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RESEARCH ARTICLE

Early-life adversity programs long-term cytokine and microglia expression within the HPA axis in female Japanese quail

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ABSTRACT

Stress exposure during prenatal and postnatal development can have persistent and often dysfunctional effects on several physiological systems, including immune function, affecting the ability to combat infection. The neuroimmune response is inextricably linked to the action of the hypothalamic–pituitary–adrenal (HPA) axis. Cytokines released from neuroimmune cells, including microglia, activate the HPA axis, while glucocorticoids in turn regulate cytokine release from microglia. Because of the close links between these two physiological systems, coupled with potential for persistent changes to HPA axis activity following developmental stress, components of the neuroimmune system could be targets for developmental programming. However, little is known of any programming effects of developmental stress on neuroimmune function. We investigated whether developmental stress exposure via elevated prenatal corticosterone (CORT) or postnatal unpredictable food availability had long-term effects on pro- (*IL-1β*) and anti-inflammatory (*IL-10*) cytokine and microglia-dependent gene (*CSF1R*) expression within HPA axis tissues in a precocial bird, the Japanese quail (*Coturnix japonica*). Following postnatal stress, we observed increased *IL-1β* expression in the pituitary gland, reduced *IL-10* expression in the amygdala and hypothalamus, and reduced *CSF1R* expression within the hypothalamus and pituitary gland. Postnatal stress disrupted the ratio of *IL-1β*:*IL-10* expression within the hippocampus and hypothalamus. Prenatal stress only increased *IL-1β* expression in the pituitary gland. We found no evidence for interactive or cumulative effects across life stages on basal cytokine and glia expression in adulthood. We show that postnatal stress may have a larger impact than elevated prenatal CORT on basal immunity in HPA-axis-specific brain regions, with changes in cytokine homeostasis and microglia abundance. These results provide evidence for postnatal programming of a pro-inflammatory neuroimmune phenotype at the expense of reduced microglia, which could have implications for central nervous system health and subsequent neuroimmune responses.

KEY WORDS: Cytokines, Developmental programming, Glucocorticoids, Neuroinflammation, Anti-inflammatory, Neuroimmune

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INTRODUCTION

Stress and activation of the hypothalamic–pituitary–adrenal (HPA) axis during development have significant and sometimes permanent effects on physiology and behaviour. Individuals exposed to stress during prenatal and/or postnatal development are more prone to develop diseases such as hypertension and diabetes, exhibit increased senescence rates and have greater cognitive impairments throughout life than do unstressed individuals (Chaby, 2016; Cottrell and Seckl, 2009; Seckl, 2004). In many cases, these effects are mediated via changes to HPA axis functioning, as developmental exposure to stress can program the sensitivity of the HPA axis to stressful stimuli as well as permanently influence feedback mechanisms within the axis (Kriengwatana et al., 2014; Maccari et al., 2003; Spencer et al., 2009).

Developmental stress impacts on several other physiological systems, including the ability to mount an immune response (Avitsur et al., 2015; Couret et al., 2009; Plant et al., 2016) within both peripheral and central systems. The central nervous system (CNS) has long been considered an ‘immune-privileged’ organ, where resident immune cells react to a range of insults including localised infections, injury, inflammation and ischaemia, as well as playing a role in regulating neurogenesis throughout life (Nimmerjahn et al., 2005; Sato, 2015; Weinstein et al., 2010; Ziv et al., 2006). Microglia are the resident immune cells of the CNS that provide the main form of active immune defence. Upon activation following an infection or other insult, microglia rapidly undergo morphological and functional changes characterised by increased production of pro-inflammatory cytokines, resulting in a pro-inflammatory signal cascade as part of the immune response (de Pablos et al., 2014; Walkera et al., 2013). Cytokines can potently activate the HPA axis and induce glucocorticoid secretion during an immune or stress response (Felger and Lotrich, 2013). In turn, cytokine release is regulated by the direct interaction of ligand-bound glucocorticoid receptor (GR) to pro-inflammatory transcription factors (Wei, 1998), leading to anti-inflammatory effects on central and peripheral tissues (Coutinho and Chapman, 2011; Miller et al., 1998; Smith and Vale, 2006; Walker and Spencer, 2018). The close links between these two physiological systems, coupled with potential for long-term alterations of the HPA axis following developmental adversity, suggest that both microglia and cytokines could be potential targets for developmental programming.

Programming effects on microglia abundance and cytokine production rates could create a shift towards an immuno-reactive phenotype that endures into adulthood with greater reactivity to subsequent stressors (reviewed in Brenhouse et al., 2018). Heightened neuroimmune responses in adulthood are a common symptom of neurodegenerative diseases (Eikelenboom et al., 2002; Spanos et al., 2015) but may also be a consequence of impaired glucocorticoid secretion. In contrast, programmed suppression of neuroimmune responses may be indicative of prolonged

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List of symbols and abbreviations

ACTB	β-actin
C_t	cycle threshold
CNS	central nervous system
CORT	corticosterone
CSF1R	colony-stimulating factor 1 receptor
GLM	generalized linear model
GOI	gene of interest
HPA	hypothalamic–pituitary–adrenal (axis)
IL-10	interleukin-10
IL-1β	interleukin 1-beta
qPCR	quantitative real-time polymerase chain reaction
RIN	RNA integrity number

glucocorticoid secretion following chronic stress, which could result in the dysfunction of both immune responses (Cherry et al., 2014; Perry and Holmes, 2014) and the HPA axis (Dhabhar, 2008; McEwen, 2017). To our knowledge, evidence supporting this programming hypothesis has only been demonstrated in peripheral immune tissues. For example, rats (*Rattus norvegicus*) exposed to both prenatal and postnatal stress (via maternal ethanol exposure) exhibit reduced peripheral T-cell populations and suppressed long-term antibody production from the lymph nodes (Seelig et al., 1999). These effects are not confined to mammals, as male European starlings (*Sturnus vulgaris*) exposed to both prenatal and postnatal stress show reduced cell-mediated immunity in adulthood in comparison with male birds that experienced stress at only one developmental stage (Love and Williams, 2008). To understand imbalanced neuroimmune and HPA responses in later life, it is important to identify which developmental stages are most susceptible to developmental programming (via glucocorticoids) and whether elevated glucocorticoids can affect long-term neuroimmune parameters.

