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Transgenerational transmission of anxiety induced by neonatal exposure to lipopolysaccharide: Implications for male and female germ lines

Adam K. Walker [a,b,*], Guy Hawkins [c], Luba Sominsky [a], Deborah M. Hodgson [a]

[a] *Laboratory of Neuroimmunology, School of Psychology, The University of Newcastle, Australia*
[b] *Laboratory of Integrative Immunophysiology, Integrative Immunology and Behavior Program, Departments of Animal Sciences and Medical Pathology, University of Illinois at Urbana-Champaign, IL 6180, USA*
[c] *Newcastle Cognition Laboratory, School of Psychology, The University of Newcastle, Australia*

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Summary Neonatal lipopolysaccharide (LPS) exposure increases anxiety-like behaviour and alters neuroendocrine responses to stress in adult rats. The current study assessed whether this anxiety-related phenotype observed in rats neonatally exposed to LPS is transferable to subsequent generations. Wistar rats were exposed to LPS (0.05 mg/kg, *Salmonella enteritidis*) or non-pyrogenic saline (equivolume) on postnatal days 3 and 5. In adulthood, animals were subjected to restraint and isolation stress or no stress, and subsequently evaluated for anxiety-like behaviours on the elevated plus maze, acoustic startle response, and holeboard apparatus. Blood was collected to examine corticosterone responses to stress and behavioural testing in adulthood. Animals from both treatment groups which exhibited the anxiety-like phenotype were bred with untreated partners. Maternal care of the second generation (F2) was monitored over the first week of life. In adulthood, the F2 generation underwent identical testing procedures as the parental (F1) generation. The F2 offspring of females exposed to LPS as neonates exhibited an anxiety-like phenotype in adulthood and a potentiated corticosterone response to stress ($p < .05$). F2 offspring of males exposed to LPS as neonates also exhibited an anxiety-like phenotype ($p < .05$), however, no differences in corticosterone responses were observed. To determine the impact of maternal care on the anxiety-like phenotype, a cross-fostering study was conducted in which offspring of LPS-treated females were fostered to saline-treated mothers and vice versa, which was found to reverse the behavioural and endocrine phenotypes of the F2 generation. These data indicate that a neonatally bacterially induced anxiety phenotype is transferable across generations in both sexes. Maternal care is the mediating mechanism along the maternal line. We suggest that transmission may be dependent upon heritable epigenetic phenomena for the paternal line. The implications of this study apply to potential neuroimmune pathways through which psychopathology may be transmitted along filial lines.

* Corresponding author at: 250 Edward R. Madigan Laboratory, 1201W. Gregory Dr., University of Illinois at Urbana-Champaign, Urbana, IL 61801-3873, USA. Tel.: +1 217 333 5142; fax: +1 217 244 5617.
E-mail address: akwalker@illinois.edu (A.K. Walker).

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1. Introduction

Psychopathology, including depression, schizophrenia and anxiety disorders, has been well-documented to transmit down filial lines (Baron et al., 1985; Cadoret et al., 1985; Holma et al., 2011; Merikangas and Swendsen, 1997) yet little clear genetic evidence has surfaced to explain this phenomenon. A range of perinatal and infant environmental factors have been reported to increase susceptibility to the onset of later life psychopathology, however, the underlying mechanisms remain largely elusive. It is now widely accepted that a combined effect of nature and nurture is likely to be responsible for this transgenerational phenomenon — a promising direction of which lies in the area of non-genomic modifications, or *epigenetics*.

Epigenetics refers to the modification of gene activation without altering the underlying DNA sequence. Chromatin remodelling and RNA transcripts have been presented as the primary avenues through which epigenetic modifications may arise, with limited evidence indicating that these changes to the epigenome may be heritable (reviewed in Meaney and Ferguson-Smith, 2010; Skinner and Guerrero-Bosagna, 2009). Certainly, environmental programming of biology in one generation has become widely accepted, whereby the perinatal environment acts as a crucial contributor to the long-term functioning of an individual. Environmental stressors during perinatal life, such as malnutrition, gestational stress, and maternal care are known to not only alter the developmental trajectory of physiological systems, but also increase the potential for later health complications (Felitti et al., 1998; Repetti et al., 2002).

In humans, a common event in foetal and neonatal life is exposure to bacteria. Perinatal exposure to bacteria permanently alters the development of critical physiological systems and programs alterations in neuroendocrine and behavioural responses. In particular, the early microbial environment influences physiological stress response systems, such as the hypothalamic—pituitary—adrenal (HPA) axis, and impacts behavioural outcomes (Bilbo et al., 2005a,b; Breivik et al., 2002; Hodgson et al., 2001; Meyer et al., 2006; Shanks et al., 1995, 2000; Shanks and Meaney, 1994; Walker et al., 2004, 2008, 2009, 2010, 2011). Recent data from this and other laboratories have indicated that exposure to bacteria early in life predisposes rodents to anxiety-like behaviour that persists into adulthood. We and others have employed animal models to investigate the underlying mechanisms of this phenomenon, and have observed robust and replicable increases in anxiety-like behaviour in rats following exposure to a bacterial mimetic, lipopolysaccharide (LPS), during the early postnatal period (Breivik et al., 2002; Shanks et al., 2000; Walker et al., 2004, 2008, 2009). Along with these changes in behaviour are associated perturbations to the HPA axis stress response (Hodgson et al., 2001; Shanks et al., 1995; Walker et al., 2009, 2010). A number of mechanisms have been proposed to account for these functional changes such as microglial priming and activation (Bilbo et al., 2005a, 2008; Bilbo and Schwarz, 2009; Sominsky et al., 2012), central cytokine regulation (Bilbo et al., 2005a; Walker et al., 2010; Kohman et al., 2008; Harre et al., 2008), glucocorticoid receptor density (Shanks et al., 1995) and epigenetic modifications.

As a step towards understanding the epigenetic inheritance of anxiety, we examined the potential for transgenerational inheritance of an anxiety-like phenotype following neonatal LPS administration to the F1 generation. Neonatal LPS exposure was employed to induce the known anxiety-like phenotype in the F1 generation (previously published in Walker et al., 2004, 2008, 2009). LPS and saline-treated males were then bred with untreated partners and the behavioural and neuroendocrine phenotypes of the F2 generation were examined. Cross-fostering was conducted to determine the relative influence of maternal care on the behavioural and endocrine profile of the F2 offspring. Identical anxiety-related variables were assessed for each generation using behavioural testing on the elevated plus maze (EPM), holeboard apparatus, and acoustic startle response (ASR). Schematics of the experimental design are provided in Fig. 1A and B.

2. Methods

2.1. Ethics statement

All experimentation occurred in accordance with the 2004 NH&MRC Australian Code of Practice for the care and use of animals for scientific practice. All efforts to reduce animal suffering, the numbers of animals used, and to utilise alternatives to in vivo techniques, if available, were made. This study was approved by The University of Newcastle Animal Care and Ethics Committee (approval number: ACEC 901).

2.2. F1 generation animals and neonatal treatment

Twenty experimentally naïve female Wistar rats obtained from the University of Newcastle animal house were bred in the University of Newcastle Psychology vivarium resulting in a total of 163 (79 male, 84 female) offspring, which were used in this experiment. At birth (postnatal day [PND] 1), whole litters were randomly assigned to either LPS or saline conditions. On PND 3 and PND 5, animals were briefly removed from their home cages, weighed, and administered either 0.05 mg/kg LPS (*Salmonella enterica*, serotype enteritidis; Sigma—Aldrich Chemical Co., USA) or an equivolume of non-pyrogenic 0.9% saline (Livingstone International, Australia) intraperitoneally. To determine that neonatal LPS administration was effective in activating the neonatal endocrine system, trunk blood was collected from a subgroup of animals (39 males and 44 females) deriving from 6 litters, on PNDs 3 and 5 following neonatal drug administration for assessment of plasma corticosterone concentrations. The remaining litters were left with their dams until weaning (PND 22) when they were separated into same-sex pair housing (41.5 cm × 28.0 cm × 22.0 cm cages; Mascot Wire Works, Sydney). Animals were left undisturbed from weaning until behavioural testing in adulthood (PND 85) except for weekly weights and observation. Housing conditions were identical to those previously reported (Walker et al., 2009).

2.3. Adult testing

2.3.1. Estrous cycle in females

Estrous cyclicity in females was monitored in adulthood using a Rat Vaginal Impedance Checker (Muromachi Kikai, Tokyo, Osaka) according to the manufacturer's instructions and is

