

Prolonged oestrogen treatment does not correlate with a sustained increase in anterior pituitary mitotic index in ovariectomized Wistar rats

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Abstract

Oestrogen is a powerful mitogen that is believed to exert a continuous, dose-dependent trophic stimulus at the anterior pituitary. This persistent mitotic effect contrasts with corticosterone and testosterone, changes in the levels of which induce only transient, self-limiting fluctuations in pituitary mitotic activity. To further define the putative long-term trophic effects of oestrogen, we have accurately analysed the effects of 7 and 28 days oestrogen treatment on anterior pituitary mitotic activity in ovariectomized 10-week-old Wistar rats using both bromodeoxyuridine (BrdU) and timed colchicine-induced mitotic arrest. An oestrogen dose-dependent increase in mitotic index was seen 7 days after the start of treatment as expected, representing an acceleration in gross mitotic activity from 1.7%/day in ovariectomized animals in the absence of any oestrogen replacement to 3.7%/day in the presence of a pharmacological dose of

oestrogen (50 mcg/rat per day: ~230 mcg/kg per day). Despite continued exposure to high-dose oestrogen and persistence of the increase in pituitary wet weight, the increase in mitotic index was unexpectedly not sustained. After 28 days of high-dose oestrogen treatment, anterior pituitary mitotic index and BrdU-labelling index were not significantly different from baseline. Although a powerful pituitary mitogen in the short term, responsible, presumably, for increased trophic variability in oestrus cycling females, these data indicate that in keeping with other trophic stimuli to the pituitary and in contrast to a much established dogma, the mitotic response to longer-term high-dose oestrogen exposure is transient and is not the driver of persistent pituitary growth, at least in female Wistar rats.

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Introduction

The anterior pituitary, like many other endocrine tissues, retains considerable plasticity throughout adult life. The precise quantitative and qualitative nature of any pituitary mitotic and/or apoptotic response is influenced by the nature of the specific stimulus or stimuli, its amplitude, duration and timing. As a trophic modulator, oestrogen is both quantitatively and qualitatively different to testosterone. Testosterone tonically inhibits pituitary mitotic activity and the withdrawal of physiological levels in male animals results in a self-limiting wave of increased pituitary mitosis lasting 2–3 weeks (Nolan & Levy 2006). Testosterone replacement during the period of increased mitosis that follows orchidectomy rapidly restores mitotic activity to levels in intact animals (Nolan & Levy 2006). Oestrogen, in contrast, is believed to exert a potent and persistent rather than self-limiting stimulatory effect on anterior pituitary mitotic activity. As a result, pharmacological doses of oestrogen have been implicated in both hyperplasia of pituitary lactotrophs and in the induction and propagation of pituitary adenomas in the

longer term. Other observations suggesting a persistent trophic influence of oestrogen are that the pituitary is slightly larger in human females than in males (Denk *et al.* 1999) and is larger again in multiparous women (Chanson *et al.* 2001). Pituitary size increases during human pregnancy by 15–36% and peaks several days *post partum* (Dinc *et al.* 1998). In both humans and rats, it has been reported that concomitant with this change in pituitary size is a marked increase in lactotrophs from around 17% antenatally (Asa *et al.* 1982) to 50% at term (Haggi *et al.* 1986). If suckling does not take place, lactotroph mass returns to almost normal within 1–3 weeks, but remains slightly higher after pregnancy than in nulliparous rats, implying that pregnancy-induced changes in the size of the prolactin immunopositive population are not entirely reversible (Asa *et al.* 1982). It has also been assumed that different patterns of physiological oestrogen exposure – stable at low levels in males and fluctuating at higher levels throughout reproductive life in females – are implicated in the sexually dimorphic characteristics of prolactinomas described by some (Ma *et al.* 2002, Nishioka *et al.* 2003, Delgrange *et al.* 2005, Schaller 2005) but not all groups (Calle-Rodrigue *et al.* 1998) and in the increased

anterior pituitary cell turnover in female compared with male rats (Oishi *et al.* 1993, McNicol & Carbajo-Perez 1999).

A peak in anterior pituitary lactotroph mitotic activity has frequently been shown to occur in oestrous, correlating with the preceding increase in oestrogen levels during the pro-oestrous phase of the female reproductive cycle (Takahashi *et al.* 1984, Oishi *et al.* 1993). Using bromodeoxyuridine (BrdU)-labelling, it has been reported that increased proliferation seen in the female rat pituitary at oestrous occurs in lactotrophs and requires central brain activity in the preceding pro-oestrous afternoon (Hashi *et al.* 1995). No statistically significant increase in the proportion of lactotrophs was observed, however, suggesting that either newly formed lactotrophs undergo early apoptosis, that other cell types were similarly influenced by oestrogen fluctuations leaving the proportion of lactotrophs unchanged, or that the overall lactotroph increase in each cycle was too small to quantify (Hashi *et al.* 1995). The latter would certainly be expected if sexually dimorphic differences in pituitary size after puberty result from the cumulative effects of small oestrogen-induced residual increases in cell number or size following each oestrous cycle. The assumption of a direct association between oestrogen exposure and pituitary size, however, and dismissal of a major contribution of oestrogen-responsive secretory cell types other than lactotrophs may be premature. Indeed, one of the most marked sexually dimorphic differences in lactotroph numbers is seen in female mice transgenic for high-level expression of bovine GH (Vidal *et al.* 1999).