To determine the long-term effects of prenatal and/or postnatal stress on important markers of the neuroimmune response, we used an avian species, the Japanese quail (*Coturnix japonica* Temminck & Schlegel 1849), which is a well-characterised neuroendocrine model (Ottinger, 2007; Ottinger et al., 2004) that has been used to investigate developmental programming (Guibert et al., 2012; Leroux et al., 2017; Marasco et al., 2016; Zimmer et al., 2013). To discriminate between the effects of stress during the prenatal stage (*in ovo* hormone manipulation) and postnatal development (unpredictable food availability), we created four distinct groups: prenatal CORT manipulation only, postnatal stress only, prenatal and postnatal stress, and control. The current study was part of a larger experiment exploring the long-term and trans-generational effects of maternal stress exposure in the Japanese quail, and tissue from females was available (Zimmer and Spencer, 2014; Zimmer et al., 2013; Zimmer et al., 2017). Therefore, this experiment focused on females and we used females from this F1 generation (Zimmer and Spencer, 2014; Zimmer et al., 2013, 2017). When all female birds reached adulthood, we isolated the pituitary gland and brain regions functionally involved in HPA axis regulation, specifically the medial amygdala, the hippocampus and the medial hypothalamus. In each region, we quantified relative mRNA expression of both pro- and anti-inflammatory cytokines, Interleukin 1-beta (*IL-1β*) and *IL-10*, respectively, and a marker of avian microglia abundance, Colony Stimulating Factor 1 Receptor (*CSF1R*) (Balic et al., 2014; Bohlen et al., 2017; Uno et al., 2012).

We expected birds exposed to increased prenatal CORT to have higher levels of microglia abundance and pro-inflammatory cytokine expression in adulthood compared with controls and birds exposed to postnatal stress. Our hypothesis was based on evidence that prenatal CORT has stronger long-term effects on stress physiology and behaviour in Japanese quail (Zimmer and Spencer, 2014; Zimmer et al., 2013, 2017) than postnatal stress. While we acknowledge that many studies report that prenatal stress induces long-term neuroinflammation more so than postnatal stress (Diz-Chaves et al., 2013; Pedersen et al., 2018), it is unclear whether these effects are mediated by increased glucocorticoid levels during development. Although there has been little investigation of the effects of stress across multiple developmental stages, we suggest that exposure to both prenatal and postnatal stress should create cumulative effects on the neuroimmune response, so that individuals with this phenotype would exhibit exacerbated effects on levels of pro-inflammatory cytokines within the brain and pituitary gland (Golovatscka et al., 2012; Reader et al., 2015; Yang et al., 2015).

MATERIALS AND METHODS

Animals

Seventy-six unrelated Japanese quail eggs were obtained from Moonridge Farm, Exeter, UK, and were incubated at 37.5°C and 55% humidity (Ova-Easy 190A incubator, Brinsea Products Ltd, UK). The birds used in this study had taken part in experiments described elsewhere (Zimmer and Spencer, 2014; Zimmer et al., 2013) and the methods will be explained briefly here.

Prenatal treatments

We manipulated stress hormones prenatally in the egg by injecting the yolk with 10 µl of 850 ng ml^{-1} CORT (Sigma-Aldrich, Poole, UK), dissolved in sterile peanut oil, at the egg apex under sterile conditions on day 5 of incubation (E5; n=38). At this developmental stage, egg fertility can be reliably determined and the yolk layers become de-stratified, allowing for passive diffusion of CORT from the outer to inner yolk layers (Almasi et al., 2012; Lipar et al., 1999). Therefore, injection at this stage ensured CORT levels were elevated in the whole yolk (Marasco et al., 2012). A pilot study using colour dye confirmed that the CORT was injected into the yolk only (C. Zimmer and K. A. Spencer, unpublished data). Radioimmunoassay and liquid chromatography-mass spectroscopy (LC-MS) determined that this dose (8.5 ng) increases endogenous CORT concentrations in the yolk within 1.8 s.d. above control yolks and increased whole yolk CORT levels to 17.1±8.3 ng ml^{-1} (mean± s.d.) (Zimmer et al., 2013). This treatment is designed to mimic the effects of maternal stress and allows for direct manipulation of the individual *in ovo* (Groothuis et al., 2005) and is in line with previous studies that show these increased CORT levels occur within physiologically relevant ranges (Love and Williams, 2008; Zimmer and Spencer, 2014; Zimmer et al., 2013). The remaining eggs (controls) were injected with sterile peanut oil on the same day (n=38). All eggs were kept in the same incubator until day 14 of incubation, when the eggs were transferred to two treatment-specific hatchers (Hatchmaker, Brinsea Products Ltd, UK) maintained at 37°C and 75% humidity until hatching. Fifty-nine eggs hatched, with a 74% hatching success rate for control eggs and an 82% success rate for CORT-injected eggs. After 24 h in the hatcher to allow their feathers to dry, each chick was given a unique nail polish mark applied every 5 days until 15 days of age, when they were large enough to be fitted with a numbered leg ring. Chicks from each prenatal treatment were then randomly allocated to four pens (114×114×58 cm) all in the same room with *ad libitum* food availability (turkey crumb, BOCM, UK), water and an electric heat lamp.

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Postnatal treatments

Once chicks had reached 4 days of age, we assigned one pen of each prenatal treatment (CORT or control) to one of the two postnatal food treatments: either removal of food for 25% of daylight hours (3.5 h) on a random daily schedule for 15 days (n=28), or *ad libitum* food at all times (n=31). Unpredictable food availability increases CORT levels in avian species (Boogert et al., 2013; Buchanan et al., 2003) without causing food restriction (Buchanan et al., 2003; Cuthill et al., 2000), and causes reduced hippocampal corticosteroid receptor gene expression in Japanese quail, which could alter HPA axis sensitivity to stressors (Zimmer and Spencer, 2014). Pens were maintained at 30°C for the first 4 days post-hatch, then reduced by 2°C per day until chicks were 10 days old, when all additional heat sources were removed and birds were moved to treatment-specific enclosures (n=8; two pens per treatment, 100×86 cm) that were maintained at 20–22°C throughout the rest of the experiment. All enclosures of each prenatal treatment were in the same room, and the birds were able to see and hear each other. From 20 days post-hatch, all birds received *ad libitum* food (standard layer pellet, BOCM). To summarise, we had four treatment groups: prenatal control/postnatal control (n=6); prenatal control/postnatal food removal (n=6); prenatal CORT/postnatal control (n=12); and prenatal CORT/postnatal food removal (n=6). The experiment was repeated with both prenatal and postnatal treatments using two batches of quail (batch 1=15 chicks; batch 2=15 chicks).

Ethical note

All experimental procedures were conducted in accordance with Home Office Animals (Scientific) Procedures Act project licence 60/4068 (K.A.S.) and personal licences 70/1364 and 60/13261 (C.Z. and K.A.S.).

