# REPRODUCTION
RESEARCH

# Effect of androgen treatment during foetal and/or neonatal life on ovarian function in prepubertal and adult rats

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## Abstract

We investigated the effects of different windows of testosterone propionate (TP) treatment during foetal and neonatal life in female rats to determine whether and when excess androgen exposure would cause disruption of adult reproductive function. Animals were killed prepubertally at d25 and as adults at d90. Plasma samples were taken for hormone analysis and ovaries serial sectioned for morphometric analyses. In prepubertal animals, only foetal + postnatal and late postnatal TP resulted in increased body weights, and an increase in transitory, but reduced antral follicle numbers without affecting total follicle populations. Treatment with TP during both foetal + postnatal life resulted in the development of streak ovaries with activated follicles containing oocytes that only progressed to a small antral (smA) stage and inactive uteri. TP exposure during foetal or late postnatal life had no effect upon adult reproductive function or the total follicle population, although there was a reduction in the primordial follicle pool. In contrast, TP treatment during full postnatal life (d1–25) resulted in anovulation in adults (d90). These animals were heavier, had a greater ovarian stromal compartment, no differences in follicle thecal cell area, but reduced numbers of anti-Mullerian hormone-positive smA follicles when compared with controls. Significantly reduced uterine weights lead reduced follicle oestradiol production. These results support the concept that androgen programming of adult female reproductive function occurs only during specific time windows in foetal and neonatal life with implications for the development of polycystic ovary syndrome in women.

## Introduction

Androgens have been shown to play important roles in ovarian function not only acting as substrates for oestradiol ($E_2$) production in preovulatory follicles but also by modulating ovarian function both directly and indirectly. Studies on both sheep (Steckler et al. 2007, 2009, Hogg et al. 2011) and primates (Abbott et al. 1998, 2002, 2007) have shown that inappropriate exposure to androgens in foetal life results in reduced or absent ovarian function in adult life and may provide models for polycystic ovary syndrome (PCOS) in women (Abbott et al. 2007, Franks 2009). Up to 22% of women may have polycystic ovaries (PCO), but only around 5–10% of women develop the syndrome (PCOS), associated with increased androgenisation, weight gain and infertility (Franks et al. 2006). Androgen receptors (ARs) are expressed in the oocyte, granulosa and thecal cells of the follicle, are highest in small follicles and expression is developmentally regulated during follicle development being down-regulated by FSH and LH (see Drummond 2006, Walters et al. 2008). Global knockout of the AR in female mice leads to reduced fertility with reduced numbers of antral follicles and increased granulosa cell (GC) apoptosis (Hu et al. 2004, Shiina et al. 2006, Walters et al. 2008). More refined studies on GC-specific AR-knockout mice suggest that these reproductive defects are attributed to lack of AR expression in GCs (Sen & Hammes 2010). Androgens promote mouse follicular development in vitro through effects on methylation and up-regulation of the FSH receptor expression in conditions that are marginal for follicle growth (Hillier & Tetsuka 1997, Murray et al. 1998a, 1998b). In contrast, excess androgens correlated with poor oocyte fertilisation and developmental rates

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(Anderiesz & Trounson 1995, Xia & Younglai 2000) and can induce atresia in developing follicles (Billig *et al.* 2003). Thus, it appears that *in vitro* the effects of androgens on follicle development in established follicle populations is dependent on the stage of follicle development and the ratio of androgens to $E_2$, while in the longer term AR expression in GCs is essential for normal follicle development (Drummond 2006).

Several studies on rats have demonstrated that continuous exposure of postpubertal animals to steroid hormones, either via regular s.c. dosing or continuous release pellet implant, produces an anovulatory phenotype in these animals (Faiman *et al.* 1988, Beloosesky *et al.* 2004, Misugi *et al.* 2006, Alexanderson *et al.* 2007, Baravalle *et al.* 2007, Manneras *et al.* 2007, 2008, Abbott *et al.* 2009). Such models recapitulate some of the features of PCOS in women who have increased ovarian and stromal volume, increased thecal 17α-hydroxylase (17αOH) activity (Nelson *et al.* 1999, 2001) and high plasma concentrations of LH, testosterone and androstenedione (Welt *et al.* 2005, Franks *et al.* 2006, Fulghesu *et al.* 2007). Sheep (Steckler *et al.* 2007) and non-human primate models of PCOS (Faiman *et al.* 1988, Abbott *et al.* 2009) focus on the adult consequences of foetal programming by administering androgen (usually testosterone) during foetal life at a time when germ cells first begin to form primordial follicles. However in rodents, this treatment period of exposure corresponds to early postnatal life (McNeilly *et al.* 2000, Pepling & Spradling 2001, Pepling 2006). Thus, previous studies on the role of androgens in rodents that concentrate on acute effects of androgens on follicle development do not address the question of the potential for long-term androgen-induced reprogramming of the ovary during foetal and/or neonatal life on adult reproductive function.

Here, we extend previous research into foetal and neonatal programming of male rats by testosterone propionate (TP; Welsh *et al.* 2008, 2010) by investigating the reproductive consequences of foetal and neonatal TP exposure in female rats. These investigations aim to determine which windows of neonatal TP treatment induce an altered reproductive phenotype in the adult females and to what extent, if any, the resulting phenotype mimics human reproductive pathophysiology such as PCOS.

## Results

### Effects of different windows of TP exposure upon female rat reproductive physiology

#### Immature females

In immature rats (d25), both foetal + postnatal and just late postnatal TP treatment resulted in a significant ($P < 0.001$) increase in body weight. Exposure to TP during any postnatal window resulted in a significant

**Table 1** Body and organ weights and plasma LH levels across TP treatment groups in prepubertal animals (d25).

| TP treatment group | Body weight (g) | Uterus weight (mg) | FSH (ng/ml) | LH (ng/ml) |
|---|---|---|---|---|
| Control oil $n=12$ | $52 \pm 2$ | $28 \pm 4$ | $2.63 \pm 0.3$ | $6.5 \pm 1.0$ |
| Foetal $n=8$ | $51 \pm 1$ | $28 \pm 2$ | $1.68 \pm 0.3$ | $3.7 \pm 0.7$ |
| Full postnatal $n=6$ | $56 \pm 3$ | $137 \pm 32^{\dagger}$ | $2.98 \pm 0.8$ | $< 0.8^{\ddagger}$ |
| Foetal + postnatal $n=5$ | $81 \pm 3^{\ddagger}$ | $121 \pm 19^{\ddagger}$ | $2.10 \pm 0.3$ | $9.6 \pm 2.3$ |
| Late postnatal $n=5$ | $77 \pm 6^{\dagger}$ | $202 \pm 6^{\ddagger}$ | $1.43 \pm 0.5$ | $< 0.8^{\ddagger}$ |

Results are mean $\pm$ s.e.m. and were analysed by Student's unpaired *t*-test ($^{\dagger}P \leq 0.01$; $^{\ddagger}P \leq 0.001$).