Oestrogen receptors, both α and β , are present in subpopulations of rat pituitary cells, particularly lactotrophs (50% express ER α and 30% express ER β) and a proportion of folliculostellate cells (Mitchner *et al.* 1998), but are also found in somatotrophs, thyrotrophs and gonadotrophs both during development (Nishihara *et al.* 2000) and in adulthood (González *et al.* 2008). Oestrogen is implicated in the stimulation of prolactin synthesis and secretion and proliferation of pituitary lactotrophs in humans and rodents. It has been suggested that paracrine interactions between lactotrophs and folliculostellate cells might regulate the mitogenic action of estradiol (Schwartz 2000, Oomizu *et al.* 2004), although the necessity for such interactions has been disputed (Ishida *et al.* 2007). In the longer-term oestrogen is thought to be ultimately responsible for the induction of prolactinomas in certain strains of rats such as male and female Fisher 344, but in general, despite the latter and a few case reports (Gooren *et al.* 1988, Garcia & Kapcala 1995), prolonged exposure to supraphysiological doses of exogenous oestrogens in humans does not inevitably result in either hyperprolactinaemia, pituitary hyperplasia or progression to prolactinoma (Melmed 2003, Ben-Jonathan *et al.* 2008).

The current study was designed to further define the short and longer term mitotic and apoptotic effects of continuous oestrogen exposure on the rat anterior pituitary. We have used both highly accurate direct histological assessment of mitotic index and BrdU incorporation *in vivo* to compare baseline anterior pituitary cell turnover and longer-term cumulative

changes in the number of dividing cells, respectively, in male and female Wistar rats. Using an ovariectomized rat model, we then further investigated the ability of exogenous oestrogen to stimulate and/or sustain an increase in mitosis in both the short term and the longer term.

Understanding the nature of pituitary mitotic and apoptotic responses to oestrogen is important as it is principally newly formed cells rather than mature pituitary cells that are sensitive to trophic stimuli (Nolan *et al.* 1999). By intermittently stimulating mitosis and accelerating apoptosis, fluctuating levels of oestrogen may be able to repopulate the nascent cell compartment and potentially sensitize the pituitary to a broad range of trophic stimuli (Aoki Mdel *et al.* 2003, Nunez *et al.* 2003, Pisera *et al.* 2004, Jaita *et al.* 2005, Candolfi *et al.* 2006). This mechanism is likely to be at least in part responsible for sexually dimorphic neuroendocrine responses such as the response to stress (Rhodes & Rubin 1999, Sandoval *et al.* 2003).

Animals and treatments

All procedures were carried out in accordance with UK Home Office animal welfare regulations. Male and female Wistar rats (Charles River, UK) were group housed and allowed to acclimatize for 1 week in our animal holding facility before the start of experimental procedures. Female rats weighed 150–175 g and male rats weighed 100–125 g on arrival. Groups of animals were killed by stunning and decapitation at the time intervals described below.

In order to determine baseline cell turnover in intact, cycling female rats, groups of animals were given a single intraperitoneal injection of 0.5 mg/100 g body weight of colchicine (Sigma) at time 0 h and killed 1.5, 3, 4.5 or 6 h later. Control animals were given saline vehicle only and killed at time 0 h (Nolan *et al.* 1999).

To follow both short- and longer-term cumulative changes in the number of dividing anterior pituitary cells (i.e. cell proliferation minus apoptosis of BrdU-labelled cells), groups of intact male and female rats received either a single intraperitoneal injection of (BrdU; 10 mg/ml in 0.007 M NaOH/0.9% (w/v) NaCl; Roche) at a dose of 200 mg/kg body weight at 1.5, 3, 6, 12 or 24 h prior to killing or daily injections of BrdU for up to 14 days.

Further groups of female Wistar rats were either surgically ovariectomized or sham operated under fluorothane anaesthesia. For post-operative pain relief, anaesthetized rats were given a s.c. injection (4 mg/kg body weight in a total volume of 0.2 ml in saline) of the non-steroidal anti-inflammatory Carprofen (Pfizer, Kent, UK). Starting 4 days after surgery, groups of ovariectomized and sham-operated rats were given daily s.c. injections of either oestrogen (17 β -oestradiol, E-8875 Sigma) or saline vehicle for 7 days. Oestrogen was either given at a dose designed to approximate physiological levels, 5 mcg/day (\sim 23 mcg/kg per day; Sakemi *et al.* 1998, Zhang *et al.* 2000) or at a higher, pharmacological dose of 50 mcg/day (230 mcg/kg per day). On day 11 after surgery,

groups of rats were given an intraperitoneal injection of 0.5 mg/100 g body weight of colchicine or saline vehicle and killed 0, 1.5, 3, 4.5 or 6 h later.

To examine the effects of longer-term high-dose oestrogen exposure in this model, additional groups of ovariectomized or sham-operated rats received the same physiological and high doses of oestrogen used above, but divided as twice weekly s.c. injections in sesame oil for 28 days before colchicine administration on day 32 after surgery. Vehicle-treated rats were given sesame oil alone. As an additional indicator of mitotic activity, 24 h before killing, some groups of rats were also given a single intraperitoneal injection of BrdU. Rats were weighed at this time point and pituitaries were weighed immediately after removal before being fixed in 4% formaldehyde in PBS.