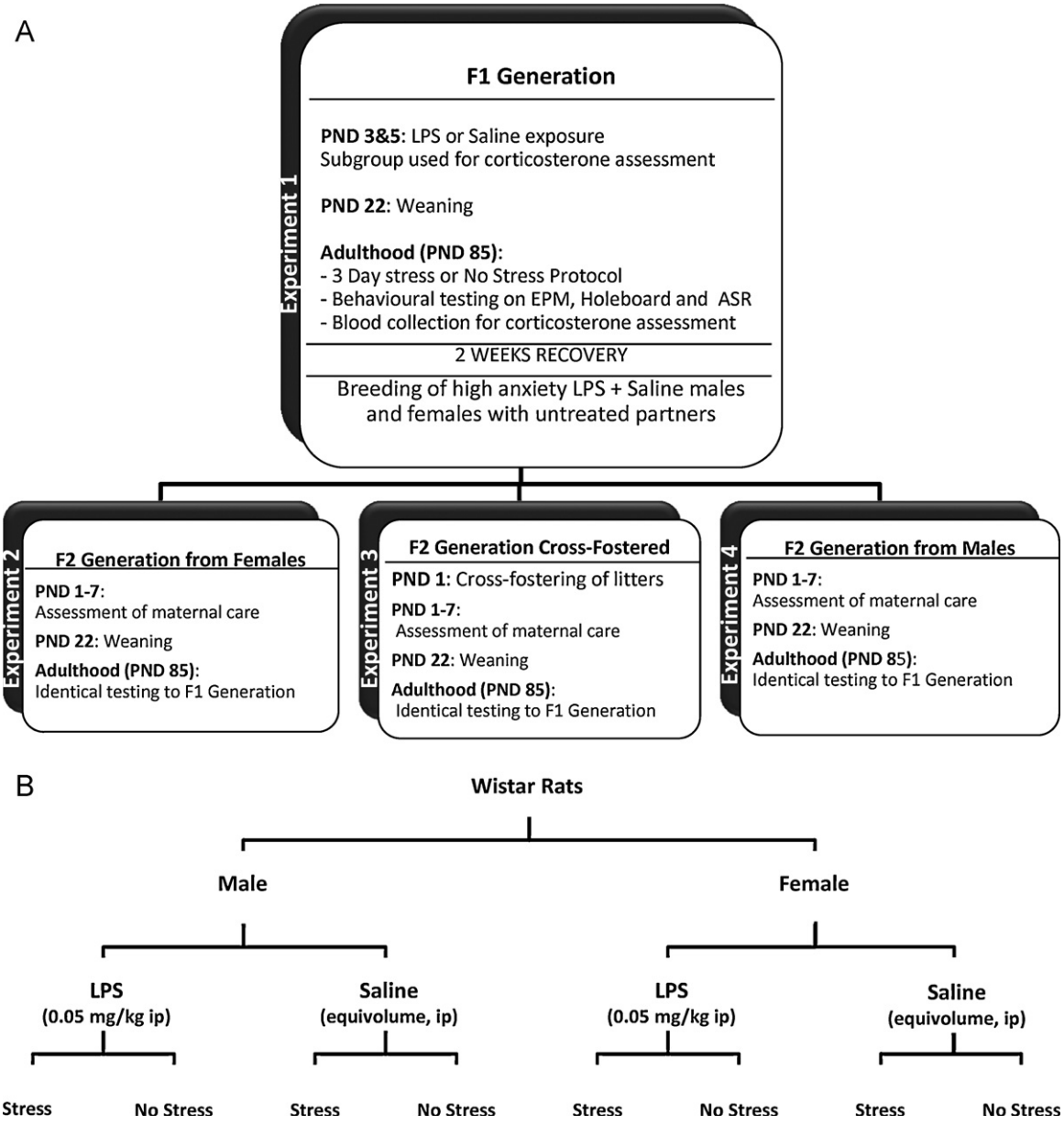

Figure 1 (A) A schematic diagram of experimental procedures conducted for each generation. (B) A schematic diagram outlining the various treatment groups employed for the F1 generation. Group allocations are applicable for all F2 cohorts except that LPS and saline groups refer to the treatment conditions of the parents.

previously described (Walker et al., 2011). Females were tested in the diestrous phase to control for natural corticosterone variation within the estrous cycle.

2.3.2. Stress protocol

Consistent with previous experiments in our laboratory, animals were randomly allocated into either a 3-day restraint and isolation stress, or no stress handled control condition described in (Walker et al., 2009) on PND 85. Hence, 4 groups for each sex existed: (1) animals exposed to LPS on PNDs 3 and 5 and stress in adulthood (LPS/Stress), (2) animals exposed to LPS on PNDs 3 and 5 and no stress in adulthood (LPS/noStress), (3) animals exposed to saline on PNDs 3 and 5 and stress in adulthood (Saline/Stress), and (4) animals exposed to saline on PNDs 3 and 5 and no stress in adulthood (Saline/noStress). Animals allocated to the stress condition underwent three consecutive days of acute stress exposure following recovery of baseline ASR testing. The stress protocol consisted of 30 min of restraint stress on the first and second day, and 30 min of isolation housing on the third day. The restraint apparatus was constructed using soft wire mesh (25.0 cm $\times$ 20.0 cm) folded around the rat and secured using butterfly clips to restrict ambulatory movement. Animals in the no stress condition remained undisturbed before behavioural testing. Behavioural testing commenced on the day following isolation, or at the equivalent time point for animals in the no stress condition. Animals underwent EPM, holeboard and ASR testing consecutively.

2.3.3. Elevated plus maze

The EPM, made of black plywood materials, consisted of two open and two enclosed arms (45.0 cm length $\times$ 10.0 cm

width) with a central square (10.0 cm × 10.0 cm), and was elevated to a height of 55.0 cm above the ground. Animals were placed in the centre square facing a closed arm (counterbalanced between animals) and allowed to explore for 5 min. Anxiety-related variables assessed included the percentage of time spent in the open arms and the number of closed and open arm entries, as well as risk assessment. Distance and activity measures were recorded for indications of locomotor activity and freezing.

2.3.4. Holeboard apparatus

The holeboard was a square arena (60.0 cm sides) enclosed by a continuous wall (40.0 cm high). The holeboard contained 4 holes (4.0 cm diameter) in the floor such that each hole was positioned at the corners of a 30.0 cm central square on the floor. A solid base was situated 5.0 cm underneath the holeboard. The apparatus was constructed of plywood materials and painted matt black, with behaviour recorded using an infrared camera mounted directly above the apparatus. Exploratory head dips, time spent in the centre square, distance travelled and activity were recorded. Animals were placed in the centre square and allowed 5 min to explore.

2.3.5. Acoustic startle response

Peak responses to startle noise were assessed using the ASR test. Animals were placed in a clear plexiglass cylinder (10.0 cm diameter × 30.0 cm length), which sat upon a spring platform with an accelerometer (Model # 353B51; PCB Piezotronics, NY, USA) attached. Acoustic stimuli consisted of 150 startle bursts of 100 dB white noise, 40 ms duration, with 0 ms rise/fall time. Inter-stimulus intervals of 6, 9 or 12 s were randomly sequenced (3/5 of the inter-stimulus intervals were 9 s, 1/5 were 6 s and 1/5 were 12 s). Peak startle amplitude in the 200 ms immediately following the first 10 and last 10 bursts were averaged to form the mean peak startle amplitude for each acoustic startle exposure. Animals were presented with a baseline session (PND 85) and allowed 1-week recovery. Subsequently, animals underwent the 3-day stress or no stress paradigm and then startle responsiveness was examined across multiple days: the first session immediately followed the EPM and holeboard exposure (session 1), followed by another session 2 days after the first (session 2).

2.3.6. Blood sampling in adulthood

Three blood samples were collected for plasma corticosterone analysis on the first day of restraint stress to assess HPA axis activity. Blood was collected in adulthood via the saphenous vein at baseline (0 min), immediately following restraint (30 min), and 30 min thereafter (60 min). Blood samples were collected at equivalent time points for animals in the no stress condition. Corticosterone responses following exposure to a novel environment were also assessed immediately following testing on the EPM and holeboard apparatus.

2.4. F2 generation animals and treatment

2.4.1. Mating of F1 females

16 Females (8 LPS, 8 saline) exhibiting the strongest anxiety-like behaviour from the LPS/Stress and SAL/Stress conditions were identified from aggregated scores averaged over key parameters: open arm entries, open arm time, risk assessment, head dips, thigmotaxis and peak startle amplitude at ASR session 1. These females were selected to produce the F2 generations of each treatment group and for maternal behaviour assessment. Following a minimum of 2 weeks recovery from their final ASR session, these females were pair-housed with another from the same neonatal treatment condition and an experimentally naïve male stud obtained from the University of Newcastle Central Animal House. After 2 weeks, the male stud was removed and the females remained pair housed for 1 week. The females were then housed individually in the maternal behaviour monitoring cages where they remained undisturbed until cessation of maternal behaviour monitoring. Breeding of these females resulted in a total of 136 animals (35 males and 30 females from LPS-treated females, and 39 males and 32 females from saline-treated females), which were used to assess anxiety-like behaviour of F2 offspring derived from the female treatment line. F2 offspring underwent no treatment intervention, thus the neonatal treatment condition of the mother is the distinguishing factor between groups (i.e., whether the mother received LPS [mLPS] or saline [mSaline] as a neonate). Importantly, we controlled for anxiety-like behaviour differences and adult treatment conditions by only selecting LPS and saline-treated females exposed to stress in adulthood with the highest indices of anxiety-like behaviour. A multivariate analysis of variance (MANOVA) was used to confirm no significant differences in regards to anxiety-related behaviours between treatment conditions of the dams.

2.4.2. Mating of F1 females for cross-fostering

Neonatal LPS exposure altered the maternal care of F2 offspring (see Section 3). Therefore, we investigated the strength of this factor in contributing to the long-term behaviour of F2 animals by cross-fostering the offspring of LPS-treated mothers with saline-treated mothers and vice versa. F1 generation females were again used to breed with experimentally naïve male studs in an identical fashion as described previously but planned so as to increase the likelihood of saline-treated and LPS-treated dams birthing on identical days. If both a saline and an LPS-treated dam gave birth on the same day (and only if within 2 h of one another), whole litters were cross-fostered. Foster dams were removed from their maternal care cages, and the offspring immersed in the bedding of the foster dam to mask the scent of the birth mother. This protocol was successful as no rejection of foster litters was observed. Breeding resulted in a total of 105 rats — 32 males and 31 females were born to saline-treated mothers and fostered to LPS-treated dams (denoted as fostered LPS animals [fLPS]), and 18 males and 24 females were born to LPS-treated mothers and fostered to saline-treated dams (denoted as fostered saline animals [fSAL]).