($P < 0.05$ and $P < 0.01$) increase in uterine weight and significant ($P < 0.001$) reduction in plasma LH levels as expected, due to aromatisation of the TP to $E_2$ (Table 1).

#### Adult females

TP administration in all treatment groups except the late postnatal group (d15–24) resulted in an absence of vaginal opening. In adult animals at d90, TP treatment in full postnatal, foetal + postnatal and late postnatal treatment groups resulted in a significant ($P < 0.05$ to $P < 0.001$) increase in body weight and reduced uterine weights (Table 2). No differences in $E_2$ or LH levels were observed across TP treatment groups. FSH levels were significantly reduced in animals from the foetal + postnatal TP treatment group ($P \leq 0.05$), and levels were undetectable ($P < 0.001$) in the full postnatal TP treatment group (Table 2).

Mean adult ovarian weight was significantly reduced ($P \leq 0.01$) in animals from the full postnatal TP treatment group due to a lack of corpora lutea (CL; Fig. 1C and Table 2). Ovaries from the foetal and late postnatal TP treatment groups appeared morphologically normal with comparable numbers of CL (data not shown) to control ovaries (Fig. 1A, B and E). Foetal + postnatal TP ovaries were very small and severely underdeveloped, embedded in fat and could not be weighed accurately. Upon histological examination (Fig. 1D), foetal + postnatal TP ovaries appeared as underdeveloped 'streak' ovaries set within the gonadal fat pad (Fig. 2B and D). These ovaries had significantly ($P < 0.01$) fewer anti-Mullerian hormone (AMH)-positive follicles compared with controls (controls: $72 \pm 20$; foetal + postnatal testosterone: $8 \pm 2$).

### Neonatal androgen exposure leads to a reduction in the pool of primordial follicles in the adult but not in the prepubertal rat

Compared with oil-treated controls, there were no significant differences in total follicle numbers in ovaries from any treatment groups in both immature d25 and adult d90 rats, except for a significant ($P < 0.05$) reduction at d90 in the foetal + postnatal group (Fig. 3B). In immature rats, both full and late postnatal

**Table 2** Body and organ weights and hormone concentrations across TP treatment groups in adult animals (d90).

| TP treatment group | Body weight (g) | Ovary weight (mg) | Uterus weight (mg) | $E_2$ (pg/ml) | FSH (ng/ml) | LH (ng/ml) |
|---|---|---|---|---|---|---|
| Control oil $n=5$ | $213\pm9$ | $95\pm4$ | $433\pm52$ | $12.1\pm2.1$ | $1.8\pm0.1$ | $5.9\pm1.0$ |
| Foetal $n=3$ | $228\pm7$ | NA | $470\pm75$ | $16.9\pm1.2$ | $2.1\pm0.7$ | $4.7\pm1.0$ |
| Full postnatal $n=4$ | $318\pm6$[‡] | $59\pm5$[†] | $307\pm15$[†] | $4.2\pm1.3$ | $<0.8$[‡] | $2.2\pm0.9$ |
| Foetal+postnatal $n=5$ | $309\pm5$[‡] | NA | $158\pm35$[‡] | $9.7\pm2.9$ | $1.3\pm0.4$[‡] | $4.3\pm1.0$ |
| Late postnatal $n=5$ | $239\pm6$[†] | $80\pm5$ | $247\pm38$[‡] | $9.3\pm3.2$ | $1.6\pm0.2$ | $5.1\pm1.2$ |

Results are mean±s.e.m. and were analysed by Student's unpaired *t*-test ([†]$P\leq0.01$; [‡]$P\leq0.001$). NA, not available.

treatment groups had a significantly ($P\leq0.05$) greater proportion of transitory and significantly ($P<0.05$) reduced proportions of antral follicles. Adults had a significantly ($P<0.01$) reduced proportion of primordial follicles in postal TP treatment groups compared with controls. Furthermore, adults from the foetal+postnatal group also had a significantly reduced number of follicles ($P\leq0.05$) and proportion of antral follicles ($P\leq0.001$), since most follicles from this treatment group did not reach large antral (lgA) stage (Fig. 2B and C).

### Assessment of adult anovular ovaries following full postnatal treatment

Full postnatal TP ovaries were morphologically cystic in appearance (Figs 1 and 4–7). To quantify this cystic phenotype, average antrum size of follicles containing an oocyte with a visible nucleus was measured in every tenth section (Fig. 4C). Full postnatal TP led to a significant ($P\leq0.001$) increase in antrum size compared with controls.

Follicular functional status in both control and full postnatal TP-treated animals was measured using semi-quantitative analysis of AMH (Fig. 4) and aromatase expression (Fig. 5). Follicles expressing these proteins were counted and categorised in every tenth serial section. Although reduced, no significant difference in the total number of AMH-positive follicles was found (Fig. 4D), but there was a significantly ($P\leq0.01$) reduced proportion of AMH-positive small antral (smA) follicles in full postnatal TP-treated ovaries (Fig. 4E). Compared with controls, the number of aromatase-positive antral follicles was significantly ($P\leq0.05$) greater in full postnatal TP-treated animals (Fig. 5D). However, no differences were found in the proportions of lgA follicles staining intensely, weakly, or not at all for aromatase (Fig. 5E).

The thecal cell layer and stromal compartment area were measured in serial tenth sections of both control and postnatal TP-treated animals using immunohisto-chemistry (IHC) for 17αOH (Fig. 6) and 3β-hydroxy-steroid dehydrogenase (3βHSD; Fig. 7) respectively. There was no difference in the proportion of 17α-OH-positive follicles (Fig. 6D) or in thecal thickness and the total area occupied by thecal cells (Fig. 6F–G) between control and full postnatal TP animals. In addition, there was no difference in the correlations

between individual thecal area and follicle diameter in either treatment group (Fig. 6E). After correction for the area of the CLs in the control ovaries, the 3βHSD-positive stromal compartment (Fig. 7C and D) of full postnatal TP ovaries was significantly ($P\leq0.05$) larger than that of control ovaries and occupied a significantly ($P\leq0.001$) greater proportion of ovarian area.