Rats in the 7-day and 28-day study received the same equivalent daily dose of 17β -oestradiol divided into the same total number of boluses over the duration of each study. A similar number of oestrogen injections between groups (8 in total) was used to ensure that the effects of stresses associated with handling and with the injections themselves were kept as similar as possible. It was also felt to be important to minimize the impact of a potentially high total number of injections for animals in the 28-day study – bearing in mind the fact that animals were also receiving relatively large volume injections of BrdU. Our previous published (Nolan & Levy 2006) and unpublished experience indicates that intermittent injections of 17β -oestradiol in sesame oil vehicle provides a very secure route to high circulating levels of oestradiol and smooth mitotic response that does not differ if results are assessed on the day of injection or on the days between injections. A high pharmacological dose of oestrogen was used for the high-dose groups to minimize the possibility that a higher dose might result in more persistent changes.

BrdU immunohistochemistry

Standard pituitary tissue sections for trophic analysis and immunohistochemistry were prepared as described (Nolan & Levy 2006). Pituitary sections were processed for BrdU immunohistochemistry according to a previously published protocol (Cameron & McKay 1999) with minor modifications (Nolan *et al.* 2004). Briefly, de-waxed and rehydrated sections were transferred to a hot antigen unmasking solution (0.01 M citric acid in water; pH 6.0) and incubated for 10 min in a microwave for 10 min in 0.001% trypsin (Roche) diluted in 0.1% (w/v) CaCl_2 /20 mM Tris buffer (pH 7.5). Following three washes in PBS, the sections were denatured in 2 N HCl in PBS for 30 min, washed again in PBS, gently agitated for 30 min in blocking serum (3% (v/v) normal horse serum, 0.5% (v/v) Triton X-100 in PBS) and incubated overnight at 4 C with monoclonal anti-BrdU antibody (Becton Dickinson #347580 Franklin Lakes, NJ, USA; 1/100 diluted in blocking serum). Sections were washed in three changes

of PBS, incubated for 1 h at room temperature with biotinylated anti-mouse IgG (Vector Labs, Peterborough, England; 1/200 diluted in blocking serum), and washed again in fresh PBS before blocking endogenous peroxidases for 30 min with 0.6% (v/v) hydrogen peroxide in PBS. Following a further three washes in PBS, sections were incubated with RTU Vectastain Elite ABC reagent (PK-7100; Vector Labs) for 1 h at room temperature, rinsed in PBS and developed for ~ 8 min in DAB substrate according to the manufacturer's instructions (SK-4100; Vector Labs). The resulting brown colour reaction was stopped in water and the sections lightly counterstained with hematoxylin.

Image analysis for mitotic activity

Mitotic event prevalence was analysed on 2 μm -thick hematoxylin and eosin-stained pituitary sections at 1000 \times magnification (Nolan *et al.* 1998) using a real-time system (AxioHOME Zeiss, Welwyn Garden City, Hertfordshire, England, Brugal *et al.* 1992) that projects an image of a computer screen fractionally above the histological section. Identifier tags placed over manually identified cells and trophic events remain in registration with the targets irrespective of subsequent stage movements and magnification changes. For each animal, three random areas of ~ 47 000 μm^2 were scored for the presence of mitotic figures. By defining counting boundaries at low power and counting events at high power, selection bias and double scoring were eliminated, allowing the error in quantifying the number of normal cells surrounding these events to be limited to $\leq 2\%$ and the overall error in estimating the prevalence of trophic events to be reduced to ~0.001%. Results were expressed as a percentage of the total cell numbers counted for each animal.

BrdU-immunopositive cell counts were performed at 1000 \times magnification. For each animal, three areas totalling 0.15 mm^2 containing an average of 2000 cells were scored as positive or negative for the presence of immunoreactive BrdU. All slides were coded and counted by one blinded observer (LAN) and the results expressed as the mean \pm s.e.m. with differences between groups evaluated using one-way ANOVA followed by Tukey–Kramer multiple comparison post-tests. $P < 0.05$ was considered statistically significant.

Results

Pituitary cell turnover under baseline conditions in young female rat anterior pituitary

In intact young (10 weeks) female rat pituitary glands, the mean prevalence of mitotic figures increased from $0.08 \pm 0.019\%$ at baseline to $0.557 \pm 0.151\%$ after 6 h colchicine exposure (Fig. 1A), indicating an average parenchymal cell turnover of 1.91% per day. Presented as a scatter plot of data

