Couse JF, Banks EP, Korach KS, Newbold RR** 2000 Expression of estrogen receptor $\beta$ is developmentally regulated in reproductive tissue of male and female mice. Biol Reprod 62:310–317
6. **Saunders PTK, Williams K, Macpherson S, Urquhart H, Irvine DS, Sharpe RM, Millar MR** 2001 Differential expression of oestrogen receptor $\alpha$ and $\beta$ proteins in the testes and male reproductive tract of human and non-human primates. Mol Hum Reprod 7:227–236
7. **Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB, Korach KS** 1996 Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. Endocrinology 137:4796–4805
8. **Hess RA, Bunick D, Lee K-H, Enmark E, Haggblad J, Nilsson S, Gustafsson J-A** 1997 A role for estrogens in the male reproductive system. Nature 390: 509–512
9. **Couse JF, Korach KS** 1999 Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 20:358–417
10. **Thompson RS, Hess DL, Binkerd PE, Hendrickx AG** 1981 The effects of prenatal diethylstilbestrol exposure on the genitalia of pubertal *Macaca mulatta*. II. Male offspring. J Reprod Med 26:309–316
11. **Arai Y, Mori T, Suzuki Y, Bern HA** 1983 Long-term effects of perinatal exposure to sex steroids and diethylstilbestrol on the reproductive system of male mammals. Int Rev Cytol 84:235–268
12. **Newbold RR, Bullock B, McLachlan JA** 1985 Lesions of the rete testis in mice exposed prenatally to diethylstilbestrol. Cancer Res 45:5145–5150
13. **Newbold RR, MaLachlan JA** 1985 Diethylstilbestrol-associated defects in murine genital tract development. In: McLachlan JA, ed. Estrogens in the environment II. New York: Elsevier; 288–318
14. **Atanassova N, McKinnell C, Walker M, Turner KJ, Fisher JS, Morley M, Millar MR, Groome NP, Sharpe RM** 1999 Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number, and the efficiency of spermatogenesis in adulthood. Endocrinology 140:5364–5373
15. **Atanassova N, McKinnell C, Williams K, Turner KJ, Fisher JS, Saunders PTK, Millar MR, Sharpe RM** 2001 Age-, cell-, and region-specific immunoexpression of ER$\alpha$ (but not ER$\beta$) during postnatal development of the epididymis and vas deferens of the rat and disruption of this pattern by neonatal treatment with diethylstilbestrol. Endocrinology 142:874–886
16. **Fisher CR, Graves KH, Parlow AF, Simpson ER** 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. Proc Natl Acad Sci USA 95:6965–6970
17. **Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, Graves KH, McLachlan RI, Simpson ER** 1999 Impairment of spermatogenesis

in mice lacking a functional aromatase (cyp19) gene. Proc Natl Acad Sci USA 96:7986–7991
18. **Hess RA, Bunick D, Lubahn DB, Zhou Q, Bouma J** 2000 Morphologic changes in efferent ductules and epididymis in estrogen receptor-$\alpha$ knockout mice. J Androl 21:107–121
19. **Hess RA, Zhou Q, Nie R, Oliveira C, Cho H, Nakaia M, Carnes K** 2001 Estrogens and epididymal function. Reprod Fertil Dev 13:273–283
20. **Sharpe RM** 1998 The roles of oestrogen in the male. Trends Endocrinol Metab 9:371–377
21. **Haavisto T, Numela K, Pohjanvirta R, Huuskonen H, El-Gehani F, Paranko J** 2001 Prenatal testosterone and LH levels in male rats exposed during pregnancy to 2,3,7,8-tetrachlorodibenzo-*p*-dixin and diethylstilbestrol. Mol Cell Endocrinol 178:169–179
22. **Prins GS, Birch L** 1995 The developmental pattern of androgen receptor expression in rat prostate lobes is altered after neonatal exposure to estrogen. Endocrinology 136:1303–1314
23. **Williams K, McKinnell C, Saunders PTK, Walker M, Fisher JS, Turner KJ, Atanassova N, Sharpe RM** 2001 Neonatal exposure to potent and environmental oestrogens and abnormalities of the male reproductive system in the rat: evidence for importance of the androgen-oestrogen balance and assessment of the relevance to man. Hum Reprod Update 7:236–247
24. **McKinnell C, Atanassova N, Williams K, Fisher JS, Walker M, Turner KJ, Saunders PTK, Sharpe RM** 2001 Suppression of androgen action and the induction of gross abnormalities of the reproductive tract in male rats treated neonatally with diethylstilbestrol. J Androl 22:323–338
25. **Panet-Raymond V, Gottlieb B, Beitel LK, Pinsky L, Trifiro MA** 2000 Interactions between androgen and estrogen receptors and the effects on their transactivational properties. Mol Cell Endocrinol 167:139–150
26. **Yeh S, Miyamoto H, Shima H, Chang CH** 2000 From estrogen to androgen receptor: a new pathway for sex hormones in prostate. Proc Natl Acad Sci USA 95:5527–5532