## Discussion

As outlined in the Introduction section, androgens can have profound positive and negative effects on ovarian follicle development as well as providing the essential substrate for $E_2$ production by preovulatory follicles. Chronic administration of steroids or steroidogenic inhibitors during postnatal life in the rat have been assessed to determine whether any altered reproductive phenotype in the adult resembles that found in women with PCOS. Steroids used include testosterone (Beloosesky *et al.* 2004), DHEA (Anderson & Lee 1997, Anderson *et al.* 1997), $E_2$ valerate (Brawer *et al.* 1986, Rosa *et al.* 2003), DHT and letrozole (Manneras *et al.* 2007) while single bolus injection of testosterone or $E_2$ on or just after the day of birth resulted in a PCO-like phenotype in the adult animal (Barraclough 1961, Brawer *et al.* 1978, Ota *et al.* 1986, Grossmann *et al.* 1987, Alexanderson *et al.* 2007). However, relatively few rodent models have fully examined the reproductive phenotype in the adult animal and contrasted it with the clinical situation (Ota *et al.* 1986, Misugi *et al.* 2006, Abbott *et al.* 2007). Follicle formation is a continuum in both humans and sheep, occurring during mid gestation (McNatty *et al.* 2000, Childs *et al.* 2010). In the rat, follicle formation occurs in the first few days after birth (Schindler *et al.* 2010), equating to approximately mid-gestation in humans. In this study, rats were exposed to TP in various time windows, including postnatal only or foetal+postnatal life. Thus, it is reasonable in our current study to assume that the exposure to TP and, potentially, any $E_2$ that may be converted from the TP (Picon *et al.* 1985), would occur over a similar time period of follicle formation and development as in the other non-rodent models of PCOS, namely sheep (Steckler *et al.* 2007) and primates (Abbott *et al.* 2009).
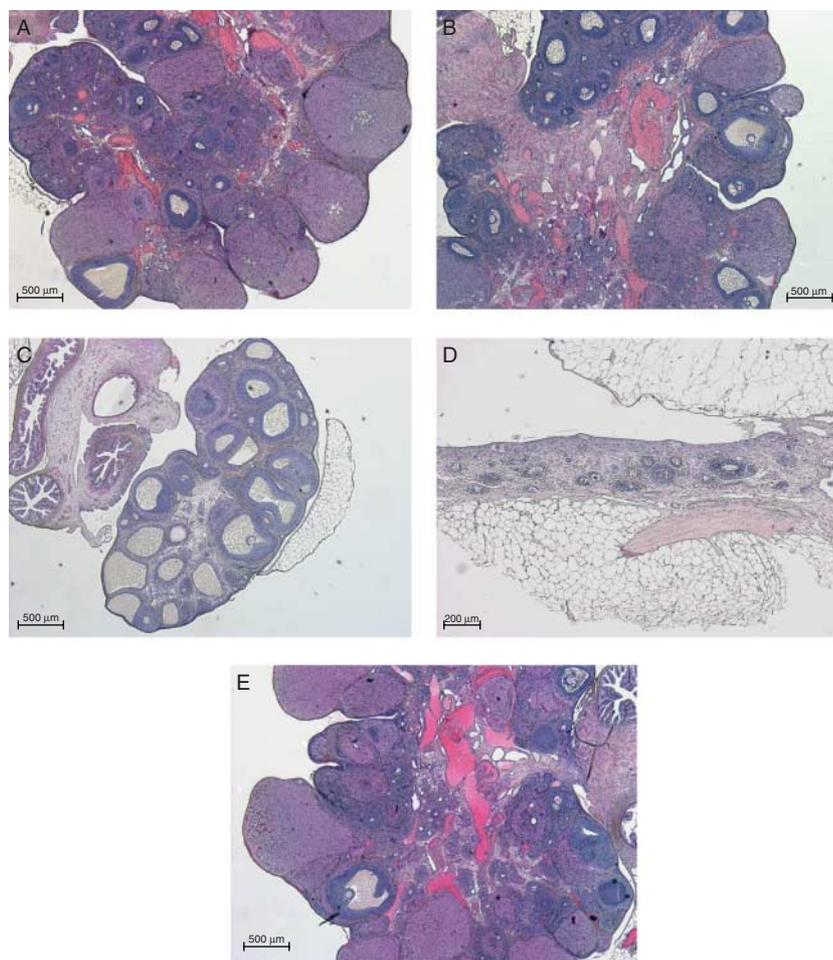
**Figure 1** Ovarian morphology of adult d90 animals from (A) control, (B) foetal, (C) postnatal, (D) foetal+postnatal and (E) late postnatal TP treatment groups. Images A–C and E taken at ×2 magnification, bar represents 500 μm. Image D taken at ×4 magnification, bar represents 200 μm.

## Foetal TP has no discernable effects upon the female rat

Foetal TP did not affect folliculogenesis or fertility in immature or adult animals, as evidenced by the presence of normal numbers of CL in the adult animals, and normal follicle numbers and proportions at both ages. Immature and adult body weights were normal, and gonadotrophin and $E_2$ levels were comparable to those seen in controls at each stage of investigation for this TP treatment group. These findings corroborate previous investigations into the effects of foetal TP upon female reproductive development (Slob *et al.* 1983). It is worth noting that there may be strain differences in the response to foetal androgen exposure as foetal TP administration in Sprague–Dawley (SD) rats in one study reduced both immature and adult body weight (Wolf *et al.* 2002, 2004). Foetal TP-treated female rats develop masculinised features of the urogenital tract, for example, an increased anogenital distance, as described in the original papers upon which these studies were based (Welsh *et al.* 2008, 2010).

## Postnatal TP treatment leads to changes in ovarian follicle composition in the immature animal

At d25, in late postnatal TP and foetal+postnatal TP (streak ovary) treatment groups, a higher proportion of activating transitory follicles and lower proportion of smA follicles was observed, with no effects upon total follicle numbers at this age. These results demonstrate the mitogenic effects of testosterone upon folliculogenesis, whereby androgens stimulate GC proliferation of smaller newly activated transitory and primary follicles as shown in graft experiments of ruminant follicles pre-exposed to testosterone and transplanted into chick ovaries; the grafts were shown to selectively increase the proportion of growing primary follicles (Qureshi *et al.* 2008). The same graft study found androgens to have a protective effect upon follicle survival, which is at odds with the reduced proportion of smA follicles found in the late postnatal and foetal+postnatal TP treatment groups of the current study. In the rat, ovarian AR expression is reduced in GCs of FSH-responsive follicles (Tetsuka *et al.* 1995) and other studies on rats have shown that
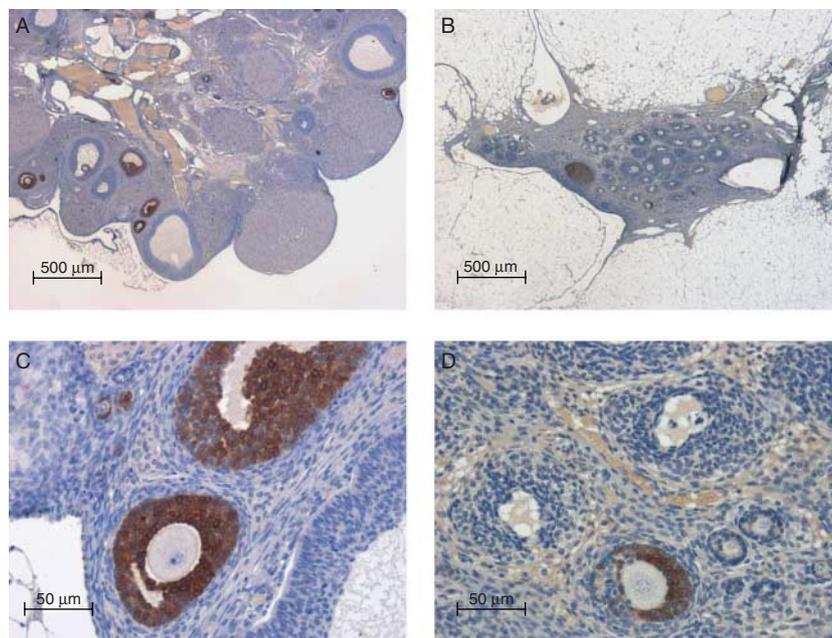
**Figure 2** Follicular functional status in control ($n = 5$) and foetal + postnatal TP-treated adult d90 animals ($n = 4$), assessed by AMH serial staining. AMH staining is shown in ovaries from control (A,C) and postnatal TP-treated (B,D) animals at (A,B) ×2 magnification, bar = 500 μm, and (C,D) ×40 magnification, bar = 50 μm.

