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Neonatal Estrogenic Effects upon the Male Rat Pituitary: Early Gonadotrophin Attenuation Precedes Long-term Recovery

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Abstract

Neonatal exposure to potent estrogenic compounds can affect multiple components of the male reproductive system causing impaired development of the epithelium and overgrowth of stromal tissue in the epididymis, vas deferens, seminal vesicles, and prostate. However, very little is known about the direct effects of estrogenic compounds on the anterior pituitary gland. In this study we have investigated the effects of neonatal estrogenic exposure upon the anterior pituitary. Both the

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early- and late-stage effects of exposure to a synthetic estrogenic agent, diethylstilbestrol (DES), upon pituitary gonadotroph cell function were assessed. We administered either a high dose (10 µg) or a low dose (0.1 µg) of DES to male rats during their neonatal period (P2–12). Gonadotroph function, cell number and morphology shortly after DES treatment (P18) and during adulthood (P90) were assessed. At P18 there was a significant decrease in follicle stimulating hormone (FSH) immunoreactivity in the pituitary gonadotroph cells in the high DES dose treated rats compared to control animals. No significant change in luteinizing hormone (LH) was observed at either DES dose. In adulthood (P90), there was no significant difference in FSH or LH gonadotroph immunoreactivity between control rats and any dose of DES-treated rats. Therefore, despite acute and selective ablation of FSH expression the gonadotrophs were able to recover in adulthood, suggesting that perinatal estrogenic exposure was only temporarily deleterious.

Keywords

Neonatal; Diethylstilbestrol; Pituitary; Gonadotroph; Follicle stimulating hormone; Luteinizing hormone; Endocrine disruptors; Testosterone; Inhibin

Introduction

It is well established that along with androgens, estrogens can also profoundly affect the development of the component parts of the male reproductive system. This information has come from two main sources. First, studies involving the administration of estrogens in fetal/neonatal life to various mammals, including humans (Arai et al. 1983; Toppari et al. 1996). Second, studies of animals in which one or both estrogen receptors (ER α , ER β) have been inactivated using transgenesis in mice (Couse and Korach 1999). However, the precise physiological role(s) played by estrogens in the development of the male reproductive system remain relatively unclear. The prevailing impression gained from the aforementioned studies, however, is that excessive exposure to estrogens, and consequent disruption of the androgen–estrogen balance, can cause permanent malformations in components of the male reproductive axis (Arai et al. 1983; Toppari et al. 1996; Prins et al. 2001; Rivas et al. 2002) and can predispose to further aberrant changes in later life (Cunha 2001; Cunha et al. 2001; Prins et al. 2001).

The aim of this study was to investigate whether neonatal estrogen disruption, using the potent synthetic estrogen diethylstilboestrol (DES), in male rodents could permanently re-programme anterior pituitary gland function. There is a substantial amount of experimental evidence that demonstrates that exposure of male rodents to high levels of exogenous synthetic estrogens results in major morphological and functional abnormalities of the hypothalamic–pituitary–gonadal (HPG) axis, including the testis and reproductive tract (Arai et al. 1983; Newbold et al. 1985; Fisher et al. 1998; Khan et al. 1998; Sharpe et al. 1998; Atanassova et al. 2005). Many of these effects have also been shown to have life-long consequences upon the structure and function of the HPG/reproductive system (Khan et al. 1998; Atanassova et al. 2000). Furthermore, studies on the sons of women who were exposed to DES during pregnancy have shown that a similar range of abnormalities can also be induced in human males exposed to high levels of exogenous estrogens in utero (Stillman

1982; Toppari et al. 1996). Due to the inherent complexity of the mammalian reproductive system, the precise mechanism(s) underlying the induction of these reproductive abnormalities are unclear. One mechanism that has been proposed so far has suggested that DES-induced effects on the testis are indirect, and result from suppression of gonadotrophin secretion by the pituitary gland during DES treatment (Brown-Grant et al. 1975; Bellido et al. 1990). Previous studies have suggested that neonatal treatment of male rats with a high dose of DES (10 µg) has permanent effects on the HPG axis in male rats (Atanassova et al. 1999). Atanassova et al. reported that neonatal treatment with 10 µg of DES caused significant reductions in: testis weight; germ cell volume; the number of Sertoli cells per testis (but no change in Leydig cells); plasma inhibin B concentrations and plasma testosterone concentrations. Additionally, this study also demonstrated significantly elevated plasma FSH levels in adulthood. These effects, although to a much lesser extent, were also obtained for neonatal rats treated with a lower dose (0.1 µg) of DES. Considering the profound connection between the peripheral reproductive organs and the activity of pituitary gonadotroph cells (Millar et al. 2004) we investigated whether the actions of neonatal DES exposure were limited to the gonads or if they extended to the pituitary. In addition, if indeed DES affects the pituitary are these actions transient or long-lasting? To address these questions we exposed male rats to multiple doses of DES during neonatal life. Simultaneous study groups of animals were employed to allow us the ability to follow the actions of DES upon pituitary structure recently after cessation of DES dosing and also later into adulthood. The data presented in this study demonstrate potent but transient actions of DES upon anterior pituitary structure and functionality. This data suggests that in the presence of potent estrogenic compounds the male pituitary gonadotrophs possess a strong regenerative capacity.

