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The acute effects of antimicrobials and lipopolysaccharide on the cellular mechanisms associated with neurodegeneration in pubertal male and female CD1 mice

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ABSTRACT

Exposure to stressors during puberty can cause enduring effects on brain functioning and behaviours related to neurodegeneration. However, the mechanisms underlying these effects remain unclear. The gut microbiome is a complex and dynamic system that could serve as a possible mechanism through which early life stress may increase the predisposition to neurodegeneration. Therefore, the current study was designed to examine the acute effects of pubertal antimicrobial and lipopolysaccharide (LPS) treatments on the cellular mechanisms associated with neurodegenerative disorders in male and female mice. At five weeks of age, male and female CD-1 mice received 200 μL of broad-spectrum antimicrobials or water, through oral gavage, twice daily for seven days. Mice received an intraperitoneal (i.p.) injection of either saline or LPS at 6 weeks of age (i.e., pubertal period). Sickness behaviours were recorded and mice were euthanized 8 h post-injection. Following euthanasia, brains and blood samples were collected. The results indicated that puberal antimicrobial and LPS treatment induced sex-dependent changes in biomarkers related to sickness behaviour, peripheral inflammation, intestinal permeability, and neurodegeneration. The findings suggest that pubertal LPS and antimicrobial treatment may increase susceptibility to neurodegenerative diseases later in life, particularly in males.

1. Introduction

Neurodegenerative disorders affect millions of individuals worldwide with a sex difference in the prevalence of disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), and multiple sclerosis (Attarian et al., 2015). Neurodegeneration profoundly impacts the central nervous system (CNS), influencing motor skills (e.g., gait, ataxia), cognition (e.g., memory, executive functions) and behaviours (e.g., disinhibition, apathy) (Haack et al., 2016; Levenson et al., 2014; Wirth et al., 2013). Although many theories have attempted to explain the causes of neurodegenerative disorders, little information exists on the etiology of these disorders.

Pubertal stress exposure may increase susceptibility to neurodegeneration later in life (Yahfoufi et al., 2020). Puberty is a critical developmental period marked by CNS remodeling and reorganization (Sisk and Foster, 2004), rendering the CNS particularly sensitive to stressors (Ismail et al., 2011; Murack et al., 2021; Murray et al., 2019, 2020). For example, exposure to a bacterial endotoxin, lipopolysaccharide (LPS), during puberty, causes enduring learning and spatial memory deficits in both male and female mice (Dinel et al., 2014; Kolmogorova et al., 2019) and increases Parkinson-like behaviours in male, but not in female mice (Girard-Joyal and Ismail, 2017). Pubertal LPS treatment has also been shown to increase cytokine concentrations in the periphery (e.g., IL1B, IL6, TNFα, IL-10, IL12, IFNγ) and cytokine mRNA expression in the brain (e.g., IL1B, IL6, TNFα) (Sharma et al., 2018). Furthermore, pubertal LPS treatment causes enduring decreases in glucocorticoid receptor expression in the paraventricular nucleus of the hypothalamus, in male, but not female adult mice (Smith et al., 2021). These findings are significant as alterations in both immune responsivity and stress reactivity have been implicated in the pathogenesis of neurodegeneration (Glass et al., 2010; Vyas and Maatouk, 2013). Taken together, these results suggest that stressors experienced during puberty can cause long-lasting changes in immune responsivity and stress reactivity, which can potentially increase susceptibility to

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neurodegeneration later in life.

The gut microbiome could mediate the effects of pubertal stress exposure on the immune system and possibly neurodegeneration. Growing evidence suggests that the gut microbiome plays a critical role in the development of the human brain and modulates immune responsivity (Borre et al., 2014; Jašarević et al., 2016). The gut microbiome hosts billions of metabolic, inflammatory, and immune regulating microorganisms (Fung et al., 2017; Qin et al., 2010). Amongst them are bacterial strains that influence neurological functioning and behavioural outcomes (Qin et al., 2010). Microbiota communicate with the brain via enteric and autonomic neuroimmune and neuroendocrine pathways referred to as the "gut-brain" axis. Alterations to gut microbiota results in 'gut dysbiosis' and may negatively affect immunomodulation and cause psychiatric disorders (Dinel et al., 2014; Girard-Joyal and Ismail, 2017; Murray et al., 2019). For example, antimicrobial-induced dysbiosis in mice increases the expression of complement 3 (C3) and the anaphylatoxin, C3a, which are associated with the onset and progression of multiple sclerosis (Yadav et al., 2017). Moreover, antimicrobial-induced dysbiosis increases oxidative stress and the concentrations of cytokines and chemokines, as well as the deposition of amyloid beta plaques in mice (Erny et al., 2015; Minter et al., 2016).

Alterations to the composition of the gut microbiome are also implicated in the modulation of several biomarkers associated with the development of neurodegenerative disorders. For example, Crohn's disease is associated with the upregulation of leucine-rich repeat kinase 2 (LRRK2), a gene that plays an important role in both sporadic and familial PD (Barrett et al., 2008; Fava et al., 2016; Liu et al., 2011). Other research has demonstrated that LPS-induced dysbiosis in mice significantly increases alpha-synuclein expression and decreases tyrosine hydroxylase (TH) expression, two proteins that are involved in the pathogenesis of PD (Hunter et al., 2009; Kelly et al., 2014). Thus, gut dysbiosis influences immune responsivity and can have far-reaching effects on cellular mechanisms associated with neurodegeneration.

Although gut dysbiosis seems to play a role in the development of neurodegenerative disorders, the mechanisms mediating these effects remain unknown. One potential explanation may be the effects of dysbiosis on intestinal permeability (Spielman et al., 2018). Gut dysbiosis has been shown to increase intestinal permeability through the effects of cytokines and chemokines on tight-junction proteins (Kacimi et al., 2011; Leclercq et al., 2014). For example, gut dysbiosis induced by a high-fat diet increases intestinal permeability by reducing the expression of the tight-junction proteins, zonula occludens-1 and occludin in mice (Cani et al., 2008). This increase in intestinal permeability coincides with an increase in plasma LPS along with an increase in serum IL1 and TNFα (Cani et al., 2008). Therefore, dysbiosis can alter intestinal permeability, allowing signalling molecules to cross the intestinal epithelial barrier, enter the circulatory system, and reach the CNS, where they can potentially play a role in the development of neurodegenerative disorders.

The majority of studies examining the effects of LPS and antimicrobial treatment on neurodegenerative disorders focus on the long-term effects of LPS and antimicrobial treatment on adult male subjects. There is a lack of knowledge on the potential mechanistic influence of LPS and antimicrobial treatment on the development of neurodegenerative disorders during critical periods of development, like puberty. Moreover, little is known about the neurodegenerative mechanisms that may affect males and females differently. Therefore, the objective of this study was to examine the acute effects of antimicrobial and LPS treatment on the cellular mechanisms associated with neurodegeneration in male and female mice. We hypothesized that antimicrobial and LPS treatment would not change the expression of TH, increase the expression of biomarkers related to neurodegeneration (i.e., SNCA, C3, LRRK2, pro-inflammatory cytokines), decrease the expression of biomarkers that can potentially slow down neurodegeneration (i.e., occludin, anti-inflammatory cytokines) and increase sickness behaviours, in a sex-dependent manner.