exogenous androgen administration at the later stages of gonadotrophin-dependent folliculogenesis can facilitate follicular atresia (Drummond 2006, Honnma *et al.* 2006). Indeed, the smA follicles present in the immature rat ovary do not express sufficient aromatase in GCs for the production of $E_2$ (Mahesh *et al.* 1987). Thus, in the current study, TP likely acts to promote GC atresia in more advanced follicles, which would account for the reduction in smA follicle proportions within these TP treatment groups at d25.

### TP treatment after birth up to d15 leads to weight gain in adult animals

Significant weight gain was observed at d25 in immature foetal + postnatal and late postnatal TP-treated animals and in adults within all postnatal TP treatment groups. It is possible that this weight gain would affect metabolic function, but this was not assessed in this study. Other studies have only examined prenatal oestrogen (Roland *et al.* 2010) and glucocorticoids (Cottrell & Seckl 2009), which can induce metabolic dysfunction in mice and rats. In the current study, the increase in body weight was much less in the late postnatal group in the adults. This indicates that the window of postnatal androgen exposure which can produce a metabolic phenotype extends for at least 14 days after birth, but the effect appears to be less if treated only in the late postnatal period from d15 to 25. Furthermore, these findings extend results from other studies in which regular neonatal administration of androgens (testosterone; Nilsson *et al.* 1998; DHT: Alexanderson *et al.* 2007) or aromatase inhibitors (Letrozole; Manneras *et al.* 2007) has been shown to program weight gain and increased

adiposity in adult animals. Recently, it has been suggested that oestrogen receptors, implying oestrogen action, exert a stronger metabolic programming effect than ARs (Beloosesky *et al.* 2004). In this study, we do not know to what extent, if at all, the TP would have been converted to $E_2$ in the neonatal treatment periods, and this will require further investigation.

### Foetal + postnatal TP treatment leads to the formation of underdeveloped streak ovaries

TP administered during both foetal and postnatal life resulted in underdeveloped streak ovaries within both immature and adult animals, which by d90 of life had fewer follicles, fewer activated AMH-expressing follicles and consequential lower circulating $E_2$ levels with significantly reduced uterine weights. Secretion of AMH by GCs of growing follicles is known to inhibit further follicle activation (Durlinger *et al.* 2002, Rey *et al.* 2003, Pellatt *et al.* 2010). It is thus conceivable that the lower expression of AMH in the streak ovaries might allow unimpeded follicular recruitment and subsequent atresia. This could account for the reduction in follicle numbers within the adult animals by d90.

The presence of streak ovaries at both d25 and d90 also implicates a combined effect of late foetal and early postnatal TP exposure, given that no effect was found in foetal TP-treated animals and a different effect was observed in the full postnatal TP-treated animals. Streak ovaries have been associated with FSH receptor (Levallet *et al.* 1999) and BMP15 (Rossetti *et al.* 2009) mutations and is associated with an autoimmune response in neonatally thymectomised mice (Scalzo & Michael 1988). The potential role of the thymus is unknown.
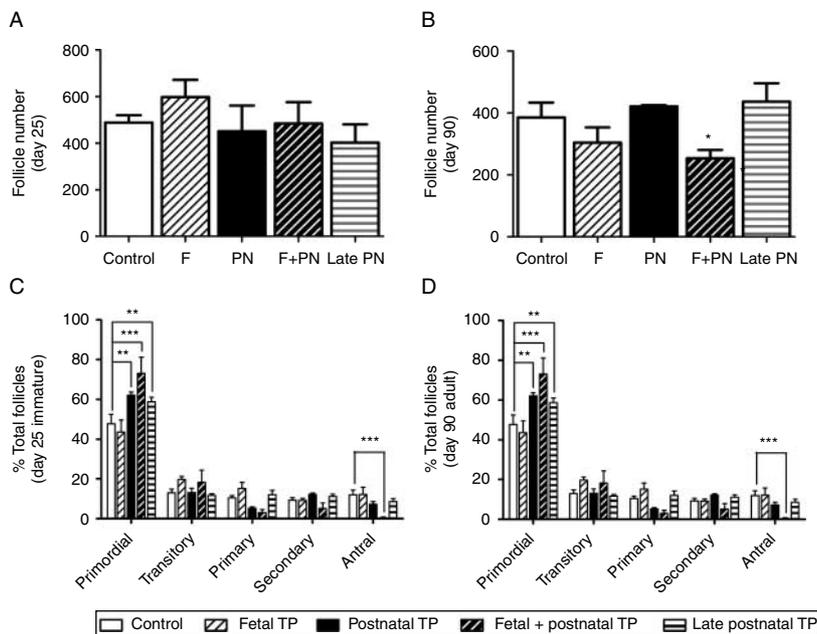
**Figure 3** Follicle count results across TP treatment groups; total follicle numbers are shown for (A) immature d25 animals and (B) adult d90 animals and were analysed by Student's *t*-test. Follicle proportions are shown for (C) immature animals and (D) adult animals and were analysed by two-way ANOVA. Values are expressed as the mean $\pm$ S.E.M. (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

