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Full Length Article

Investigating the gut-brain axis in a neurodevelopmental rodent model of schizophrenia

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ABSTRACT

Background: Although the aetiology of schizophrenia remains unknown, it has been suggested that it might occur in response to alterations in the gut-brain axis (GBA), the bi-directional communication system between the gut and the brain. The current study aimed to determine whether the "two-hit" animal model of neuropsychopathology (maternal immune activation combined with adolescent cannabinoid exposure), produced abnormalities in the GBA

Method: Pregnant Wistar rats were administered the viral mimetic polyI:C on gestational day 19 and offspring were administered the synthetic cannabinoid HU210 from postnatal days 35–48. Evidence of GBA activation was assessed in the hypothalamus, colon and fecal samples from male and female offspring at adolescence and adulthood

Results: Findings were sex-specific with adolescent female offspring exhibiting an increased hypothalamic inflammatory profile, increased hypothalamic CRHR1 mRNA, and decreased fecal expression of *Bifidobacterium longum*, however, no changes were detected in colonic inflammation or integrity.

Conclusion: These results indicate that the rat two-hit model, documented to produce behavioural and neuroanatomical abnormalities, also produces hypothalamic and microbiota abnormalities. The results also demonstrate significant sex differences, suggesting that this model may be useful for investigating the role of the GBA in the aetiology of neurodevelopmental disorders such as schizophrenia.

1. Introduction

Schizophrenia is a severely debilitating, complex neuropsychiatric disorder with an onset during early adulthood. It is characterised by a diverse range of symptoms and is considered to be a disorder of neurodevelopment, attributed to the action and interaction of multiple genes, as well as environmental factors. While numerous environmental risk factors have been identified to play a role in the development of schizophrenia, research suggests that a combination of these factors occurring during sensitive periods of neurodevelopment are more likely to have an adverse outcome (Feigenson et al., 2014).

To study the effect of combined environmental insults on the mammalian brain, a "two-hit" model can be used. In such models, exposure to environmental risk factors during the perinatal period can alter neuronal circuits, enhancing susceptibility to neurological disorders. It is hypothesized that a further second "hit" during adolescence, another period of critical brain development, can then activate and amplify symptoms, inducing the onset of disease. Maternal immune activation (MIA) and adolescent cannabinoid exposure(ACE) have each been identified as major environmental risk factors for altered neurodevelopment and psychiatric disorders such as schizophrenia, bipolar disorder, depression and autism spectrum disorders (Depino, 2018; Hollins et al., 2014, 2016b; Mednick, Huttunen, & Machon, 1994; Moore et al., 2007; Spear, 2000). Furthermore, studies show that a combination of both these risk factors further exacerbates neuropathology and associated behavioural abnormalities (Dalton et al., 2012; Hollins et al., 2014, 2016b), however, the majority of these studies only investigate the effects of these risk factors in males. This study will be the first to look at

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the effect of a dual stressor on components of the gut-brain axis (GBA) in both sexes.

The GBA is a bi-directional communication system between the gut and the brain. Gastrointestinal disorders have been identified as a significant comorbidity (Cucino and Sonnenberg, 2001; Gupta et al., 1997) in schizophrenia patients, with up to 92% of sufferers exhibiting severe gastrointestinal abnormalities, such as barrier dysfunction and inflammation (Nemani et al., 2015). Moreover, there is evidence that the hypothalamic-pituitary-adrenal (HPA) axis, a vital component of the GBA, is dysregulated in patients with schizophrenia. This dysregulation is thought to be mediated by increased levels of proinflammatory cytokines such as interleukin 6 (IL6) (Gaughran and Welch, 2008).

Further evidence of a dysregulated GBA in schizophrenia comes from research identifying altered gut bacteria, also a crucial component of the GBA, in patients with the disorder (Zheng et al., 2019). Studies also indicate that gut bacteria can affect the development and regulation of the HPA axis and in turn, influence brain function and behaviour, including neuroendocrine responses to stress (Hollins et al., 2018). Although this may occur via many different mechanisms, one possible option is that this may arise through a weakened intestinal barrier, with evidence of increased markers of intestinal inflammation and bacterial translocation in schizophrenia patients (Dickerson et al., 2017; Nemani et al., 2015).

Although the role of the GBA has not been identified in schizophrenia, the immune system provides bidirectional communication between the gut and the brain. Moreover, the immune system is closely connected with interactions between the gut microbiota and the HPA axis (Aidy et al., 2015) (Fig. 1). Evidence suggests that altered immune functioning may contribute to the aetiology and pathogenesis of schizophrenia, with patients exhibiting an inflammatory profile that correlates with symptom severity (Bergink et al., 2014; Gaughran and Welch, 2008). In the present study, we examine the effect of combined environmental stressors on inflammation, the HPA axis, intestinal barrier and gut bacteria. We report that prenatal polyI:C in combination with adolescent cannabinoid treatment induces inflammation and alterations in the HPA axis, as well as decreases in levels of bacteria known to be involved in aberrant mental health. There was no observable change in expression of intestinal barrier genes or in intestinal inflammation. These findings have implications for our understanding of the GBA in response to environmental stressors and the use of the two-hit model in investigating psychiatric disorders.