Materials and Methods

Animals and Treatments

Wistar rats, bred in the University of Edinburgh MFAA, were maintained under standard conditions. They were maintained under controlled conditions at 22–23 °C ambient temperature, 55–60% relative humidity, 12L:12D cycle, and were maintained on a soy-free diet (rat and mouse soya-free breeding diet; SDS, Dundee, Scotland). On the day after birth, rats from several litters were cross-fostered into all-male litters of 8–12 pups, and these animals were then allocated to different treatments ($n = 4/5$ per group) which were then treated by subcutaneous injection as follows. Experimental male rat groups were either injected sub cutaneously (s.c.) with a low (0.1 µg) or high (10 µg) dose of DES (in a vehicle of 20 µl of corn oil: Sigma, UK) on days 2, 4, 6, 8, 10 and 12 post parturition. Rats were injected on alternating days as DES is sufficiently long-acting to allow this. Vehicle control animals were injected with 20 µl of corn oil on days 2, 4, 6, 8, 10 and 12 post parturition. Two major sets of experimental rat groups were set up containing the aforementioned low dose DES, high dose DES and vehicle-treated groups that allowed us to euthanize a primary set of treated groups at day 18 post parturition and at day 90 post parturition (see Fig. 1).

For blood chemistry measurements, animals were anesthetized with flurothane and blood samples were collected from the tail vein on day 26 (early puberty), day 35 (mid puberty),

and day 90 (adulthood), as shown in Fig. 1, and were assayed for LH, FSH, inhibin B, and testosterone by RIA and ELISA, respectively. Rats were either euthanized on day 18 or 90 by carbon dioxide inhalation followed by cervical dislocation and the whole pituitary gland was removed. The pituitaries were either snap frozen on dry ice and stored at -80°C for mRNA analysis or they were subsequently fixed for 5 h in Bouin's fixative for immunohistochemical analysis. After Bouin's fixation, the pituitaries were transferred into 70% ethanol before being processed for 17.5 h in an automated TP1050 processor (Leica Corp., Deerfield, IL, USA) and embedded in paraffin wax. Sections of 5- μm thickness were cut, floated onto slides coated with 3% 3-aminopropyltriethoxysilane (Sigma, UK), and dried overnight at 50°C before being used for immunohistochemical analysis and cell enumeration.

Enumeration of LH β and FSH β Immunoreactive Cells in the Pituitary Gland

Paraffin-embedded pituitary glands were sectioned transversely until approximately the midline of the tissue was reached. Consecutive sections from the midline region were then subsequently immunostained for LH β and FSH β using specific antibodies as reported previously (Brown et al. 2001). FSH β was detected using a polyclonal rabbit antibody raised against human FSH β (M94, gift from Dr S. Lynch, Birmingham, UK) and LH β using a monoclonal mouse antibody raised against bovine LH β (518B7, gift from Dr J.F. Roser, University of California Davis, USA). Unless otherwise stated, all incubations were performed at room temperature. The slides were de-waxed, rehydrated and endogenous peroxidase was blocked using 3% (volume/volume: v/v) hydrogen peroxide in methanol. After washing in water, the sections were washed twice (5 min each) in Tris-buffered saline (0.05 M TBS, pH 7.4 and 0.85% NaCl). For FSH β , slides were then blocked for 30 min with normal swine serum (NSS; Diagnostics Scotland, Carlisle, UK) diluted 1:5 in TBS containing 5% BSA; for LH β , slides were blocked with normal rabbit serum (NRS) diluted 1:5 in TBS with 5% BSA. The primary antibodies were diluted in the appropriate blocking solution (FSH β 1:1000; LH β 1:5000) and 100 μl was added to each slide before incubation at 4°C overnight in a light-proof humidity chamber. The slides were then washed in TBS (2×5 min) before incubation for 30 min with a biotinylated second antibody, namely swine anti-rabbit (Dako, Ely, UK) for FSH β or rabbit anti-mouse (Dako) for LH β , diluted 1:500 in the appropriate blocking serum. After two washes in TBS (2×5 min), avidin-biotin conjugated horse-radish peroxidase (ABC-HRP; Dako) was applied for 30 min. After two final washes (2×5 min) in TBS a solution of diaminobenzidine (DAB) was applied (liquid DAB; Dako). The slides were developed until the color reached the required intensity in the control sections and then terminated by immersion in distilled water. The slides were counterstained with hematoxylin before being dehydrated by immersion in a graded series of ethanols and then being cleared in xylene. A coverslip was fixed over the sections using Pertex mounting medium (Cell Path, Hemel Hempstead, UK). Sections from control and treated animals were prepared in parallel at all times. Immunostained sections were examined on a Zeiss Axioskop light microscope using a $\times 40$ objective. The number of immunoreactive cells was determined by placing a 21 mm diameter graticule over a randomly chosen area of the anterior pituitary. Within that graticule, the number of immunoreactive (nucleated) cells was counted and the total number of non-immunoreactive cells was also counted and the percentage immunoreactive cells then calculated. This was done for four randomly chosen

areas on each slide and repeated once. The counting procedure was then repeated by another independent researcher with no knowledge of each section identity. Final data for each animal were then computed as the average of the two runs and between the two researchers.

Measurement of Plasma Hormone Levels

Plasma concentrations of FSH and LH were measured by RIA as previously described (McNeilly et al. 1996), using agents supplied by Dr A. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA, USA). The reference preparations were AFP-5308D and rLH-RP-1, and the minimum detectable concentrations were 1 ng/ml and 200 pg/ml, for FSH and LH, respectively. The intra- and inter-assay coefficients of variation were <10% for both assays.

Plasma levels of inhibin B were measured using a two-site enzyme-linked immunoassay that uses a capture antibody directed against the C-terminal portion of the human β_B -subunit and the F(ab) fraction of a mouse monoclonal antibody (R1) to the N-terminal portion of the inhibin α -subunit conjugated to alkaline phosphatase. The assay has been previously validated for measurement of inhibin B in the rat (Illingworth et al. 1996; Woodruff et al. 1996), and in the present studies it was confirmed that rat plasma diluted in parallel with the inhibin B standard and that levels of inhibin B in castrate adult male rat plasma were reduced to undetectable levels (<60 pg/ml).