2. Methodology

2.1. Animals

Ninety-six male and female CD-1 mice were shipped from Charles River Laboratories (Saint-Constant, Québec, Canada) at three weeks of age (see Fig. 1). Mice were pair-housed in sex-specific rooms and were kept on a reversed light cycle (lights off at 1000 h) under standard conditions (14 h:10 h light/dark cycle; 24 ± 2 °C; relative humidity of 40 ± 5). Mice were housed in polycarbonate Lexan housing cages (17 cm wide × 28 long × 12 cm high) that were bedded with Teklad Corn Cob bedding (Harlan Laboratories, Inc., Madison, WI, USA) and enriched with one square piece of Nestlet (Ancare Corp., Bellmore, NY, USA) and a cardboard refuge hut (Ketchum Manufacturing, Inc., Brockville, ON, Canada). Food (Harlan Laboratories, Inc., Madison, WI, US, T2018 – Global 18% rodent) and water were available ad libitum. All observational tests were completed during the dark phase under dim red light unless specified. Under our housing conditions, CD-1 female mice demonstrate vaginal opening at approximately 30 days following birth and begin estrous cycling around 60 days of age (Murray, Butcher, Kearns, Lamba, Liang, Stintzi, and Ismail, *under review*). Measurements of preputial separation in male mice are difficult to analyze, however, measurements of scrotum width in six-week-old male mice indicate that the scrotum has not reached adult size (Murray, Butcher, Kearns, Lamba, Liang, Stintzi, and Ismail, *under review*). Therefore, our six-week-old male and female mice are pubescents. All experiments were approved by the Animal Care Committee of the University of Ottawa.

2.2. Antimicrobial treatment

At five weeks of age, mice were administered 200 μL of mixed broad-spectrum antimicrobial solution or water through gavage twice daily for 7 day as described in (Esposito et al., 2022). Briefly, the antimicrobial solution was made fresh daily and contained 15 mg/mL of ampicillin (No. BP1760-5, Fisher Scientific, Geel, Belgium), neomycin (No. 480125 GM, EMD Millipore Corp, MA, USA), streptomycin (NO. BP910-50, Alfa Aesar, Fisher Scientific, Ottawa, ON), and 10 mg/mL of metronidazole (No. AC210340050, Acros Organics, New Jersey, USA) in distilled water. The treatments were administered at 0600 h and 1800 h, respectively. This dosage and treatment regimen have been shown to sufficiently suppress total microbial content (Zarrinpar et al., 2018).

2.3. Lipopolysaccharide administration

Six-week-old mice received an intraperitoneal (ip) injection of either 1.5 mg/kg of LPS (*Escherichia coli* seroptype O26:B6; L#3755; Sigma Chemical Co., St. Louis, MO, USA) or an equivalent volume of 0.9% sterile saline at the end of the light cycle. This dose of LPS was chosen because it has been previously shown to induce sexually dimorphic sickness behaviours for approximately 24–48 h (Cai et al., 2016).

2.4. Sickness monitoring

Sickness monitoring was conducted at 2, 4, 6, and 8 h after injection. Assessment of the progression of sickness behaviours followed a non-invasive and unbiased approach with two raters blind to the experimental conditions (as described in Kolmogorova et al., 2017). The raters visually assessed the mice for symptoms including lethargy (reduced locomotion), huddling (curled body posture), ptosis (drooping eyelids), and pilo-erection (erection of fur). At each time-point, the raters scored the total number of symptoms displayed by each mouse (one symptom = 1, two symptoms = 2, three symptoms = 3, four symptoms = 4). Sickness scores at each time-point from the two raters were averaged and used in statistical analyses.

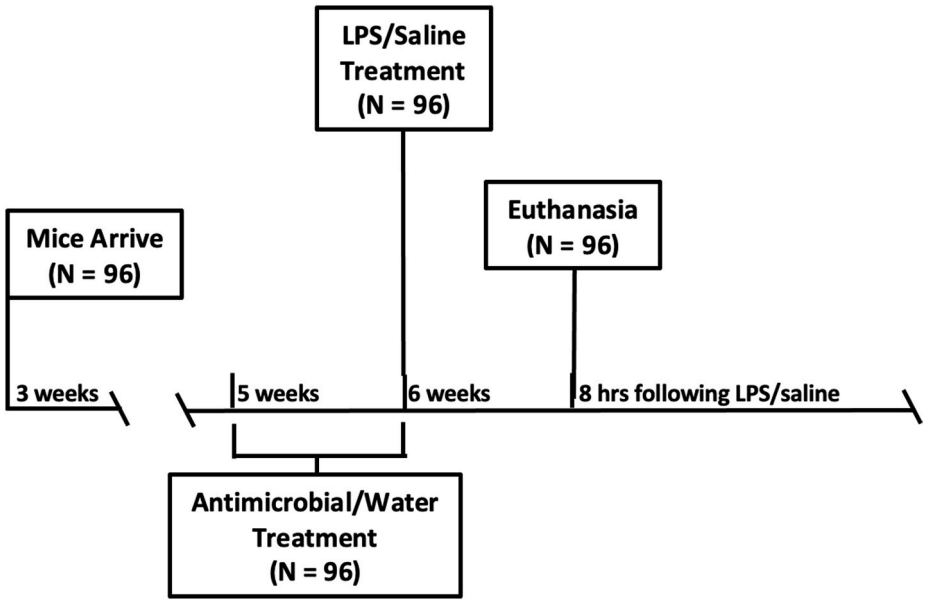

Fig. 1. Experimental timeline of mice examined for the acute effects of pubertal LPS and antimicrobial treatment on cellular mechanism associated with neurodegeneration.

2.5. Plasma extraction

Eight hours following the saline or LPS treatment, mice were anesthetized with Euthanyl (Sodium pentobarbital; 500 mg/kg, ip). Mice were assessed for motor reflexes by gently pinching their feet. Once no motor reflexes were detected, blood was collected by cardiac puncture and placed into Microvette CB 300 K2E blood extraction tubes (Sarstedt AG & Co, Nümbrecht, Germany) that were coated with an anticoagulant, EDTA. Tubes were kept at 4 °C until plasma extraction. Within 3 h of blood collection, samples were centrifuged at 1000×*g* at 20 °C for 15 min to separate plasma. Plasma was extracted and stored in aliquots at −80 °C.

2.6. Brain tissue extraction

Following blood collection, mice were decapitated and brains were extracted and flash-frozen in liquid nitrogen and stored at −80 °C until processing. The brain tissue was sliced with a LEICA CM1950 cryostat at 300 μM, and tissue from the caudate-putamen (CP) and substantia nigra (SN) were dissected and placed into RNA-free tubes. Tubes were stored at −80 °C until RNA extraction.

2.7. Ileum tissue extraction

The ileum was extracted and stored at −80 °C until processing. The ileum tissue was dissected open longitudinally on ice and washed with Phosphate-Buffered saline (PBS; 3.45 gm Na2HPO4, 0.78 gm NaH2-PO4•H2O, 24 gm NaCl, 0.6 gm KCl, 3L dH2O). Following the washing step, the luminal surface of the ileum was scraped off and was stored at −80 °C until protein extraction.

2.8. mRNA extraction and cDNA synthesis

PureLink RNA Mini Kit (No. 12183020; Thermo-Fisher Scientific) was used according to the manufacturer's instructions to extract mRNA from CP and SN tissue. Extracted mRNA was then incubated with gDNA wipeout buffer to remove genomic DNA prior to cDNA synthesis. cDNA was synthesized with the QuantiTect Reverse Transcription kit (No. 205311; QIAGEN). The products of the cDNA synthesis step were used in subsequent real-time quantitative PCR.