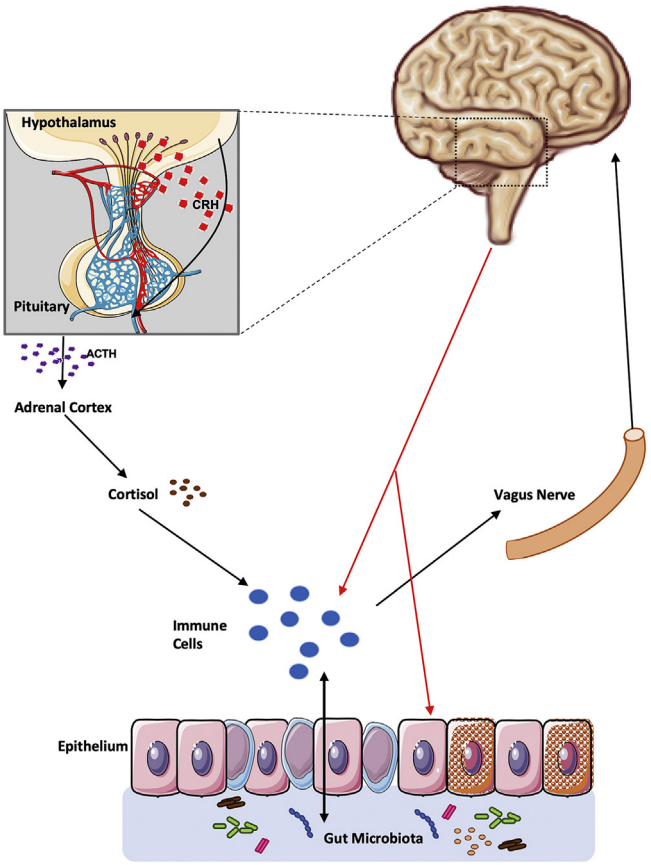

Fig. 1. The HPA axis and the gut-brain axis. In response to stress, parvocellular neurons of the paraventricular hypothalamus increase secretion of corticotropin-releasing hormone (CRH), which is released into portal vessels activating secretion of adrenocorticotrophic hormone (ACTH) from anterior pituitary cells. In turn, ACTH enters the circulation and stimulates the release of glucocorticoids, cortisol in humans and corticosterone in rodents, from the adrenal gland. Cortisol and the gut microbiota can influence immune responses and signalling via the vagus nerve. This in turn can trigger a neural response which then acts in a feedback loop. Figure adapted from (Hollins et al., 2018).

2. Methods and materials

2.1. Animals

Male and nulliparous female Wistar rats (n = 4, n = 38, respectively) were obtained from the Animal Resource Centre (ARC, Western Australia) at 8–10 weeks of age. Housing (41.5 × 28 × 22 cm wire-top cages; Mascot Wire Works, Australia) and food (Rat Chow, Specialty Feeds, Western Australia) were consistently maintained with a 12-h light/dark schedule (lights on at 7:00 a.m.) and a constant temperature of 20 °C ± 2. The animals were acclimated for 1 week before daily monitoring of the female oestrous cycle began, using an impedance probe. On the day of proestrus females were mated with male rats overnight. The following morning, the presence of sperm via vaginal smear was used to identify the day of conception, identified as gestational day (GD) 0. Pregnant dams were then randomly allocated to a treatment group (MIA, n = 20 or saline (SAL), n = 18). All handling of animals and procedures were approved by the University of Newcastle Animal Care and Ethics Committee (A-2016-610).

2.2. Prenatal polyI:C treatment

On gestational day (GD) 19, pregnant rats received either a single intravenous injection of 5 mg/kg polyinosinic:polycytidylic acid (polyI:C; Sigma, Australia) dissolved in phosphate buffered saline (PBS) or an equivalent volume of PBS. Two hours post anaesthesia, blood samples were collected from the saphenous vein of pregnant dams, placed in EDTA-coated tubes, then centrifuged at 1000×*g* for 20 min at 4 °C. Plasma was stored at −20 °C until assayed. Circulating levels of the pro-inflammatory cytokine Il6 were assessed using a Quantikine IL-6 enzyme-linked immunosorbent assay (ELISA, R&D Systems Inc., Minneapolis, MN, USA), as per the manufacturer's instructions, to confirm activation of the maternal immune system following polyI:C administration (Harvey and Boksa, 2012). Pups were weaned on postnatal day (PND) 21 and littermates left together until PND 27 to prevent heat loss when the mother is removed (Allmann-Iselin, 2000). On PND 27, pups were sexed and pair-housed with animals of the same sex and prenatal treatment group: (1) vehicle only: prenatal PBS with adolescent vehicle exposure (Veh-Veh); and (2) two-hit group: prenatal polyI:C with adolescent HU210 (MIA-ACE). Animals were allocated from different litters to avoid litter effects. Specifically, each group contained 4–6 animals, from a total of 20 Veh-Veh and 17 MIA-ACE separate litters for tissue collection, and 11 Veh-Veh and 11 MIA-ACE litters for fecal collection.