Plasma levels of testosterone were measured using an enzyme-linked immunosorbent assay adapted from an earlier RIA method (Corker and Davidson 1981). Plasma, to which was added trace amounts of [3 H]testosterone (Amersham International, Aylesbury, UK), was extracted twice with 10 volumes of hexane-ether (4:1, v/v), and the organic phase was dried down under nitrogen at 55°C. The efficiency of extraction averaged 75%. The second antibody was immobilized to an enzyme-linked immunosorbent assay plate by addition of 100 μ l acid purified donkey anti-goat/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 μ l) were assayed after dilution in 0.1 M PBS, pH 7.4, containing 0.1% gelatin (Sigma Chemical Co.) and incubated overnight at 4°C with 50 μ l sheep antitestosterone-3-carboxymethyloxide-BSA diluted 1:100,000 plus 50 μ l testosterone-3-carboxymethyloxide labeled with 1:20,000 diluted horseradish peroxidase (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 Chemical Co.) and 0.03% hydrogen peroxide diluted in 0.1 M citrate-phosphate, pH 5.0, to each well. The plate was then incubated in the dark for 10–30 min until the color reaction was optimal. The reaction was stopped by the addition of 50 μ l 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.

Quantitative RT-PCR

Levels of mRNA for LH β and FSH β were measured using quantitative Taqman RT-PCR. This was performed as described previously for LH β (Nicol et al. 2002). Specific primer and

probe sequences were designed using Primer Express software (PE Biosystems, Warrington, UK) and were synthesized either by PE Biosystems or by Biosource Europe (Nivelles, Belgium). Exhaustive evaluation of the specificity of each primer and probe set was performed before experimental utilization. The most selective and accurate probe and primer sets used for both LH β and FSH β sequences are shown in Table 1.

Statistical Analyses

Comparison of the different parameters for the various treatment groups was made using simple non-paired Student's *t*-tests and analysis of variance (ANOVA) using the statistical suite of GraphPad Prism v. 3.0. (GraphPad Software, La Jolla CA).

Results

Neonatal DES Treatment Causes Acute Alterations in Gonadotrophin Levels in the Male Pituitary

To investigate both the short- and long-term effects of neonatal DES exposure upon reproductive capacity, we employed multiple groups of male rats injected for multiple days nearly immediately post partum (day 2) up until day 12, and then their gonadotrophin and gonadal function was assessed either 6 or 78 days later. Initially, we studied the actions of the DES doses 6 days after cessation of injection upon the number of LH or FSH positive cells in the anterior pituitary of the test animals. Six days after the end of the DES injection regime, we observed no alteration in the number of LH or FSH immunoreactive cells in the pituitary of rats from the control (vehicle-treated) or 0.1 μ g DES group (Fig. 2a). However, there was a significant reduction in the percentage of FSH immunoreactive cells ($P < 0.05$) for the 10 μ g DES-treated rats (Fig. 2a). There was also a small reduction in FSH immunoreactive cells for the low dose DES-treated rats, but this reduction was not statistically significant. There was no significant change in the percentage of LH immunostained cells for both doses of DES, though values were consistently lower in the DES-treated animals. When expressed as a ratio between the FSH:LH immunoreactive cells, there was a clear disruption of gonadotrophin expression ratio in the anterior pituitary of the 10 μ g DES-treated rats only 6 days after treatment cessation (Fig. 2b). Visual inspection of the pituitary sections themselves demonstrated the clear acute and selective ablation of FSH immunoreactive cells without any significant disruption of gross pituitary morphology (Fig. 3).

Recovery of Pituitary Gonadotrophin Expression After Extended Recovery from Neonatal DES Exposure

In adult rats treated neonatally with either the low dose or high dose of DES, there was no difference in the expression of LH or FSH immunoreactive cells (Fig. 4a) or their ratio to each other (Fig. 4b) in the anterior pituitary 78 days post cessation of DES exposure. Visual inspection of the pituitary sections themselves demonstrated that the clear acute and selective ablation of FSH immunoreactive cells seen at 6 days post DES treatment cessation was absent in the adult rats (Fig. 5). In addition, there were no changes in LH or gross pituitary morphology. As in adulthood there was no longer any suppression of FSH

immunoreactive staining (as we measured at day 18), this suggests that this DES-induced FSH reduction was acute and did not persist into adulthood.

Effects of Neonatal DES Treatment on LH and FSH mRNA Levels in the Anterior Pituitary Gland

Real-Time PCR analysis of mRNA levels for LH and FSH in pituitaries from rats 6 days after cessation of DES treatment revealed a significant reduction in FSH mRNA levels ($P < 0.01$) only in animals treated with 10 μg DES. There was no significant change in LH mRNA levels in either of the DES dosage groups (Fig. 6a). This reduction in FSH mRNA correlated with the significant reduction in FSH immunoreactive cells we measured 6 days after DES cessation (Fig. 3d). PCR analysis of mRNA levels for LH and FSH in the anterior pituitary glands of adult rats (78 days after DES cessation) revealed that there was no significant difference between neonatally DES-treated rats (0.1 or 10 μg -treated) and age-matched control rats (vehicle-treated; Fig. 6b). This correlates with the results we obtained from our immunohistochemical analyses we performed for LH and FSH cells in these rats (Fig. 5b, d), as we found no significant alteration in the number of LH or FSH immunoreactive cells in the anterior pituitary. This again demonstrates that neonatal DES treatment only had an acute effect on FSH mRNA levels and that this effect no longer persisted into adulthood.