2.4.3. Mating of F1 males

As with F1 females, F1 males that exhibited the strongest anxiety-like behaviour from the LPS/Stress ($n = 8$) and SAL/Stress conditions ($n = 8$) were selected for breeding with experimentally naïve dams obtained from the University of Newcastle Central Animal House. Following a minimum of 2 weeks recovery from their final ASR session, these males were single-housed with the female for 2 weeks, after which

the females were housed individually in the maternal behaviour monitoring cages where they remained undisturbed until cessation of maternal behaviour monitoring. Breeding resulted in a total of 33 animals (17 males and 16 females) from LPS-treated males and 44 animals (26 males and 18 females) from saline-treated males, which were used to assess anxiety-like behaviour of second-generation offspring derived from the male treatment line. Again, F2 offspring underwent no treatment intervention, thus the treatment condition from the paternal line distinguishes the F2 groups (i.e., whether the father received LPS [pLPS] or saline [pSaline] as a neonate). Mean litter size and male to female ratios between pLPS and pSaline litters were not significantly different.

2.5. Assessment of maternal behaviour

Given that neonatal LPS exposure has been shown to produce long-term changes in behaviour (Breivik et al., 2002; Shanks et al., 2000; Walker et al., 2004, 2009), maternal care of second generation offspring was observed to determine whether this might be a contributing factor in any behavioural changes observed in the F2 generation. Maternal care cages (40.0 cm × 32.0 cm × 17.0 cm) consisted of a steel base, sides and rear. A clear plexiglass front and lid were used to allow clear visual access for filming and observing maternal care behaviour. Paper confetti was provided for nest building. Maternal care was recorded with a closed circuit four channel digital video recorder (DVR4-1000 Swann Security, Melbourne, Australia). The behavioural parameters detailed in Table 1 were monitored over six 60 min observation periods on PNDs 1, 3, 5, and 7 at 0800, 1100, 1400, 1700, 2000 and 0500 h. Maternal behaviour data was transformed into a percentage of all recorded behaviours for each day. Data were then averaged across PNDs 1, 3, 5, and 7 to form an aggregate score for each maternal behaviour. The behaviours and protocol used for assessment of maternal care have been previously employed (Liu et al., 1997; Bosch et al., 2007; Caldji et al., 1998; Champagne and Meaney, 2007; Champagne et al., 2001; Myers et al., 1989).

2.6. Assessment of F2 generation behaviour and corticosterone responsivity

Second-generation animals were left completely undisturbed with the dam in the maternal care cages to minimise any effects intervention may have on maternal care of offspring. Animals were weaned on PND 22, and all subsequent protocols, including stress versus no stress exposure, behavioural testing and blood sampling occurred identically to that of the F1 generation. Each group within each F2 generation cohort contained 8 rats or greater.

2.7. Assessment of F2 generation corticosterone concentrations during neonatal life

A subgroup of F2 generation rats from both maternal and paternal lines ($n \geq 11$ for all groups) was euthanised during neonatal life in order to assess circulating plasma corticosterone concentrations. Note that whole litters were sacrificed so as not to interfere with the maternal care status of litters allocated for testing in adulthood. Whole litters were briefly removed from their home cages on PND 3 and PND 5, weighed and trunk blood was collected via rapid decapitation and collected into EDTA-coated tubes. Neonatal weights and blood samples were not collected for the animals in the cross-fostering study so as not to further disturb fostered litters and increase the chance of litter rejection.

2.8. Radioimmunoassay procedures

All blood samples were centrifuged at 1000 × *g* for 20 min at 4 °C, and plasma stored at −20 °C until assayed. Plasma corticosterone concentrations were assessed using a rat corticosterone ^{125}I radioimmunoassay kit (MP Biomedicals, USA). The recovery of free corticosterone is 100%, with an inter- and intra-assay variability of 4.4% and 6.5%, respectively.

2.9. Data analysis

Data were analysed using the Statistical Package for the Social Sciences for Windows, Version 17. Analyses of variance (ANOVA) were conducted for all analyses. We controlled for potential litter effects by nesting litter into treatment for each ANOVA model. Planned comparisons were performed when interactions were significant using Bonferroni's α correction to 0.05 and *t* test analyses corrected to 0.05 to minimise family-wise error. Data were analysed in the same manner for all experiments.

Table 1 Maternal behaviours selected for analysis.

Behaviour	Description
Licking-grooming	When the dam licked or groomed any pup
Arched-back nursing	Dam nursing with limbs outstretched supporting an arched back over the pups
Licking-grooming while arched-back nursing (LG-ABN)	Dam nursing with limbs outstretched supporting an arched back over the pups while licking or grooming any pup
Blanket nursing	Dam nursing by lying flat over her pups
Passive nursing	Dam lying on her side or back as the pups nurse
Nest building	Dam tending to her nest, either by moving materials or carrying pups
Non pup-directed behaviours	Any behaviour not directed towards pups or the nest (eating, drinking, self-grooming, exploring or resting off pups)

3. Results

3.1. Impact of neonatal LPS exposure on F1 and F2 anxiety-like behaviour in adulthood

3.1.1. Resistance to restraint

The level of activity during restraint and isolation was assessed using a computer automated behavioural tracking system (Motion Mensura Pty Ltd, Australia; described in Walker et al., 2009). Data for the time spent resisting restraint on day 1 and day 2 of stress exposure for all generational cohorts can be found in Table 2. As measured by the tracking system's computational measures of pixel density and movement, LPS-treated animals (F1) spent significantly less time resisting restraint on day 1 and day 2 of stress exposure compared to saline controls, $F(8,27) = 2.35$, $p < .05$ and $F(1,27) = 2.68$, $p < .05$, respectively. No differences in activity or distance travelled were observed during isolation for the F1 generation.

F2 offspring of LPS-treated mothers spent significantly less time resisting the restraint on day 1 and day 2 of stress exposure compared to saline controls, $F(4,36) = 6.50$, $p < .001$ and $F(4,35) = 3.17$, $p < .05$, respectively. Males exhibited significantly reduced activity and distance travelled during isolation compared to females, $F(1,39) = 4.93$, $p < .05$ and $F(1,35) = 2.17$, $p < .01$, respectively (data not shown).

No differences were observed in regards to activity in restraint or distance travelled in isolation for neither the offspring of LPS-treated or saline-treated males nor the cross-fostered cohort.

3.1.2. Behaviour in the elevated plus maze

3.1.2.1. F1 generation. A significant interaction between neonatal treatment and sex was observed for the percentage of open arm entries and time spent in the open arms, $F(5, 43) = 3.28$, $p < .05$ and $F(5, 41) = 3.95$, $p < .01$, respectively for the F1 generation. Planned comparisons revealed that LPS-treated males spent significantly less time in the open arms ($M = 51.62\%$, $SEM = 4.03$) compared to saline-treated males ($M = 63.37\%$, $SEM = 3.76$), $t_{.05}(32) = 2.37$, $p < .05$. However, no differences were observed between treatment groups for females. This effect was similarly observed for open arm entries, whereby LPS-treated males exhibited fewer entries ($M = 48.89\%$, $SEM = 3.23$) compared to their saline counterparts ($M = 53.87\%$, $SEM = 3.13$), $t_{.05}(34) = 3.03$, $p < .05$. Again, no differences existed between females. Trends indicated that LPS-treated animals engaged in more risk-assessment behaviour compared to saline-treated controls, which approached significance ($p = .1$), Fig. 2A.

Significant effects of adult treatment on EPM behaviour were also observed. Animals exposed to stress in adulthood displayed a significantly reduced percentage of time spent in the open arms ($M = 52.87\%$, $SEM = 2.75$) compared to no stress controls ($M = 64.52\%$, $SEM = 2.55$), $F(1, 41) = 6.65$, $p < .05$. Trend analysis revealed animals exposed to stress in adulthood made fewer open arm entries ($M = 51.25\%$, $SEM = 2.38$) compared to controls ($M = 58.37\%$, $SEM = 2.13$) which approached significance ($p = .1$). In addition, animals exposed to stress in adulthood engaged in significantly more counts of risk assessment behaviour ($M = 4.17$, $SEM = 0.46$) than animals exposed to no stress in adulthood ($M = 2.34$, $SEM = 0.41$), $F(1, 43) = 6.50$, $p < .05$.

3.1.2.2. F2 generation from males. Paternal neonatal LPS exposure significantly affected both time spent in the open arms and risk assessment behaviour on the EPM, $F(6,48) = 8.18$, $p < .001$ and $F(5,43) = 2.55$, $p < .05$, respectively. F2 offspring of males exposed to LPS demonstrated a significantly lower percentage of time spent in the open arms ($M = 44.24\%$, $SEM = 3.33$) compared to F2 offspring of males exposed to saline ($M = 47.16\%$, $SEM = 2.89$). F2 offspring of males exposed to LPS also exhibited significantly more risk assessment behaviour ($M = 7.45$, $SEM = 0.45$) compared to controls ($M = 3.54$, $SEM = 0.39$), Fig. 2B. No differences in open arm entries were observed.

3.1.2.3. F2 generation from females. A significant effect of maternal treatment was observed for F2 offspring in regards to the proportion of time spent in the open arms of the EPM. Offspring of neonatally LPS-treated mothers spent significantly less time in the open arms ($M = 53.38\%$, $SEM = 3.42$) compared to offspring of neonatally saline-treated mothers ($M = 55.19\%$, $SEM = 3.32$) in adulthood, $F(4,70) = 2.45$, $p = .05$. No differences in the number of open arm entries were observed. Maternal treatment also affected risk assessment behaviour in F2 offspring. Fig. 2C shows that the offspring of LPS-treated mothers engaged in significantly more risk assessment behaviour than offspring of saline-treated mothers, $F(6,61) = 4.54$, $p = .001$.