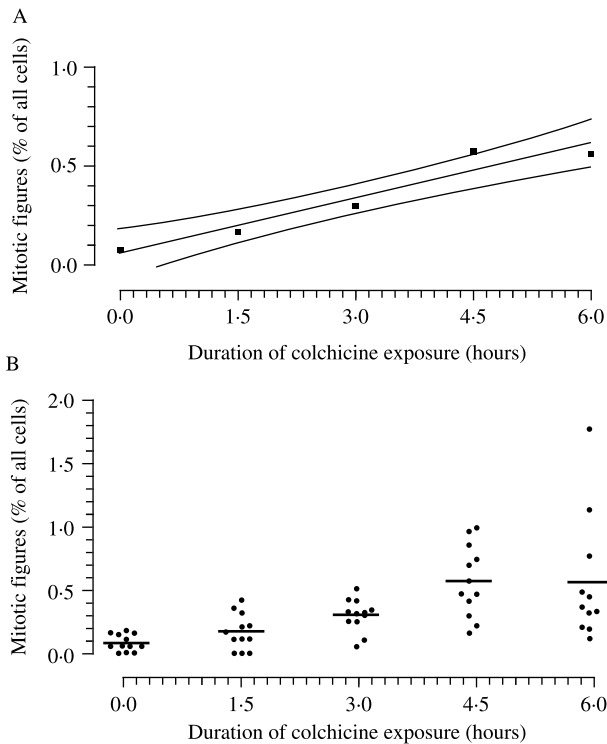


Figure 1 Changes in the prevalence of mitotic figures with time in intact female rats given a single intraperitoneal injection of colchicine. (A) Mean values with 95% confidence limit lines (upper panel) and (B) A scatter plot of values obtained from individual rats (lower panel). $n=11-12$ at all time points.

derived from individual animals (Fig. 1B), the data reveal striking variability in pituitary trophic activity, greatly in excess of that seen in intact male rats at any age (an approximately three fold difference in S.E.M. at the 6-hour time point (Nolan *et al.* 1999)).

A direct comparison between gross mitotic rate measured using colchicine-induced mitotic arrest over 6 h and net trophic activity (mitosis minus apoptosis over time) within the anterior pituitary of male and female rats was made using cumulative BrdU-labelling index measured over both the first 24-h period of BrdU exposure and over an extended 14-day period. Individual BrdU-labelling indices measured over the first 24-h period were much more variable in female than male rats (Fig. 2A and B) in keeping with the 6-hour data presented after colchicine treatment. In terms of net trophic activity, which is mitosis minus apoptosis of nascent cells over the following 14 days, however, there was no overall difference (Fig. 2C). This suggests that recurrent waves of increased mitotic activity are interspersed with periods of very little mitotic activity, although the possibility that increased mitotic activity occurs concurrently with increased apoptotic activity, and vice versa, although unlikely, cannot be confidently excluded from these data.

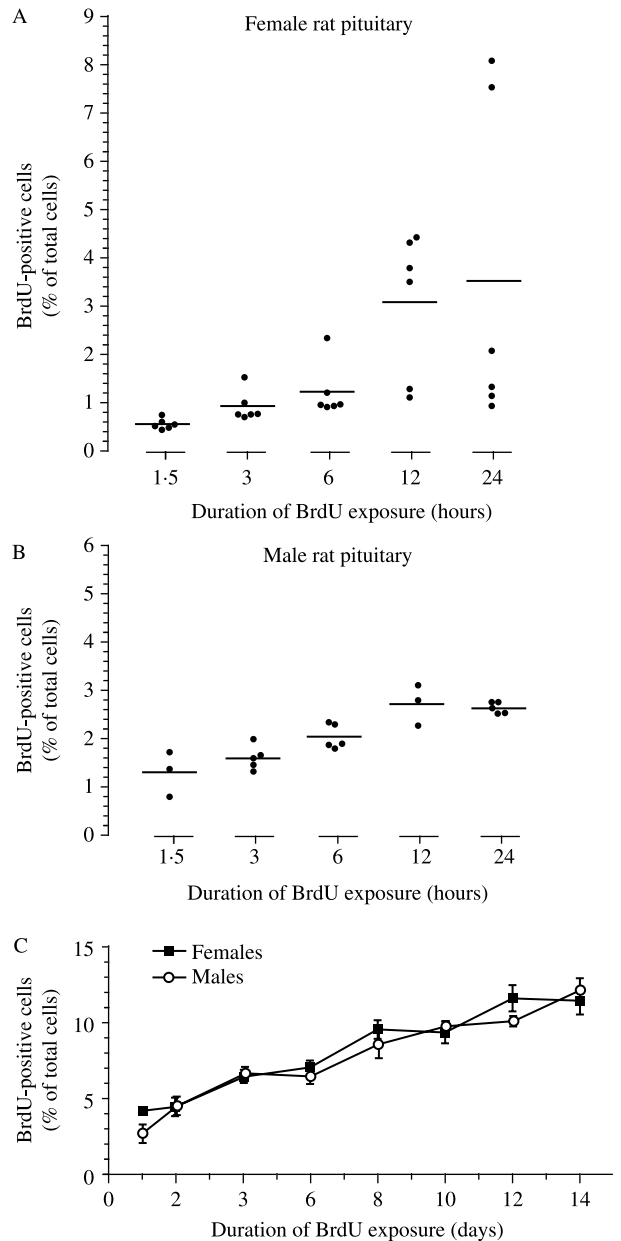


Figure 2 Cumulative increase in bromodeoxyuridine (BrdU) immunopositive cells as a percentage of total parenchymal cells in intact (A) female (B) and male Wistar rats during the first 24 h after a single BrdU injection. Data points are shown for each individual animal together with means. $n=3-6$ at all time points. (C) Comparison of the cumulative increases in BrdU immunopositive cells in male and female rats receiving daily injections of BrdU for up to 14 days. Means \pm S.E.M.s are shown; $n=3-7$ at all time points.

Acute oestrogen exposure increases pituitary cell turnover in female rats

Female rats were either ovariectomized or sham-operated and left to recover for 4 days before being treated with daily injections of oestrogen or vehicle for the next 7 days.

Colchicine was then administered on the following day as described.