Neonatal DES Exposure Causes Significant Alterations in Circulating Gonadotrophin Levels After Cessation of Estrogenic Exposure

Neonatal treatment with DES had significant effects on FSH levels, which were both age- and dose-dependent (Fig. 7a). Treatment with 10 μg , but not with 0.1 μg DES, caused a significant suppression in plasma FSH levels ($P < 0.01$) at 26 days of age (14 days post DES cessation). In contrast, at later times following DES dosing cessation both the low dose and high dose DES treatments resulted in an elevation of plasma FSH levels at days 35 and 90, though this was only statistically significant for the two DES doses at day 90 ($P < 0.05$). The neonatal treatment with DES also had significant age-dependent effects on plasma LH levels (Fig. 7b). Treatment with either 0.1 or 10 μg DES caused only a significant elevation in plasma LH concentrations in adulthood (day 90: 78 days post DES treatment cessation). LH concentrations were suppressed slightly immediately after neonatal treatment with both the high and low DES doses at days 26 and 35 of age; however, this suppression was small and not statistically significant.

Despite Pituitary Recovery from Neonatal DES Exposure, Gonadal Functionality is Severely Affected

Our current data has demonstrated that in the anterior pituitary, at the morphological and gonadotrophin expression level, the effects of neonatal DES exposure can be reversed with aging into adulthood. Given that previous investigations have seen long lasting effects upon gonadal function with DES treatment, we decided to assess two endocrinological factors crucially related to gonadal activity, inhibin B and testosterone. Plasma inhibin B levels (Fig. 8a) were significantly suppressed 78 days post DES cessation for both 0.1 μg ($P < 0.01$) and 10 μg ($P < 0.05$) DES-treated rats. This corresponds with the significantly elevated FSH levels (Fig. 7a) observed for these DES-treated rats, as plasma inhibin B negatively regulates

plasma FSH levels. Plasma testosterone levels (Fig. 8b) were significantly suppressed in adulthood in a dose-dependent manner (0.1 µg DES $P < 0.05$; 10 µg DES $P < 0.001$). As also the plasma LH levels at day 90 (Fig. 7b) in these rats were significantly elevated, this suggests that neonatal DES treatment could have affected long-term Leydig cell function in the testes.

Discussion

The main aim of this study was to determine whether neonatal treatment with the potent estrogenic agent, DES, could induce significant alterations in anterior pituitary gland function, and whether potential pituitary effects would be long-lasting. There have been numerous studies performed to date showing that neonatal treatment of male rats with high doses of DES can induce a range of developmental abnormalities, including inhibition of Sertoli cell proliferation (Atanassova et al. 1999), suppression of Leydig cell development and function (Sharpe et al. 2003), and abnormal development of the rete testis (Aceitero et al. 1998; Fisher et al. 1999; McKinnell et al. 2001), efferent ducts (Fisher et al. 1997, 1999), epididymis (Atanassova et al. 2001; McKinnell et al. 2001), vas deferens (Atanassova et al. 2001; McKinnell et al. 2001), seminal vesicles (Williams et al. 2000, 2001), and the prostate (Prins and Birch 1995). However, in contrast, studies using lower (but still physiologically substantial) doses of DES have failed to induce most of the gross structural changes in the testis, caused by administering a high dose of DES (Atanassova et al. 2001; Rivas et al. 2002). Coincident with the DES induction of these testicular abnormalities, neonatal treatment with high, but not low, doses of DES have also been shown to grossly suppress expression of the androgen receptor (AR) in all of the testicular tissues affected adversely by DES treatment (McKinnell et al. 2001).

However, until now, it was unclear whether neonatal DES treatment in rodents had any direct effects on the function of the gonadotroph cells, present in the anterior pituitary gland. The results from this study have demonstrated that shortly after neonatal high dose DES treatment, at 18 days of age, DES significantly reduced the number of FSH immunoreactive gonadotroph cells ($P < 0.01$) in the anterior pituitary gland (Fig. 3d). This significant change was only induced by neonatal administration of the high dose (10 µg) DES treatment regime and not by the neonatal administration of the low dose (0.1 µg) DES regime. Interestingly, we found there to be no significant alteration in the number of LH immunoreactive gonadotroph cells at 18 days of age, for both doses of DES (Fig. 2a). As the vast majority of gonadotroph cells in the anterior pituitary are bi-hormonal (i.e., they produce and secrete both LH and FSH) (Currie and McNeilly 1995) and there was no significant alteration in the total number of gonadotrophin-positive cells present within the anterior pituitary gland, this suggests that the neonatal 10 µg DES treatment depleted FSH from the gonadotroph cells, without abolishing the entire gonadotroph cell.

The mRNA analyses of the pituitary glands collected from our neonatally DES-treated rats revealed that there was a significant reduction in FSH mRNA levels in these rats shortly after high dose DES treatment (at 18 days of age) ($P < 0.01$), but there was no significant alteration in LH mRNA levels (Fig. 6a). Plasma hormone levels measured shortly after the treatment period, on day 26 (early puberty) and on day 35 (mid puberty) of age, showed that