3.1.2.4. F2 generation cross-fostered. A significant sex $\times$ stress in adulthood $\times$ cross-fostering effect was observed for open arm entries ($F(5,52) = 2.55$, $p < .05$) and risk assessment behaviour ($F(5,54) = 4.71$, $p = .001$). Planned comparisons revealed that F2 males born to saline-treated mothers but fostered to LPS-treated dams made significantly fewer open arm entries ($M = 44.47\%$, $SEM = 3.25$) compared to F2 males born to LPS-treated mothers but fostered to saline-treated dams when exposed to stress in adulthood ($M = 57.82\%$, $SEM = 3.31$), $t_{.05}(20) = 3.13$, $p < .05$. This effect was similarly observed for time engaged in risk assessment

Table 2 Mean time (%) $\pm$ *SEM* spent resisting restraint.

	Restraint day 1	Restraint day 2
F1 generation		
LPS	43.91% (4.0)*	38.96% (3.90)*
Saline	61.91% (3.95)	56.44% (3.85)
F2 generation from males		
pLPS	22.44% (5.99)	16.27% (5.46)
pSaline	26.98% (6.90)	27.48% (8.02)
F2 generation from females		
mLPS	40.18% (6.60)*	29.50% (6.60)*
mSaline	59.4% (6.26)	41.45% (6.33)
F2 generation cross-fostered		
fLPS	9.09% (1.88)	8.36% (1.89)
fSaline	14.23% (3.52)	12.15% (3.33)

* $p < .05$.

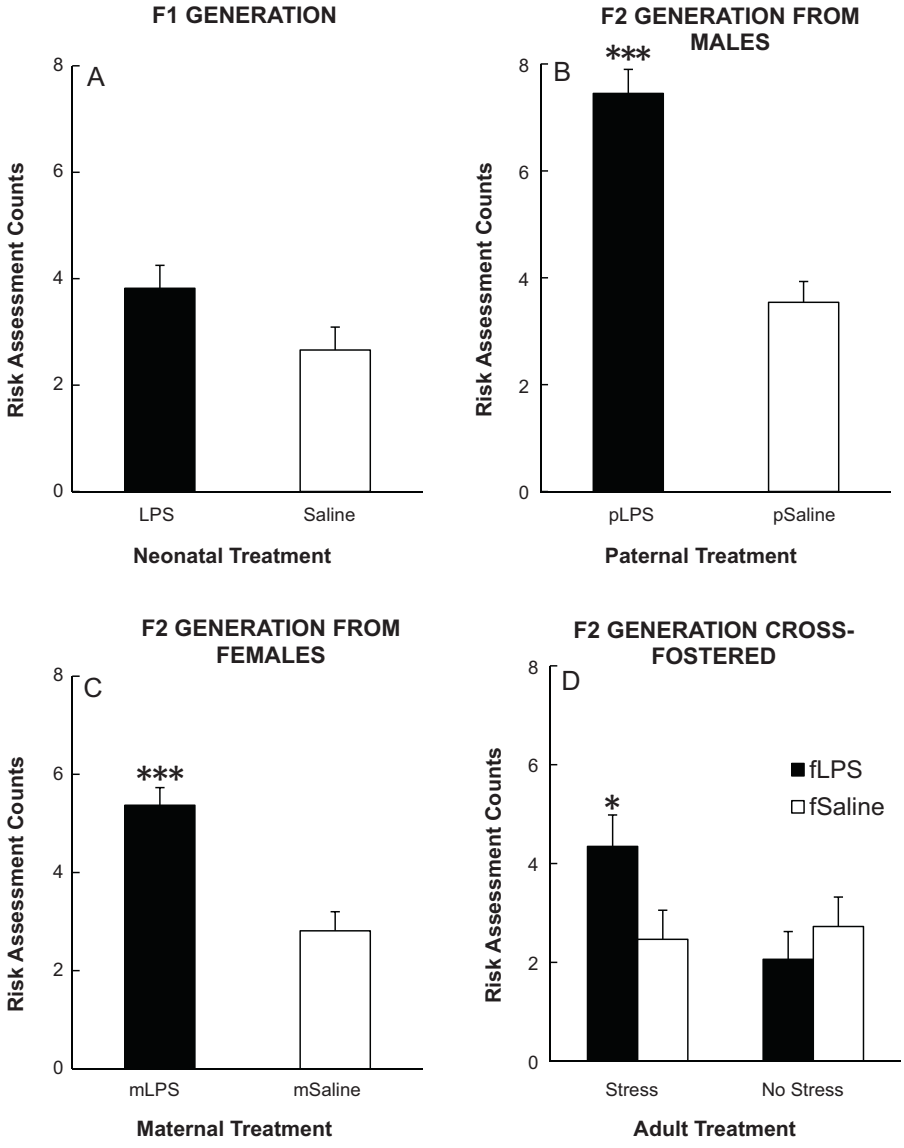

Figure 2 Mean number of risk-assessment behaviours (±*SEM*) for each generation in the elevated plus maze as assessed by nested ANOVA. Filled bars represent neonatally challenged LPS animals for the F1 generation, F2 generation offspring of males (pLPS) and females (mLPS) neonatally exposed to LPS, and males born to mothers exposed to neonatal saline treatment but fostered to LPS-treated mothers (fLPS) exposed to stress and no stress in adulthood. Hollow bars represent neonatally challenged saline animals for the F1 generation, F2 generation offspring of males (pSaline) and females (mSaline) neonatally exposed to saline, and males born to mothers exposed to neonatal LPS treatment but fostered to saline-treated mothers (fSaline) exposed to stress and no stress in adulthood; $n \geq 9$ for all groups, *$p < .05$ and ***$p < .001$.

behaviour ($t_{.05}(19) = 2.33$, $p < .05$), Fig. 2D. No differences were observed for time spent in the open arms.

3.1.3. Behaviour in the holeboard apparatus

3.1.3.1. F1 generation. Significant main effects of neonatal treatment and adult treatment were observed in regards to exploratory head dips in the holeboard apparatus. Fig. 3A shows that LPS-treated animals exhibited significantly fewer head dips than saline-treated animals, $F(10, 43) = 3.36$, $p < .01$. Animals exposed to stress in adulthood exhibited significantly fewer head dips ($M = 3.94$, $SEM = 0.39$) than no stress controls ($M = 5.26$, $SEM = 0.39$), $F(1, 43) = 7.66$, $p < .01$. No significant differences were observed in regards to time spent in the centre square.

3.1.3.2. F2 generation from males. Significant main effects of paternal neonatal treatment and sex were observed for exploratory head dips in the holeboard apparatus. Fig. 3B shows that F2 offspring of males exposed to LPS demonstrated significantly fewer exploratory head dips compared to F2 offspring of males exposed to saline, $F(5, 50) = 2.66$, $p < .05$. Males also demonstrated fewer exploratory head dips ($M = 4.18$, $SEM = 0.36$) compared to females ($M = 5.37$, $SEM = 0.39$), $F(1, 50) = 4.83$, $p < .05$.

Main effects of paternal neonatal treatment, adult treatment, and sex were observed for the percentage of time spent in the centre square. F2 offspring of males exposed to LPS spent significantly less time in the centre square ($M = 4.08\%$, $SEM = 0.82$) compared to F2 offspring of males