However, the involvement of alterations in FSH receptor function was not examined in the current study, while BMP15 does not appear to play a significant role in rodent follicle formation or development (Yan *et al.* 2001). Neonatal $E_2$ exposure in rodents prevents germ cell nest breakdown in the ovary (Kezele & Skinner 2003, Jefferson *et al.* 2006) and effects involving ESR1 (ERα), ESR2 (ERβ) and the membrane-bound estrogen receptor (Chen *et al.* 2009). It is possible that in our study, circulating $E_2$ resulting from the placental or foetal and neonatal ovarian aromatisation of TP (Picon *et al.* 1985) and/or levels of TP acting purely as an androgen in animals exposed as foetuses were high enough to persist into early neonatal life and increase the sensitivity of the postnatal animal to further androgen exposure. This notion is supported by another study in which TP administered to pregnant SD rats during the late foetal period (e17–e18) had a priming effect upon female animals, leading to the masculinisation of female behaviour and physiology in animals, which subsequently received a small postnatal dose of 5 μg TP (Hoepfner & Ward 1988). Animals in the foetal and foetal+postnatal TP treatment group were cross fostered to untreated dams that had recently littered to avoid pup death (Wolf *et al.* 2002, Welsh *et al.* 2008, 2010). Evidence suggests that inadequate maternal care, in addition to differences in litter size during the rodent neonatal period, can affect hypothalamic–pituitary axis development (Champagne *et al.* 2003, Roma *et al.* 2007). Cross fostering of certain litters (foetal and foetal +postnatal TP) and not others (full postnatal and late postnatal TP) may have had an additional environmental effect upon endocrine development and function in animals from different litters, potentially accounting for the differences in ovarian morphology and reproductive dysfunction observed between treatment groups.

However, in the previous studies, using the same time windows of TP treatment did not identify any significant endocrine or phenotypic differences after cross fostering compared with normal controls (Welsh *et al.* 2008, 2010).

### Postnatal TP treatment leads to changes in ovarian follicle composition in the adult animal

One unexpected finding of these investigations was the increase in the proportion of primordial follicles consistently observed in all animals that received TP postnatally. This result is at odds with previous observation of an increase in primary follicles and decrease in the primordial follicle pool in ovaries of women with PCOS (Webber *et al.* 2003). At present, it is unclear clear how neonatal TP affects the rat follicle pool in this way, although recent research in mice has shown that oestrogen acting via both ESR1 and ESR2 can affect primordial follicle populations by inhibiting oocyte apoptosis before birth and preventing germ cell nest breakdown after birth (Chen *et al.* 2009). Thus, aromatisation of TP into $E_2$ within the neonate could account for the effects seen, as aromatase activity has been shown in the neonatal pituitary (Carretero *et al.* 2003) and ovaries (Picon *et al.* 1985) of female rats. Additionally, reduced primordial follicle recruitment could play a part in the conservation of the primordial pool documented in the current study.

### The postnatally androgenised rat as a PCOS model

Full postnatal TP (d1–25) induced a PCOS-like phenotype in the adult rat and was evaluated as a PCOS model.
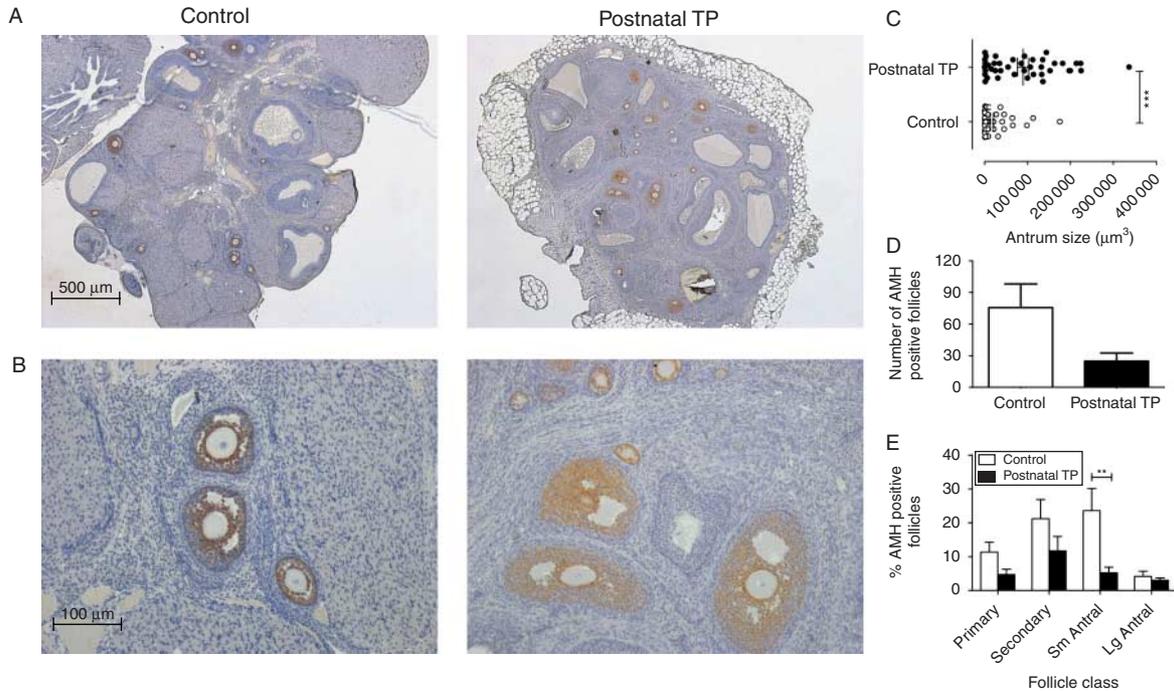
**Figure 4** Follicular functional status in control ($n=5$) and postnatal TP-treated adult d90 animals ($n=4$), assessed by AMH serial staining and antrum size. AMH staining is shown in ovaries from control and postnatal TP-treated animals at (A) $\times2$ magnification, bar$=500$ μm, and (B) $\times20$ magnification, bar$=100$ μm. Follicles were analysed based on (C) antrum size, shown as a scatter plot with lines indicating the mean$\pm$s.e.m., (D) the number of AMH-positive follicles and (E) which follicles of different sizes stained positively for AMH follicles. (D–E) Values are expressed as the mean$\pm$s.e.m. (**$P\leq0.01$; ***$P\leq0.001$).

Animals from this treatment group had reduced ovarian weights due to anovulation and lack of CL in addition to low uterine weights that, as a bioassay for oestrogens in the rat, indicate low oestrogen levels. However, it should be noted that plasma $E_2$ levels were comparable to controls in this group. An unexpected effect was that plasma FSH levels were undetectable, and LH levels were low in this treatment group. Given that the bioassay for $E_2$ levels indicated reduced levels (though when measured plasma $E_2$ was within normal range), it seems reasonable to expect that this would result in an increase in both LH and FSH secretion (McNeilly *et al.* 2003). The fact that this has not occurred suggests that the TP treatment has altered the negative feedback regulation of gonadotrophin secretion and will require further investigation.