circulating LH levels were slightly suppressed in both the low dose and high dose DES-treated rats on days 26 and 35, although this was not statistically significant (Fig. 7b). FSH concentrations on the other hand, were significantly suppressed ($P < 0.001$) in the 10 μg DES-treated animals at 26 days of age (Fig. 7a). Both the high dose and low dose DES treatments resulted in a slight elevation of plasma FSH at 35 days of age, but this change was not statistically significant. In adulthood, at 90 days of age, there was no longer a significant suppression of FSH immunoreactive staining or suppression of FSH mRNA levels, suggesting that the neonatal DES treatment did not have any significant long-lasting effects on the number of FSH immunoreactive cells or on FSH mRNA levels in the anterior pituitary gland. Actually we noted a modest increase in the amount of circulating FSH instead. Additionally, the circulating plasma levels of LH were profoundly elevated for both the low and high doses of DES ($P < 0.01$) at day 90. Interestingly, we noted both at day 18 and 90 that there was no direct correlation between circulating gonadotrophin levels and gonadotrophin immuno-expression/mRNA levels. These discrepancies clearly indicate that the gonadotrophin regulatory system is more complex than previously thought and requires further investigation in the future. LH acts primarily on the Leydig cells of the testes to promote the synthesis of testosterone, and subsequently testosterone exerts negative feedback on hypothalamic gonadotrophin-releasing hormone (GnRH) release and on the pituitary gonadotroph cells (Matsumoto and Bremner 1984; Millar et al. 2004). In adulthood, neonatal DES treatment caused a marked reduction in circulating testosterone levels in both the high dose and low dose DES-treated rats (0.1 μg DES $P < 0.05$ and 10 μg DES $P < 0.001$). Interestingly, previous studies performed by Sharpe and colleagues have shown that neonatal DES treatment causes no significant DES-induced alteration in the number of Leydig cells present per testis (Sharpe et al. 2003). This suggests that the DES-induced suppressed testosterone concentrations in adulthood were caused by a lack of the Leydig cells to produce and secrete testosterone, and not due to a reduction in the total number of Leydig cells present per rat testis. Since testosterone exerts negative feedback at the level of the pituitary and hypothalamus, and as testosterone levels were suppressed after DES treatment (Fig. 8b), this may have caused altered feedback to both the hypothalamus and pituitary and as a result plasma LH levels were elevated in adulthood ($P < 0.01$). FSH on the other hand, acts primarily on testicular Sertoli cells to promote the synthesis of inhibin B, which subsequently negatively regulates FSH production and secretion from the pituitary gland (Jensen et al. 1997; Nachtigall et al. 1996). In adult rats (90 days old), we found a significant increase in circulating FSH levels for both high dose and low dose DES-treated rats ($P < 0.05$). Neonatal DES treatment has been shown by Sharpe and colleagues to cause a significant reduction in the number of Sertoli cells per testis and a significant suppression of plasma inhibin B levels (Sharpe et al. 1998). This suggests that the suppression of inhibin B levels in our neonatally DES-treated rats was possibly caused by a reduction in the number of Sertoli cells per testis, which resulted in a significant increase in plasma FSH levels, as inhibin B negatively regulates FSH production and secretion from the pituitary gland.

The hormones FSH and testosterone play important roles in spermatogenesis, although in non-seasonal animals testosterone is thought to be the most important regulatory factor (Sharpe et al. 1994). In addition, the most important factor that determines the ceiling of sperm production and output in all mammals is the number of Sertoli cells per testis (Sharpe

et al. 1994). Sperm production in adult life is dependent upon neonatal Sertoli cell proliferation. The number of Sertoli cells in the adult testis determines both testis size and daily sperm production. This relationship occurs because each Sertoli cell has a fixed capacity for the number of germ cells that it can support (Orth et al. 1988). As the number of Sertoli cells per testis determines the number of spermatozoa produced per day, it is vital that the correct number of Sertoli cells is generated in neonatal life (Sharpe et al. 1994). Previous studies have shown that both androgen and FSH concentrations are very important for the regulation of Sertoli cell proliferation and number (Hess et al. 1993; De Franc, a et al. 1995; Sharpe et al. 2003; Scott et al. 2007). Thus, as plasma FSH levels were significantly reduced at day 26 in the DES-treated rats, this suggests that this may have caused a decrease in Sertoli cell proliferation during the neonatal period and hence resulted in there being a lower number of mature Sertoli cells present per testis in adulthood in these treated animals. The reduced number of Sertoli cells in adulthood subsequently caused a suppression of circulating inhibin B levels (and hence altered negative feedback on the pituitary gland), resulting in permanently elevated plasma concentrations of FSH. Treatment with high doses of DES has been shown to induce widespread structural abnormalities of the testis, including loss of immunoexpression of the androgen receptor (AR) (Williams et al. 2001). Interestingly, in this study we did not observe any significant DES-induced alterations in AR immunoexpression in the pituitary gonadotrophs (data not shown).

In conclusion, we have shown in this study that neonatal DES treatment can have direct effects on anterior pituitary gonadotroph cell function during the neonatal period. Neonatal administration of a high dose of DES (10 µg) resulted in a significant reduction in the number of FSH immunoreactive gonadotroph cells and in a significant suppression of FSH mRNA levels and plasma FSH concentrations at 18 days of age. These changes in the number of FSH immunoreactive gonadotrophs and in FSH mRNA levels could have caused reduced proliferation of testicular Sertoli cells during the neonatal period and hence caused a permanently lowered number of Sertoli cells per testis in adulthood. This would then cause a life-long suppression of plasma inhibin B levels and a permanent elevation of plasma FSH levels. Interestingly, in adulthood, the pituitary gonadotroph cells appeared to recover from this significant neonatal DES insult, as there was no longer a reduction in FSH immunoreactive cells and in FSH mRNA levels. However, circulating levels of inhibin B remained suppressed and circulating levels of FSH remained elevated in adulthood, which was likely due to a permanent alteration in Sertoli cell number. The neonatal DES treatment also appeared to have direct effects on testicular function, as neonatal DES treatment caused a permanent reduction in the ability of the Leydig cells to produce and secrete testosterone.

Our study has clearly demonstrated that high doses of a potent estrogenic agent, DES, can cause significant direct effects on gonadotroph cell function in the anterior pituitary gland, including significant alterations in the number of FSH immunoreactive cells, mRNA levels and in circulating levels of LH, FSH, testosterone and inhibin B. Interestingly the direct effects of neonatal DES treatment on the gonadotroph cell function appear to be acute, as they are only present shortly after DES administration (at 18 days of age) and these alterations do not persist into adulthood (at 90 days of age). Our results suggest that gonadotroph cell function is robust and that gonadotrophs are capable of overcoming potent

neonatal insults. Future studies are needed to investigate the direct effects of endocrine disrupting compounds on pituitary function.

