REPRODUCTION
RESEARCH

Neonatal programming by immunological challenge: effects on ovarian function in the adult rat

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Abstract

Neonatal exposure to an immunological challenge (lipopolysaccharide, LPS) increases the activity of hypothalamo-pituitary–adrenal axis and sensitises the GNRH pulse generator to the inhibitory influence of stress in adult rats. We investigated the effects of neonatal exposure to LPS on various reproductive parameters during puberty and into adulthood in female rats. LPS (50 μg/kg, i.p.) or saline was administered on postnatal days 3 and 5. Vaginal opening was recorded, and oestrous cyclicity was monitored immediately post puberty and again at 8–9 weeks of age. At 10 weeks of age, the ovaries were removed and the number of follicles was counted, together with the thickness of the theca interna of the largest antral follicles. Ovarian sympathetic nerve activity was assessed immunohistochemically by measurement of the levels of ovarian low-affinity receptor of nerve growth factor (p75NGFR). In rats exposed to LPS in early life, there was a significant delay in puberty and disruption of oestrous cyclicity immediately post puberty, which persisted into adulthood. The follicle reserve was decreased, the thickness of the theca interna increased and the expression profile of ovarian p75NGFR increased in the neonatal LPS-treated animals. These data suggest that exposure to LPS during early neonatal life can have long-term dysfunctional effects on the female reproductive system, which might involve, at least in part, increased ovarian sympathetic nerve activity.

Reproduction (2011) **141** 241–248

Introduction

It is well known that stressful stimuli can have profound suppressive effects on the activity of the hypothalamo-pituitary–gonadal axis, especially the GNRH pulse generator, the central regulator of reproduction (Ferin 2006). We have previously shown that neonatal exposure to lipopolysaccharide (LPS) as an immunological challenge programmes enhanced stress responsiveness of the hypothalamo-pituitary–adrenal (HPA) axis in adult rats, with increases in basal corticosterone pulse frequency and amplitude, as well as marked increases in stress-induced corticosterone release (Shanks *et al*. 1995, 2000). These changes are due, in part, to decrease in hippocampal and hypothalamic glucocorticoid receptors, which attenuate corticosterone negative feedback (Shanks *et al*. 1995, 2000). We have recently shown that exposure to LPS in early neonatal life results in long-term sensitisation of the GNRH pulse generator to the inhibitory influence of stress in adulthood (Li *et al*. 2007). Perinatal endotoxin exposure has also been shown to decrease circulating levels of progesterone and to increase testosterone in adult female rats (Nillson *et al*. 2001). Endotoxin, or interleukin 1, a key mediator of endotoxin effects, has been found to disrupt ovarian cyclicity in the adult of various species including the rat, cow, sheep and monkey (Peter *et al*. 1989, Rivest *et al*. 1993, Xiao *et al*. 1998, Battaglia *et al*. 2000). We have recently shown that the critical period for a significant delay in puberty (as defined by vaginal opening and first vaginal oestrus) as a result of neonatal LPS exposure is before 7 days of age (Knox *et al*. 2009), but there have been no reported studies on whether ovarian cyclicity is affected or whether reproductive effects persist into later life.

In addition to activation of the HPA axis, stressors activate the sympathetic nervous system (Zhou & Jones 1993, Xia & Krukoff 2003). Central administration of endotoxin has been demonstrated to activate the catecholaminergic system in the hypothalamus and

DOI: 10.1530/REP-10-0252
ISSN 1470–1626 (paper) 1741–7899 (online)
Online version via www.reproduction-online.org