In sham-operated rats receiving vehicle only, i.e. exposed to endogenous oestrogen dictated by the normal oestrous cycle, the mean prevalence of mitotic figures increased from $0.121 \pm 0.028\%$ at baseline to $0.699 \pm 0.272\%$ after 6 h colchicine exposure representing a cell turnover of 2.31% per day (Fig. 3A). These data confirmed the variability in baseline cell turnover previously observed in cycling female rats.

In ovariectomized rats in the absence of oestrogen replacement, the mean prevalence of mitotic figures increased from $0.122 \pm 0.027\%$ at baseline to $0.548 \pm 0.107\%$ after 6 h representing a slightly decreased cell turnover of 1.70% per day (Fig. 3B). Removal of oestrogen, for 4 days at least, decreased but did not completely abolish the variability of cell turnover between individual animals seen in females but not males.

In ovariectomized rats receiving daily injections of oestrogen to approximate circulating physiological levels, the mean prevalence of mitotic figures increased from $0.172 \pm 0.031\%$ at baseline to $0.945 \pm 0.119\%$ after 6 h representing a small increase in cell turnover to 3.09% per day at the lower dose of exogenous oestrogen (Fig. 3C). The mean prevalence of mitotic figures increased from $0.21 \pm 0.033\%$ at baseline to $1.145 \pm 0.19\%$ after 6 h representing a greater increase in cell turnover to 3.74% per day when the rats received a pharmacological dose of exogenous oestrogen (Fig. 3D).

Using direct morphological identification of apoptotic bodies in H&E-stained tissue sections, there were no measurable differences in the very low apoptotic indices between any of the experimental groups at baseline, i.e. prior to colchicine exposure (Fig. 4). After 6 h of colchicine treatment however, there was a measurable increase in the mean apoptotic index in rats that had been exposed to oestrogen that reached statistical significance in the ovariectomized animals treated with the physiological dose of oestrogen (Fig. 4).

An increase in mitotic index is not sustained during longer-term oestrogen exposure

The mean body weight of ovariectomized rats not supplemented with oestrogen was significantly higher than that of intact cycling animals after 28 days and significantly lower in ovariectomized rats given the highest dose of oestrogen (Fig. 5A; Bryzgalova *et al.* 2008). Pituitary wet weight was significantly increased in ovariectomized rats treated with the higher dose of oestrogen (Fig. 5B).

Contrary to expectations, the increases in mitotic index seen after 7 days of oestrogen treatment were not sustained after 28 days of oestrogen treatment and at this time there were no significant differences in the cumulative mitotic indices measured after colchicine-induced metaphase arrest (Fig. 6).

These data were confirmed by the significant reduction of BrdU-labelled cells following a 24-hour exposure immediately prior to killing at 28 days compared with that found at

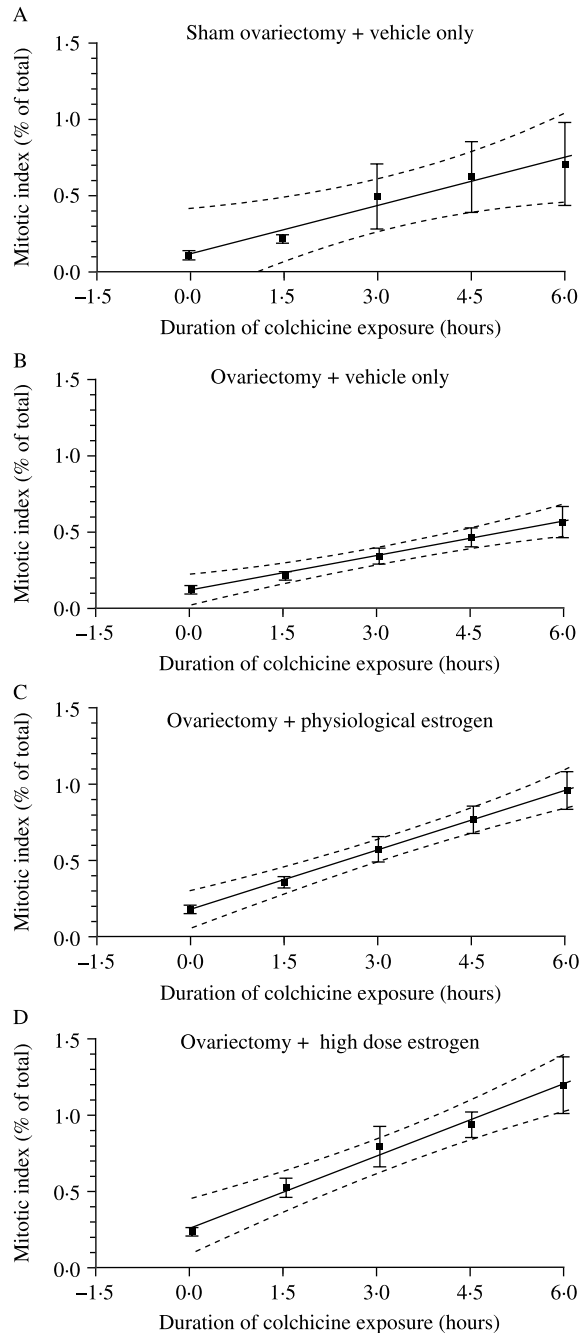


Figure 3 Comparison of the prevalence of mitotic figures with time in rats given a single intraperitoneal injection of colchicine 7 days after the start of oestrogen or vehicle treatment. (A) Control, sham-operated-vehicle treated rats; (B) ovariectomized-vehicle treated rats; (C) ovariectomized rats receiving physiological oestrogen replacement injections and (D) ovariectomized rats receiving high-dose oestrogen injections. Mean values with 95% confidence limit lines are shown. $n=4-6$ at all time points.