2.9. Real-time quantitative polymerase chain reaction (RT-qPCR)

Relative gene expression was assessed using the SsoAdvanced Universal SYBR Green Supermix (No. 1725274; Bio-Rad) in triplicates of 10 μL reactions on the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Primer efficiency was determined using the slope between RNA quantity and cycle thresholds with CFX Maestro software (Bio-Rad). All primer pairs achieved reaction efficiency between 90 and 110%. β-actin was used as a housekeeping gene for all samples and did not change significantly across experimental conditions. For each reaction, the quantitative threshold amplification cycle number (CQ) was determined, and log transformed as previously described (Taylor et al., 2019). The primers were ordered from Integrated DNA technologies and the primer sequences are displayed in Table 1.

2.10. Multiplex immunoassay

Plasma concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 2 (IL2), interleukin 23 (IL23), interleukin 10 (IL10), interleukin 12 (p70) (IL12), and interleukin 17A (IL17A) were measured with a multiplex bead-based Luminex immunoassay. Multiplex kits (No. MTH17MAG-47K; Millipore-Sigma) were used according to the supplier's instructions, and plasma samples were measured in duplicates. Each plate contained one pooled sample to monitor the inter-assay variation. The MAGPIX system was used to measure the final cytokine concentrations.

2.11. Enzyme-linked immunosorbent assay (ELISA)

Plasma concentrations of fatty acid binding protein 2 (FABP2) were measured with an ELISA. The ELISA kit (No. SEA559Mul; Cloud-Clone Corp.) was used according to the manufacturer's instructions and plasma samples were measured in duplicates. Each plate contained one

Table 1
Summary of primer sequences.

Target Gene	Forward	Reverse
β-actin	GAACCCTAAGGCCAACCGTG	GGTACGACCAGAGGCATACAGG
LRRK2	GCCACGAATCTCAATAGCAAG	CCAAAGCCAAGCACAGTATTC
SNCA	CTTTAGCCATGGATGTGTTCA	TTGTCTTTCCAGCTGCCTCT

pooled sample to monitor inter-assay variation. All plates were read with Biotek Powerwave XS2 and analyzed with the Gen 5 V2.0 software. Three samples had intra-assay CVs greater than 15% and were excluded from the analyses.

2.12. Quantitative western blot analyses

The dissected CP, SN and ileum tissues were first homogenized in tissue protein extraction reagent buffer (T-PER; Thermo Scientific, ref: 78510) containing the protease inhibitors Roche PhosSTOP™ (Millipore Sigma; cat: 04001) and Roche cOmplete™ ULTRA Tablets EDTA-free (Millipore Sigma; cat: 05001). The brain homogenates were incubated on ice for 10 min, then centrifuged at 4 °C at 19,000 *g* for 20 min. The ileum homogenates were incubated on ice for 10 min, then centrifuged 3 times at 4 °C at 21,000 g for 10 min. The supernatants were then collected to assay total protein concentrations using the Pierce™ Bicinchoninic Acid Assay (BCA) protein assay kit (Thermo Fisher Scientific). A total of 15 μg (brain samples) and 17 μg (ileum samples) of protein were mixed with a loading buffer containing 2-Mercaptoethnol and then heated to 95 °C for 5 min. Samples were electrophoresed on a 12% polyacrylamide gel (TGX Stain-Free™ FastCast™ Acrylamide Kit; Bio-Rad; cat: 1610185) using Mini-PROTEAN® 3 Dodeca™ Cell system (Bio-Rad). A pooled protein sample was loaded to each of the gels to account for inter-gel variation. The gel image was then collected with ChemiDoc™ XRS + System (Bio-Rad). These images were used for protein loading normalization instead of running a housekeeping protein as referenced (Eaton et al., 2013; Fosang and Colbran, 2015). The separated proteins were transferred onto 0.2 μm nitrocellulose membranes with Trans-Blot® Electrophoretic Transfer Cell system (Bio-Rad). The nitrocellulose membrane for brain samples was blocked for 1 h at room temperature in a blocking solution containing 5% skim milk in PBS. The nitrocellulose membrane for ileum samples were treated with 4% paraformaldehyde (PFA) at room temperature for 30 min and were then blocked for 1 h in a blocking solution containing 5% skim milk in PBS. The nitrocellulose membranes were then incubated overnight at 4 °C in a solution of 5% milk/Tris-buffered saline in 0.1% Tween® 20 detergent (TBST; 20 mM Tris base, 137 mM NaCl, 0.1% Tween 20) containing rabbit anti-C3 (1/2000; Abcam; cat: AB200999) and rabbit anti-TH (1/20000; Abcam; cat: AB137869) (brain samples), or rabbit anti-occludin (1/400; Abcam; cat: AB167161) (ileum samples). The membranes underwent 3 × 10-min washes with TBST and were then incubated for 1 h at room temperature with relevant goat anti-rabbit IRDye® 800CW secondary antibodies (LiCor Biosciences; cat: 92632211) in a 5% skim milk/TBST solution. Following another set of 3 × 10-min washes with TBST, protein intensity was quantified using Li-Cor Biosciences Odyssey® software. The intensity was then normalized using the loaded protein measurement from the gel image. Finally, to control for inter-gel variation, the ratio of the normalized protein intensity between each sample to their intra-gel pooled sample was calculated and presented as a fold change (mean of duplicate ± SEM), which was used for relative protein abundance comparison between groups.

2.13. Statistical analyses

All statistical analyses were performed using IBM SPSS v20 software. Cases that exceeded the 1.5 interquartile range in boxplot analyses (western blot, RT-qPCR, multiplex, ELISA, and sickness behaviour data) were considered statistical outliers and were limited, by winsorization to the next outer-most score within the 1.5 interquartile range (Hastings et al., 1947). For measures of sickness behaviours, a four-way mixed analysis of variance (ANOVA) was used to measure the within-subject effects of time (2, 4, 6, and 8 h) and the between-subject effects of sex (male or female), antimicrobial treatment (AMNS or water), and LPS treatment (LPS or saline). Greenhouse-Geisser corrections were applied to F-values that violated Mauchly's test of sphericity. For all other measures (i.e., western blot, RT-qPCR, multiplex, ELISA), a 2 × 2 x 2 ANOVA was performed for sex (male or female), antimicrobial treatment (AMNS or water), and LPS treatment (LPS or saline). When appropriate statistically significant effects were followed by pairwise comparisons with Bonferroni corrections. Measures of effect sizes were estimated using partial eta-squared (η_p^2). Statistical significance was set to $p < 0.05$.