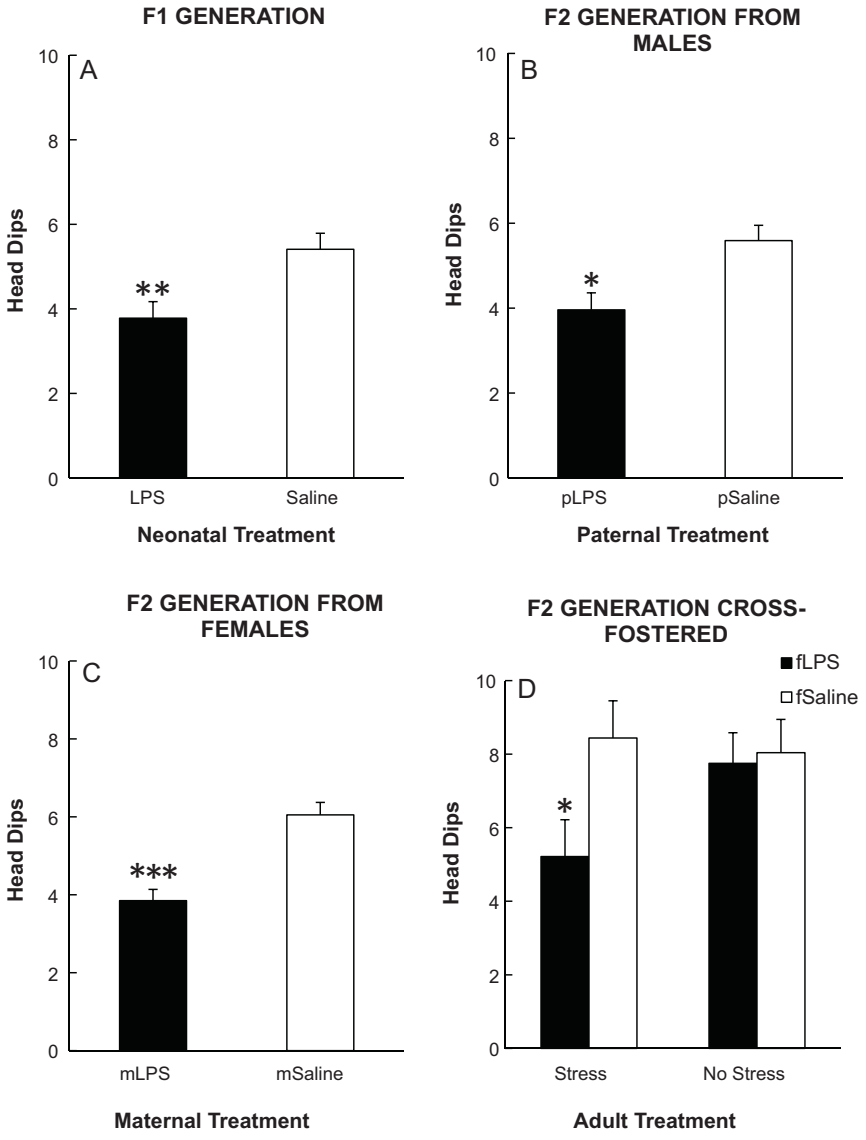

Figure 3 Mean number of exploratory head dips (±*SEM*) for each generation in the holeboard apparatus as assessed by nested ANOVA. Filled bars represent neonatally challenged LPS animals for the F1 generation, F2 generation offspring of males (pLPS) and females (mLPS) neonatally exposed to LPS, and males born to mothers exposed to neonatal saline treatment but fostered to LPS-treated mothers (fLPS) exposed to stress and no stress in adulthood. Hollow bars represent neonatally challenged saline animals for the F1 generation, F2 generation offspring of males (pSaline) and females (mSaline) neonatally exposed to saline, and females born to mothers exposed to neonatal LPS treatment but fostered to saline-treated mothers (fSaline) exposed to stress and no stress in adulthood; $n \geq 10$ for all groups, *$p < .05$, **$p < .01$, and ***$p < .001$.

exposed to saline ($M = 8.19\%$, $SEM = 0.78$), $F(5, 45) = 6.30$, $p < .001$. Animals exposed to stress in adulthood spent significantly less time in the centre square ($M = 5.01\%$, $SEM = 0.86$) compared to no stress controls ($M = 7.26\%$, $SEM = 0.74$), $F(1, 45) = 3.93$, $p = .05$. Males spent significantly less time in the centre square ($M = 4.43\%$, $SEM = 0.78$) compared to females ($M = 7.84\%$, $SEM = 0.83$), $F(1, 45) = 9.04$, $p < .01$. No significant interactions were observed.

3.1.3.3. F2 generation from females. Again, the neonatal treatment of the mother affected the behaviour of F2 offspring on the holeboard apparatus. The offspring of LPS-treated mothers displayed significantly fewer exploratory head dips compared to the offspring of saline-treated mothers, $F(6,62) = 9.70$, $p = .001$ (Fig. 3C). These animals also spent less time in the centre square ($M = 3.26\%$, $SEM = 0.44$) compared to offspring of saline-treated mothers ($M = 3.51\%$, $SEM = 0.48$), $F(6,64) = 2.38$, $p = .05$.

A significant adult treatment × sex interaction was observed for head dips in the holeboard apparatus, whereby males exposed to stress in adulthood displayed significantly more head dips compared to their ''no stress'' counterparts ($F(1,62) = 4.56$, $p < .05$), but no difference existed between females (data not shown). A main effect of sex was also observed for time spent in the centre square with males spending less time in the centre square compared to females, $F(1,64) = 5.69$, $p < .05$ (data not shown).

3.1.3.4. F2 generation cross-fostered. A significant sex - $\times$ stress in adulthood $\times$ cross-fostering effect was observed for the number of head dips, $F(6,59) = 7.86$, $p < .05$. Planned comparisons revealed that F2 females born to saline-treated mothers but fostered to LPS-treated dams exhibited significantly fewer head dips compared to F2 females born to LPS-treated mothers but fostered to saline-treated dams when exposed to stress in adulthood, $t_{.05}(19) = 2.63$, $p < .05$ (Fig. 3D). There were no differences among males. No significant differences in time spent in the centre square were observed.

3.1.4. The acoustic startle response

3.1.4.1. F1 generation. Assessment of startle amplitude indicated a significant effect of neonatal treatment across ASR sessions, whereby LPS-treated males and females demonstrated significantly increased startle amplitudes compared to saline-treated controls across all three ASR exposures ($F(20, 62) = 1.89$, $p < .05$), Fig. 4A and B.

3.1.4.2. F2 generation from males. No significant differences in peak startle amplitudes were observed during any ASR session.

3.1.4.3. F2 generation from females. A significant maternal treatment $\times$ sex effect was observed for the peak startle amplitude across ASR sessions, $F(8,96) = 8.03$, $p < .001$. Planned comparisons revealed that F2 males born to neonatally saline-treated mothers displayed significantly higher ASR amplitudes at baseline ($t_{.05}(35) = 6.26$, $p < .05$) and at session 2 ($t_{.05}(35) = 2.95$, $p < .05$) compared to males born to neonatally LPS-treated mothers. F2 females born

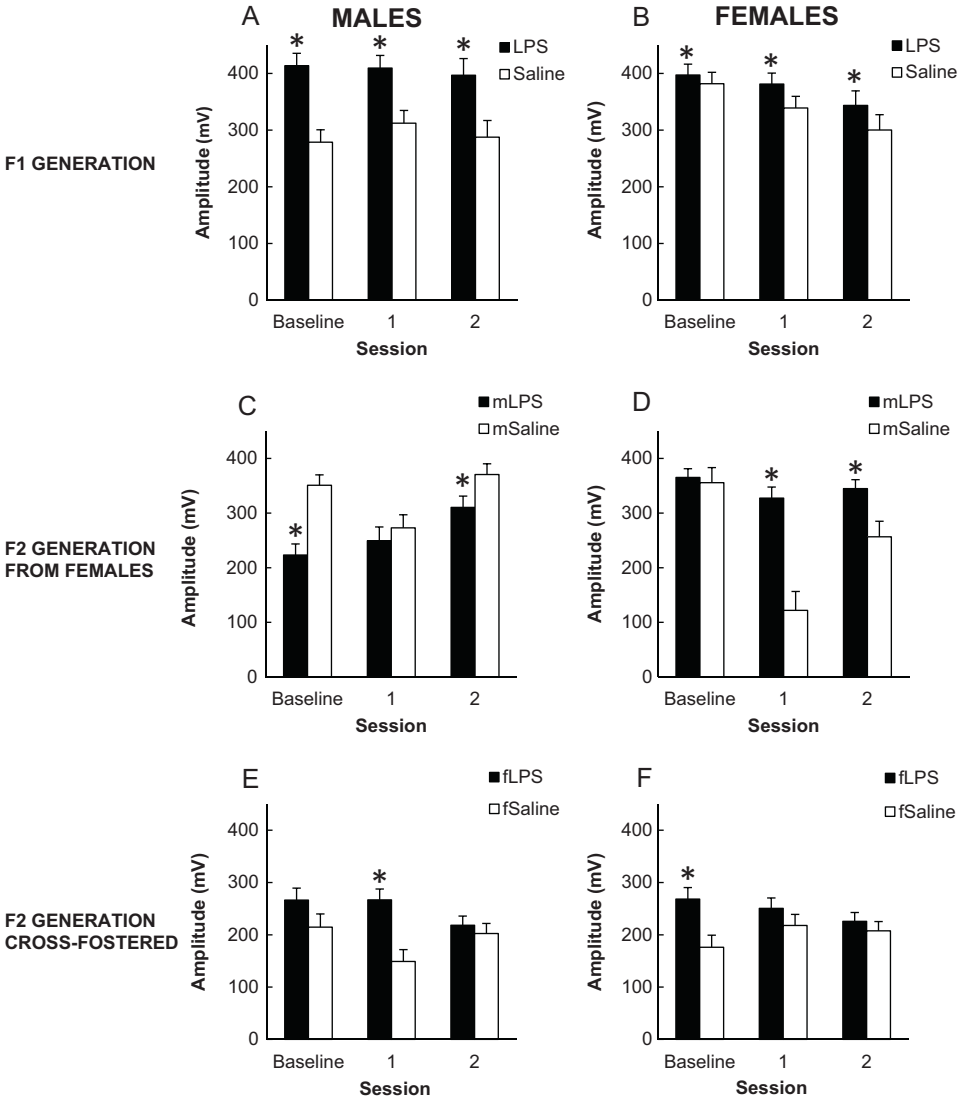

Figure 4 Mean peak acoustic startle response (ASR) amplitudes (mV; $\pm$*SEM*) at baseline, and ASR sessions 1 and 2 as assessed by nested ANOVA. Filled bars represent neonatally challenged LPS animals for the F1 generation, F2 generation offspring of males (pLPS) and females (mLPS) neonatally exposed to LPS, and offspring born to mothers exposed to neonatal saline treatment but fostered to LPS-treated mothers (fLPS). Hollow bars represent neonatally challenged saline animals for the F1 generation, F2 generation offspring of males (pSaline) and females (mSaline) neonatally exposed to saline, and offspring born to mothers exposed to neonatal LPS treatment but fostered to saline-treated mothers (fSaline); $n \geq 9$ for all groups, $^*p < .05$.

to neonatally saline-treated mothers exhibited higher amplitudes at session 1 ($t_{.05}(29) = 9.17$, $p < .05$) and session 2 ($t_{.05}(29) = 6.92$, $p < .05$) compared to females born to neonatally LPS-treated mothers, Fig. 4C and D.

3.1.4.4. F2 generation cross-fostered. A significant sex × stress in adulthood × cross-fostering effect was observed for the mean startle amplitude across time, $F(8,98) = 2.62$, $p < .05$. ASR effects are depicted in Fig. 4E and F. Planned comparisons revealed no differences between groups that received stress in adulthood at any time point for either males or females due to clear ceiling effects created by the adult stress protocol (data not shown). However, F2 males born to saline-treated mothers but fostered to LPS-treated dams exhibited significantly higher startle amplitudes compared to F2 males born to LPS-treated mothers but fostered to saline-treated dams at session 1, $t_{.05}(17) = 4.97$, $p < .05$. Similarly, F2 females born to saline-treated mothers but fostered to LPS-treated dams exhibited significantly higher peak startle amplitudes compared to F2 females born to LPS-treated mothers but fostered to saline-treated dams at baseline, $t_{.05}(19) = 4.05$, $p < .05$.