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References

- Aceitero J, Llanero M, Parrado R, Peña E, Lopez-Beltran A. Neonatal exposure of male rats to estradiol benzoate causes rete testis dilation and backflow impairment of spermatogenesis. *The Anatomical Record*. 1998; 252:17–33. [PubMed: 9737741]
- Arai Y, Mori T, Suzuki Y, Bern HA. Long-term effects of perinatal exposure to sex steroids and diethylstilbestrol on the reproductive system of male mammals. *International Reviews of Cytology*. 1983; 84:235–268.
- Atanassova N, McKinnell C, Fisher J, Sharp RM. Neonatal treatment of rats with diethylstilboestrol (DES) induces stromal-epithelial abnormalities of the vas deferens and cauda epididymis in adulthood following delayed basal cell development. *Reproduction*. 2005; 129:589–601. [PubMed: 15855622]
- Atanassova N, McKinnell C, Turner KJ, Walker M, Fisher JS, Morley M, et al. Comparative effects of neonatal exposure of male rats to potent and weak (environmental) estrogens on spermatogenesis at puberty and the relationship to adult testis size and fertility: Evidence for stimulatory effects of low estrogen levels. *Endocrinology*. 2000; 141:3898–3907. [PubMed: 11014247]
- Atanassova N, McKinnell C, Walker M, Turner KJ, Fisher JS, Morley M, et al. Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number, and the efficiency of spermatogenesis in adulthood. *Endocrinology*. 1999; 140:5364–5373. [PubMed: 10537168]
- Atanassova N, McKinnell C, Williams K, Turner KJ, Fisher JS, Saunders PT, et al. Age-, cell- and region-specific immunoeexpression of estrogen receptor alpha (but not estrogen receptor beta) during postnatal development of the epididymis and vas deferens of the rat and disruption of this pattern by neonatal treatment with diethylstilbestrol. *Endocrinology*. 2001; 142:874–886. [PubMed: 11159861]
- Bellido C, Pinilla L, Aguilar R, Gaytan F, Aguilar E. Possible role of changes in post-natal gonadotrophin concentrations on permanent impairment of the reproductive system in neonatally oestrogenized male rats. *Journal of Reproduction and Fertility*. 1990; 90:369–374. [PubMed: 2123500]
- Brown P, McNeilly JR, Evans JG, Crawford GM, Walker M, Christian HC, et al. Manipulating the in vivo mRNA expression profile of FSH beta to resemble that of LH beta does not promote a concomitant increase in intracellular storage of follicle-stimulating hormone. *Journal of Neuroendocrinology*. 2001; 13:50–62. [PubMed: 11123515]
- Brown-Grant K, Fink G, Greig F, Murray MA. Altered sexual development in male rats after oestrogen administration during the neonatal period. *Journal of Reproduction and Fertility*. 1975; 44:25–42. [PubMed: 1151930]
- Corker CS, Davidson DW. Radioimmunoassay of testosterone in various biological fluids without chromatography. *Journal of Steroid Biochemistry*. 1981; 9:319–323.
- Couse JF, Korach KS. Estrogen receptor null mice: What have we learnt and where will they lead us? *Endocrine Reviews*. 1999; 20:358–417. [PubMed: 10368776]
- Cunha GR. Evidence that epithelial and mesenchymal estrogen receptor- α mediates effects of estrogen on prostatic epithelium. *Developmental Biology*. 2001; 229:432–442. [PubMed: 11150243]
- Cunha GR, Wang YZ, Hayward SW, Risbridger GP. Estrogenic effects on prostatic differentiation and carcinogenesis. *Reproduction, Fertility, and Development*. 2001; 13:285–296.
- Currie RJ, McNeilly AS. Mobilization of LH secretory granules in gonadotrophs in relation to gene expression, synthesis and secretion of LH during the preovulatory phase of the sheep oestrous cycle. *Journal of Endocrinology*. 1995; 147:259–270. [PubMed: 7490556]