2.3. Adolescent HU210 treatment of offspring

The synthetic cannabinoid HU210 (Sapphire Laboratories, Australia) was dissolved in a vehicle solution of Tween 80: dimethyl

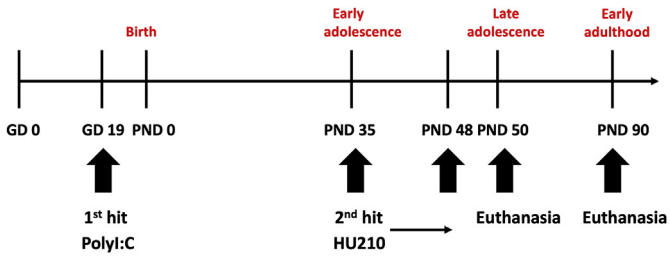

Fig. 2. Experimental Timeline. Pregnant Wistar rats were administered polyI:C or vehicle on GD 19 and offspring were administered the synthetic cannabinoid HU210 or vehicle for 14 days from postnatal days 35–48. Animals were euthanised on PND 50 or PND 90.

sulfoxide:saline (1:1:98). On PND 35 rats in the MIA-ACE group received daily intraperitoneal injections of HU210 (males: 100 μg/kg, females: 75 μg/kg, differing dosage to account for drug sensitivity in females-even when controlled for weight), for 14 days to replicate chronic drug exposure during a critical period of neurodevelopment. Rats in the Veh-Veh group received vehicle solution for the treatment period (Hollins et al., 2014, 2016b) (Fig. 2).

2.4. Plasma and tissue collection

Animals were euthanised at either PND 50 or PND 90 (between 9:00 a.m. and 3:00 p.m.) via isoflurane inhalation (5% induction) and rapid decapitation. These time points are equivalent to late adolescence and early adulthood in humans (Sengupta, 2013). Immediately following euthanasia, blood was collected via cardiac puncture, placed in EDTA-coated tubes, then centrifuged at 1000×*g* for 20 min at 4 °C. Plasma was stored at −20 °C until used for radioimmunoassay. Whole brains were removed and the hypothalamus dissected according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998), snap-frozen on dry ice and stored at −80 °C until assayed. The intestinal tract was removed, washed with PBS and a 1 cm piece of colon (large intestine) was excised, snap-frozen on dry ice and stored at −80 °C until assayed.

2.5. Radioimmunoassay

Plasma corticosterone levels were assessed using a rat ImmuChem™ Double Antibody Corticosterone ^{125}I RIA kit (MP Biomedicals, California, USA) following the manufacturer's instructions. Corticosterone (CORT) levels were determined using a gamma counter (Wallac 1470 Wizard, Turku, Finland) and reported as ng/mL. The recovery of free corticosterone is 100%, with an inter- and intra-assay variability of 4.4% and 6.5%, respectively. Notably, the method of euthanasia used in this study is associated with increasing corticosterone in females without altered gene expression which will be taken into account (Bekhbat et al., 2016).

2.6. Tissue homogenisation and RNA isolation

Colon samples were removed from −80 °C storage and stored at −20 °C for 24 h. Tissue was homogenised with the TissueLyzer® (Qiagen, The Netherlands; 10 min at 50 Hz) in an RNAse-free microtube containing 1 mL of QIAzol® Lysis Reagent and two 7 mm stainless steel beads (Qiagen Pty Ltd). Hypothalamus samples were removed from −80 °C storage and homogenised in a RNAse-free microtube containing 1 mL of QIAzol® Lysis Reagent. Total RNA was extracted from all samples using the RNeasy mini kit and DNase I reagents (Qiagen) according to the manufacturer's instructions. RNA concentration was assessed using the NanoDrop Spectrophotometer 2000c (Thermo Fisher Scientific, Waltham, MA, USA).

Table 1
Primers used in real-time qPCR.