27. **Williams K, Fisher JS, Turner KJ, McKinnell C, Saunders PTK, Sharpe RM** 2001 Relationship between expression of sex steroid receptors and structure of the seminal vesicles after neonatal treatment of rats with potent or weak estrogens, tamoxifen, flutamide or a GnRH antagonist. Environ Health Perspect 109:1227–1235
28. **Sharpe RM, Atanassova N, McKinnell C, Parte P, Turner KJ, Fisher JS, Kerr JB, Groome NP, Macpherson S, Millar MR, Saunders PTK** 1998 Abnormalities in functional development of the Sertoli cells in rats treated neonatally with diethylstilbestrol: a possible role for estrogens in Sertoli cell development. Biol Reprod 59:1084–1094
29. **Imperato-McGinley J, Sanchez RS, Spencer JR, Yee B, Vaughan ED** 1992 Comparison of the effects of the 5$\alpha$-reductase inhibitor finasteride and the antiandrogen flutamide on prostate and genital differentiation: dose-response studies. Endocrinology 131:1149–1156
30. **Fisher JS, Turner KJ, Brown D, Sharpe RM** 1999 Effect of neonatal exposure to estrogenic compounds on development of the excurrent ducts of the rat testis through puberty to adulthood. Environ Health Perspect 107:397–405
31. **Norton AJ, Jordan S, Yeomans P** 1994 Brief, high-temperature heat denaturation (pressure cooking): a simple and effective method of antigen retrieval for routinely processed tissues. J Pathol 173:371–379
32. **Corker CS, Davidson DW** 1981 Radioimmunoassay of testosterone in various biological fluids without chromatography. J Steroid Biochem 9:319–323
33. **Fisher JS, Millar MR, Majdic G, Saunders PTK, Fraser HM, Sharpe RM** 1997 Immunolocalisation of oestrogen receptor-$\alpha$ (ER$\alpha$) within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. J Endocrinol 153:485–495
34. **Hess RA, Gist DH, Bunick D, Lubahn DB, Farrell A, Bahr J, Cooke PS, Greene GL** 1997 Estrogen receptor ($\alpha$ and $\beta$) expression in the excurrent ducts of the adult male rat reproductive system. J Androl 18:602–611
35. **Lee KH, Hess RA, Bahr JM, Lubahn DB, Taylor J, Bunick D** 2000 Estrogen receptor $\alpha$ has a functional role in the mouse rete testis and efferent ductules. Biol Reprod 63:1873–1880
36. **McPherson SJ, Wang H, Jones ME, Pedersen J, Iismaa TP, Wreford N, Simpson ER, Risbridger GP** 2001 Elevated androgens and prolactin in aromatase-deficient mice cause enlargement, but not malignancy, of the prostate gland. Endocrinology 142:2458–2467
37. **Mathur R, Braunstein GD** 1997 Gynecomastia: pathology, mechanisms and treatment strategies. Horm Res 48:95–102
38. **Braunstein GD** 1999 Aromatase and gynecomastia. Endocr Relat Cancer 6:315–324
39. **Giwercman A, Kledal T, Schwartz M, Giwercman YL, Leffers H, Zazzi H, Wedell A, Skakkebaek NE** 2000 Preserved male fertility despite decreased androgen sensitivity caused by a mutation in the ligand-binding domain of the androgen receptor gene. J Clin Endocrinol Metab 85:2253–2259
40. **Bennett HW, Underwood EJ, Shier FL** 1946 A specific breeding problem of sheep on subterranean clover pastures in western Australia. Austr Vet J 22:2–12
41. **Chamley WA, Findlay JK, Nairn ME** 1977 The effect of testosterone and an anti-oestrogen on hypertrophy of bulbourethral glands of wethers grazing oestrogenic pastures. Austr Vet J 53:476–477
42. **Randles JL** 1990 Clinical, pathological and histopathological findings in lambs implanted with a growth promoting product containing progesterone and oestradiol. J South Afr Vet Assoc 61:126–127
43. **Prins GS, Birch L** 1997 Neonatal estrogen exposure up-regulates estrogen receptor expression in the developing and adult rat prostate lobes. Endocrinology 138:1801–1809
44. **Prins GS, Birch L, Couse JF, Choi I, Katzenellenbogen B, Korach KS** 2001 Estrogen imprinting of the developing prostate gland is mediated through stromal estrogen receptor $\alpha$: studies with $\alpha$ERKO and $\beta$ERKO mice. Cancer Res 61:6089–6097
45. **Zhou J, Siu NG, Adesanya-Famuiya O, Anderson K, Bondy CA** 2000 Testosterone inhibits estrogen-induced mammary epithelial proliferation and suppresses estrogen receptor expression. FASEB J 14:1725–1730
46. **Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Barone MV, Ametrano D, Zannini MS, Abbondanza C, Auricchio F** 2000 Steroid-induced androgen receptor-oestradiol receptor $\beta$-Src complex triggers prostate cancer cell proliferation. EMBO J 20:5406–5417
47. **Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC** 2001 Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 104:719–730
48. **Turner KJ, Morley M, MacPherson S, Millar MR, Wilson JA, Sharpe RM, Saunders PTK** 2001 Modulation of gene expression by androgen and oestrogens in the testis and prostate of the adult rat following androgen withdrawal. Mol Cell Endocrinol 178:73–87