- De França LR, Hess RA, Cooke PS, Russell LD. Neonatal hypothyroidism causes delayed Sertoli cell maturation in rats treated with propylthiouracil: Evidence that the Sertoli cell controls testis growth. *The Anatomical Record*. 1995; 242:57–69. [PubMed: 7604982]
- Fisher JS, Millar MR, Majdic G, Saunders PT, Fraser HM, Sharpe RM. Immunolocalisation of oestrogen receptor-alpha within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. *Journal of Endocrinology*. 1997; 153:485–495. [PubMed: 9204003]
- Fisher JS, Turner KJ, Brown D, Sharpe RM. Effect of neonatal exposure to estrogenic compounds on development of the excurrent ducts of the rat testis through puberty to adulthood. *Environmental Health Perspectives*. 1999; 107:397–405. [PubMed: 10210696]
- Fisher JS, Turner KJ, Fraser HM, Saunders PT, Brown D, Sharpe RM. Immunoexpression of aquaporin-1 in the efferent ducts of the rat and marmoset monkey during development, its modulation by estrogens, and its possible role in fluid resorption. *Endocrinology*. 1998; 139:3935–3945. [PubMed: 9724049]
- Hess RA, Cooke PS, Bunick D, Kirby JD. Adult testicular enlargement induced by neonatal hypothyroidism is accompanied by increased Sertoli and germ cell numbers. *Endocrinology*. 1993; 132:2607–2613. [PubMed: 8504761]
- Illingworth PJ, Groome NP, Bryd W, Rainey WE, McNeilly AS, Mather JP, et al. Inhibin-B: A likely candidate for the physiologically important form of inhibin in man. *Journal of Clinical Endocrinology and Metabolism*. 1996; 81:1321–1325. [PubMed: 8636325]
- Jensen TK, Andersson AM, Hjollund NH, Scheike T, Kolstad H, Giwercman A, et al. Inhibin B as a serum marker of spermatogenesis: Correlation to differences in sperm concentration and follicle-stimulating hormone levels. A study of 349 Danish men. *Journal of Clinical Endocrinology and Metabolism*. 1997; 82:4059–4063.
- Khan SA, Ball RB, Hendry WJ 3rd. Effects of neonatal administration of diethylstilbestrol in male hamsters: Disruption of reproductive function in adults after apparently normal pubertal development. *Biology of Reproduction*. 1998; 58:137–142. [PubMed: 9472934]
- Matsumoto AM, Bremner WJ. Modulation of pulsatile gonadotrophin secretion by testosterone in man. *Journal of Clinical Endocrinology and Metabolism*. 1984; 58:609–614. [PubMed: 6421864]
- McKinnell C, Atanassova N, Williams K, Fisher JS, Walker M, Turner KJ, et al. Suppression of androgen action and the induction of gross abnormalities of the reproductive tract in male rats treated neonatally with diethylstilbestrol. *Journal of Andrology*. 2001; 22:323–338. [PubMed: 11229807]
- McNeilly JR, Brown P, Mullins J, Clark AJ, McNeilly AS. Characterization of the ovine LH beta-subunit gene: The promoter is regulated by GnRH and gonadal steroids in transgenic mice. *Journal of Endocrinology*. 1996; 151:481–489. [PubMed: 8994393]
- Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley S. Gonadotropin-releasing hormone receptors. *Endocrine Reviews*. 2004; 25:235–275. [PubMed: 15082521]
- Nachtigall LB, Boepple PA, Seminara SB, Khoury RH, Sluss PM, Lecain AE, et al. Inhibin B secretion in males with gonadotropin-releasing hormone (GnRH) deficiency before and during long-term GnRH replacement: Relationship to spontaneous puberty, testicular volume, and prior treatment—a clinical research center study. *Journal of Clinical Endocrinology and Metabolism*. 1996; 81:3520–3525. [PubMed: 8855795]
- Newbold RR, Bullock BC, McLachlan JA. Lesions of the rete testis in mice exposed prenatally to diethylstilbestrol. *Cancer Research*. 1985; 45:5145–5150. [PubMed: 4027990]
- Nicol L, McNeilly JR, Stridsberg M, Crawford JL, McNeilly AS. Influence of steroids and GnRH on biosynthesis and secretion of secretogranin II and chromogranin A in relation to LH release in LbetaT2 gonadotroph cells. *Journal of Endocrinology*. 2002; 174:473–483. [PubMed: 12208668]
- Orth JM, Gunsalus GL, Lamperti AA. Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. *Endocrinology*. 1988; 122:787–794. [PubMed: 3125042]
- Prins GS, Birch L. The developmental pattern of androgen receptor expression in rat prostate lobes is altered after neonatal exposure to estrogen. *Endocrinology*. 1995; 136:1303–1314. [PubMed: 7867585]

- Prins GS, Birch L, Habermann H, Chang WY, Tebeau C, Putz O, et al. Influence of neonatal estrogen on prostate development. *Reproduction, Fertility, and Development*. 2001; 13:241–252.
- Rivas A, Fisher JS, McKinnell C, Atanassova N, Sharpe RM. 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. *Endocrinology*. 2002; 143:4797–4808. [PubMed: 12446607]
- Scott HM, Hutchison GR, Mahood IK, Hallmark N, Welsh M, De Gendt K, et al. Role of androgens in fetal testis development and dysgenesis. *Endocrinology*. 2007; 148(5):2027–2036. [PubMed: 17289843]
- Sharpe RM, Atanassova N, McKinnell C, Parte P, Turner KJ, Fisher JS, et al. Abnormalities in functional development of the Sertoli cells in rats treated neonatally with diethylstilbestrol: A possible role for estrogens in Sertoli cell development. *Biology of Reproduction*. 1998; 59:1084–1094. [PubMed: 9780313]
- Sharpe RM, Kerr JB, McKinnell C, Millar M. Temporal relationship between androgen-dependent changes in the volume of seminiferous tubule fluid, lumen size and seminiferous tubule protein secretion in rats. *Journal of Reproduction and Fertility*. 1994; 101:193–198. [PubMed: 8064681]
- Sharpe RM, Rivas A, Walker M, McKinnell C, Fisher JS. Effect of neonatal treatment of rats with potent or weak (environmental) oestrogens, or with a GnRH antagonist, on Leydig cell development and function through puberty into adulthood. *International Journal of Andrology*. 2003; 26:26–36. [PubMed: 12534935]
- Stillman RJ. In utero exposure to diethylstilbestrol: Adverse effects on the reproductive tract and reproductive performance and male and female offspring. *American Journal of Obstetrics and Gynecology*. 1982; 142:905–921. [PubMed: 6121486]
- Toppiari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ Jr, et al. Male reproductive health and environmental xenoestrogens. *Environmental Health Perspectives*. 1996; 104:741–803. [PubMed: 8880001]
- Williams K, Fisher JS, Turner KJ, McKinnell C, Saunders PT, Sharpe RM. Relationship between expression of sex steroid receptors and structure of the seminal vesicles after neonatal treatment of rats with potent or weak estrogens. *Environmental Health Perspectives*. 2001; 109:1227–1235. [PubMed: 11748029]
- Williams K, Saunders PT, Atanassova N, Fisher JS, Turner KJ, Millar MR, et al. Induction of progesterone receptor immunoexpression in stromal tissue throughout the male reproductive tract after neonatal oestrogen treatment of rats. *Molecular and Cellular Endocrinology*. 2000; 164:117–131. [PubMed: 11026564]
- Woodruff TK, Besecke LM, Groome NP, Draper LB, Schwartz NB, Weiss J. Inhibin A and inhibin B are inversely correlated to follicle-stimulating hormone, yet are discordant during the follicular phase of the rat estrous cycle, and inhibin A is expressed in a sexually dimorphic manner. *Endocrinology*. 1996; 137:5463–5467. [PubMed: 8940372]