3. Results

3.1. Sickness behaviours

The four-way mixed ANOVA violated Mauchly's Test of Sphericity ($p < 0.05$), and all within-subject effects were assessed with Greenhouse-Geisser corrections. The ANOVA found a significant within-subjects main effect of time ($F_{(2.6,48.5)} = 52.51$, $p < 0.01$, $\eta_p^2 = 0.34$) and a time x LPS interaction ($F_{(2.6,48.5)} = 52.51$, $p < 0.01$, $\eta_p^2 = 0.34$). The three-way mixed ANOVA also found a significant between subjects' main effect of LPS ($F_{(1, 104)} = 674.80$, $p < 0.01$, $\eta_p^2 = 0.87$) and a significant sex x LPS interaction ($F_{(1, 104)} = 4.64$, $p = 0.40$, $\eta_p^2 = 0.04$). Pairwise comparisons showed that regardless of sex and AMNS treatment, mice treated with LPS displayed significantly greater sickness behaviours in comparison to their SAL-treated counterparts ($MD = 2.42$, $SE = 0.09$, $p < 0.01$; Fig. 2A and B). Mice treated with LPS displayed significantly less sickness behaviours 2 h following treatment in comparison to 4 ($MD = 0.62$, $SE = 0.07$, $p < 0.01$), 6 ($MD = 1.04$, $SE = 0.09$, $p < 0.01$) and 8 h ($MD = 1.22$, $SE = 0.09$, $p < 0.01$; Fig. 2A and B) following treatment. Moreover, LPS-treated males displayed significantly more sickness behaviours then LPS-treated females at 2 ($MD = 0.61$, $SE = 0.14$, $p < 0.01$), 6 ($MD = 0.50$, $SE = 0.16$, $p = 0.02$), and 8 ($MD = 0.41$, $SE = 0.09$, $p = 0.12$) hours following treatment.

3.2. Peripheral plasma GM-CSF concentrations

The ANOVA found a significant main effect of sex ($F_{(1, 72)} = 4.49$, $p = 0.04$, $\eta_p^2 = 0.06$). Pairwise comparisons showed that regardless of LPS and AMNS treatments, males displayed significantly greater GM-CSF concentrations in comparison to their female counterparts ($MD = 6.23$, $SE = 3.13$, $p = 0.04$). Furthermore, LPS-treated females had significantly greater GM-CSF concentrations in comparison to their SAL-treated counterparts ($MD = 9.79$, $SE = 4.23$, $p = 0.03$). LPS-treated males did not show any significant difference in GM-CSF concentration in comparison to their SAL-treated counterparts ($MD = 0.63$, $SE = 4.23$, $p = 0.89$; Fig. 3A).

3.3. Peripheral plasma IL2 concentrations

The ANOVA found significant sex x AMNS treatment ($F_{(1, 72)} = 5.89$, $p = 0.02$, $\eta_p^2 = 0.08$), sex x LPS ($F_{(1, 72)} = 10.41$, $p < 0.01$, $\eta_p^2 = 0.13$), and AMNS treatment x LPS ($F_{(1, 72)} = 5.89$, $p = 0.02$, $\eta_p^2 = 0.08$) interactions. Pairwise comparisons showed that regardless of sex, CTL-LPS treated mice displayed significantly less IL2 concentrations in comparison to CTL-SAL treated mice ($MD = 0.57$, $SE = 0.26$, $p = 0.03$). CTL-treated males and SAL-treated males displayed significantly greater IL2 concentrations in comparison to their female counterparts ($MD = 0.57$, $SE = 0.26$, $p = 0.03$; $MD = 0.71$, $SE = 0.26$, $p < 0.01$, respectively). Moreover, LPS-treated males displayed significantly less IL2 concentrations in comparison to their SAL-treated counterparts ($MD = 0.71$, $SE = 0.26$, $p = 0.01$). LPS-treated females did not show any significant differences in IL2 concentrations in comparison to their SAL-treated counterparts ($MD = 0.45$, $SE = 0.26$, $p = 0.08$; Fig. 3B).

3.4. Peripheral plasma IL10 concentrations

The ANOVA found a significant main effect of LPS ($F_{(1, 72)} = 129.79$, $p < 0.01$, $\eta_p^2 = 0.64$) and significant sex x AMNS treatment ($F_{(1, 72)} =$

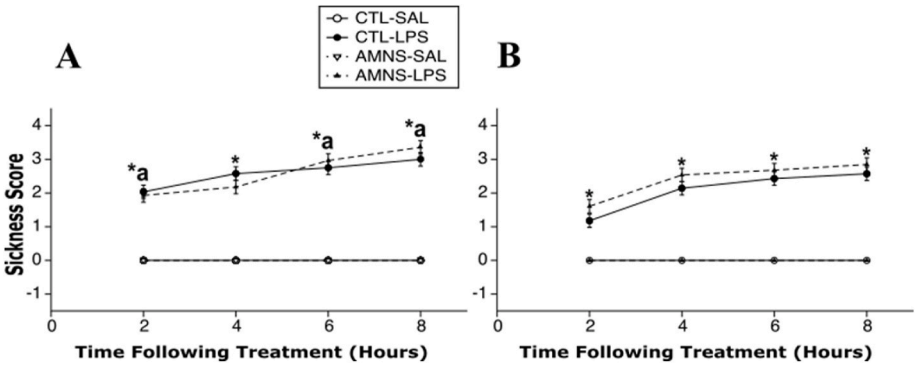

Fig. 2. Mean (±SEM) sickness scores of six-week-old (A) male and (B) female mice treated with either saline (SAL) or lipopolysaccharide (LPS), and with either water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS), n = 10/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$). (a) denotes a significant difference between male and female counterparts ($p < 0.05$).