3.2. Assessment of maternal care

3.2.1. Impact of neonatal LPS exposure on maternal behaviour

Litter size and male-to-female ratio of the F2 litter did not significantly differ between LPS-treated and saline-treated dams' litters. While there was no significant difference between LPS and saline-treated dams in regards to the total time spent nursing, LPS-treated dams spent significantly less time arched-back nursing (ABN) than dams treated with saline, $F(1, 13) = 14.24$, $p < .01$, as shown in Fig. 5A. Furthermore, LPS-treated dams engaged in significantly less LG-ABN ($F(1, 13) = 20.36$, $p < .001$) and significantly more blanket nursing ($F(1, 12) = 6.26$, $p < .05$) compared to saline-treated dams after accounting for the significant covariate of litter size ($p < .05$). No significant difference emerged for time spent passive nursing. Fig. 5B shows the results of pup-directed behaviour, where LPS-treated dams spent significantly less time licking and grooming their pups and tending to their nest compared to saline-treated dams, $F(1, 13) = 53.27$, $p < .001$ and $F(1, 13) = 6.39$, $p < .05$, respectively. Furthermore, LPS-treated dams spent significantly more time engaged in off-nest behaviours (resting off pups, exploring, eating, drinking, self-grooming) than saline treated dams, $F(1, 13) = 21.95$, $p < .001$. No rejection was observed for cross-fostered litters.

For untreated females bred with LPS or saline-treated males, no differences were observed for any of the maternal care behaviours measured.

4. Neonatal corticosterone concentrations

4.1. F1 generation

Neonatal LPS injections resulted in a significant increase in corticosterone four hours following administration on PND 3 (LPS: $M = 6.88$ ng/ml, $SEM = 0.38$; SAL: $M = 5.25$ ng/ml, $SEM = 0.31$) and PND 5 (LPS: $M = 7.79$ ng/ml, $SEM = 0.36$; SAL: $M = 6.78$ ng/ml, $SEM = 0.34$), $F(5, 39) = 6.67$, $p < .01$ and $F(5, 38) = 4.83$, $p < .05$, respectively.

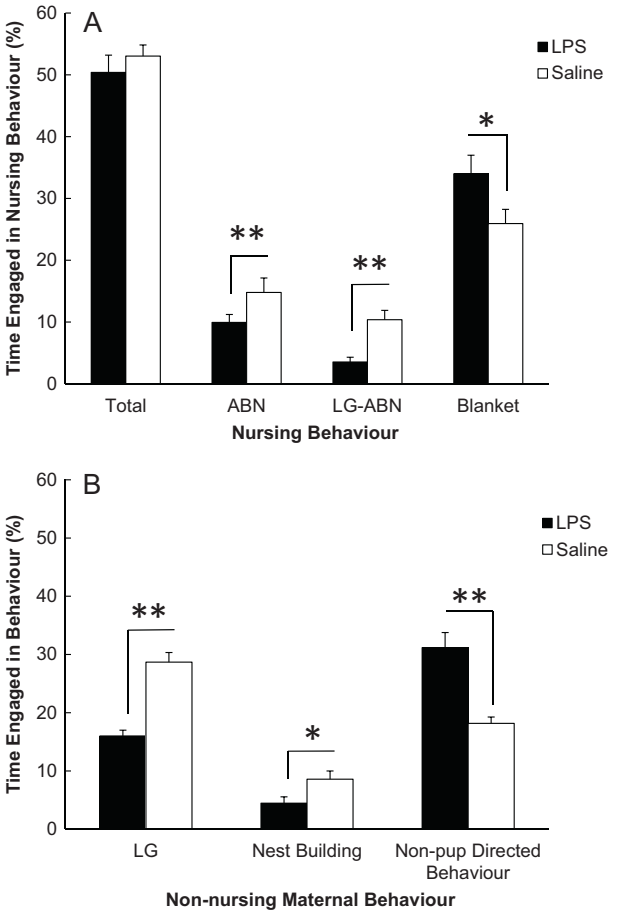

Figure 5 Effect of neonatal treatment on maternal care of F2 offspring. (A) Represents mean proportion of time (%; ±*SEM*) engaged in all nursing behaviours (Total), arched-back nursing (ABN), licking and grooming while arched-back nursing (LG-ABN) and blanket nursing (Blanket) as assessed by ANOVA. (B) Represents mean proportion of time (%; ±*SEM*) engaged in non-nursing maternal care behaviours such as licking and grooming (LG), nest building, and non-pup directed behaviour. Filled bars represent neonatally challenged LPS females and the hollow bars represent neonatally challenged saline females; $n = 8$ for all groups, $^*p < .05$ and $^{**}p < .01$.

4.2. F2 generation from males

No significant differences in plasma corticosterone were observed for F2 generation animals from the paternal line were observed on PND 3 or PND 5.

4.3. F2 generation from females

On PND 3, F2 offspring of mothers neonatally exposed to LPS had significantly higher plasma corticosterone concentrations ($M = 39.3$ ng/ml, $SEM = 2.51$) than F2 offspring of mothers neonatally exposed to saline ($M = 30.25$ ng/ml, $SEM = 2.32$), $F(1, 18) = 7.03$, $p < .05$. Males also exhibited significantly higher corticosterone concentrations ($M = 39.87$ ng/ml, $SEM = 2.51$) compared to females ($M = 30.49$ ng/ml,

SEM = 2.32), $F(1, 18) = 6.32$, $p < .05$. No differences were observed on PND 5.

5. Adult corticosterone responses to restraint stress

5.1. F1 generation

Assessment of corticosterone responses to the first day of restraint stress revealed an effect of stress in adulthood across time, $F(1.7, 90.16) = 18.26$, $p < .001$. Planned comparisons revealed no differences between groups at baseline, however, animals exposed to stress in adulthood exhibited significantly higher plasma corticosterone concentrations at 30 min and 60 min following baseline compared to no stress controls ($t_{.05}(82) = 9.99$, $p < .05$ and $t_{.05}(82) = 6.31$, $p < .05$) respectively, Notably, trends indicated that LPS-treated animals displayed higher corticosterone concentrations at each time point compared to saline controls when exposed to stress in adulthood (Fig. 6A and B), however, this did not reach significance due to the stringencies of statistical nesting. No differences were observed between LPS and saline-treated rats exposed to no stress in adulthood. A main effect of sex was also observed, whereby females exhibited significantly higher corticosterone concentrations (*M* = 436.57 ng/ml, *SEM* = 24.22) compared to males (*M* = 230.51 ng/ml, *SEM* = 23.95), $F(1, 53) = 23.06$, $p < .001$.

5.2. F2 generation from males

Significant effects of sex and adult treatment were observed in response to acute restraint stress in adulthood.