Gene	Forward primer sequence (5' - 3')	Reverse Primer Sequence (5' – 3')
Il6	TGCCTTCCCTACTTCACAAG	CCATTGCACAACTCTTTTCTCA
Il1b	AACATAAGCCAACAAGTGGT	TTCATCACACAGGACAGGTA
Tnf	CGAGATGTGGAACTGGCAGA	CGATCACCCCGAAGTTCAGT
Tjp1	GCCCAGAGTGAAGGCAATTCC	TGAGATGGGGGTGGGTCTGG
Tjp2	AGAGCCGCCCAAGGTCAGAAA	TGGATCGCCCAAAGGCAGGT
Cldn4	CAACGTCATCCGCGACTTCT	CCCAGCCGATGTAAAGCGAG
Ocln	TTGGGTTTGAATTCATCCGGC	GAGAGCGATCTAGAGCCTGGAG
Crh	GCAACATTTCATTTCCCGATAATC	TGGAACCTCATCTCGGCTTT
Crhr1	GAAGGCTACCAGACTTGCTC	GCCATTGTTTGTCGTGTTGT
Actb	TCTGTGTGGATTGGTGGCTCTA	CTGCTTGCTGATCCACATCTG
Gapdh	GGCTGGCATTGCTCTCAA	GAGGTCCACCACCCTGTTG
B. longum	CGGGTGAGTAATGCGTGACC	TGATAGGACGCGACCCCA

The table lists forward and reverse primer sequences used in qPCR reactions.

2.7. Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was performed to determine the mRNA expression levels of markers of inflammation: *Il6,* interleukin 1 beta (*Il1b*) and tumor necrosis factor (*Tnf*); markers of GI barrier integrity: tight junction protein 1 (*Tjp1*), tight junction protein 2 (*Tjp2*), Claudin 4 (*Cldn4*), and occludin (*Ocln*); and markers of HPA axis activity: corticotrophin-releasing hormone (*Crh*) and corticotrophin-releasing hormone receptor 1 (*Crhr1*). Briefly, 1000 ng of total cellular RNA was used for a random-primed reverse transcription with SuperScript® IV VILO™ cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Triplicate reactions were performed using 25 ng of cDNA combined with Fast SYBR® Mix (Thermo Fisher Scientific) and 1 μM of forward and reverse primers (Sigma-Aldrich) in a final volume of 12.5 μL. Reactions were carried out using a 7500 Fast real-time PCR System (Applied Biosystems, Foster City, CA). The amplification conditions were 20 s at 95 °C (1 cycle), followed by 3 s at 95 °C and 30 s at 60 °C (40 cycles) followed by a melting step. Gene expression was normalised to the combined geometric mean of two endogenous controls, actin beta (*Actb*) and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), and differential expression of a given gene was determined using the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001). The choice of an appropriate reference gene as an endogenous control is a critical step for the normalization of PCR results. The geometric mean of multiple housekeeping genes is validated as an accurate normalization factor as compared to the use of a single housekeeping gene for normalization, which can lead to relatively large errors (Livak and Schmittgen, 2001). Primer sequences are detailed in Table 1.

2.8. DNA isolation and purification

Fresh fecal pellets were collected from PND 50 and PND 90 offspring into sterile 1.8 mL cryogenic vials (Eppendorf, Macquarie Park, Australia). This was achieved by massaging the abdomen of each rodent until defecation. Pellets were snap frozen on dry ice and stored at −80 °C. 100–200 mg of frozen sample was suspended in 1.4 mL buffer ASL (Qiagen, Hilden, Germany) in a microtube containing one 5 mm steel bead (Qiagen). Samples were then homogenised using a TissueLyser LT (Qiagen) for 4 min at 50 Hz. DNA extraction and purification was performed using the QIAamp DNA Stool Mini Kit (Qiagen) as per manufacturer's instructions. The DNA eluate was stored at −20 °C until further analysis.

2.9. Real-time PCR of fecal microflora

Real-Time PCR analysis of *B. longum* was performed using a 7500 Fast real-time PCR System (Applied Biosystems). Each reaction was carried out in a total volume of 25 μL containing Fast SYBR® Mix (Thermo Fisher Scientific), 200 nM of forward (5′-CGGGTGAGTAATGCGTGACC-3′) and reverse (5′-TGATAGGACGCGACCCCA-3′); primers (Sigma-Aldrich), 7 μL

of diluted fecal DNA and 0.1 mg/mL bovine serum albumin (New England Biolabs, Ipswich, MA, USA). The amplification conditions were 10 min at 95 °C (1 cycle), followed by 30 s at 95 °C (40 cycles) and 1 min at 60 °C (1 cycle) followed by a melting step. Gene expression was normalised to the endogenous control *Gapdh*, and differential expression of a given gene was determined using the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001). Gapdh is considered the gold standard reference gene for fecal qPCR (Stauber et al., 2016).

2.10. Statistical analyses

Statistical analyses were performed using SPSS version 24 (SPSS Inc., IBM Corporation). Data was analysed using univariate analysis of variance, with treatment group (Veh-Veh and MIA-ACE), postnatal day (PND 50 and PND 90) and sex (male and female) as between subject's factors. Normality and homegeneity of variance were assessed using Shapiro-Wilks test and Levene's test respectively ($p > 0.05$). A log transformation was performed to correct for violations of normality and homogeneity where needed. Statistical significance was set at $p < 0.05$. Post hoc pairwise comparisons were used to explore significant main effects and interactions and SPSS Bonferroni adjusted *p*-values reported.