brainstem, including increasing tyrosine hydroxylase (*TH*) mRNA levels in locus coeruleus, the central noradrenergic system of the brain (Xia & Krukoff 2003). Furthermore, endotoxin challenge increases circulating levels of noradrenaline and adrenaline in the rat (Zhou & Jones 1993). Stress-induced increase in systemic sympathetic activity can also increase sympathetic tone in the ovary. Chronic intermittent cold or restraint stress-induced increase in sympathetic nerve activity is associated with increased noradrenaline, nerve growth factor (NGF) and its receptor (p75NGFR) in the ovary (Paredes *et al*. 1998, Dorfman *et al*. 2003). Furthermore, the well-established polycystic ovary (PCO) rat model induced by oestradiol valerate administration, which is associated with increased ovarian sympathetic tone, shown by an overproduction of noradrenaline (Lara *et al*. 1993), NGF and p75NGFR in the ovary (Lara *et al*. 2000), is accompanied by irregular oestrous cycles and infertility (Lara *et al*. 2000). Neonatal exposure to oestradiol valerate similarly increases ovarian sympathetic activity resulting in ovarian dysfunction in adult rats (Sotomayor-Zarate *et al*. 2008).

The aim of this study is to test the hypothesis that exposure to the bacterial endotoxin, LPS, in early neonatal life, which delays puberty and enhances stress responsiveness, is associated with ovarian dysfunction in adulthood, as manifested by alterations in ovarian cyclicity and morphology and expression of ovarian p75NGF, a marker of sympathetic nerve activity.

Results

Neonatal LPS exposure delayed vaginal opening and first vaginal oestrus

Neonatal exposure to LPS resulted in a significant delay in both the day of vaginal opening (neo-LPS: 40.3 ± 0.8; neo-saline: 38.2 ± 0.5; $P<0.05$) and the day of first vaginal oestrus (neo-LPS: 41.2 ± 0.7; neo-saline: 38.6 ± 0.6; $P<0.05$). However, there was no significant difference in body weight gain throughout the experimental period between neonatal LPS- and neonatal saline-treated groups (Fig. 1).

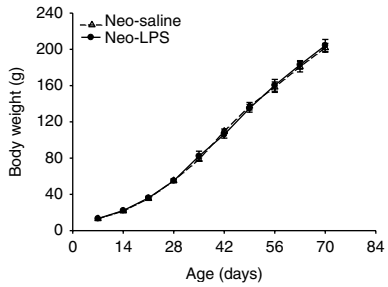

Figure 1 Effects of neonatal exposure to LPS (LPS, 50 μg/kg i.p.) on postnatal day 3 and 5 on body weight. There was no significant difference in body weight gain between neonatal LPS (neo-LPS)-treated and neonatal saline (neo-saline) controls ($n=37$–42 per group).

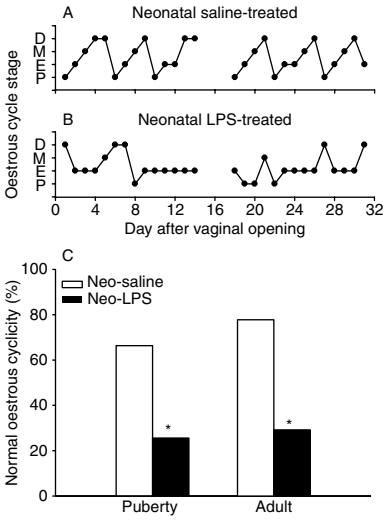

Figure 2 Effects of neonatal exposure to LPS on oestrous cyclicity immediately post puberty and in adulthood. Representative examples of oestrous cyclicity are illustrated in (A) and (B). Normal oestrous cyclicity is defined as having at least two consecutive normal cycles (i.e. cycle length 4–5 days; oestrus phase 1–2 days). The percentage of normal oestrous cyclicity was decreased at puberty and in adulthood of neonatal LPS-treated rats compared with neonatal saline controls (C). P, prooestrus; E, oestrus; M, metoestrus; D, dioestrus. *$P<0.05$ versus neonatal saline controls ($n=37$–42 per group).