Tissue collection

The method was the same as described in Zimmer and Spencer (2014). Briefly, adult females (246.5±1.4 days old, mean±s.e.m.) were euthanized by injection of an overdose of sodium pentobarbital (Dolethal; Vetoquinol, UK). Brains and pituitary glands were quickly removed (within 2 min) and were frozen on dry ice, then stored at −80°C. For dissection, we placed brains ventral side up in a brain matrix (Roboz Surgical Instrument Co., USA) embedded on a mixture of wet and dry ice to keep the tissue optimally frozen. From each brain, we cut a 2-mm-thick coronal section using two razor blades positioned approximately 4 mm from the rostral pole and 2 mm from the cerebellum (Zimmer and Spencer, 2014). We obtained two identical bilateral punches (1 mm diameter each) from the hippocampus and amygdala, and a single punch from the medial hypothalamus that spanned the third ventricle. We isolated total RNA using an Absolutely RNA Miniprep kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. We measured RNA concentration and integrity with an RNA 6000 Pico assay kit for the amygdala, hippocampus and hypothalamus and a RNA 6000 Nano kit for pituitary gland using an Agilent 2100 bioanalyzer (Agilent Technologies, UK) according to the manufacturer's instructions. The mean RNA integrity number (RIN) of these samples was 8.2± 0.1 (range: 5.2–10).

Genes of interest

IL-1β is a pro-inflammatory cytokine that contributes to innate immune defence and potently activates the HPA axis upon stress exposure (Schmidt et al., 1995). *IL-1β* secretion is specifically regulated by the anti-inflammatory cytokine *IL-10*, which promotes adaptive immune responses and prevents both glial apoptosis and the hypersecretion of hormones during HPA axis activation (Smith et al., 1999). As the ratio of pro-:anti-inflammatory cytokines has been used as an indicator of neurodegeneration, we also examined the effects of early-life stress on the *IL-1β*:*IL-10* ratio (Remarque et al., 2001; You et al., 2011). *CSF1R* is exclusively expressed by microglia, and microglia are physiologically reliant upon its expression (Elmore et al., 2014; Erblich et al., 2011; Ginhoux et al., 2010). This gene has been used as a proxy for microglia abundance from tissue extracts (Bohlen et al., 2017) and was selected to investigate basal microglia expression and not microglia activity, as our treatments did not reflect a specific infection or insult that would activate the immune response. Because of the size of each sample, we chose to measure gene expression and not protein levels of both cytokines and *CSF1R* where mRNA levels of these genes have previously been shown to mirror protein levels under experimental conditions (Pohlers et al., 2005; Soares et al., 2009).

We used gene-specific primers for detection of each gene of interest (GOI) (*IL-1β*, *IL-10*, *CSF1R*) and the housekeeping gene β-actin (*ACTB*), which was determined in a previous study (Zimmer and Spencer, 2014) as the best candidate housekeeping gene in this population (M=0.30, other candidates $M \geq 0.34$) after using a chicken (*Gallus gallus domesticus*) Genorm kit (Primerdesign, Southampton, UK). Specific PerfectProbe™ primers (Primerdesign) were designed for *IL-1β* (Entrez gene ID: AB559570), *IL-10* (Entrez gene ID: AB559574) and *ACTB* (Entrez gene ID: AB199913) based on published chicken nucleotide sequences and were validated using quail cDNA by PrimerDesign© (full primer sequences are available in Table 1). Chicken RT^2 qPCR Primer Assays (Qiagen; catalogue no. 330001; PPG01594A) were used for detection of *CSF1R* (Entrez gene ID: 396406) with *ACTB* (GenBank accession no. NM_205518) used as the corresponding housekeeping gene. BLAST analysis indicated that the chicken *CSF1R* sequence shares 95% sequence homology with that of the draft Japanese quail genome, which was the closest reference genome available at that time (Kawahara-Miki et al., 2013). All primer sequences and accession numbers are provided in Table 1.

cDNA synthesis and quantitative PCR

We used an Affinity Script Multiple Temperature cDNA Synthesis Kit (Agilent Technologies) to synthesise first-strand cDNA, which was diluted to 25 pg µl^{-1}. This concentration was determined as the optimal detection limit for each of our GOI following standard curve

Table 1. Forward and reverse primer sequences for genes used in qPCR data collection (chicken primer sequences not supplied by Qiagen)

Species	Gene	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Product length (bp)	Accession number
Coturnix japonica (Japanese quail)	*IL-1β*	AGAAGTGCTTCGTGCTGGAG	ACTTTCTGGCTGGAGGAAGG	76	AB559570
	IL-10	CCAGCACCAGCCACCAG	CGTCGCATCGTCATCTTCAG	73	AB559574
	ACTB	CAGCAAGCAGGAGTATGATGAA	AAGGGTGTGGGTGTTGGTAA	87	AB199913
Gallus gallus domesticus (chicken)	*CSF1R*				396406
	ACTB				NM_205518

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assays using two-fold serial dilutions from a subset of cDNA samples from each treatment group and each tissue. Each cDNA sample was measured for mRNA abundance by quantitative real-time PCR (qPCR) using, for detection of *IL-1β*, *IL-10* and its corresponding *ACTB* in a 20 µl reaction volume containing: 5 µl of cDNA, 10 µl of 2× Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies), 1 µl of gene-specific primer at a working concentration of 300 nmol l^{-1}, 0.3 µl of reference dye and 3.7 µl of RNAse/DNase-free water. For *CSF1R* and its corresponding *ACTB*, reactions of 25 µl were performed using 5 µl of cDNA template, 12.5 µl RT^2 SYBR Green ROX FAST Mastermix (Qiagen), 1 µl of gene-specific primer and 6.5 µl of RNAse/DNase-free water. All reactions were performed in duplicate and each run contained blanks and non-template controls. Reactions were performed on a Stratagene MX3005P with *IL-1β* and *IL-10* (95°C for 3 min, then 50 cycles of 95°C for 20 s, 60°C for 20 s) and *CSF1R* (95°C for 10 min, then 50 cycles of 95°C for 10 s, 60°C for 30 s) reactions run on separate 96-well plates. Relative expression of each GOI was quantified using the Delta C_t method (ΔC_t) relative to *ACTB* : $2^{-(C_{t,GOI/ACTB}-C_{t,ACTB})}$ (Pfaffl, 2004).

Statistical analysis

To compare the overall relative expression of each GOI across tissue types, we used generalized linear models (GLMs) in SPSS with tissue and GOI as fixed factors. To determine the effects of the prenatal and postnatal treatments on the relative expression of each of our dependent variables – *IL-1β*, *IL-10*, *CSF1R* and the *IL-1β*:*IL-10* ratio within each tissue – we used GLMs with prenatal and postnatal treatment and their interaction as fixed factors. Batch number was included as a random effect to account for individual differences across batches. GLMs were fitted with a gamma distribution as the residuals of the linear models were not normally distributed. For multiple comparisons, Šidák adjustment was applied to obtain a corrected *P*-value. Data presented are means±s.e.m.