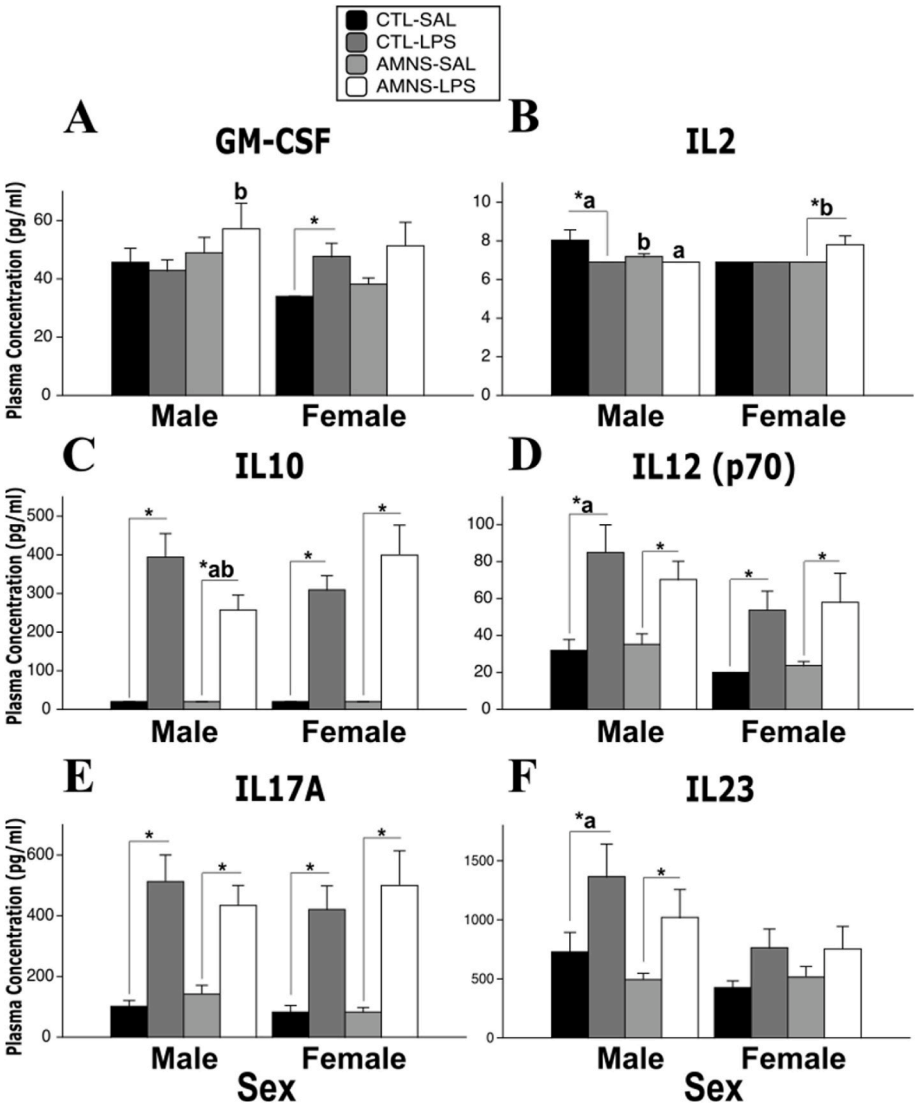

Fig. 3. Mean (±SEM) acute plasma (A) GM-CSF concentrations, (B) IL2 concentrations, (C) IL10 concentrations, (D) IL12 (p70) concentrations, (E) IL17A concentrations, and (F) IL23 concentrations of six-week-old mice treated with either saline (SAL) or lipopolysaccharide (LPS), and either water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS), n = 20/group. Data represented as mean fold change (±SEM), n = 10/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (a) denotes a significant difference between male and female counterparts ($p < 0.05$) and (b) denotes a significant difference between water and antimicrobial treatments ($p < 0.05$).

4.08, $p = 0.05$, $\eta_p^2 = 0.05$), and sex x AMNS treatment x LPS ($F_{(1, 72)} = 4.08$, $p = 0.05$, $\eta_p^2 = 0.05$) interactions. Pairwise comparisons showed that regardless of sex and AMNS treatment, LPS-treated mice displayed significantly greater IL10 concentrations in comparison to their SAL-treated counterparts ($MD = 319.84$, $SE = 28.07$, $p < 0.01$). Furthermore, AMNS-LPS treated males displayed significantly less IL10 concentrations in comparison to their CTL-LPS treated counterparts ($MD = 136.84$, $SE = 56.15$, $p = 0.02$). AMNS-LPS- treated females did not display any significant differences in comparison to their CTL-LPS treated counterparts ($MD = 89.94$, $SE = 56.15$, $p = 0.11$). Lastly,

AMNS-LPS treated males displayed significantly less IL10 concentrations in comparison to their AMNS-LPS treated female counterparts ($MD = 141.97$, $SE = 56.15$, $p = 0.01$; Fig. 3C).

3.5. Peripheral plasma IL12 (p70) concentrations

The ANOVA found significant main effects of LPS ($F_{(1, 72)} = 35.75$, $p < 0.01$, $\eta_p^2 = 0.33$) and sex ($F_{(1, 72)} = 8.05$, $p < 0.01$, $\eta_p^2 = 0.07$). Pairwise comparisons showed that regardless of sex and AMNS treatment, LPS-treated mice displayed significantly greater IL12 (p70) concentrations in comparison to their SAL-treated counterparts ($MD = 38.58$, $SE = 6.45$, $p < 0.01$). Furthermore, LPS-treated males displayed significantly greater IL12 (p70) concentrations in comparison to their LPS-treated female counterparts ($MD = 23.44$, $SE = 9.13$, $p = 0.01$; Fig. 3D).

3.6. Peripheral plasma IL17A concentrations

The ANOVA found a significant main effect of LPS ($F_{(1, 72)} = 67.89$, $p < 0.01$, $\eta_p^2 = 0.49$). Pairwise comparisons showed that regardless of sex and AMNS treatment, LPS-treated mice displayed significantly greater IL17A concentrations in comparison to their SAL-treated counterparts ($MD = 350.29$, $SE = 42.51$, $p < 0.01$; Fig. 3E).

3.7. Peripheral plasma IL23 concentrations

The ANOVA found a significant main effect of LPS ($F_{(1, 72)} = 8.54$, $p < 0.01$, $\eta_p^2 = 0.11$). and of sex ($F_{(1, 72)} = 6.03$, $p = 0.02$, $\eta_p^2 = 0.08$). Pairwise comparisons showed that regardless of sex and AMNS treatment, LPS-treated mice displayed significantly greater IL23 concentrations in comparison to their SAL-treated counterparts ($MD = 382.10$, $SE = 130.74$, $p < 0.01$). Furthermore, LPS-treated males displayed significantly greater IL23 concentrations in comparison to LPS-treated females ($MD = 434.27$, $SE = 184.90$, $p = 0.02$; Fig. 3F).

3.8. Assessment of FABP2 concentrations with ELISA

The ANOVA found a significant main effect of LPS ($F_{(1, 69)} = 7.73$, $p = 0.01$, $\eta_p^2 = 0.10$) and significant sex x AMNS treatment ($F_{(1, 69)} = 4.79$, $p = 0.03$, $\eta_p^2 = 0.07$) and sex x AMNS treatment x LPS ($F_{(1, 69)} = 13.14$, $p < 0.01$, $\eta_p^2 = 0.16$) interactions. Pairwise comparisons showed that regardless of sex and AMNS treatment, LPS-treated mice displayed significantly less FABP2 concentrations in comparison to their SAL-treated counterparts ($MD = 1.42$, $SE = 0.51$, $p = 0.01$). AMNS-SAL treated males displayed significantly greater FABP2 concentrations in comparison to their AMNS-SAL treated female counterparts ($MD = 2.59$, $SE = 1.10$, $p = 0.02$). AMNS-LPS treated males displayed significantly less FABP2 concentrations in comparison to their AMNS-SAL treated counterparts ($MD = 2.94$, $SE = 1.03$, $p = 0.01$). Furthermore, CTL-LPS treated females displayed significantly less FABP2 concentrations in comparison to their CTL-SAL treated counterparts ($MD = 3.62$, $SE = 1.03$, $p < 0.01$; Fig. 4).

3.9. C3 and TH protein expressions in the CP and SN

The ANOVA did not show any significant difference in C3 or TH expression in the CP (Fig. 5A and B). Furthermore, no significant difference in TH expression was found in the SN (Fig. 5D). However, a significant main effect of LPS ($F_{(1, 48)} = 5.11$, $p = 0.03$, $\eta_p^2 = 0.10$) and a significant sex x LPS ($F_{(1, 58)} = 6.84$, $p = 0.01$, $\eta_p^2 = 0.13$) interaction was found for C3 in the SN. Pairwise comparisons for C3 expression in the SN showed that regardless of sex and AMNS treatment, LPS-treated mice displayed significantly less C3 expression in comparison to their SAL-treated counterparts ($MD = 1.31$, $SE = 0.58$, $p = 0.03$). SAL-treated males displayed significantly greater C3 expression in comparison to their SAL-treated female counterparts ($MD = 2.02$, $SE = 0.82$, $p = 0.02$). Furthermore, LPS-treated males displayed significantly less C3 expression in comparison to their SAL-treated counterparts ($MD = 2.82$, $SE = 0.82$, $p = 0.01$). LPS-treated females did not show any significant in C3 expression in comparison to their SAL-treated counterparts ($MD = 0.21$, $SE = 0.82$, $p = 0.80$; Fig. 5C).