Neonatal LPS exposure disrupted oestrous cyclicity immediately post puberty and in adulthood

Representative examples of oestrous cycles in each treatment group are illustrated in Fig. 2A and B. The majority (66.4%) of the neonatal saline-treated rats showed normal, 4–5 days oestrous cycles immediately post puberty, and the percentage of normal cyclicity increased to 77.8% in adulthood (Fig. 2C). In neonatal LPS-treated rats, only 26.2% showed normal cyclicity immediately post puberty, and this disruption persisted into adulthood, with only 29.2% of them showing normal cyclicity (Fig. 2C). The percentage of normal oestrous cycles was significantly lower in neonatal LPS-treated rats, both immediately post puberty and in adulthood compared with saline controls (Fig. 2C; $P<0.05$).

Effects of neonatal LPS exposure on ovarian weight and morphology in adulthood

The mean ± S.E.M. of ovarian weight at 10 weeks of age for the neonatal saline- and neonatal LPS-treated groups was not significantly different (70.3 ± 3.1 and 72.9 ± 3.2 mg respectively). There was a significant decrease in the total number of primordial, primary, preantral and antral follicles in neonatal LPS-treated females, compared with neonatal saline-treated control animals (Table 1). In addition, the thickness of the theca interna layer in the largest antral follicle was significantly

Table 1 Number of different types of follicles in the ovaries of adult rats treated neonatally (postnatal day 3+5) with saline (neo-saline) or lipopolysaccharide (neo-LPS).

Follicle type	Neo-saline ($n=4$)	Neo-LPS ($n=4$)	*P* value
Primordial	355.0±7.3 (344–369)	210.0±18.8 (176–241)*	0.002
Primary	431.0±20.1 (394–463)	254.3±41.1 (172–297)*	0.018
Preantral	63.3±5.8 (56–75)	39.0±6.2 (27–48)*	0.047
Antral	28.6±1.7 (26–32)	14.0±2.8 (9–14)*	0.012

Values are mean±S.E.M. (range). *$P<0.05$ versus neonatal saline controls (Student's *t*-test).

increased at oestrus, metoestrus and dioestrus in the ovaries from neonatal LPS-treated rats compared with neonatal saline controls (Fig. 3).

Effects of neonatal LPS exposure on sympathetic tone in the adult ovary

The immunohistochemical staining for p75NGFR was generally confined to the theca cells, especially the theca interna of growing follicles (preantral and antral follicles; Fig. 4A-D). Neither primordial nor primary follicles had specific staining for p75NGFR. There was no obvious specific staining for p75NGFR in the granulosa cells and corpora lutea, but there was some staining in the interstitial tissue. There was no significant difference in staining intensity between the different phases of the oestrous cycle in the neonatal LPS-treated rats or neonatal saline controls (Fig. 4E and F). However, the intensity of staining for p75NGFR in the theca interna of preantral (Fig. 4E) and antral (Fig. 4F) follicles was significantly enhanced in neonatal LPS-treated rats compared with neonatal saline controls ($P<0.05$).

Discussion

This study demonstrates that neonatal exposure to LPS in female rats can have long-term effects on reproductive function. Not only were markers of puberty onset (vaginal opening and first vaginal oestrus) delayed (in agreement with the findings of Knox *et al.* (2009)), but in addition, oestrous cyclicity was disrupted both immediately post puberty and into adulthood. The disruption to oestrous cyclicity was characterised by a persistent or prolongation of vaginal oestrus. Although the mechanisms underlying this disruption remain to be established, it has been reported that corticosterone administered on postnatal days (pnd) 3 and 6 induced disruption of oestrous cycles immediately post puberty and in adulthood, which again was characterised by persistent vaginal oestrus (Turner & Taylor 1977). It has been shown that increased levels of glucocorticoid can also block LH surges or ovulation in rats (Baldwin 1979, Roozendaal *et al.* 1997) and sheep (Macfarlane *et al.* 2000). Hypercortisolism is associated with functional hypothalamic amenorrhoea in women (Suh *et al.* 1988, Brundu *et al.* 2006). Since we have previously shown that adult rats treated neonatally with LPS (using an identical protocol to this study) are known to exhibit hypercorticosteronaemia (Shanks *et al.* 2000), it is possible that this may underlie the disruption of oestrous cyclicity observed in this study. We have recently shown that there is a critical time window for neonatal LPS treatment to delay puberty in the rat, with treatment delayed to pnd 7 and 9 or pnd 14 and 16 no longer effective (Knox *et al.* 2009), consistent with the observation that oestrous cyclicity was unaffected in adult rats administered with LPS on pnd 10 (Iwasa *et al.* 2009). It is possible that a similar critical time window may operate for long-term programming effect of an immunological challenge on oestrous cyclicity.