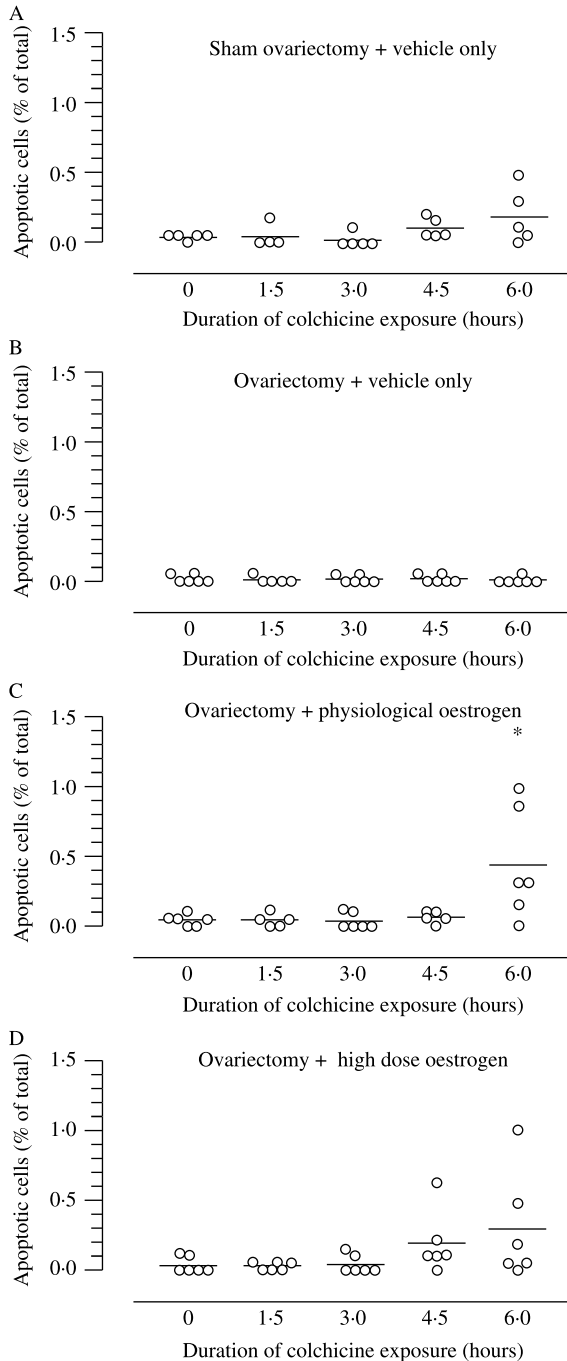


Figure 4 Comparison of the prevalence of apoptotic figures with time in rats given a single intraperitoneal injection of colchicine 7 days after the start of oestrogen or vehicle treatment. (A) Control, sham-operated-vehicle treated rats; (B) ovariectomized-vehicle treated rats; (C) ovariectomized rats receiving physiological oestrogen replacement injections and (D) ovariectomized rats receiving high-dose oestrogen injections. Mean values with the scatter of individual data points are shown. $n=4-6$ at all time points. * $P<0.05$ compared with all other time points.

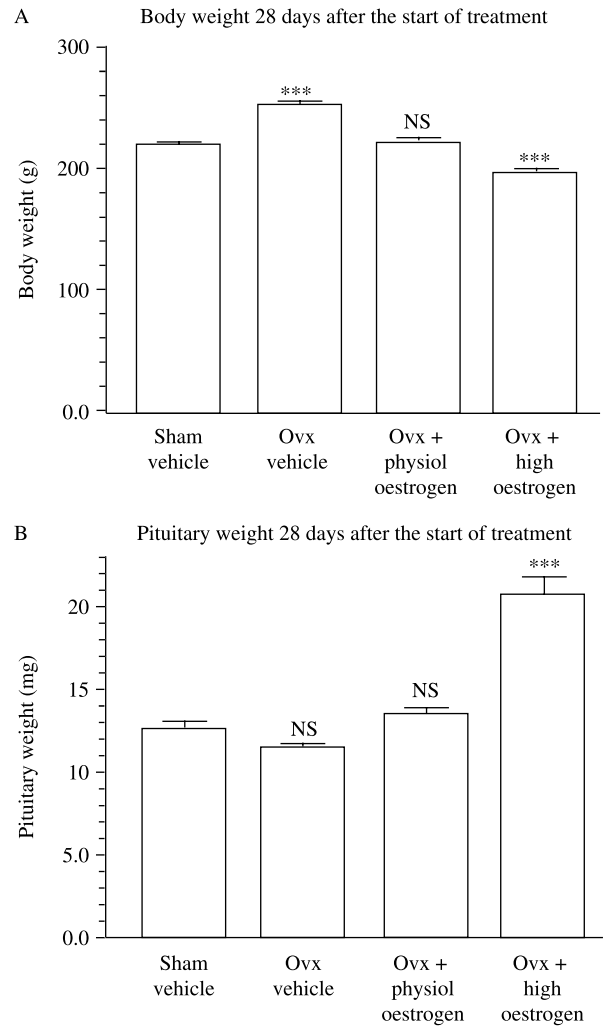


Figure 5 The effects of ovariectomy (ovx) and/or oestrogen treatments (Es) on (A) total body weight and (B) pituitary wet weight 28 days after the start of oestrogen or vehicle treatment. Means \pm S.E.M.S are shown; $n=27-30$; *** $P<0.001$ and NS not significant, compared with sham-operated, vehicle-treated controls.