3. Results

3.1. Plasma IL-6 cytokine concentrations in pregnant dams

To determine whether polyI:C at the dose of 5 mg/kg i.v. provoked a systemic immune response in the pregnant rat, plasma Il6 concentrations were assayed in pregnant rats 2 h after polyI:C treatment. MIA dams exhibited a significant increase (M = 1191.2119 pg/ml, SEM = 135.605) in plasma Il6 ($t_{(1,45)} = -8.294$, $p < 0.001$) when compared to saline-treated controls (M = 41.0655 pg/ml, SEM = 4.910), indicative of a systemic immune response.

3.2. MIA-ACE does not affect the integrity of the intestinal barrier in offspring

To assess the integrity of the gastrointestinal barrier, mRNA expression levels of *Tjp1, Tjp2, Cldn4* and *Ocln*, the major components that are critical in the maintenance of epithelial barrier function and control of paracellular permeability, were measured by qPCR in the colon of MIA-ACE offspring. Compared to controls, MIA-ACE offspring at PND 50 and PND 90 exhibited no significant interactions between treatment and age, or treatment, age and sex ($p > 0.05$) for markers of gastrointestinal integrity (Table 2).

3.3. MIA-ACE offspring do not exhibit evidence of intestinal inflammation

qPCR was performed to investigate the expression of pro-inflammatory cytokine mRNA: *Il6, Tnf*, and *Il1b* in the colon. Compared to controls, MIA-ACE offspring at PND 50 and PND 90 exhibited no significant interactions between treatment and age or treatment, age and sex ($p > 0.05$) for markers of gastrointestinal inflammation (Table 2).

3.4. Adolescent MIA-ACE offspring display age- and sex-dependent alterations in hypothalamic inflammation

To investigate inflammation in the hypothalamus, the mRNA expression level of the pro-inflammatory cytokine *Il6* was investigated. *Il6* mRNA expression was significantly increased in the hypothalamus with an overall significant interaction between sex, age and treatment ($F_{(1,\ 21)} = 33.548$, $p < 0.001$). *Il6* mRNA expression was significantly upregulated in adolescent female MIA-ACE offspring compared to non-treated controls, with pairwise comparison mean difference of 1.320, $p < 0.001$. However, in adolescent male MIA-ACE offspring *Il6* mRNA expression was significantly decreased in the hypothalamus, with pairwise comparison mean difference of −0.425, $p = 0.008$ (Fig. 3A).

Table 2
Treatment effect of MIA-ACE on gastrointestinal barrier integrity and inflammation.

		Mean ± SEM				
		PND 50		PND 90		
Gene	Sex	Veh-Veh	MIA-ACE	Veh-Veh	MIA-ACE	Treatment effect
TJP1	Female	0.023047± 0.005890	0.033738± 0.044795	0.039873± 0.015877	0.035787± 0.003896	F (1, 52) = 0.081, p = 0.777
	Male	0.039408± 0.009936	0.034682± 0.005039	0.062202± 0.010510	0.052359± 0.012590	
TJP2	Female	0.079734± 0.019647	0.069644± 0.019720	0.023871± 0.009734	0.020856± 0.005402	F (1, 52) = 1.456, p = 0.233
	Male	0.106015± 0.041856	0.076823± 0.012778	0.584087± 0.673025	0.271678± 0.374764	
CLDN4	Female	0.016036± 0.007354	0.016213± 0.006308	0.031301± 0.012927	0.028236± 0.007713	F (1, 52) = 0.487, p = 0.488
	Male	0.037155± 0.012952	0.038426± 0.012813	0.031324± 0.019172	0.026842± 0.009737	
OCLN	Female	0.027053± 0.005688	0.023813± 0.012271	0.046753± 0.017659	0.043254± 0.020506	F (1, 52) = 0.062, p = 0.805
	Male	0.044813± 0.023489	0.022699± 0.003467	0.108441± 0.046803	0.093847± 0.047376	
IL6	Female	0.000012± 0.000006	0.000009± 0.000006	0.000034± 0.000027	0.000050± 0.000044	F (1, 52) = 0.366, p = 0.548
	Male	0.000035± 0.000049	0.000014± 0.000006	0.000171± 0.000074	0.000195± 0.000137	
IL1B	Female	0.000807± 0.000415	0.000645± 0.000397	0.000744± 0.000473	0.001029± 0.000603	F (1, 52) = 0.011, p = 0.548
	Male	0.001252± 0.000728	0.000591± 0.000172	0.000572± 0.000324	0.000659± 0.000237	
TNF	Female	0.000095± 0.000052	0.000054± 0.000025	0.000168± 0.000121	0.000144± 0.000108	F (1, 52) = 0.743, p = 0.393
	Male	0.002149± 0.003104	0.000800± 0.000655	0.001013± 0.000556	0.001098± 0.000439	

Data presented as mean ± SEM. Treatment effect shows values for treatment*age*sex.