RESULTS

Cytokine and microglia mRNA expression in HPA axis regions

Relative expression of both *IL-1β* and *IL-10* varied across all brain regions (gene×tissue $F_{3,30}$=11.78, P<0.0001; Fig. 1), and *IL-10* expression was greater than that of *IL-1β* in the hippocampus (t=3.84, P=0.0042), the hypothalamus (t=7.28, P<0.0001) and the pituitary gland (t=6.55; P<0.0001; Fig. 1). *IL-1β* relative expression was higher in the pituitary gland than it was in the hypothalamus (t=−3.08, P=0.015), whereas *IL-10* relative expression was higher in the hypothalamus and the pituitary gland than it was in the hippocampus (t=−4.41, P<0.0001; t=−0.657, P<0.0001, respectively) or the amygdala (t=−9.05, P<0.0001; t=−10.78; P<0.0001, respectively; Fig. 1).

Relative *CSF1R* expression also varied across the regions, with the lowest expression observed in the pituitary gland ($F_{3,29}$=76.3, P<0.0001) compared with all other tissues (Fig. 2). Tissue sample sizes differed across genes as some samples did not yield a ΔC_t from the qPCR reaction.

Effects of early-life stress

In the pituitary gland, relative *IL-1β* expression was lower in birds exposed to increased prenatal CORT compared with controls ($\chi^2_{1,21}$=6.2, P=0.013), while relative expression was higher in response to postnatal stress compared with postnatal controls ($\chi^2_{1,21}$=14.1, P<0.0001; Fig. 3D). In the other HPA-associated tissues, relative *IL-1β* expression did not differ significantly between control and stress conditions in both prenatal and postnatal treatment groups. We also observed no significant interaction between treatments across all tissues (Fig. 3A–C, Table 2).

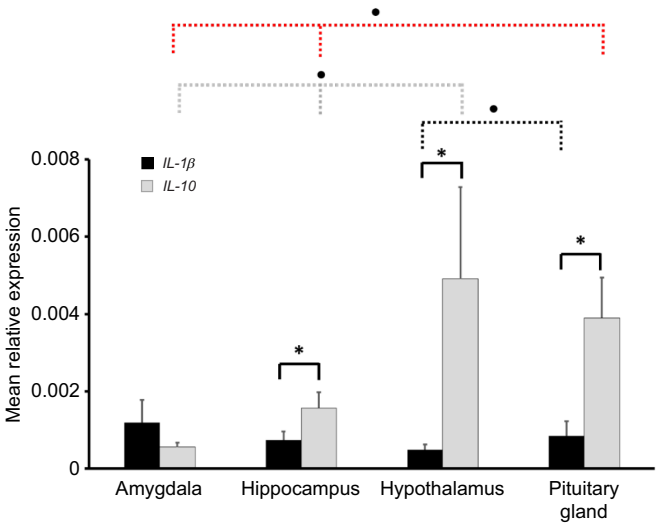

Fig. 1. Mean±s.e.m. relative gene expression of *IL-1β* and *IL-10* in female Japanese quail. Solid lines and asterisks denote significant differences (P<0.05) in expression between *IL-1β* and *IL-10* within the specific tissues (*IL-1β*: amygdala n=29; hippocampus n=27; hypothalamus n=27; pituitary n=21; *IL-10*: amygdala n=28; hippocampus n=25; hypothalamus n=28; pituitary n=22). Dashed black line and circles indicate a significant difference in *IL-1β* expression between the hypothalamus and the pituitary gland. Grey dashed line and black circle denote significant differences in *IL-10* expression between the hypothalamus and both the hippocampus and amygdala. Red dashed line and black circle indicate a significant difference in *IL-10* expression between the pituitary gland and both the hippocampus and the amygdala.

Individuals subjected to postnatal stress had lower relative *IL-10* expression in the amygdala ($\chi^2_{1,28}$=6.05, P=0.014) and the hypothalamus ($\chi^2_{1,28}$=8.52, P=0.004) than did the control birds (Fig. 4A and C, respectively). These birds also tended to have lower relative *IL-10* expression in the hippocampus ($\chi^2_{1,27}$=3.64, P=0.056; Fig. 4B). There was no effect of prenatal CORT or an interaction

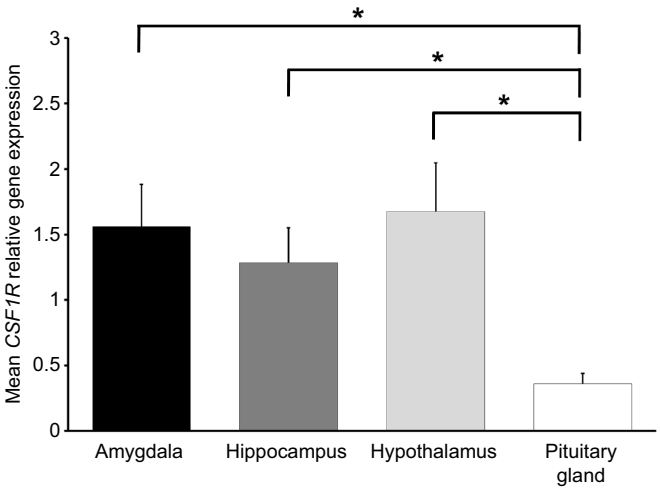

Fig. 2. Mean±s.e.m. relative gene expression of *CSF1R* in female Japanese quail. Asterisks denote significant differences (P<0.05) in the expression of *CSF1R* between tissues (amygdala n=28; hippocampus n=28; hypothalamus n=27; pituitary n=22).