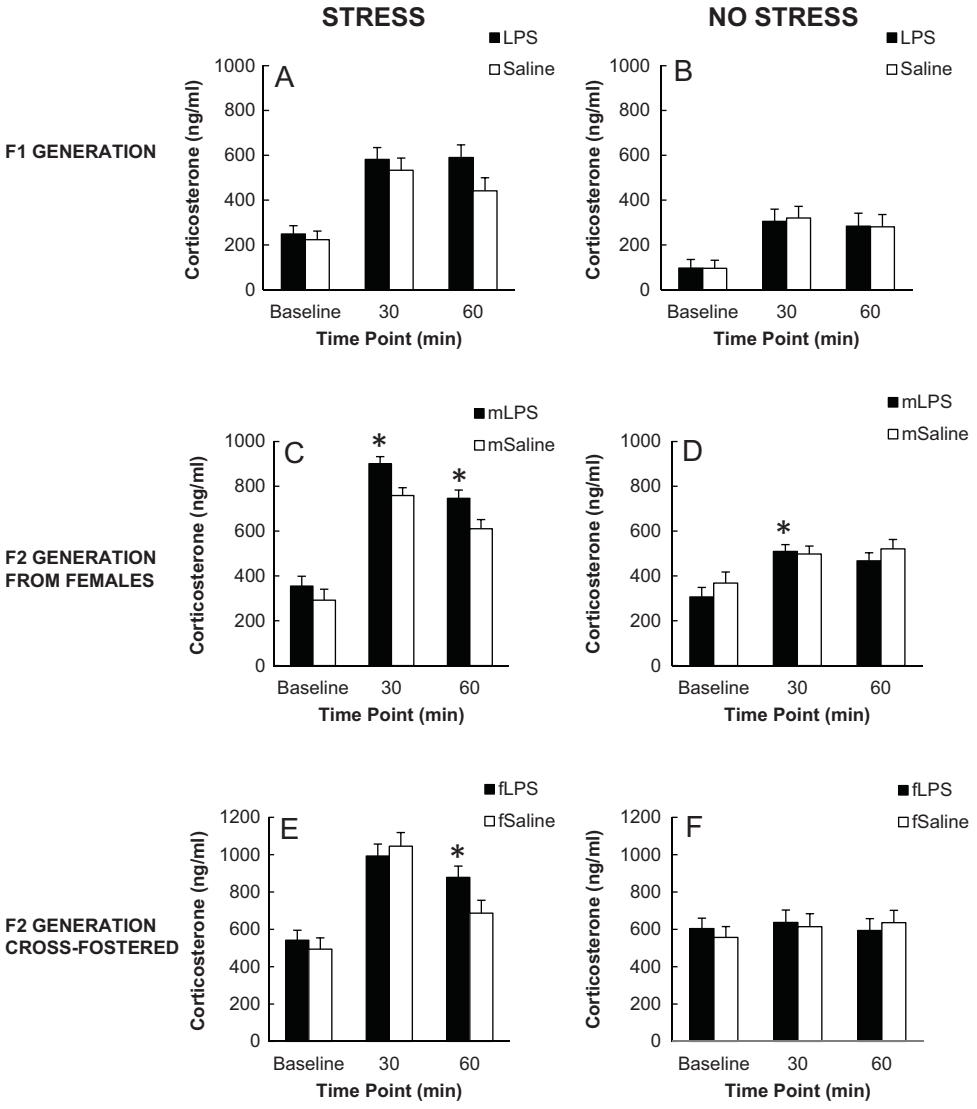

Figure 6 Mean plasma corticosterone responses to acute restraint stress or no stress in adulthood (ng/ml; ±*SEM*) as assessed by nested ANOVA. Filled bars represent neonatally challenged LPS animals for the F1 generation, F2 generation offspring of males (pLPS) and females (mLPS) neonatally exposed to LPS, and females born to mothers exposed to neonatal saline treatment but fostered to LPS-treated mothers (fLPS). Hollow bars represent neonatally challenged saline animals for the F1 generation, F2 generation offspring of males (pSaline) and females (mSaline) neonatally exposed to saline, and females born to mothers exposed to neonatal LPS treatment but fostered to saline-treated mothers (fSaline); $n \geq 11$ for all groups, $^*p < .05$.

Animals exposed to stress in adulthood exhibited significantly higher corticosterone concentrations (M = 609.43 ng/ml, SEM = 34.72) compared to no stress controls (M = 378.02 ng/ml, SEM = 34.39), $F(1, 55) = 22.42$, $p < .001$. Females had significantly higher corticosterone concentrations (M = 598.11 ng/ml, SEM = 37.16) compared to males (M = 389.33 ng/ml, SEM = 31.75), $F(1, 55) = 18.25$, $p < .001$. No effect of paternal treatment was observed.

5.3. F2 generation from females

Corticosterone responses to restraint stress revealed significant effects of maternal and adult treatments across time, $F(12, 138) = 2.17$, $p < .05$ and $F(2, 138) = 25.68$, $p < .05$, respectively, as shown in Fig. 6C and D. Planned comparisons revealed that offspring of neonatally LPS-treated mothers had significantly higher corticosterone responses at 30 min compared to saline controls ($t_{.05}(94) = 1.93$, $p < .05$). Similarly, animals exposed to stress in adulthood had higher plasma corticosterone levels at 30 min and 60 min compared to no stress animals ($t_{.05}(94) = 10.08$, $p < .05$ and $t_{.05}(94) = 6.02$, $p < .05$, respectively). A main effect of sex revealed that females exhibited significantly higher corticosterone concentrations (M = 700.46 ng/ml, SEM = 19.73) compared to males (M = 377.66 ng/ml, SEM = 19.07), $F(1, 69) = 258.87$, $p < .001$.

5.4. F2 generation cross-fostered

A significant sex × stress in adulthood × cross-fostering effect across time was observed in regards to corticosterone responses to acute restraint stress, $F(10, 108) = 2.23$, $p < .05$. Planned comparisons revealed no differences among males, however, females born to saline-treated mothers but fostered to LPS-treated dams displayed significantly higher corticosterone concentrations at 60 min when exposed to stress in adulthood compared to their LPS born but saline fostered counterparts, $t_{.05}(21) = 2.94$, $p < .05$ (Fig. 6E and F).

6. Adult corticosterone responses to anxiety testing

6.1. F1 generation

As shown in Fig. 7A, there was a significant interaction between sex and neonatal treatment for corticosterone concentrations following EPM and holeboard testing, $F(5, 71) = 2.79$, $p < .05$. Planned comparisons revealed no group differences between males, however, females exposed to neonatal LPS demonstrated a significantly blunted corticosterone response compared to saline-treated females ($t_{.05}(33) = 2.82$, $p < .05$).

6.2. F2 generation from males

In regards to the response to anxiety testing, only a significant effect of sex was observed whereby females exhibited significantly higher corticosterone concentrations (M = 928.38 ng/ml, SEM = 25.84) compared to males

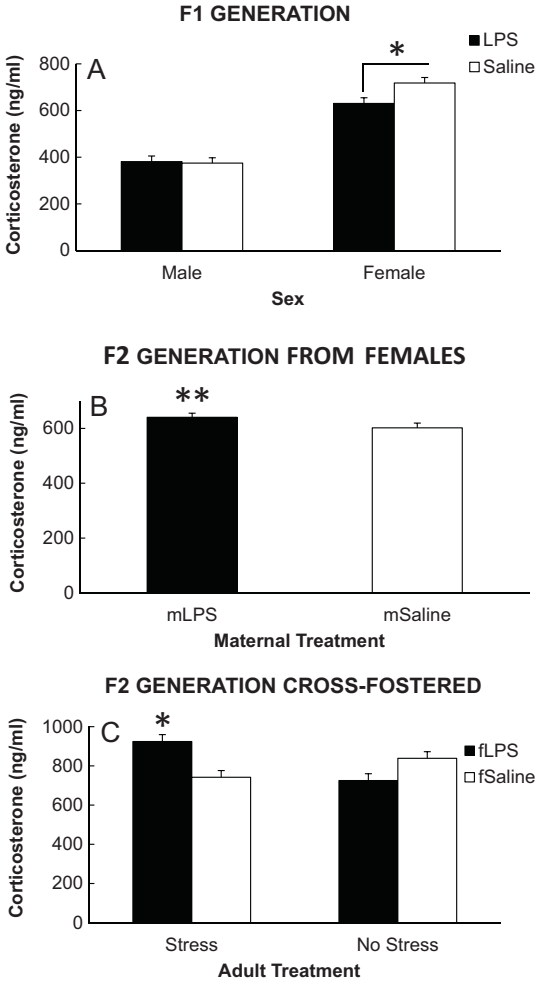

Figure 7 Mean plasma corticosterone responses to behavioural testing of anxiety in adulthood (ng/ml; ±SEM) as assessed by nested ANOVA. Filled bars represent neonatally challenged LPS animals for the F1 generation, F2 generation offspring of males (pLPS) and females (mLPS) neonatally exposed to LPS, and females born to mothers exposed to neonatal saline treatment but fostered to LPS-treated mothers (fLPS) exposed to stress or no stress. Hollow bars represent neonatally challenged saline animals for the F1 generation, F2 generation offspring of males (pSaline) and females (mSaline) neonatally exposed to saline, and females born to mothers exposed to neonatal LPS treatment but fostered to saline-treated mothers (fSaline) exposed to stress or no stress; $n \geq 10$ for all groups, *$p < .05$ and **$p < .01$.

(M = 594.59 ng/ml, SEM = 21.07), $F(1, 51) = 100.23$, $p < .001$. No effect of paternal treatment was observed.

6.3. F2 generation from females

Main effects of maternal treatment, sex and adult treatment were observed for corticosterone responses to EPM and holeboard exposure. Offspring of mothers neonatally exposed to LPS exhibited significantly higher corticosterone responses to the behavioural testing compared to offspring of neonatally saline-treated mothers, $F(6, 69) = 4.88$, $p < .001$ (Fig. 7B). Animals exposed to stress in adulthood (M = 668.65 ng/ml,

SEM = 16.12) exhibited significantly higher corticosterone responses to the EPM and holeboard compared to non-stressed controls (*M* = 577.51 ng/ml, *SEM* = 16.03), $F(1, 69) = 10.88$, $p < .01$. Females had significantly higher corticosterone levels (*M* = 853.52 ng/ml, *SEM* = 16.37) than males (*M* = 412.36 ng/ml, *SEM* = 15.82), $F(1, 69) = 357.81$, $p < .001$.

6.4. F2 generation cross-fostered

A significant stress in adulthood × cross-fostering effect was observed for plasma corticosterone concentrations in response to behavioural testing on the EPM and holeboard apparatus, $F(8, 51) = 2.40$, $p < .05$. Planned comparisons revealed that animals born to saline-treated mothers but fostered to LPS-treated dams who were exposed to stress in adulthood displayed significantly higher corticosterone concentrations than animals born to LPS-treated mothers but fostered to saline-treated dams who were exposed to stress in adulthood ($t_{.05}(41) = 4.19$, $p < .05$), and animals born to saline-treated mothers but fostered to LPS-treated dams who underwent no stress in adulthood, $t_{.05}(42) = 4.62$, $p < .05$ (Fig. 7C). Females exhibited significantly higher corticosterone concentrations (*M* = 1007.89 ng/ml, *SEM* = 24.6) than males (*M* = 549.66 ng/ml, *SEM* = 24.3), $F(1,51) = 152.02$, $p < .001$.

7. Discussion

We and others have previously shown that neonatal exposure to a bacterial mimetic increases anxiety-like behaviour and alters neuroendocrine functioning in later life (Breivik et al., 2002; Shanks et al., 2000; Walker et al., 2004, 2008, 2009, 2010). Here, we have expanded upon previous findings to demonstrate that the early microbial environment has the potential to not only alter the individual directly exposed, but also their offspring. The data suggest that two potentially independent and distinguishable mechanisms may be responsible for this transgenerational phenomenon depending on whether the mother or the father was exposed to the mimetic. The characteristic changes in peripheral endocrine function also appear reliant upon this.