Histological examination of ovaries from full postnatal TP-treated animals showed that they were anovular, with large fluid filled antral follicles apparent on subjective inspection. The reduction in the proportion of smA AMH-positive follicles in animals given postnatal TP may have contributed towards a larger mean number of antral follicles staining positive for aromatase found in adjacent sections and supports the idea that the functional status of follicles was altered, particularly given the low levels of FSH. This is supplemented by the fact that follicles within the ovaries of adult postnatal

TP-treated animals had larger mean antrum sizes. Further studies will be required to determine whether the altered ovarian phenotype that recapitulates some of the features of PCOS are related only to long-term effects on the ovary of TP exposure in the prenatal period or to altered gonadotrophin levels with or without a presumed metabolic effect related to weight gain.

No difference was observed between ovaries from the full postnatal TP-treated animals and control animals with regard to the status of the theca. Indeed, the thecal compartment was of a comparable size, surrounded a comparable number of follicles and the 17αOH stained area was similar between control and TP-treated animals. Given that the theca only develops around follicles as they grow beyond the primary stage (Young & McNeilly 2010), these results suggest that in the rat, at least there is no long-term effect on any thecal precursor cells in the stroma in adult life. In contrast, a novel finding of this study was the increased amount and proportion of 3βHSD-positive stroma found in full postnatal TP-treated animals, which further recapitulates clinical observations in women with PCOS (Fulghesu *et al.* 2007, Welt *et al.* 2005). As no differences in circulating gonadotrophins were observed in adult full postnatal TP-treated animals, this would indicate that this increased stromal compartment could be due to primary programming of non-thecal precursor cells, as hypothesised by other investigators (Franks 2009).
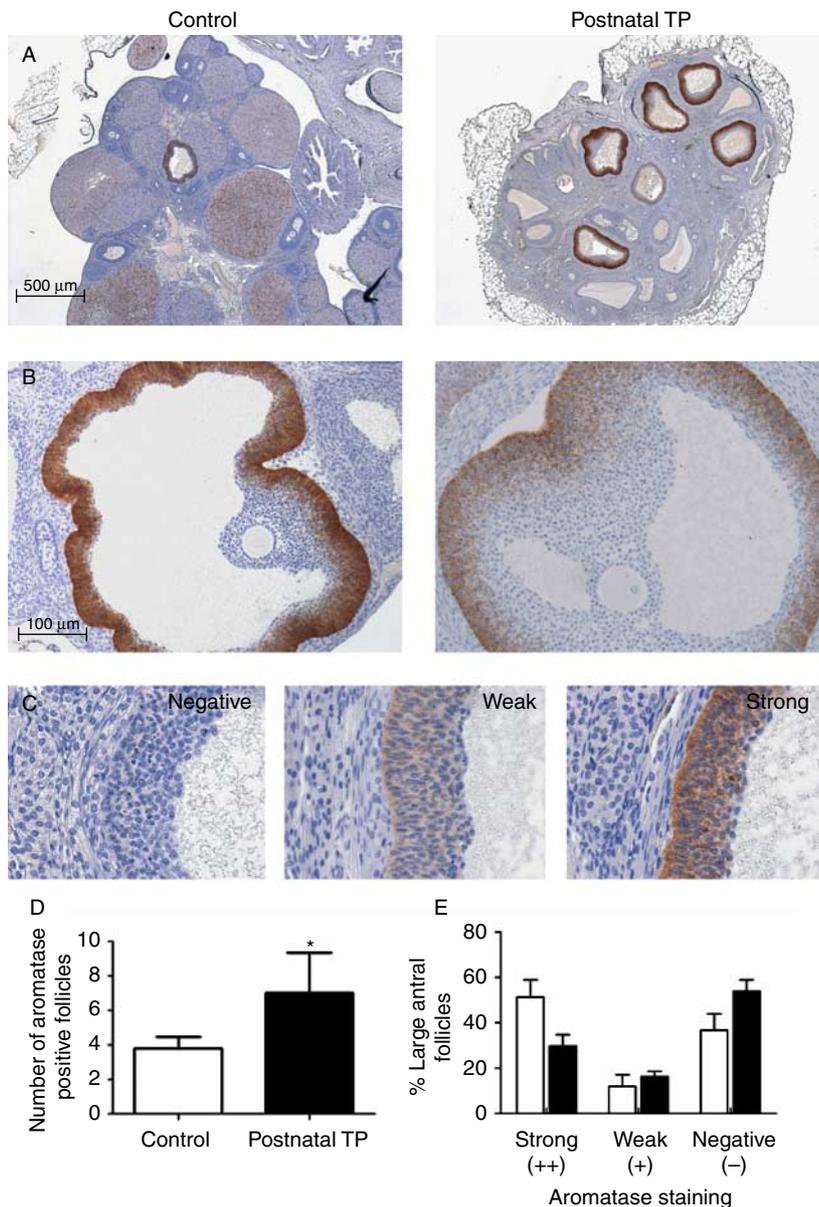
**Figure 5** Follicular functional status in control ($n=5$) and postnatal TP-treated adult d90 animals ($n=4$) assessed by aromatase serial staining and antrum size. Aromatase staining is shown in ovaries from control and postnatal TP-treated animals at (A) $\times 2$ magnification, bar=500 μm, and (B) $\times 20$ magnification, bar=100 μm, and (C) at different staining intensities; (D) the number of follicles expressing aromatase and (E) the proportions of follicles expressing either strong, weak or negative aromatase levels. Values are expressed as the mean$\pm$s.e.m. (*$P \leq 0.05$).

In conclusion, the results from the foetal + postnatal TP group, in agreement with the findings of other studies (Hoepfner & Ward 1988), demonstrate that foetal TP may reduce the sensitivity threshold to neonatal testosterone exposure in female animals, providing one explanation for the failure of normal ovarian development observed in this treatment group. Exposure to TP during the first 25 days of life additionally led to a significant increase in both immature and adult body weight through an as yet unknown mechanism. Finally, TP administration during the first 15 days of life resulted in a PCOS-like phenotype in the female: overweight, anovulatory and with larger than normal follicles as well as an increased ovarian stromal compartment. However, these phenotypic changes occur in the absence of the abnormalities of

FSH and LH typically associated with PCOS and suggest that neonatal TP exposure in the rat at least does not provide an adequate model for all aspects of PCOS.