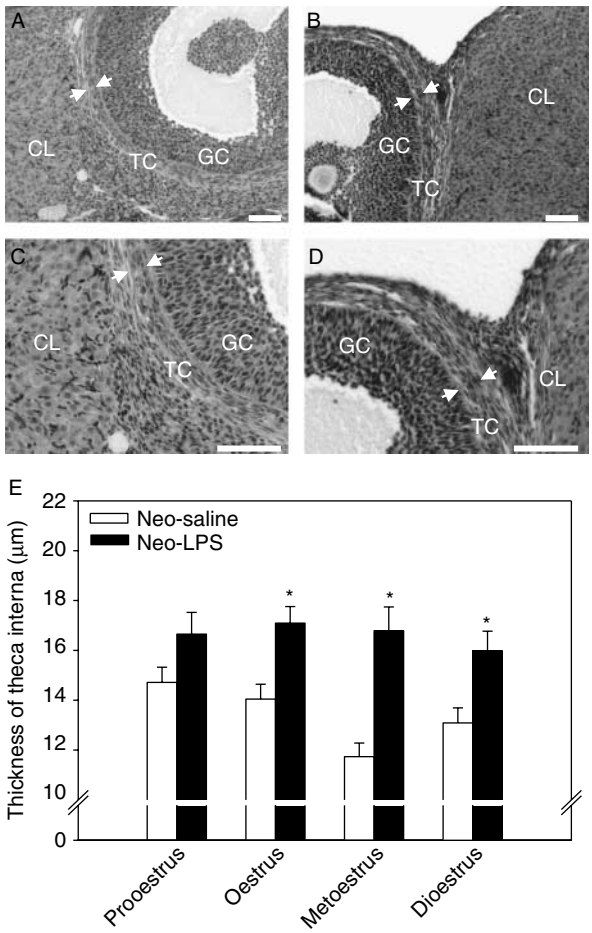

Figure 3 Effects of neonatal exposure to LPS on the thickness of the theca interna layer of the largest follicles in adult ovary. Representative examples of the theca interna layer of the largest follicle in adult ovaries are illustrated in A and C (neonatal saline controls) and B and D (neonatal LPS-treated rats). The thickness of the theca interna layer of the largest follicles was increased at oestrus, metoestrus and dioestrus in neonatal LPS-treated rats compared with neonatal saline controls (E). CL, corpus luteum; GC, granulosa cell; TC, theca cell; arrows show the theca interna layer. Photomicrographs illustrate dioestrus stage of oestrous cycle. Scale bar=50 µm. *$P<0.05$, versus neonatal saline controls ($n=8$–11 for each group).