7 days (Fig. 7). The smaller reduction in BrdU-labelled cells in ovariectomized rats treated with vehicle is presumably due to a period of time in excess of 7 days required to stabilize anterior pituitary proliferation following withdrawal of endogenous oestrogen at the time of surgery.

Discussion

Histologically, the adult anterior pituitary appears to be relatively trophically quiescent in comparison with many other tissues. However, the pars distalis is not only mitotically active under basal conditions but has been shown to be highly responsive to changes in ambient glucocorticoid, male

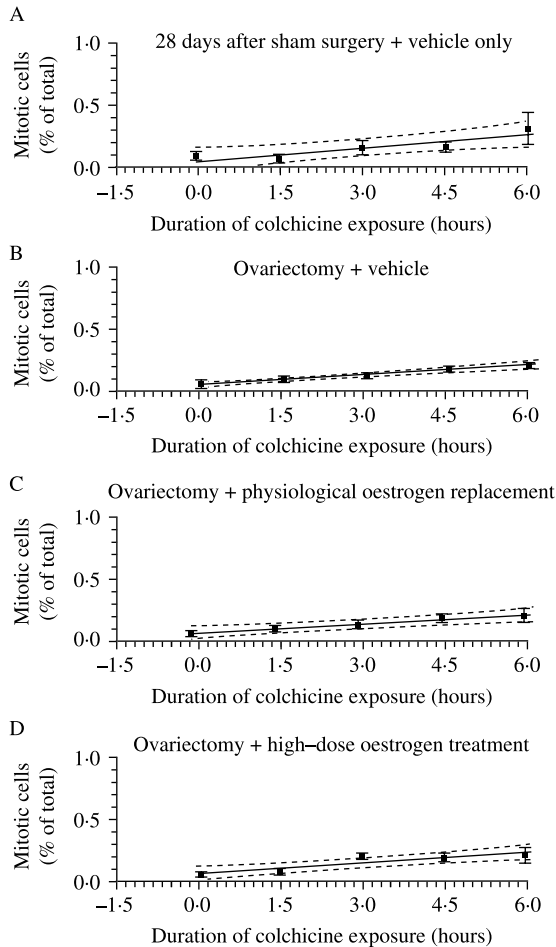


Figure 6 Lack of effect of sustained oestrogen treatment on the prevalence of mitotic figures with time in rats given a single intraperitoneal injection of colchicines 28 days after the start of oestrogen or vehicle treatment. (A) Control, sham-operated-vehicle treated rats; (B) ovariectomized-vehicle treated rats; (C) ovariectomized rats receiving physiological oestrogen replacement injections and (D) ovariectomized rats receiving high-dose oestrogen injections. Mean values with 95% confidence limit lines are shown. $n=4-6$ at all time points.

hormone and thyroid hormone levels (Nolan *et al.* 1998, 1999, 2004). Several studies have also been sensitive enough to suggest a diurnal variation in mitotic activity in the pituitary, although there is some disagreement about the exact number and timing of peak/s and nadirs (Nouet & Kujas 1975, Carbajo-Perez & Watanabe 1990, Carbajo-Perez *et al.* 1991, Oishi *et al.* 1993, McNicol & Carbajo-Perez 1999; for review (Levy 2002). The control of trophic activity in the pituitary and other endocrine organs is of particular interest because of the high prevalence and unusual behaviour of nodule and adenoma formation in these tissues. After induction and an initial period of growth, net cell turnover in these trophic anomalies returns to normal in almost all cases. They subsequently remain static from a net trophic

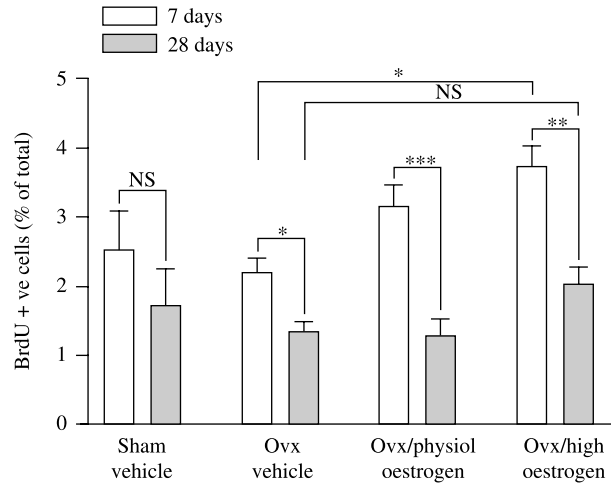


Figure 7 The prevalence of bromodeoxyuridine (BrdU) immunopositive cells as a percentage of total parenchymal cells in ovariectomized (Ovx) or sham-operated rats treated with oestrogen or vehicle for 7 days (open bars) and 28 days (grey bars). BrdU was given for 24 h before killing in all groups. Means \pm S.E.M.s are shown; $n=4-7$; * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared with the 7-day treatment point. NS not significant.

point of view for long periods of time or even, occasionally, resolve of their own accord. Progressive growth, whilst familiar in the clinical setting, is numerically relatively unusual. Nevertheless, the modest increase in size of the pituitary in females compared with males and in parous compared with nulliparous females, and the dramatic trophic effects of high-dose oestrogen exposure in some rat strains such as the Fischer 344 (Shull *et al.* 1998), has led to the belief that oestrogen is not only a powerful pituitary mitogen, but that its effects are also particularly persistent.