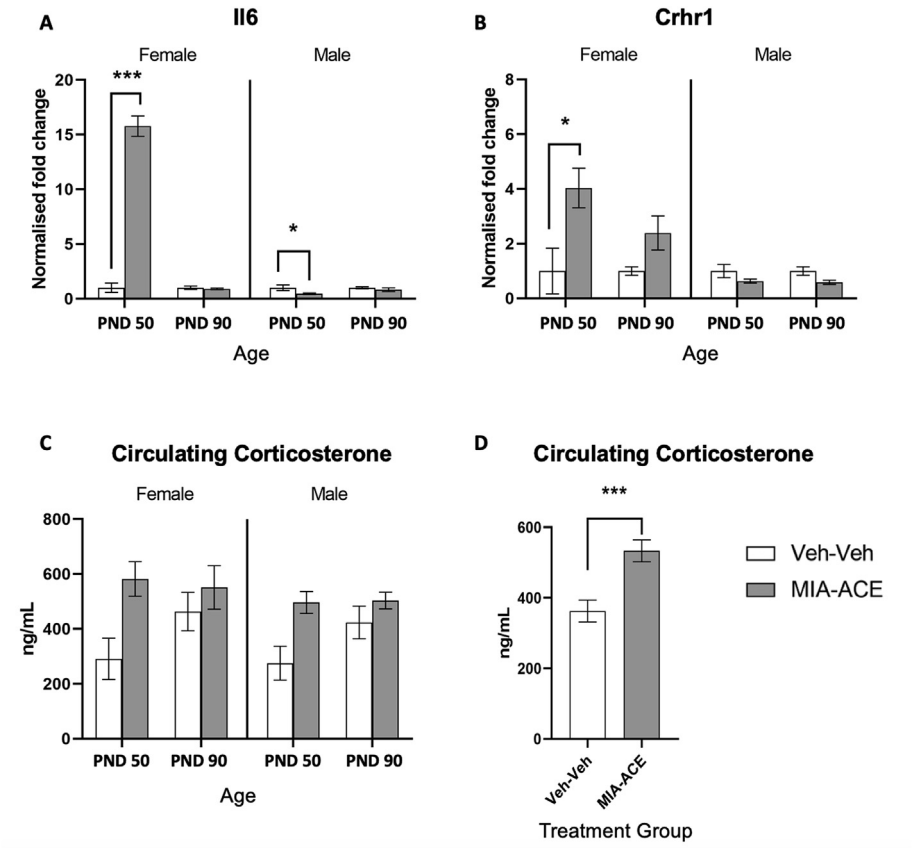

Fig. 3. Hypothalamic inflammation and HPA axis activation. A. qPCR of Il6 mRNA expression in the hypothalamus. B. qPCR of Crhr1 mRNA expression in the hypothalamus. C. Corticosterone levels in males and females during adolescence and adulthood. D. The overall effect of treatment on levels of corticosterone. A–B: The control was set at 1. A–D: Error bars indicate ±SEM. $^{*}p < 0.05$; $^{***}p < 0.001$ (n = 4–6 per group).

3.5. Adolescent MIA-ACE offspring display alterations in the HPA axis

To assess HPA axis activation, expression levels of *Crh* and *Crhr1* mRNA, as well as plasma levels of circulating corticosterone were investigated. Significantly increased *Crhr1* mRNA levels were evident in the hypothalamus of MIA-ACE adolescent females, with a pairwise comparison mean difference of 0.865, $p = 0.007$ (Fig. 3B). However, there was no significant difference in the expression of *Crhr1* mRNA levels in MIA-ACE-treated males, with a significant interaction between sex and treatment group ($F_{(1,\ 21)} = 4.601$, $p = 0.044$). There was no significant difference in the expression of *Crh* mRNA between groups ($p > 0.05$). Assessment of circulating corticosterone levels in MIA-ACE offspring revealed a trending but non-significant interaction between treatment group and age ($F_{(1,24)} = 3.869$, $p = 0.061$) with adolescent males and females having elevated circulating corticosterone levels (Fig. 3C). There was also a significant main effect of treatment group ($F_{(1,\ 24)} = 15.142$, $p = 0.001$) whereby circulating corticosterone levels in MIA-ACE offspring was significantly elevated regardless of age and sex (Fig. 3D).

3.6. Fecal microbiota aligns with previously reported age- and sex-dependent alterations in MIA-ACE offspring

When used as a probiotic, the bacteria *Bifidobacterium longum* (*B. longum*) appears to display anxiolytic-like activity in rats and beneficial psychological effects in healthy human volunteers (Messaoudi et al., 2010). The expression levels of this bacteria were examined in fecal samples of adolescent and adult MIA-ACE offspring using qPCR. This method has been previously validated as a sensitive and precise technique to detect bacteria (Delroisse et al., 2008). Pairwise comparisons identified a significant down-regulation and mean difference of 1.365 in female MIA-ACE offspring at PND 50 ($p = 0.009$) and a trending but non-significant downregulation in female MIA-ACE offspring at PND 90 ($p = 0.068$). There was no significant effect of MIA-ACE treatment in males at any age group. A significant two-way interaction ($F_{(1,\ 21)} = 9.095$, $p = 0.007$) was found between sex and treatment for *B. longum* expression in fecal samples (Fig. 4).