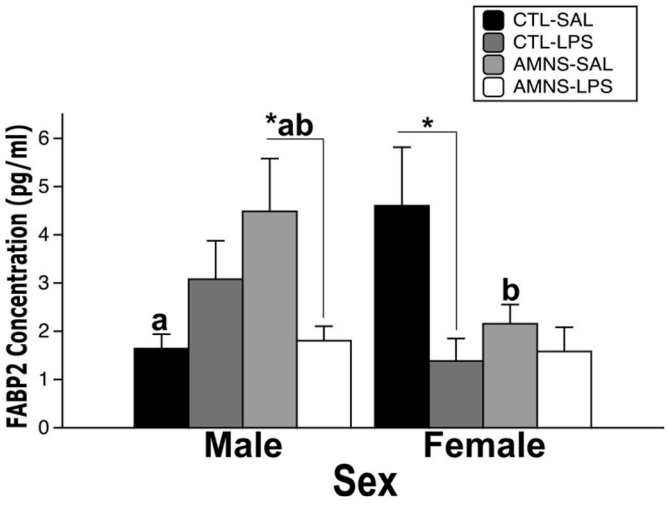

Fig. 4. Mean (±SEM) acute plasma FABP2 concentrations of six-week-old mice treated with either saline (SAL) or lipopolysaccharide (LPS), and either water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS), n = 10/group. The asterisks (*) denote a significant difference between LPS and saline counterparts ($p < 0.05$), (a) denotes a significant difference between male and female counterparts ($p < 0.05$) and (b) denotes a significant difference between water and antimicrobial treatments ($p < 0.05$).

3.10. Occludin protein expression in the ileum

The ANOVA found significant main effects of LPS ($F_{(1, 48)} = 13.60$, $p < 0.01$, $\eta_p^2 = 0.22$) and AMNS treatment ($F_{(1, 48)} = 7.20$, $p = 0.01$, $\eta_p^2 = 0.13$). The ANOVA also found a significant sex x LPS ($F_{(1, 48)} = 1.70$, $p = 0.05$, $\eta_p^2 = 0.08$) interaction. Pairwise comparisons showed that regardless of sex and AMNS treatment, LPS-treated mice displayed significantly greater occludin expression in comparison to their SAL-treated counterparts ($MD = 0.64$, $SE = 0.17$, $p < 0.01$). AMNS-treated mice displayed significantly greater occludin expression in comparison to their CTL-treated counterparts ($MD = 0.50$, $SE = 0.17$, $p = 0.01$). Furthermore, LPS-treated females displayed significantly greater occludin expression in comparison to their LPS-treated male counterparts ($MD = 0.69$, $SE = 0.25$, $p = 0.01$; Fig. 5E).

3.11. SNCA and LRRK2 mRNA expressions in the CP and SN

The ANOVA found a significant main effect of LPS for SNCA and LRRK2 in the CP ($F_{(1, 48)} = 7.33$, $p = 0.001$, $\eta_p^2 = 0.13$; $F_{(1, 48)} = 9.23$, $p < 0.001$, $\eta_p^2 = 0.16$; respectively). No significant difference was shown for SNCA or LRRK2 mRNA expression in the SN. Pairwise comparisons showed that regardless of sex and AMNS treatment, LPS-treated mice displayed significantly greater SNCA and LRRK2 mRNA expressions in the CP in comparison to their SAL-treated counterparts ($MD = 0.68$, $SE = 0.25$, $p = 0.001$; $MD = 0.85$, $SE = 0.28$, $p < 0.001$; respectively; see Fig. 6).

4. Discussion

Neurodegenerative disorders are one of the leading causes of mortality and morbidity worldwide. Although the etiology of neurodegenerative disorders is unknown, alterations to the gut microbiome during puberty may increase susceptibility to neurodegeneration later in life. Several studies have linked LPS and antimicrobial treatment with

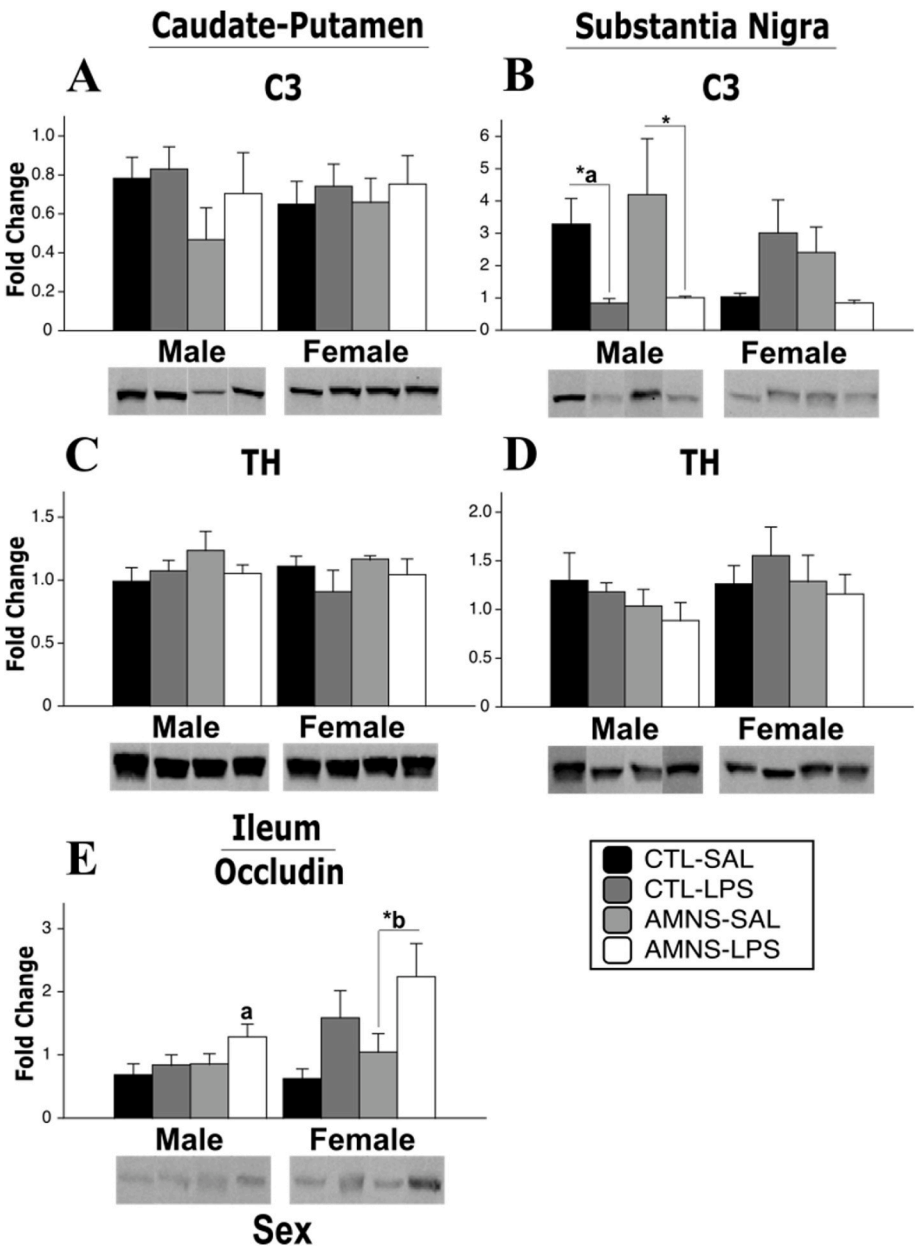

Fig. 5. Mean (±SEM) acute protein (A) C3 expression in the CP, (B) C3 expression in the SN, (C) TH expression in the CP, (D) TH expression in the SN, and (E) occludin expression in the ileum of six-week-old mice treated with either saline (SAL) or lipopolysaccharide (LPS), and either water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS), n = 7/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (a) denotes a significant difference between male and female counterparts ($p < 0.05$) and (b) denotes a significant difference between water and antimicrobial treatments ($p < 0.05$).