Fig. 1. DES dosing, blood sampling and euthanization programs

a Male rats were split up into control vehicle (corn oil), 0.1 µg or 10 µg DES treatment groups. After multiple injections, the animals were euthanized on day 18 post partum, at which time a blood sample was taken. **b** Male rats were split up into control vehicle (corn oil), 0.1 µg or 10 µg DES treatment groups. After multiple injections, blood samples were collected at day 18, 26, 35 and 90 post partum. At day 90 post partum, the animals were euthanized

Fig. 2. DES treatment of male rats causes an immediate reduction in pituitary FSH

a After 12 days of vehicle (control), 0.1 µg or 10 µg DES treatment, a significant reduction ($P < 0.01$) in the number of FSH immunoreactive cells in the 10 µg DES group was observed in the anterior pituitary of the test animals when measured at day 18 post partum. **b** Expressed as a ratio at day 18, the male rat anterior pituitary possessed a significantly ($P < 0.01$) lower ratio of FSH:LH immunoreactive cells in the anterior pituitary for the 10 µg DES test group only. Values are the mean $\pm$ SEM, ** $P < 0.01$

Fig. 3. Immunohistochemical analysis of anterior pituitary expression of LH or FSH immunoreactive cells 6 days after cessation of DES treatments

a LH immunoreactive cells in vehicle-treated male rats euthanized on day 18 post partum. **b** LH immunoreactive cells in 10 µg DES-treated male rats euthanized on day 18 post partum. **c** FSH immunoreactive cells in vehicle-treated male rats euthanized on day 18 post partum. **d** FSH immunoreactive cells in 10 µg DES-treated male rats euthanized on day 18 post partum

Fig. 4. Recovery of FSH protein expression in the anterior pituitary in male rats after neonatal DES treatment. **a** After 12 days of vehicle (control), 0.1 or 10 μ g DES treatment there was no significant reduction in the number of FSH- or LH immunoreactive cells observed in the anterior pituitary of the test animals in any treatment group when examined at 90 days post partum. **b** Expressed as a ratio on day 90 post partum, there were no apparent differences in the FSH:LH immunoreactive cell populations in the anterior pituitary among any of the experimental groups. Values are the mean $\pm$ SEM

Fig. 5. Immunohistochemical analysis of anterior pituitary expression of LH or FSH immunoreactive cells 78 days after cessation of DES treatments

a LH immunoreactive cells in vehicle-treated male rats euthanized on day 90 post partum. **b** LH immunoreactive cells in 10 µg DES-treated male rats euthanized on day 90 post partum. **c** FSH immunoreactive cells in vehicle-treated male rats euthanized on day 90 post partum. **d** FSH immunoreactive cells in 10 µg DES-treated male rats euthanized on day 90 post partum

Fig. 6. RT-PCR analysis of LH/FSH expression in male rat anterior pituitary following neonatal exposure to DES

a Ratios of LH and FSH mRNA over 18S mRNA expression are depicted for pituitary extracts from control (vehicle-treated), 0.1 and 10 µg DES-treated animals after euthanization 6 days post treatment cessation. The 10 µg DES group displayed a significant ($P < 0.01$) reduction in the levels of FSH mRNA. **b** Ratios of LH and FSH mRNA over 18S mRNA expression are depicted for pituitary extracts from control (vehicle-treated), 0.1 and 10 µg DES-treated animals after euthanization 78 days post treatment cessation. None of the experimental groups displayed any significant reductions in the levels of gonadotrophin mRNA. Values are the mean $\pm$ SEM, ** $P < 0.01$

Fig. 7. Plasma gonadotrophin levels in male rats treated neonatally with DES

a Plasma FSH levels measured in control (vehicle-treated) 0.1 or 10 µg DES-treated rats at days 18, 26, 35 and 90 post partum. **b** Plasma LH levels measured in control (vehicle-treated) 0.1 or 10 µg DES-treated rats at days 18, 26, 35 and 90 post partum. Values are the mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Fig. 8. Neonatal DES exposure significantly affects plasma inhibin B and testosterone levels in male rats

a Plasma levels of inhibin B were measured in male rats 78 days post cessation of vehicle (control), 0.1 or 10 µg DES treatment. DES at 0.1 and 10 µg significantly ($P < 0.01$ and < 0.05 , respectively) reduced circulating inhibin B levels at day 90 post partum. **b** Plasma levels of testosterone were measured in male rats 78 days post cessation of vehicle (control), 0.1 or 10 µg DES treatment. DES at 0.1 and 10 µg significantly ($P < 0.05$ and < 0.001 , respectively) reduced circulating testosterone levels at day 90 post partum. Values are the mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 1

Sequences of primers and probes used for Taqman RT-PCR analysis

Gene	Forward primer	Reverse primer	Probe (FAM labeled)
FSH β	ggagagcaatctgtgccata	Gcagaaacggcactcttct	ctgtgaattgaccaacatcaccatctcagtaga
LH β	tgtcctagcatggtccgagtact	Agggctacaggaaaggagactatg	cggctgctttgcctcctgtgc