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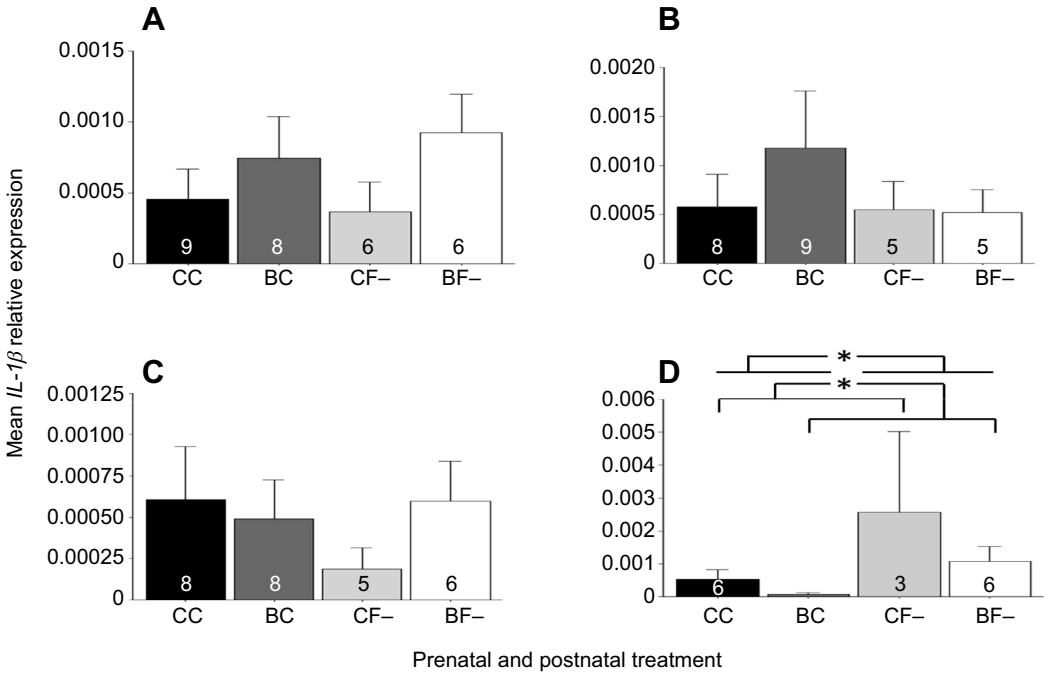

Fig. 3. Mean±s.e.m. relative gene expression of *IL1-β* in female Japanese quail exposed to the four treatment groups: prenatal and postnatal control (CC), prenatal CORT and postnatal control (BC), prenatal control and postnatal unpredictable food availability (CF–), and both stress treatments (BF–). (A) Amygdala, (B) hippocampus, (C) hypothalamus and (D) pituitary gland. Sample sizes for each condition are shown within the corresponding bars (for the BC treatment in D, n=6). Asterisks denote significant differences (P<0.05) in expression of *IL-1β* between stress and control conditions within each developmental stage.

between the prenatal and postnatal treatments in any tissues (Table 2).

The ratio of *IL-1β*:*IL-10* in the hippocampus ($\chi^2_{1,26}$=10.3, P=0.0013) and in the hypothalamus ($\chi^2_{1,27}$=4.29, P=0.038) of postnatally stressed birds was higher than it was in control birds (Fig. 5B and C, respectively) and we saw no effects of prenatal CORT or interaction between treatments across tissues (Table 2).

Relative *CSF1R* expression in the hypothalamus and the pituitary decreased as a result of postnatal stress (hypothalamus: $\chi^2_{1,27}$=7.22, P=0.007; pituitary gland: $\chi^2_{1,22}$=5.84, P=0.016; Fig. 6C and D, respectively). However, we observed no effect of prenatal CORT nor any interaction between the prenatal and postnatal treatments on relative *CSF1R* expression (Table 2).

DISCUSSION

We show that prenatal exogenous CORT and physiological/psychological stress from post-hatch day 5 to 20 had long-term effects on cytokine and microglia expression within key tissues involved in HPA axis functioning in adult female Japanese quail. These effects were observed more than 200 days following stress exposure, demonstrating the potential for programming long-term changes in the neuroimmune phenotype following exposure to a non-pathogenic stressor during development. Although in this experiment, prenatal stress had minimal statistically significant effects (birds had lower *IL-1β* expression in the pituitary gland), we found that postnatal stress appears to be a more potent mediator of these long-term effects on neuroimmune markers. Birds exposed to postnatal unpredictable food availability had reduced *IL-10* mRNA expression in the

Table 2. Statistical output from GLMs for the effects of prenatal CORT, postnatal stress and their interaction for *IL-1β*, *IL-10*, *IL-1β*:*IL-10* and *CSF1R* relative gene expression in the amygdala, hippocampus, hypothalamus and pituitary gland

Tissue	Treatment	*IL-1β*			*IL-10*			*IL-1β*:*IL-10*			*CSF1R*		
		χ^2	d.f.	*P*	χ^2	d.f.	*P*	χ^2	d.f.	*P*	χ^2	d.f.	*P*
Amygdala	Prenatal	1.7	1, 29	0.193	1.64	1, 28	0.2	0.042	1, 28	0.837	0.029	1, 28	0.866
	Postnatal	0.077	1, 29	0.781	6.054	1, 28	**0.014**	0.136	1, 28	0.712	2.67	1, 28	0.102
	Interaction	0.43	1, 29	0.512	0.004	1, 28	0.947	0.202	1, 28	0.653	0.005	1, 28	0.943
Hippocampus	Prenatal	0.515	1, 27	0.473	2.13	1, 27	0.145	0.34	1, 26	0.561	0.009	1, 28	0.924
	Postnatal	0.54	1, 27	0.462	3.64	1, 27	0.056	10.3	1, 26	**0.0013**	3.18	1, 28	0.075
	Interaction	0.659	1, 27	0.829	1.911	1, 27	0.167	0.14	1, 26	0.706	0.132	1, 28	0.716
Hypothalamus	Prenatal	2.06	1, 27	0.151	1.946	1, 28	0.163	0.95	1, 27	0.33	0.192	1, 27	0.661
	Postnatal	0.472	1, 27	0.492	8.52	1, 28	**0.004**	4.29	1, 27	**0.038**	7.22	1, 27	**0.007**
	Interaction	2.47	1, 27	0.116	0.269	1, 28	0.604	0.084	1, 27	0.772	0.469	1, 27	0.493
Pituitary gland	Prenatal	6.2	1, 21	**0.013**	1.79	1, 22	0.18	0.8	1, 21	0.371	0.369	1, 22	0.543
	Postnatal	14.1	1, 21	**<0.0001**	0.016	1, 22	0.899	2.31	1, 21	0.129	5.84	1, 22	**0.016**
	Interaction	0.942	1, 21	0.332	0.071	1, 22	0.79	0.34	1, 21	0.562	0.109	1, 22	0.741

Significant *P*-values are highlighted in bold.