## Materials and Methods

### Animals and treatments

Wistar rats were bred and maintained in the University of Edinburgh animal facility under standard conditions with licensed approval from the University Ethical Review Process and a UK Home Office Project Licence. Animals had access to water and a soy-free breeding diet made available *ad libitum* (SDS; Dundee, Scotland). Matings were timed and presence of a vaginal plug designated embryonic day 0.5 (e0.5). TP treatment regimes are summarised in the original paper
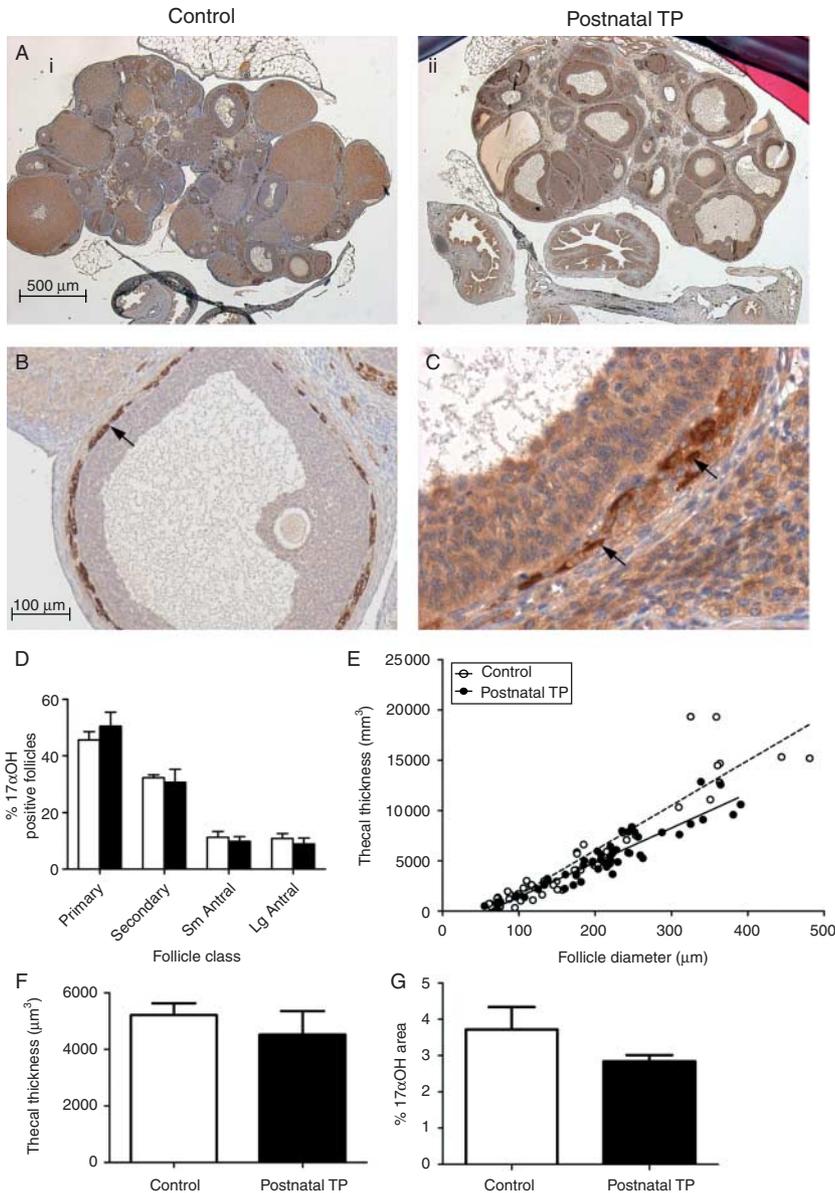
**Figure 6** 17α-Hydroxylase (17αOH) staining in ovaries from (A) control (i; *n*=5) and postnatal (ii; *n*=4) TP-treated adult d90 animals, bar= 500 μm. Theca interna 17αOH staining at (B) ×20 magnification, bar=100 μm, and (C) ×40 magnification. (D) The proportions of follicles expressing 17αOH in their theca interna were classified and analysed by two-way ANOVA with Bonferroni *post hoc* test, (E) thecal thickness was correlated with follicle diameter for both control and TP-treated animals and analysed by linear regression (control $R^2$=0.875, postnatal $R^2$= 0.883). A Fisher-*Z* transformation tested for differences between these two correlations; control *n*=43 follicles and postnatal TP *n*=44 follicles yielded *P*=0.876. Differences in (F) thecal thickness and (G) proportional 17αOH-positive area were analysed by Student's *t*-test. Bar graph values are expressed as the mean±s.e.m.

(Welsh *et al.* 2010). Briefly, for foetal TP treatment, pregnant dams were injected s.c. with 20 mg/kg TP in 0.4 ml corn oil daily between e14.5 and 21.5. To avoid foetal mortality that can be brought about by dystocia (Welsh *et al.* 2008), foetuses from TP-treated dams were delivered by caesarean and cross-fostered to untreated dams that had delivered within the last 6 h (Welsh *et al.* 2010). For postnatal TP treatment, pups were injected with 20 mg/kg TP every 3 days (d) in three different treatment groups: i) late postnatal TP treatment, from d15 to 24; ii) foetal+postnatal TP treatment, from foetal e14.5 to e21.5, then from d1 (the day after birth) to 24; and iii) full postnatal TP treatment, from d1 to 24. At least three litters were used per treatment group. Control animals received the same volume of oil as for each treatment period and were treated in foetal and neonatal life. This treatment had previously been shown to have no effect when compared with untreated controls

(Welsh *et al.* 2010). Animals were killed by inhalation of carbon dioxide and cervical dislocation on d25 (immature) and d90 (adult). Body and organ weights were documented and a blood sample collected by cardiac puncture for hormone analysis. Ovaries and uteri were fixed in Bouins for 6 h, transferred into 70% ethanol and processed into paraffin wax using standard methods.

## Ovarian IHC

One ovary from each animal was serial sectioned (5 μm thickness) and mounted onto permafrost slides for analysis using multiple IHC markers. Every tenth section was used for each set of IHC analysis. Sections were stained with haema-toxylin and eosin (H&E) for follicle count analysis, or DAB IHC was performed as described previously (McNeilly *et al.* 2000).
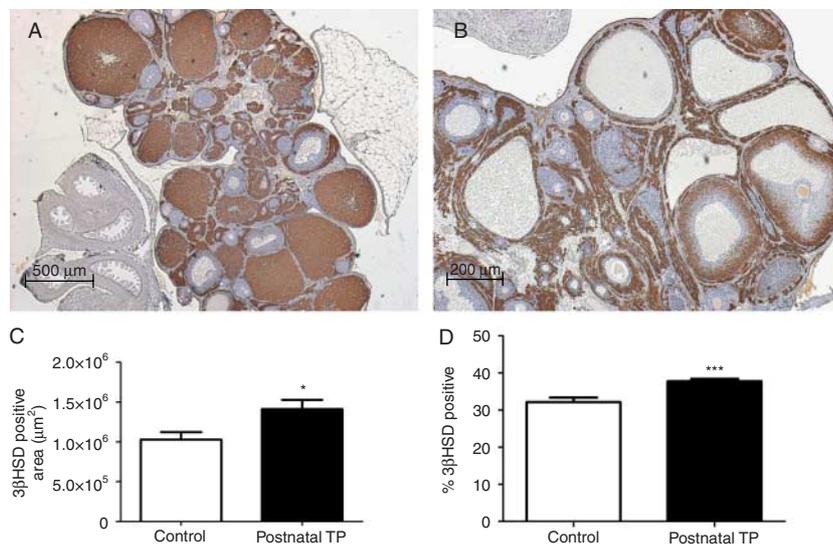
**Figure 7** 3β-Hydroxysteroid dehydrogenase (3βHSD) staining in ovaries from (A) control ($n = 5$), bar $= 500$ μm, and (B) postnatal TP-treated adult d90 animals ($n = 4$), bar $= 200$ μm. Mural granulosa, theca and CL 3βHSD staining was accounted for, and (C) 3βHSD-positive stromal area was measured in μm$^2$, and (D) taken as a proportion of ovarian area (minus CL area in controls). Values are expressed as the mean $\pm$ s.e.m. (*$P \leq 0.05$; ***$P \leq 0.001$).