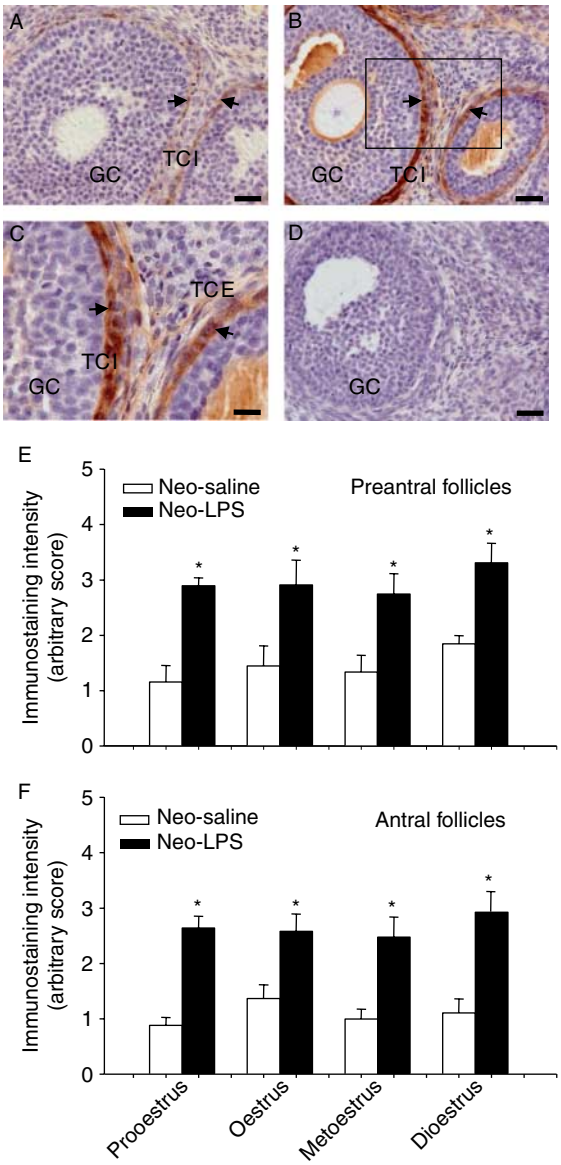

Figure 4 Effects of neonatal exposure to LPS on the immunoreactivity of p75NGFR in theca interna cells of the adult ovary. Low intensity of immunoreactivity for p75NGFR was detected in theca cells, especially theca interna (arrows) of growing follicles (preantral and antral follicles) from neonatal saline controls (A). The immunostaining for p75NGFR in the theca interna cells of preantral and antral follicles was significantly higher in the neonatal LPS-treated rats (B). (C) shows a higher magnification of the boxed area in (B). (D) is a negative control with no staining in the adjacent section incubated in absence of primary antibody. *H*-scores of immunostaining intensity for p75NGFR in theca interna cells are summarised in (E) and (F). There was no significant difference in staining intensity between different phases of the oestrous cycle. GC, granulosa cell; TCI, theca interna cell; TCE, theca externa cell. Photomicrographs illustrate dioestrus stage of oestrous cycle. Scale bars: A, B and D = 20 μm; C = 10 μm. *$P<0.05$, versus neonatal saline controls ($n=8$–11 for each group).

In contrast to our findings, Nillson *et al.* (2002) failed to show an effect of neonatal LPS treatment on pnd 3 and 5 on oestrous cyclicity in adult rats. The reason for this discrepancy is unknown, although different strains of rat and different routes of LPS were used.

The neuropeptide kisspeptin (KISS1) and its receptor (KISS1R) have been identified as an essential part of the hypothalamic circuits that govern the initiation of puberty (Navarro *et al.* 2007). Additionally, KISS1/KISS1R signalling plays a critical role in controlling normal ovarian cyclicity in the adult through its regulation of the GNRH pulse generator (Li *et al.* 2009) and the GNRH surge generator (Kinoshita *et al.* 2005, Pineda *et al.* 2010). We found that neonatal LPS administration caused a significant decrease in *Kiss1* mRNA expression and increase in *Kiss1r* mRNA expression in medial preoptic area (mPOA) in adult animals (Knox *et al.* 2009). A similar change in the *Kiss1*/*Kiss1r* expression profile was observed in the mPOA in response to both acute or chronic physiological stress levels of corticosterone (Kinsey-Jones *et al.* 2009). Thus, the altered hypothalamic *Kiss1*/*Kiss1r* expression by neonatal exposure to LPS may account for, at least in part, the disruption of oestrous cyclicity observed in this study.