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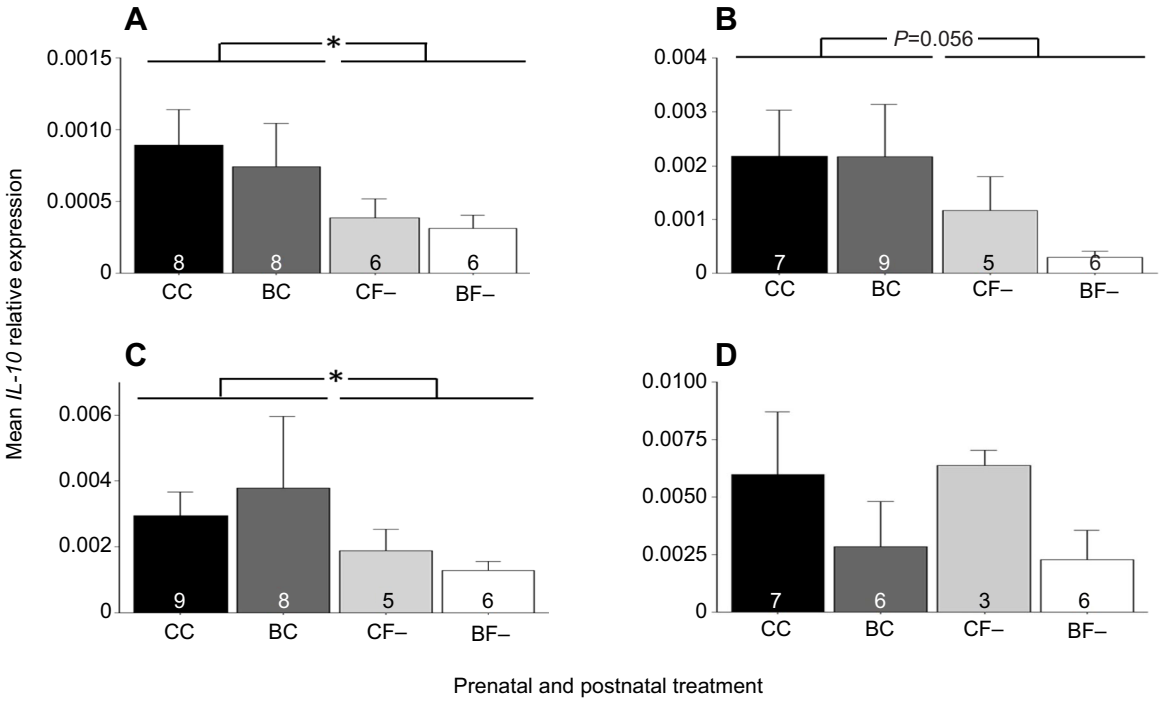

Fig. 4. Mean±s.e.m. relative gene expression of *IL-10* in female Japanese quail exposed to the four treatment groups: prenatal and postnatal control (CC), prenatal CORT and postnatal control (BC), prenatal control and postnatal unpredictable food availability (CF–) and both stress treatments (BF–). (A) Amygdala, (B) hippocampus, (C) hypothalamus and (D) pituitary gland. Sample sizes for each condition are shown within the corresponding bars. Asterisks denote significant differences (P<0.05) in expression of *IL-10* between stress and control conditions within each developmental stage.

amygdala and the hypothalamus, a higher *IL-1β:IL-10* ratio in the hippocampus and hypothalamus, and reduced *CSF1R* mRNA expression in the hypothalamus and pituitary gland. We found no compelling evidence for a cumulative effect of stress on neuroimmune factors at different developmental stages. To our knowledge, this is the first demonstration that exposure to a non-pathogenic stressor during development has programming effects on both neural cytokine and microglia expression that persist into adulthood.

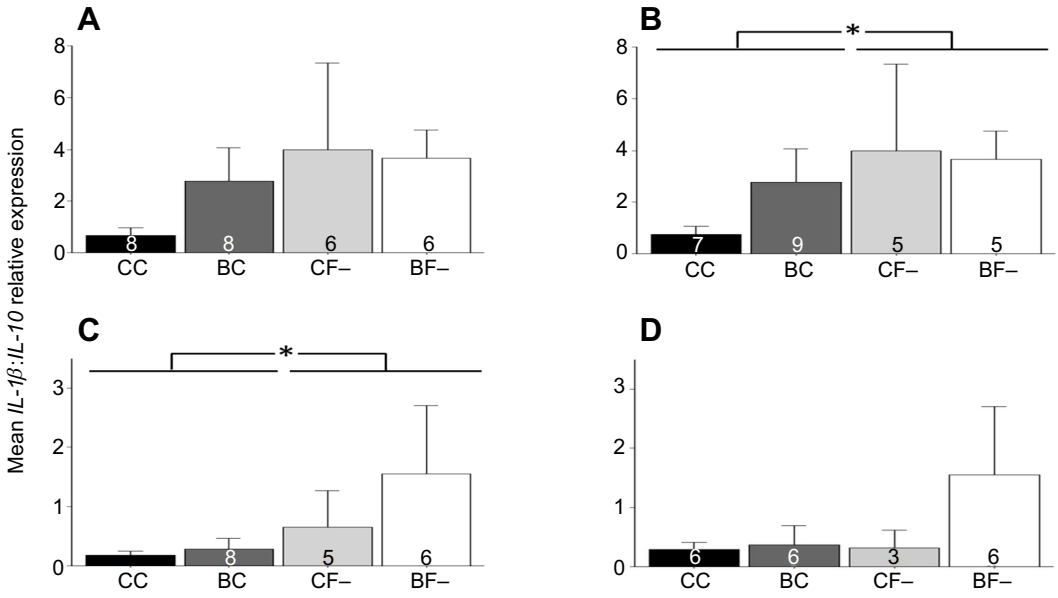

Fig. 5. Mean±s.e.m. *IL-1β:IL-10* expression ratio in female Japanese quail exposed to the four treatment groups: prenatal and postnatal control (CC), prenatal CORT and postnatal control (BC), prenatal control and postnatal unpredictable food availability (CF–) and both stress treatments (BF–). (A) Amygdala, (B) hippocampus, (C) hypothalamus and (D) pituitary gland. Sample sizes for each condition are shown within the corresponding bars (for the CC treatment in C, n=8). Asterisks denote significant differences (P<0.05) in the expression of *IL-1β:IL-10* between stress and control conditions within each developmental stage.