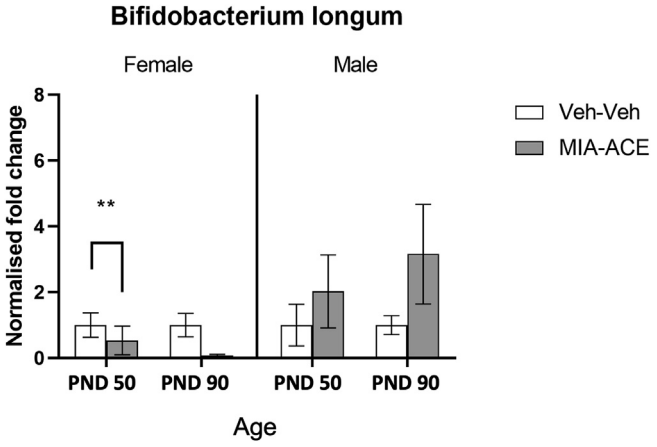

Fig. 4. Expression of fecal microflora. qPCR of *bifidobacterium longum* expression in fecal samples of male and female MIA-ACE and Veh-Veh treated offspring at PND 50 and PND 90. The control was set at 1. Error bars indicate ±SEM. $^{**}p < 0.01$ (n = 4–6 per group).

4. Discussion

There is growing and compounding evidence of the comorbidity of psychiatric disorders and perturbations in the GBA. Furthermore, environmental factors have been shown to cause alterations in neurodevelopment that are linked to the pathogenesis of neuropsychiatric disorders. Despite this, there have been relatively few studies that have examined the relationship between environmental risk factors for schizophrenia and the GBA. Research has shown that activation of the HPA axis during critical periods of fetal brain maturation, such as with in utero infection, can alter the programing of the HPA axis, impairing feedback regulation in later life (Mednick et al., 1994). Furthermore, we have previously identified that immune challenge in utero can affect glutamatergic signalling predominantly in male adult offspring (Rahman et al., 2017); can increase interstitial white matter neuron (IWMN) density in adult male and female offspring (Duchatel et al., 2016); and can induce schizophrenia-related behaviours including prepulse inhibition (PPI) deficits in male offspring, and working memory impairment in male and female offspring (Meehan et al., 2017). We have further identified that immune challenge in utero combined with adolescent exposure to cannabinoids can induce neural changes in offspring with possible outcomes relevant to neurological disorders in adulthood, such as altered gene transcription (Hollins et al., 2014, 2016b; Hollins, Walker and Cairns, 2016a) and altered neurotransmitter density and binding in males (Dalton et al., 2012). In the current study, we sought to investigate whether exposure to the dual insult of MIA and ACE produces alterations in components of the GBA.

In this study, our results show that the hypothalamus exhibited significant alterations in IL6 mRNA that were both age and sex dependent. Female MIA-ACE offspring had a significant increase (>15-fold) in expression levels of IL6 mRNA during adolescence but not adulthood. On the other hand, expression levels of IL6 mRNA were significantly decreased in male MIA-ACE offspring during adolescence but not adulthood. Increased levels of IL6 are found in schizophrenia, with levels highest in individuals experiencing acute episodes and associated with longer duration of illness and treatment resistance (Gaughran and Welch, 2008). Furthermore, IL6 is demonstrated to reduce interstitial dopamine levels and to modulate the serotonin response in the rat hypothalamus (Gaughran and Welch, 2008). In support of our findings, we have previously shown in the rat that a combination of MIA and ACE can induce abnormal elevation of serotonin $5HT_{1A}$ receptor binding in the hippocampus of late adolescent, young adult and adult offspring (Dalton et al., 2012). Altered dopamine receptor levels have also been observed in response to ACE (Dalton and Zavitsanou, 2010).

There is some evidence to suggest that HPA axis activation may be mediated by proinflammatory cytokines known to be increased in schizophrenia, such as IL1, IL6, and TNF (Gaughran and Welch, 2008). Indeed, there is a close relationship between and immune activity, the HPA axis and glucocorticoid status. Infection in utero has been demonstrated to increase plasma glucocorticoid levels and impair regulation of the HPA axis in the offspring (Hollins and Cairns, 2016). Furthermore, approximately two-thirds of patients with chronic schizophrenia exhibit abnormal salivary cortisol levels, which are both higher in adolescents, and linked to overall symptom severity (Weinstein et al., 1999).