In the present study, the observation of sexually dimorphic pituitary mitotic activity with increased individual variability of pituitary mitotic activity in intact female rats strongly implicates fluctuating oestrogen levels associated with the oestrous cycle in rapid changes in pituitary mitotic activity. However, administration of daily BrdU injections over a 14-day period, which permitted analysis of cumulative mitotic activity in Wistar rats, showed that there was absolutely no evidence of a sex difference in net mitotic activity after the first 24 h. This finding may contrast with the findings of one other group who examined Sprague-Dawley rats and found 'cumulative incorporation of BrdU in females to be consistently twice as high as in males' but nevertheless concluded that 'cell renewal occurs at a doubled rate in the pituitary of female rats' (Oishi *et al.* 1993), implying once again that there may be a relationship with cell turnover but without a net gain in comparison with males.

Exogenous oestrogen given to intact, young male rats results in an increase in mitotic activity that peaks after 3 days and appears to drift back towards baseline by 7 days despite continuing exposure without any evidence of a concurrent

increase in apoptotic activity detectable by direct observation of apoptotic bodies (Nolan & Levy 2006). In the present study, 7 days after the start of oestrogen treatment in ovariectomized rats, we observed a significant, dose-dependent increase in cell proliferation measured by colchicine-induced metaphase arrest. After 28 days of continuous exogenous oestrogen exposure at physiological or supra-physiological levels, anterior pituitary mitotic rate was indistinguishable from that found in either sham-operated cycling rats or ovariectomized rats receiving vehicle alone, despite an increase in pituitary wet weight over that period. The exact timing of oestrogen injection did not affect the mitotic or apoptotic indices measured as no differences were found when the last dose of oestrogen was given either 3 days or 1 day prior to the administration of colchicine (data not shown).

The self-limiting nature of longer term mitotic and apoptotic responses to persistent stimuli is a consistent characteristic of pituitary trophic activity and may be related to receptor down-regulation (Shupnik 2002) or differential modulation of downstream co-activators or repressors and/or autocrine/paracrine growth factors such as galanin, transforming growth factor α (TGF α) and TGF β (Chun *et al.* 1998, Denef 2003). Sudden changes rather than persistent hormonal abnormalities appear to be the key to induction of pituitary trophic activity. Certainly, exposure to oestrogen for 28 days results in a significant increase in pituitary wet weight in both female (Fig. 5B) and male Wistar rats (our own unpublished data). This does not, however, correlate necessarily with an increase in total pituitary cell number, which might be accounted for by increased cell size, increased vascularity and decreased apoptosis of post-mitotic cells.

It has been suggested that peaks of circulating oestrogen might sensitize pituitary cells to pro-apoptotic signals such as lipopolysaccharide, FasL and dopamine (Pisera *et al.* 2004, Jaita *et al.* 2005, Radl *et al.* 2008). Induction of apoptosis is frequently mediated through the Fas/FasL system during physiological cell turnover in cycling hormone-dependent tissues. Both the Fas receptor and ligand are expressed in lactotrophs and although apoptosis in these cells has been shown in some studies to be enhanced by oestrogen (Jaita *et al.* 2005), others have suggested that the cells that undergo cyclical apoptotic changes during the oestrous cycle are mainly gonadotrophs and that there is no direct effect of oestrogen involved (Yin & Arita 2002).

After 6 h of colchicine treatment, there was an increase in the mean prevalence of apoptotic cells in both sham-operated intact rats and ovariectomized rats treated with oestrogen at both endpoints. In the absence of oestrogen, there was no measurable increase in apoptosis, suggesting that oestrogen can sensitize a small population of anterior pituitary cells to the pro-apoptotic effects of colchicine. The significance of this finding is unclear. We did not detect any significant differences in the very low baseline prevalence of apoptotic events following oestrogen treatment for either 7 or 28 days, but it is important to point out that at these very low levels small changes, with potentially very considerable biological

significance, are likely to have been undetectable. Unfortunately, in our experience, histological methods with potentially greater sensitivity than direct morphological identification but relatively low specificity such as TUNEL and caspase-3 immunocytochemistry are non-contributory in these circumstances.

In summary, oestrogen is believed to exert powerful trophic effects on the pituitary. It is thought to be responsible for the increase in pituitary size during pregnancy in humans, and to lead to dramatic pituitary hyperplasia and adenoma formation in some strains of rat. This study was designed to quantify these potent and persistent trophic effects of oestrogen. In complete contrast to expectations, continuous exposure to high-dose oestrogen for 28 days resulted in a return of anterior pituitary mitotic activity to baseline. In female Wistar rats at least, high-dose oestrogen is not sufficient to induce persistent pituitary mitotic activity.

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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