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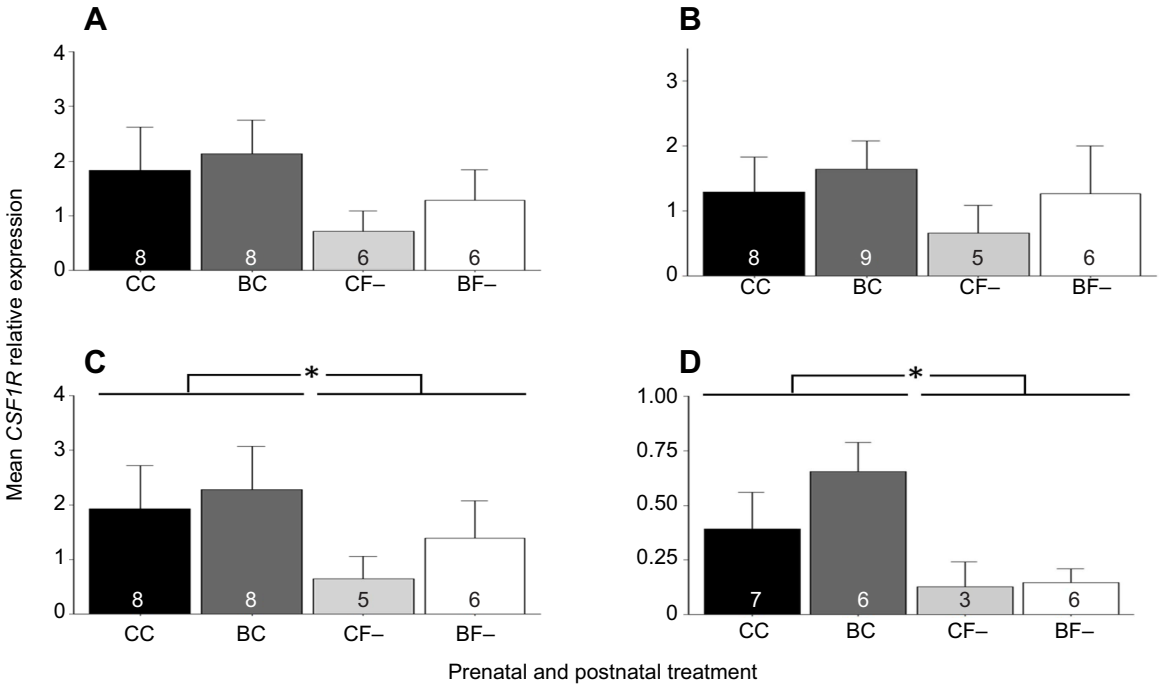

Fig. 6. Mean±s.e.m. relative gene expression of *CSF1R* in female Japanese quail exposed to the four treatment groups: prenatal and postnatal control (CC), prenatal CORT and postnatal control (BC), prenatal control and postnatal unpredictable food availability (CF–) and both stress treatments (BF–). (A) Amygdala, (B) hippocampus, (C) hypothalamus and (D) pituitary gland. Sample sizes for each condition are shown within the corresponding bars. Asterisks denote significant differences (P<0.05) in expression of *CSF1R* between stress and control conditions within each developmental stage.

We first determined the expression patterns of *IL1β*, *IL-10* and *CSF1R* across each tissue irrespective of treatment. *IL-1β* expression was highest in the pituitary gland relative to the hypothalamus and *IL-10* was predominantly expressed in both the hypothalamus and the pituitary gland. These expression patterns match those in mammals (Quan et al., 1998), where both cytokines function to enhance glucocorticoid production downstream of the hypothalamus and pituitary gland (Sapolsky et al., 1987). *CSF1R* expression was lowest in the pituitary gland, confirming previous observations of small numbers of microglia populations within the neurohypophysis and pituitary stalk (Moffett and Paden, 1994). Microglia abundance did not vary between the amygdala, hippocampus and hypothalamus, which contrasts with the reported irregular distribution of microglia observed in later life (Wong, 2013). This finding suggests that microglia colonisation of the CNS may be geared towards occupying these specific brain regions in adulthood as they may be the most susceptible to infection (Grabert et al., 2016).

Postnatal stress induced long-term changes in the expression of all markers that we measured with reduced *IL-10* and *CSF1R* mRNA expression and an increase in the ratio of *IL-1β*:*IL-10* compared with postnatal controls. Postnatal unpredictable food availability has been shown to enhance antibody responses, increase haemolysis and haemagglutination in grey partridges (*Perdix perdix*) (Homberger et al., 2013). In black-capped chickadees (*Poecile atricapillus*), this same treatment leads to a reduction in body mass during the fever response following lipopolysaccharide infection (Cornelius et al., 2017). As the unpredictable food availability treatment induces stress by increasing baseline CORT levels (Buchanan et al., 2003; Fokidis et al., 2012), its effects on immune function may be attributed to the fact that stress can induce large-scale trafficking of immune cells to target tissues that are vulnerable to infection, including the brain (Dhabhar et al., 2012; Ziv et al., 2006). In the short term, glucocorticoids suppress peripheral immune responses by increasing the expression of anti-inflammatory cytokines in rodents, such as *IL-10* (Coutinho and Chapman, 2011; Mozo et al., 2004). However, we show that over the long term, postnatal stress has the opposite effect within the neuroimmune system, as *IL-10* expression significantly declined in the amygdala and hypothalamus, with a similar trend in the hippocampus. Repeated early-life stress can impair the intracellular communication between amygdala and hypothalamic neurons in the processing of stressful stimuli of rats during adulthood (Kaur and Salm, 2008). This suggests that both the amygdala and the hypothalamus are crucial in initiating the cytokine response to stress in the adult brain (Kaur and Salm, 2008). Low *IL-10* expression within these regions is associated with increased adrenal gland and decreased thymus relative mass in rodents (Mesquita et al., 2008; reviewed by Smith et al., 1999), indicating impaired negative feedback action of glucocorticoids on HPA axis functioning (Pariante and Miller, 2001; Sorrells and Sapolsky, 2007). Therefore, our results suggest that low *IL-10* expression in these regions may impair activity of the HPA axis cascade.

The pro-:anti-inflammatory cytokine ratio is an important indicator of excessive inflammation or immune hypo-responsiveness (Hernández Cruz et al., 2008), which are important markers of host defences to infection and disease (Adelman et al., 2013; Vinkler et al., 2018). The reduction in *IL-10* expression in response to postnatal stress explains the increased *IL-1β*:*IL-10* ratio in both the hippocampus and the hypothalamus, as *IL-1β* expression was not affected by postnatal stress in these tissues. This increase is consistent with effects seen in rodents exposed to various chronic mild stressors (e.g. food deprivation, heat stress, cage tilt) (You et al., 2011). Higher ratios of peripheral pro-:anti-inflammatory cytokines are observed in several clinical populations even under healthy, normal physiological conditions (Gogos et al., 2000; Hou et al., 2017). However, maintaining the ratio between these cytokines is important in mediating not only a healthy immune response, but also in the

maintenance of CNS sensitivity to autoimmune conditions and neurodegenerative diseases such as dementia, Alzheimer's disease and Parkinson's disease (Elenkov and Chrousos, 1999; Remarque et al., 2001; Rocha-Ferreira et al., 2017). Our data show that these effects can be longer-lived, especially when experienced during development, although the factors that contribute to the persistence of this pro-inflammatory state and its effect on neurogenesis are not yet clear.