neurodegeneration in adult male subjects (Batista et al., 2019; Brandscheid et al., 2017; Fröhlich et al., 2016; Wang et al., 2019; Zhao et al., 2019). However, the effects of LPS and antimicrobial treatment on neurodegeneration in pubertal males and females are unknown. Thus, this study was designed to investigate the acute effects of pubertal LPS and antimicrobial treatment on the cellular mechanisms associated with neurodegeneration in male and female mice. Our results showed that LPS and antimicrobial treatments were associated with increased sickness behaviours and sex-specific alterations in peripheral inflammation (i.e., IL12(p70), IL17A, IL23, IL10), intestinal permeability (i.e., occludin and FABP2), and markers related to neurodegeneration (i.e., C3, LRRK2, SNCA).

AMNS-LPS treated male and female mice displayed increased sickness behaviours. Moreover, AMNS-LPS treated male mice displayed greater plasma pro-inflammatory cytokines (e.g., IL12 (p70), IL17A, IL23) and less plasma anti-inflammatory cytokines (e.g., IL10) in comparison to their female counterparts. These findings support our hypotheses and are consistent with previous research linking LPS with alterations in immune responsivity and sickness behaviours (Cai et al., 2016; Kolmogorova et al., 2017; Sharma et al., 2018). IL17A and IL23 are pro-inflammatory cytokines that have been shown to play a role in the development of neurodegeneration (Brigas et al., 2021; Chen et al., 2020; Nitsch et al., 2021; Zheng et al., 2016). IL17A is commonly believed to be produced by $CD4^+$ lymphocytes (i.e., Th17 cells) (Ruiz de Morales et al., 2020). Th17 cells also express IL23-receptors and require IL23 for cell proliferation and survival (Gaffen et al., 2014). However, IL17A can also be produced in the absence of IL23 by $CD8^+$ lymphocytes as well as some natural killer cells which are rapidly activated after an infection or injury (Srenathan et al., 2016). It is theorized that in the absence of IL23, IL17A has a non-pathogenic phenotype that promotes tissue repair and regulates barrier functions (Wu et al., 2018). However, in the presence of IL23, IL17A exhibits a pathogenic phenotype that can produce a detrimental immune response (McAleer and Kolls, 2011). Therefore, while females possess a non-pathogenic phenotype, increases in IL17A and IL23 concentrations following AMNS-LPS treatment in males suggest that they possess a pathogenic phenotype that may increase their susceptibility to neurodegeneration later in life.

The sex-specific effects of AMNS-LPS treatment on plasma cytokine

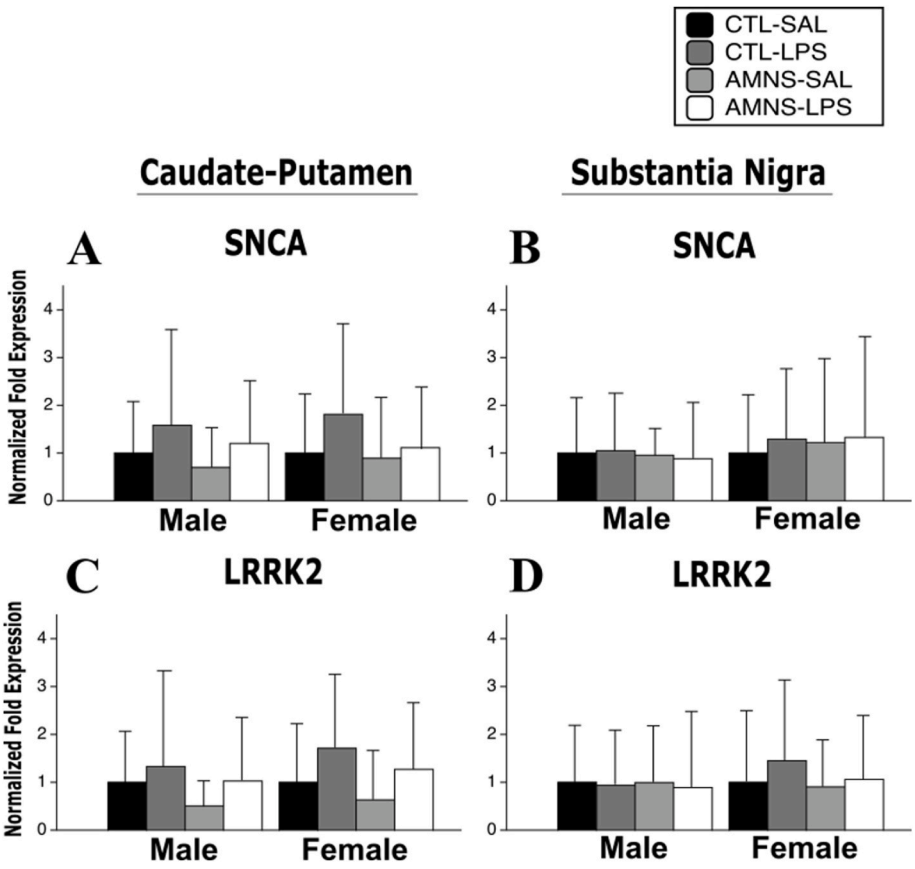

Fig. 6. Geomean (±SEM) of acute mRNA (A) SNCA expression in the CP, (B) SNCA expression in the SN, (C) LRRK2 expression in the CP, and (D) LRRK2 expression in the SN of six-week-old mice treated with either saline (SAL) or lipopolysaccharide (LPS), and either water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS), n = 7/group. The asterisks (*) denote a significant difference between LPS and saline counterparts ($p < 0.05$), and (b) denotes a significant difference between water and antimicrobial treatments ($p < 0.05$).

concentrations may be due to the sex differences in circulating gonadal hormones levels. Estradiol is believed to enhance the immune response while testosterone has a negative impact on it (Taneja, 2018). For example, estradiol replacement in ovariectomized rats treated with formalin significantly decreases pro-inflammatory cytokine (i.e., TNFα and IL1β) expression and increases anti-inflammatory cytokine (i.e., IL10) expression (Shivers et al., 2015). Conversely, dihydrotestosterone treatment promotes a harmful inflammatory response in rats by increasing nuclear factor kapa B activation and increasing the production of pro-inflammatory markers such as cyclooxygenase-2 and inducible nitrous oxide synthase (Gonzales et al., 2009). Taken together, these results suggest that females may have a more adaptive immune response to AMNS-LPS treatment compared to males due to differences in circulating gonadal hormones.