It is well established that normal ovarian cyclicity and ovulation rely on both normal pulsatile and preovulatory surge release of FSH and LH. Increased levels of corticosterone have been shown to decrease levels of FSH in rats (Valli *et al.* 2000, Baravalle *et al.* 2007). It is possible that the increased basal levels of corticosterone in adult rats treated neonatally with LPS (Shanks *et al.* 2000) may decrease FSH secretion, leading to the decreased number of preantral and antral follicles observed in this study. We have previously shown that neonatal LPS treatment sensitises the GNRH pulse generator to the inhibitory effect of stress in adulthood (Li *et al.* 2007), although basal pulsatile LH secretion was not affected. However, in this study we used gonadal-intact rats instead of ovariectomised, oestradiol-replaced animals as in our previous study. Other studies have already shown that increased corticosterone levels in ovary-intact rats were accompanied by a reduction in LH secretion (Valli *et al.* 2000, Baravalle *et al.* 2007). Additionally, elevated levels of glucocorticoid or restraint stress have been shown to delay, attenuate or block the preovulatory LH surge in sheep (Wagenmaker *et al.* 2009) and rats (Baldwin 1979, Roozendaal *et al.* 1997). Whether the LH surge is also affected by the administration of LPS in early life needs to be investigated further.

The total number of follicles, in particular primordial follicles, was decreased in adult rats treated neonatally with LPS. This suggests that early life exposure to an immunological challenge may affect the earliest stages of folliculogenesis, when primordial follicles are organising. It has been demonstrated that follicles are assembled and differentiated after pnd 2 in rats (Rajah *et al.* 1992,

Kezele *et al*. 2002), so the administration of LPS on pnd 3 and 5 may coincide with the critical developmental time of primordial follicles assembly and transition. Therefore, we speculate that neonatal exposure to LPS reduced the reserve of ovarian follicles and impaired fertility later in life by interfering with the assembly of primordial follicles and, consequently, transition from primordial to secondary follicles. Indeed, a reduced follicular pool size has been shown to result in an increased incidence of irregular cyclicity (Anzalone *et al*. 2001).

Whether the increase in p75NGFR, a marker of ovarian sympathetic nerve activity (Lara *et al*. 2000), observed in this study, is due to the up-regulation of central sympathetic activity or local peripheral regulatory mechanism and its role in controlling ovarian function need further studies. The thickness of the theca interna layer of the largest follicle in adult ovaries was increased by neonatal LPS treatment. This may be due to increased ovarian sympathetic tone since previous studies using chronic intermittent cold stress, which classically enhances ovarian sympathetic nerve activity, as measured by increased noradrenaline and NGF in the ovary, are similarly accompanied by increased thickness of the theca layer in the rat ovary (Dorfman *et al*. 2003). Interestingly, it was recently shown that exercise or acupuncture reversed the increased thickness of the theca interna in the dihydrotestosterone-induced PCOS rat ovary (Manneråas *et al*. 2009). In this study, the immunoreactivity for p75NGFR, the common receptor for neurotrophins, which are necessary for growth and differentiation of ovarian sympathetic nerves (Dissen *et al*. 2002), was increased in ovaries from neonatal LPS-treated rats. These data suggest that ovarian sympathetic activity may have been increased by neonatal exposure to LPS. The ovarian sympathetic innervation has been shown to participate in the control of ovarian function such as follicular development, steroid secretion and ovulation (Ojeda & Lara 1989, Lara *et al*. 1990). However, increased sympathetic activity in the ovary has been shown to derange follicular development, disrupt oestrous cyclicity and reduce ovulation in stress animal models (Paredes *et al*. 1998, Dorfman *et al*. 2009) and oestradiol valerate-induced PCO rats (Rosa-E-Silva *et al*. 2003, Greiner *et al*. 2005). Transgenic mice with increased NGF level in the ovary show delay in puberty and disruption of oestrous cyclicity (Dissen *et al*. 2009). Furthermore, immunoneutralising NGF action in conjunction with blocking the synthesis of p75NGFR restored oestrous cyclicity and ovulatory capacity in oestradiol valerate-treated rats (Lara *et al*. 2000). Moreover, there is an increased density of ovarian catecholaminergic nerves (Heider *et al*. 2001) and increased sympathetic nerve activity measured by direct intraneural recordings in PCOS patients (Sverrisdöttir *et al*. 2008). The increase in p75NGFR, a key marker of sympathetic activity observed in the ovary in this study, may therefore account for the derangement of follicular development, increased thickness of the theca interna and disruption of oestrous cyclicity.