We observed a long-term reduction in basal microglia abundance as a result of postnatal exposure to a non-pathogenic stressor. To our knowledge, such a loss in basal, not phagocytic or immune-activated microglia has only been found using either pharmacological inhibitors of activated microglia or *CSF1R* knockout mice (*Mus musculus*) to investigate microglia depletion in disease models (Elmore et al., 2014; Han et al., 2017; Spangenberg et al., 2016). Furthermore, these studies often adopt histological or immunohistochemical approaches to observe microglia *in situ* rather than the quantitative gene expression approach used in our study. The reduction in *CSF1R* expression also accompanied an increase in the *IL-1β*:*IL-10* ratio, suggesting that there may be a compensatory mechanism at play within the brain. As pro-inflammatory cytokines are predominantly secreted by microglia in the CNS (Felger and Lotrich, 2013), postnatal stress may program these low numbers of microglia to produce fewer anti-inflammatory cytokines in order to both elicit an innate neuroimmune response and stimulate HPA axis activity. This may represent mechanisms whereby the neuroimmune system is compensating for microglia senescence by accumulating inflammatory cytokines within the CNS during ageing (Streit and Xue, 2010). Alternatively, it may indicate that microglia fail to revert to basal levels following stress exposure throughout life (Ajami et al., 2007). However, our data contribute to the emerging evidence that developmental stress may shift the CNS phenotype towards a more immune-sensitive state (Williamson et al., 2011). One way this may occur is via an increase in the expression of receptors for immune molecules on microglia in regions such as the hippocampus and the pre-frontal cortex (Viviani et al., 2014). Basal microglia crucially survey the microenvironment for pathogens, clear cellular debris and maintain synaptic connections required for neurogenesis by secreting neuroprotective cytokines (Gemma and Bachstetter, 2013; Nimmerjahn et al., 2005; Sato, 2015). This critical role for basal microglia suggests a skew towards an anti-inflammatory phenotype, so postnatal stress may have induced the transition in basal microglia from its anti-inflammatory, neuroprotective role to that of a pro-inflammatory phenotype.

Prenatal and postnatal stress had opposing effects on *IL-1β* mRNA expression, where prenatal CORT induced a decline in *IL-1β* mRNA while postnatal stress increased expression. The prenatal effects we observed contrast with mammalian studies in which prenatal stress was shown to increase pro-inflammatory cytokine expression, resulting in long-term programming of a pro-inflammatory phenotype (Shanks and Lightman, 2001; Vanbesien-Mailliot et al., 2007). Our data are consistent with the restrictions prenatal elevations in CORT have on T-cell immune responses at day 10 post-hatch in yellow-legged gull chicks (*Larus michahellis*) (Rubolini et al., 2005). However, those cytokine levels followed an immune challenge, and while the reaction to an immune challenge is important, understanding cytokine levels under normal physiological conditions can give insight into animal health, owing to their potent effects on a range of neurological factors. For example, increases in *IL-1β* alter synaptic plasticity (Schneider et al., 1998), trigger neuronal cell death (Viviani et al., 2006) and promote the potential for neurological disorders. In some altricial species, such as zebra finches (*Taeniopygia guttata*), cortisol is present at concentrations similar to that of CORT in the brain and is considered to be the primary glucocorticoid present during lymphoid organ development (Schmidt et al., 2010). Therefore, it is possible that cortisol may be the salient glucocorticoid that affected the immune system during early development, which may explain why we saw relatively minimal effects of elevated prenatal CORT compared with postnatal stress in our GOIs. The majority of HPA axis maturation in precocial birds is thought to occur pre-hatch (Carsia et al., 1987; Ericsson and Jensen, 2016; Jenkins and Porter, 2004), when glucocorticoids are present in the blood of the chick embryo around E10 (Henriksen et al., 2011; Tona et al., 2005) and can influence the activity of the cutaneous immune response (Rubolini et al., 2005). However, the postnatal period represents a more critical period for HPA axis maturation in most mammals and some bird species (Kapoor et al., 2006; Sims and Holberton, 2000; Wada et al., 2009), when both CORT and cortisol are present in equal amounts (Jenkins and Porter, 2004). As the effects of developmental stress can vary depending on the duration of early stress exposure, more work is required to establish any vulnerable windows of sensitivity to prenatal elevations in glucocorticoids (Walker and Spencer, 2018).

Taken together, our results suggest that prenatal CORT manipulation does not promote activation of the innate neuroimmune system in the Japanese quail. Instead, we showed that prenatal exposure to CORT programs a blunted pituitary *IL-1β* response in adulthood. This could be interpreted in one of two ways. (1) Prenatal CORT may program a weakened pro-inflammatory response, resulting in a poor innate neuroimmune response. This may impact on HPA axis functioning, as these birds had an attenuated stress responses, suggesting increased negative feedback efficiency (Zimmer et al., 2013). Alternatively, (2) prenatal CORT may prolong the immediate immunosuppressive state typically observed in animals exposed to stress in the short term (Dimatelis et al., 2012; Wei et al., 2012). The importance of this is that developmental stress may program different neuroimmune responses depending on the developmental stage at which an individual is exposed to stress, the type of stressor experienced and the quality of the environment to which the individual is exposed in later life (Walker and Spencer, 2018). Such effects may be adaptive or transmitted through generations (Ericsson et al., 2016; Grindstaff et al., 2006).

We show that the development of the neuroimmune system in the Japanese quail was susceptible to stress mainly during the postnatal period, and that prenatal development had few long-term effects. In addition, we found no evidence for cumulative effects of stress over different developmental stages. These changes also suggest developmental programming of a pro-inflammatory phenotype with the potential for increased neuroinflammation that could negatively impact on CNS health. However, we suggest that this postnatal effect may be adaptive in ensuring an appropriate inflammatory neuroimmune response in adulthood at the expense of reduced microglia number. Such programming effects could have significant and permanent impacts on HPA axis functioning in later life.

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Competing interests
The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.J.W., C.Z., K.A.S.; Methodology: D.J.W., C.Z., K.A.S.; Software: D.J.W.; Validation: D.J.W., C.Z., K.A.S.; Formal analysis: D.J.W.; Investigation: D.J.W.; Resources: D.J.W., C.Z., M.L.; Data curation: D.J.W.; Writing - original draft: D.J.W.; Writing - review & editing: D.J.W., C.Z., M.L., S.D.H., K.A.S.; Visualization: D.J.W.; Supervision: C.Z., S.D.H., K.A.S.; Project administration: C.Z., S.D.H., K.A.S.; Funding acquisition: D.J.W., C.Z., S.D.H., K.A.S.

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Data availability

Data are available from Mendeley (Walker, 2019): http://dx.doi.org/10.17632/6r7d2pg2zk.1

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