Primary antibodies were diluted in normal serum: mouse anti-aromatase at 1:50 μl (Gift – Prof. N Groome, Oxford Brookes University); goat anti-AMH at 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA: MIS C-20); rabbit anti-17αOH at 1:500 (Gift– Dr D Hales Southern Illinois University); and rabbit anti-3βHSD at 1:250 (Gift–Prof. I Mason, University of Edinburgh). Slides were counterstained with haematoxylin, dehydrated through alcohols and xylene and mounted in pertex (Histolab, Gothenburg, Sweden) for analysis.

### Morphological and stereological analysis

Every tenth section of each ovary was H&E stained and examined at ×20 and ×40 magnification under a light microscope. Follicles were counted and classified according to a previously published method, allowing for a realistic estimate of follicle proportions with minimal data manipulation (Hirshfield & Midgley 1978, McNeilly et al. 2011). Only follicles containing a visible oocyte nucleus were included in the count, and these were classified based on their GC morphology. A layer of flat squamous GCs indicated a primordial follicle. Two or more cuboidal GCs indicated a transitory follicle. If two or less than two layers of cuboidal GCs were present around the oocyte, the follicle was primary and if more than two layers of cuboidal GCs were present around the oocyte, the follicle was secondary. If there were more than two layers of cuboidal GCs and an antrum had formed, the follicle was antral. In subsequent analyses, antral follicles with clear cumulus–oocyte complex formation and >5 GC layers were classified as lgA follicles and those with <4 GC layers were classified as smA follicles.

The number of cells per follicles expressing AMH or aromatase as well as their staining intensity was used to semi-quantitatively assess follicle morphology. Morphometric analysis of antrum size, 17αOH staining and 3βHSD staining was completed using stereological equipment comprising an Olympus BH-2 microscope fitted with a Prior automatic stage (Prior Scientific Instruments Ltd., Cambridge, UK). For 17αOH and 3βHSD, stained area below 10 μm$^2$ was not included,

in order to discount any background. CL and areas of non-specific staining were also masked using Image-Pro Plus version 6.2 with Stereologer-Pro 5 plug-in software (Media Cybernetics UK, Wokingham, Berkshire, UK), as published previously (McNeilly et al. 2011). Figure images were taken with a Provis AX70 (Olympus Optical, London, UK) microscope fitted to a Canon DS6031 camera (Canon Europe, Amsterdam, The Netherlands).

### Hormone RIA and ELISA

Plasma E$_2$ was assayed following solvent extraction using a sensitive RIA modified for mouse plasma as described previously (McNeilly et al. 2000, 2011). All samples were assayed in a single assay with a minimum detectable concentration of 4.6 pg/ml and an intra-assay coefficient of variation of <8%. The in-house ELISAs using the same general method were performed to assess plasma FSH and LH. Capture antibody diluted in buffer (50 μl of 0.2 M sodium bicarbonate–carbonate buffer) was adsorbed onto plastic plates (Nunc Biologicals, Thermo Scientific, Epsom, Surrey, UK) overnight. Plates were then washed with wash buffer (0.1 M Tris–HCl pH 7.5) and then washed with assay buffer (Tris–HCl, pH 7.5) containing 0.001% Tween20, 1% BSA (Sigma A3294) and 0.1% bovine-γ-globulin for a further 1 h. After a further wash, 5 μl standards, quality controls and unknown samples were added to the plate in duplicate and incubated with 95 μl assay buffer overnight at 4 °C. Between steps, plates were washed using 200 μl wash buffer and dried and all antibodies/samples were diluted in assay buffer.

For the FSH ELISA, mouse FSH (NIDDK AFP5308D; Dr A Parlow, Harbor-UCLA Medical Center, Torrance, CA, USA) was used as standard (range 0.78–50 ng/ml) with anti-human FSH MAB (4 μg/ml; MedixBiochemica 6602, BiosPacific, Inc., Emeryville, CA, USA) used as capture antibody. The second antibody was biotinylated rabbit anti-rat α-subunit IC1 (NIDDK AFP66P99860; 5 μg/ml) and anti-rabbit HRP conjugate (1:10 000; Thermo Scientific #31458) was used for signal detection.

For the LH ELISA, mouse LH (NIDDK AFP5306A) was used as standard (range 0.78–25 ng/ml) with anti-bovine LH chain MAB (2 µg/ml; 518B7; Gift – Dr J Roser, UCLA) used as capture antibody. The second antibody was biotinylated anti-human LH MAB (MedixBiochemica 5303, Kauniainen, Finland; 1 µg/ml) and Amdex streptavidin-HRP conjugate (1:10 000; GE Healthcare, Chalfont St Giles, Bucks, UK) RPN4401Vq was used for signal detection.

After a final wash cycle, both ELISAs were visualised with 100 µl 3′,5′,5′-tetramethylbenzidine micro-well peroxidase blue chromogen solution for 30 min (KPL). In both assays, oxidation was stopped by addition of 6% phosphoric acid. Absorbance was read using a plate spectrophotometer as $\lambda$ 450 nm minus $\lambda$ 620 nm to eliminate any background from the results that were analysed using AssayZap (Biosoft, Cambridge, UK).

## Statistical analysis

All data shown are expressed as mean ± S.E.M. and analysed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Two-way ANOVA was performed between any grouped data, for example, follicle counts, with a Bonferroni *post hoc* analysis to assess for specific in-group differences. Unless otherwise states, a two-tailed unpaired Student's *t*-test was used to evaluate the differences between rodent treatment groups in all other circumstances. To determine the correlation between two sets of data, the Pearson product–moment correlation (PPMC) was used. To assess for differences between two correlation coefficients, a Fisher *r*-to-*Z* transformation was performed and applied to the sample correlation coefficients (*r*) calculated by PPMC. In Graphpad Prism, a *P* value for a Fisher transformation was then calculated to test for the significant differences between two sets of overlapping correlative data.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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