Several stressors, including LPS, have been shown to increase sympathetic nerve activity in adult rats (Zhou & Jones 1993, Xia & Krukoff 2003). It has been shown that the autonomic nervous systems play an important role in the regulation of ovarian physiology (Agudo 2002, Dissen *et al*. 2002). Viral transneuronal tracing studies have provided morphological evidence for direct innervation of the ovary via multisynaptic neuronal pathways, including the noradrenergic nucleus, the locus coeruleus (Gerendai *et al*. 1998). Cold stress, which promotes PCO through up-regulation of ovarian sympathetic activity in rats (Dorfman *et al*. 2003, 2009), is also accompanied by an increased activation of TH-immunoreactive neurones in the locus coeruleus (Bernuci *et al*. 2008, Anselmo-Franci *et al*. 2009). Moreover, lesions in the locus coeruleus can reduce the increased ovarian noradrenergic activity, decrease the thickened theca interna layer and restore oestrous cycles (Bernuci *et al*. 2008).

In conclusion, this study shows that neonatal exposure to LPS leads to long-term dysfunction of the reproductive system at the ovarian level in rats, especially disruption of oestrous cyclicity and thickening on the theca interna, which might be associated with the increased ovarian sympathetic tone. Therefore, in addition to increasing the activity of the HPA axis (Shanks *et al*. 2000) and sensitising the hypothalamic GNRH pulse generator to the inhibitory effects of stress in adulthood (Li *et al*. 2007), immunological challenge in early life can have a long-lasting suppressive effect on ovarian function.

Materials and Methods

Animals and neonatal endotoxin exposure

Pregnant Sprague–Dawley rats (Charles River, Margate, UK) were housed under controlled conditions (12 h light:12 h darkness cycle, with lights on at 0700 h; temperature, 22 ±2 °C) and supplied with *ad libitum* food and water. On the day of birth (pnd 0), litters were culled to 10–12 pups. On pnd 3 and 5, pups were injected i.p. with 50 μg/kg endotoxin in 0.05 ml sterile saline (LPS, serotype *Escherichia coli* 055:B5; Sigma–Aldrich), a dose known to provoke permanent HPA axis activation in adulthood (Shanks *et al*. 1995, 2000) but to cause no discernable change in the general health of the pups or maternal behaviour (Shanks *et al*. 2000). Control pups received saline (0.05 ml). Animals were randomly assigned to LPS or vehicle in the same litter. All litters were weaned at pnd 21, and female offspring were housed four to six per cage until they reached 10 weeks of age. All animal procedures were conducted under the British Home Office Animal Scientific Procedure Act 1986 (Project Licence 70/6237) and in accordance with accepted standards of the local ethical review committee.

Puberty onset and oestrous cyclicity monitoring

Animals were monitored daily for vaginal opening from pnd 28. Once vaginal opening occurred, vaginal smears were taken and monitored daily for 2 consecutive weeks and again at 8–9 weeks of the age. The different stages of the oestrous cycle were determined according to the predominant cell type present in the vaginal smears under light microscope (Marcondes *et al.* 2002). Normal oestrous cyclicity was defined as having at least two consecutive normal oestrous cycles, which lasts for 4–5 days with 1–2 days of oestrus. The cycle length was determined by the number of consecutive days from the last day of a cornified smear to the last day of an oestrus smear in the subsequent cycle. Animals were weighed weekly until the end of the experiment.

Ovarian morphology

At 10 weeks of age, rats were ovariectomised. The right ovary was cleaned of fat tissue, weighed and fixed in 10% formaldehyde buffer for 20 h at room temperature. Wax-embedded ovaries were sectioned longitudinally at 4 μm; every tenth section (six sections from each ovary) was mounted on a glass slide and stained with haematoxylin–eosin. The thickness of the theca interna layer of the largest follicle was determined with a calibrated scale bar in the microscope. For counting the total number of different types of follicles, the ovaries from a separate group of animals (neonatal LPS- or neonatal saline-treated rats) were completely sectioned at a thickness of 8 μm per section, and each was subjected to analysis. Only follicles in which the nucleus of the oocyte was visible were counted (Lara *et al.* 1990). The sections were examined under a light microscope (Zeiss Axioskop 2 plus, Oberkochen, Germany) with an image analysis system (Axiovision 2.05; Zeiss) by two independent investigators blind to the treatment group.