7.1. Transgenerational effects of anxiety-like behaviour

Neonatal LPS exposure resulted in an increased adult anxiety-like phenotype in animals on all behavioural tests utilised, which is consistent with our previous findings (Walker et al., 2004, 2008, 2009; Sominsky et al., 2012). The observation that neonatal exposure to LPS produces reliable increases in anxiety-like behaviour is not novel. Of primary interest here are the previously unobserved transgenerational effects. When females that were neonatally exposed to LPS and demonstrated an anxiety-like phenotype in adulthood were bred with untreated males, we observed an increase in anxiety-like behaviour in their (F2) offspring in adulthood. This behavioural phenotype was evident in all testing apparatuses including restraint, the EPM, holeboard apparatus and ASR.

An increased anxiety-like phenotype was also observed in the F2 generation offspring of males neonatally exposed to LPS. These offspring demonstrated reduced time in the open arms and increased risk assessment in the EPM. Similarly, fewer exploratory head dips and reduced time spent in the centre square of the holeboard apparatus were observed compared to the offspring of saline-treated fathers. These findings suggest that neonatal immune stimulation is sufficient not only to induce an increased anxiety-like phenotype in males and females, but also has the capacity to induce an increased anxiety-related phenotype in the subsequent generation.

7.2. Transgenerational effects of corticosterone responses to stress

The HPA axis has been shown to be particularly susceptible to long-term functional alterations following neonatal immune challenge (Shanks et al., 1995, 2000; Shanks and Meaney, 1994; Walker et al., 2009, 2010). The trends of these animals indicate replication of such changes in endocrine stress responsivity following neonatal LPS treatment in Wistar rats that are consistent with our previously reported findings (Walker et al., 2009, 2010). For more in-depth discussion regarding the behavioural and endocrine alterations in rats exposed to LPS during neonatal life please refer to the above-mentioned publications. Again, the findings of interest in the current study are those that pertain to the transgenerational transmission of such alterations in neuroendocrine responsivity. Offspring of mothers exposed to neonatal LPS demonstrated significantly increased corticosterone concentrations on PND 3, indicative of increased activation of the HPA axis during the early postnatal period, and reflective of the increased neonatal corticosterone responses to LPS exhibited by their mothers following LPS challenge. Importantly, the F2 offspring were not directly exposed to the LPS themselves and were not disturbed whatsoever until the time of blood collection. This increased corticosterone activity was maintained in adulthood, whereby F2 generation offspring of mothers neonatally treated with LPS displayed elevated corticosterone concentrations following restraint stress and in response to testing on the EPM and holeboard apparatus. While the LPS-treated females of the F1 generation displayed a blunted response to anxiety behaviour testing, their offspring maintained a potentiated corticosterone response. This would indicate that the exact signature of the endocrine perturbations of the mothers is not transferred; rather a more general perturbation to endocrine stress responsivity is transmitted to the subsequent generation nonetheless.

The corticosterone signature of F2 generation rats along the paternal line was markedly different. No difference in circulating corticosterone was observed in these neonates, nor were there differences in corticosterone responses to acute stress or behavioural testing in adulthood. Hence, while the behavioural phenotype was transmitted to the F2 generation, the endocrine perturbations experienced following neonatal LPS exposure in the father were not. It should be noted however, that only circulating corticosterone was assessed in each of these experiments, and thus we can only assert that the downstream peripheral release of corticosterone does not appear to be inherited. The possibility remains that changes in HPA axis neural circuitry known to

occur following neonatal LPS exposure (Shanks et al., 2000; Iwasa et al., 2009a,b; Reul et al., 1994; Spencer et al., 2006) are transferred to the second generation.

7.3. Mechanisms of transgenerational inheritance

It would appear that the mechanisms of transference across generations for the anxiety-related phenotype and endocrine perturbations differ depending on the source of transmission — that is, whether the transference occurs across the maternal or paternal line. In regards to the maternal line, it is clear that the mechanism responsible for the transference of increased anxiety-like behaviour and hyper-responsive corticosterone activity is maternal care. We observed a reduction in the quality of maternal care and nursing behaviours for females neonatally treated with LPS towards their F2 offspring. Given that maternal care has been shown to differentially impact on long-term stress responsivity in rats via non-genomic mediation (Liu et al., 1997; Francis et al., 1999; Weaver et al., 2006), it was necessary to include an additional cross-fostered cohort.

Cross-fostering revealed that the increase in anxiety-like behaviour seen in the F2 offspring of LPS-treated mothers was a behaviourally mediated effect due to the reduction of maternal care experienced by the F2 animals. Offspring of saline-treated mothers fostered to LPS-treated dams exhibited reduced open arm entries and increased risk assessment in the EPM. Similarly, a reduction in head dips was observed in these animals in the holeboard apparatus, as well as increases in the mean peak startle amplitude on the ASR. These findings show a clear reversal of the transgenerational effect of anxiety-like behaviour seen in the offspring of neonatally LPS-treated F1 females. Furthermore, the data demonstrate that the offspring of saline-treated F1 females also show a reversal of their behavioural phenotype in adulthood, exhibiting increased anxiety-like behaviour, when fostered to LPS-treated dams who demonstrate lower quality maternal care. The fact that the group differences have been reversed is conclusive evidence that maternal care is the primary mediator across the maternal line.

The neuroendocrine perturbations observed in the offspring of mothers neonatally exposed to LPS also appear to be mediated via their experienced reduction in maternal care. Following cross-fostering, it was the offspring of mothers neonatally exposed to saline which demonstrated increased corticosterone responses at 60 min following baseline of the acute restraint stress protocol. Again, corticosterone responses following anxiety behaviour testing revealed these offspring of saline-treated mothers to exhibit a potentiated corticosterone response following stress in adulthood. Thus a reversal of the endocrine phenotype was observed when animals were cross-fostered, indicating the primary mediator of the endocrine perturbations of F2 generation of LPS-treated mothers is the quality of maternal care.

The findings we report here clearly expand on those of Michael Meaney's laboratory, which has demonstrated that poor maternal care results in increased stress-related behaviours and neuroendocrine responses (Liu et al., 1997; Francis et al., 1999; Weaver et al., 2004, 2006). The mechanisms responsible involve epigenetic modulation of HPA axis circuitry, whereby offspring of low quality nursing mothers exhibit decreased acetylation within the CpG dinucleotides of the exon 1_7 promoter on the glucocorticoid receptor (GR) promoter sequence (Weaver et al., 2006). These rats also show hypermethylation of the exon 1_7 GR promoter associated with hypoacetylation of histone H3-lysine (K)-9 and reduced binding to nerve growth factor-inducible protein-A (NGFI-A) (Weaver et al., 2004). Thus GR abundance is down-regulated and negative feedback is impaired. Importantly, these findings are reversible when cross-fostering of litters born to mothers providing higher quality of maternal care with litters born to mothers providing lower quality of maternal care is provided (Weaver et al., 2004).

Here, we have extended beyond Meaney's work to show that neonatal LPS exposure is sufficient to not only increase anxiety-like behaviour in adulthood but also impair maternal care behaviour provided to the next generation, which directly regulates long-term stress responsivity and behaviour of the F2 generation. Thus, these data would suggest that a cycle is created of direct epigenetic modification to each potential generation via reduced maternal care quality, which was initiated by neonatal exposure to immunological stress in the F1 generation. It would appear that we have isolated a neuroimmune pathway through which maternal care of subsequent generations can be modulated, and produce the repeated changes in phenotype reported in studies demonstrating the impact of low maternal care and neglect. Human studies have also indicated poor attachment care-giving in infancy to increase the predisposition to a range of psychiatric illness including anxiety, as well as alter HPA axis stress activity (Anda et al., 2006; Pierrehumbert et al., 2003; Weiss et al., 1999; Essex et al., 2002; Buss et al., 2007).

Unlike the maternal line, transference of the anxiety-like phenotype across the paternal line cannot be explained by maternal care. Males were bred with untreated females and no differences in the maternal care of these females to their F2 offspring were observed. These findings are particularly novel, and suggest that non-genomic modifications may be responsible for transmission of the anxiety-like phenotype observed along the paternal line. Supportive of this hypothesis is our recently published findings regarding subtle morphological changes to testicular development in male rats exposed postnatally to LPS (Walker et al., 2011). LPS-treated rats display reduced gonocyte presence and delayed testicular development during early neonatal life following exposure to the bacterial mimetic. This delay appears persistent given that classification of the spermatogenic cycle indicated that the majority of seminiferous tubules in LPS-treated rats were at an earlier stage compared to controls in adulthood. Increased epithelial disorganisation of tubules was also observed in these animals. Thus, we have evidence of a vulnerability of developing gametes to immune stimulation. This is especially pertinent given that the LPS exposure occurs during the period of early postnatal mitotic division in the male rat — a period surrounding vulnerability to epigenomic erasure and imprinting of the gametes (Skinner and Guerrero-Bosagna, 2009; Surani, 2001). Confirmation of epigenetic imprinting to the gametes is still required, and we propose that assessment of the global methylation status of the testes or gonocytes in these animals will provide elucidation of this hypothesis given that this period surrounds the

time of global germ cell demethylation and remethylation in the rat (Skinner and Guerrero-Bosagna, 2009).

8. Conclusion

Our data suggest that neonatal bacterial exposure can produce increased susceptibilities to anxiety-like behaviour in the F1 and subsequent generations. Notably, it would appear that maternal care is the primary mediator for these effects along the maternal line. To date we have not identified a specific mechanism to account for the findings along the paternal line but our ongoing studies are investigating the role of germ line modifications to the epigenome — in particular methylation status of gonocytes. Our findings support epidemiological studies that show enhanced susceptibility to psychopathology along specific filial lines, and suggest that the perinatal microbial environment of one generation can contribute to this phenomenon.

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Conflict of interest

The authors declare there are no conflicts of interest.

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