We find a trend for an increase in circulating plasma corticosterone during adolescence in both male and female MIA-ACE offspring, with an overall significant increase in treatment rats compared to controls. Although it has been suggested RIA would produce sex-specific (females only) and generally higher basal, corticosterone, no sex specific changes were identified (Bekhbat et al., 2016; Bekhbat et al., 2018). Although we found no changes in the expression levels of CRH mRNA, studies have shown that within the hypothalamus, CRH mRNA is repressed by glucocorticoids (Jeanneteau et al., 2012) which have dual regulatory actions on CRH mRNA levels by inhibiting transcription and decreasing mRNA stability (Ma et al., 2001). Interestingly, we found an increase in the expression of the CRH receptor, CRHR1, in the hypothalamus of adolescent female MIA-ACE offspring. This is in line with our previous findings of increased expression levels of CRHR1 mRNA in adulthood following neonatal infection that correlate with increased glucocorticoid receptor mRNA levels, without changes in CRH mRNA expression (Sominsky et al., 2013). The interaction of glucocorticoids and CRH with their respective receptors during critical periods of brain maturation can alter the programing of the HPA axis (Ulupınar, 2009) with studies suggesting that CRHR1 is essential in mediating behavioural responses to stress and susceptibility to neuropathology. Alterations in this receptor in female adolescents following MIA-ACE as found in this study may indicate downstream alterations or exacerbations in schizophrenia-related behaviours such as sensorimotor gating deficits, sensitivity to psychomimetic drugs, and working memory impairments.

This finding also suggests that a second regulatory pathway may be promoting the increase in CRHR1 mRNA. In the hypothalamus, the neurotrophin brain-derived neurotrophic factor (BDNF) along with glucocorticoid signalling maintain CRH and glucocorticoid bioavailability (Jeanneteau et al., 2012). Of particular interest, research has found that gut microbiota are involved in regulating the expression of BDNF (Nemani et al., 2015), with alterations in both BDNF expression and gut microbiota found in patients with schizophrenia (reviewed in (Hollins et al., 2018)). Research shows that the bacteria *B. longum* can increase BDNF levels (Alam et al., 2017), decrease circulating corticosterone levels, and reduce schizophrenia-like behaviour (Orikasa et al., 2016). In addition to decreasing schizophrenia-like behaviours, a combination of *L. hevecticus* and *B. longum* decreased neuroinflamation in response to an environmental insult (Mohammadi et al., 2019). Furthermore, *B. longum* can suppress the progression of gastrointestinal inflammation, regulate neuroinflammation and attenuate cognitive decline, with impaired cognition now recognised as one of the core symptoms of schizophrenia (Lee et al., 2019). We therefore investigated whether the expression levels of *B. longum* would be altered in adolescent or adult offspring following exposure to MIA-ACE. We found that levels of *B. longum* were significantly decreased in fecal samples from adolescent female MIA-ACE offspring. Similarly, the female adult offspring exhibited a similar result to the adolescent offspring, although not significant. These findings may demonstrate an amelioration in microbiotic alterations by adulthood. As previous research suggests, a decrease in this bacteria could possibly account for the increased levels of circulating corticosterone and IL6 and CRHR1 mRNA found in the adolescent female MIA-ACE offspring in this study (Cussotto et al., 2018; Hollins et al., 2018; Neuman et al., 2015).

As alterations in gut microbiota can occur via a permeable intestinal barrier, we next examined the integrity of the intestinal barrier in the colon, as *B. longum* is known to be present in the distal gut of healthy humans (Gill et al., 2006). We examined mRNA expression of the epithelial tight junctions that maintain the intestinal barrier, a critical determinant in the development of intestinal inflammation and previously associated with a multitude of physiological and pathological conditions (Lee, 2015). Although previous studies have found evidence of intestinal inflammation and bacterial translocation in schizophrenia patients, indicating structural damage to the gastrointestinal barrier (Dickerson et al., 2017; Nemani et al., 2015), contrary to our expectations, we did not find any effects of the combined MIA-ACE on either intestinal barrier proteins, or inflammatory cytokines in the colon. This finding aligns with a previous report that suggests *B. longum* may signal to the CNS by activating vagal pathways at the level of the enteric nervous system to influence behavioural and neuroendocrine responses (Bercik et al., 2011).

5. Conclusions

The primary finding of this study is that immune challenge in utero combined with adolescent exposure to cannabinoids has an effect on the HPA axis in adolescent offspring, an effect that seems to be largely restricted to, or more extreme in, female offspring. To our knowledge this

is the first study to look at sex differences in this two-hit model. These alterations are accompanied by a reduction in the gut bacteria *B. longum* of adolescent female offspring which research shows could account for the HPA axis alterations and may influence the vulnerability to behavioural abnormalities in schizophrenia. Future research could explore alternative brain regions, such as The bed nucleus of the stria terminalis which provides tonic inhibitory tone over the CRH neurons in the PVN that constitute the apex of the HPA axis; expanded 16S microbiota profiling, protein confirmation and behavioural assessment. Our results suggest that this two-hit model of neuropathology may be a promising model for studying sex differences in schizophrenia, particularly during the prodromal stage, with emphasis on the role of the HPA axis.

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