The follicles were classified as following: primordial – follicles with oocytes surrounded by one layer of flattened pregranulosa cells; primary – follicles with oocytes surrounded by no more than two layers of cuboidal granulosa cells; preantral – follicles without any antral cavity and with two or more layers of granulosa cells; antral – follicles with apparent cavity (Lara *et al.* 1990, 2000).

Immunohistochemistry

Immunohistochemical staining for p75NGFR was performed on 4 μm paraffin embedded sections from ovaries of 10-week-old rats. The sections were deparaffinised in xylene, hydrated in descending concentration of ethanol and incubated with 0.3% Triton X-100 (Sigma–Aldrich) in PBS to increase permeability before incubated with 0.3% H_2O_2 to inhibit endogenous peroxidases and with non-immune goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS to reduce background staining. Thereafter, the sections were incubated in 1:1000 monoclonal mouse anti-p75NGFR primary antibody (Chemicon International, Temecula, CA, USA) containing 2% normal goat serum at 4 °C for 24 h. The sections were then rinsed before incubation in 1:150 biotinylated goat anti-mouse IgG (Vector Laboratories) for 90 min at room temperature followed by 1:200 conjugated avidin–biotin complex (Vector Laboratories) for an additional 45 min at room temperature. Visualisation of p75NGFR immunoreactivity was achieved using the 3′,3′-diaminobenzidine (Sigma–Aldrich). The sections were finally counterstained with 10% haematoxylin and coverslipped with DPX. Negative controls were run by incubating adjacent sections with non-immune serum and omission of the primary antibody. The sections from control and LPS-treated animals were run together in the same batch. Immunohistochemical detection of rat spinal cord was included as a positive control for p75NGFR in each batch of experiments. In each immunohistochemical experiment, two control sections from the same ovary tissue were included as immunostaining quality controls for the variation between different batches of experiments.

Semi-quantitative analyses of immunostaining intensity for p75NGFR were carried out on a Zeiss AxioVision microscope image system (Zeiss). All analyses were performed on coded slides by two independent investigators blind to the treatment groups.

Statistical analyses

Comparisons between neonatal LPS and neonatal saline treatment groups on vaginal opening, first oestrus and body weight were made by subjecting data to one-way ANOVA followed by Dunnett's test. The percentage of normal oestrous cycles between groups was compared using χ^2 test. Comparisons between groups on thickness of the theca interna layer and follicle number were made by subjecting data to the Mann–Whitney *U* test and Student's *t*-test respectively. All data are shown as mean ± S.E.M.

The intensity of immunostaining for p75NGFR was assessed by using a modification of a semi-quantitative *H*-score method described by Akercan *et al.* (2008). Semi-quantitative immunohistochemical *H*-score values were calculated from the intensity and percentage of cells staining at each intensity. Intensities were classified as 0 (no staining), +1 (weak staining), +2 (weak–moderate staining), +3 (moderate staining), +4 (strong staining) and +5 (very strong staining). For each slide, the *H*-score value was calculated using the equation: $\Sigma(I \times PC)$, where *I* and *PC* represent intensity and percentage of cells that stain at each intensity respectively. Data were expressed as mean ± S.E.M. The different groups were analysed by the Mann–Whitney *U* test. *P* values <0.05 were considered statistically significant.

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.

Funding

This work was supported by Wellcome Trust and BBSRC. X-Q Wu sponsored by a KC Wong PhD studentship.

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Received 25 May 2010
First decision 25 June 2010
Revised manuscript received 1 November 2010
Accepted 17 November 2010