LPS treatment was also associated with sex-specific changes in intestinal permeability. Females treated with LPS displayed significantly greater occludin expression in the ileum in comparison to their LPS-treated male counterparts. This finding does not support our hypothesis and is not consistent with previous research examining the effects of LPS and antimicrobial treatment on intestinal permeability. Occludin is a tight-junction protein that plays a critical role in regulating intestinal permeability (Cummins, 2012). C57BL/6 mice treated with ampicillin via gavage for 14 days display significant decreases in occludin levels in the colon, suggesting an increase in intestinal permeability (Shi et al., 2018). This discrepancy with our findings may be due to differences in the length of antimicrobial treatment, the species that were utilized, the age of the mice, and the tissue that was analzed (i.e., ileum vs. colon).

The increase in occludin levels in female mice observed in our study could be a protective response to LPS treatment that is mediated by IL17A and IL23 concentrations. Research in mice has shown that the early production of IL17A, in the absence of IL23, protects the intestinal barrier following dextran sodium sulfate injury (Lee et al., 2015). Therefore, the significant increases in occludin expression and IL17A concentration, in the absence of IL23, in females, suggest that they have a greater ability to protect the intestinal barrier in response to LPS treatment. In contrast, the significant increases in IL17A and IL23 concentrations in males suggest that they may be more susceptible to intestinal barrier dysfunctions in response to LPS treatment. The sex difference in occludin concentration could also be due to differences in circulating gonadal hormones. Research in rats has shown that ovariectomy increases intestinal permeability while treatment with 17β-oestradiol upregulates the expression of occludin and junctional adhesion molecule A in the colon, indicating a decrease in intestinal permeability (Braniste et al., 2009).

LPS-treated male and female mice showed decreases in plasma FABP2 concentrations. These results are not in line with our hypothesis but are partially consistent with previous research examining plasma FABP2 concentrations. FABP2 is an intracellular protein that is abundantly expressed in the epithelial cells located in the small and large intestine (Pelsers et al., 2003). Under normal conditions, plasma FABP2 concentrations are thought to reflect the physiological turnover rate of enterocytes (Bischoff et al., 2014; Uhde et al., 2016). However, elevated levels of plasma FABP2 could indicate excessive damage to enterocytes and an increase in intestinal permeability (Bischoff et al., 2014). Previous research has shown that gut dysbiosis, induced by chronic unpredictable stress in rats, significantly increases the expression of plasma FABP2 (Lv et al., 2019). However, other research with rats has shown that dysbiosis induced by a high-fat diet results in a decrease in plasma FABP2 concentrations (Lau et al., 2016). It remains unclear as to why plasma FABP2 concentrations would decrease following LPS treatment in our current study. It is possible that the decrease in plasma FABP2 concentrations is an adaptive response to LPS treatment, where the turnover rate of enterocytes is decreased to maintain intestinal barrier integrity. It is also possible that the stressors used in this study (LPS and

antimicrobials) have unique acute effects on plasma FABP2 concentrations which have yet to be elucidated. However, further research is required to determine the acute and enduring effects of LPS and antimicrobial treatment on enterocytes and plasma FABP2 concentrations.

Our results also showed that LPS-treated males displayed significantly less C3 expression in the SN in comparison to their SAL-treated counterparts, while no significant differences were observed in females. C3 is a protein that plays a central role in the complement system and has various functions including eliminating foreign pathogens, synaptic pruning, tissue regeneration, and clearing debris from cells and tissues (Ricklin et al., 2016). Dysregulation of C3 expression can contribute to the development of neurodegenerative (i.e., amyotrophic lateral sclerosis, AD, PD) and neurodevelopmental disorders (i.e., autism spectrum disorder, schizophrenia) (Loeffler et al., 2006; Mayilyan et al., 2008; Warren et al., 1994; Woodruff et al., 2014; T. Wu et al., 2019). For example, elevated levels of C3 have been reported in the brains of patients suffering from Huntington's disease, AD, and PD (Fatoba et al., 2021). However, C3-deficient mice show an increase in amyloid beta deposition and neuronal loss, suggesting a protective role of C3 in the brain (Maier et al., 2008). Moreover, C3 knockdown mice show decreases in synaptic pruning and increases in social interaction impairments and repetitive behaviours, deficits that are associated with autism spectrum disorder (Fagan et al., 2017; Magdalon et al., 2020). Therefore, decreased C3 expression in male mice following pubertal LPS treatment may indicate deficits in synaptic pruning or a decreased ability to remove invading pathogens, potentially increasing susceptibly to neurodegenerative and/or neurodevelopmental disorders.

LPS-treated mice also showed significant increases in SNCA and LRRK2 expression in the CP. These results are consistent with our hypothesis and with previous research examining the effects of LPS on SNCA and LRRK2 expression. An accumulation of SNCA and LRRK2 in the brain has been associated with the pathology of several neurodegenerative disorders such as PD, AD, dementia with Lewy bodies, and multiple system atrophy (Meade et al., 2019; Santpere and Ferrer, 2009; Siddiqui et al., 2016). Although the mechanisms mediating the effects of SNCA and LRRK2 on neurodegeneration are unclear, it is believed that SNCA and LRRK2 play a role in the regulation of neuroinflammation. For example, LPS-treatment in transgenic mice overexpressing alpha-synuclein results in enduring neuroinflammation (i.e., Iba-1, Mac1, inducible NO synthase, cyclooxygenase-2, and gp91phox) in both the SN and striatum, 5-months following LPS-treatment (Gao et al., 2011). Moreover, this persistent neuroinflammation was also associated with progressive degeneration of the nigrostriatal dopamine pathway and Lewy-body inclusions in nigral neurons (Gao et al., 2011). Other research has shown that the inhibition of LRRK2 in LPS-treated microglial cells results in a decrease in interleukin-1β and cyclooxygenase-2 expression along with the downregulation in nuclear factor kapa B signaling (Russo et al., 2015). Therefore, the acute increase of SNCA and LRRK2 expression following pubertal LPS treatment in our study could indicate the beginning of an enduring neuroinflammatory response that is mediated by SNCA and LRRK2.

No significant difference in TH expression was observed in the SN and CP of pubertal male and female mice following AMNS-LPS treatment. These findings support our hypothesis and are consistent with previous research examining the effects of LPS treatment on TH expression in pubertal mice. TH is an enzyme that catalyzes the hydroxylation of tyrosine to L-DOPA, a precursor of dopamine, epinephrine, and norepinephrine (Daubner et al., 2011). Research from our laboratory has shown that LPS treatment increases TH expression in adult mice, but not in pubertal mice (Girard-Joyal and Ismail, 2017). This age difference may be due to a compensatory response to LPS in adult mice, which pubertal mice may lack due to lower levels of circulating gonadal hormones (Girard-Joyal and Ismail, 2017). Alternatively, given that TH is also a precursor of norepinephrine, it is possible that TH remains unaltered following pubertal AMNS-LPS treatment due to the known hypo-responsiveness of pubertal mice to stressors (Cai et al., 2016; Girard-Joyal et al., 2015; Sharma et al., 2018).

4.1. Limitations and future directions

Given that exposure to an immune challenge and antimicrobials can cause gut dysbiosis and can impact the functioning of various systems like metabolic function, endocrine function, immune function, sexual development, brain function, and more (Clarke et al., 2014; Dinan and Cryan, 2017; Kennedy et al., 2018), it is difficult to determine the precise mechanisms underlying the effects observed in the current study. Secondly, all mice were euthanized 8 h post-injection at six-weeks of age. Therefore, no inferences about brain functioning can be made beyond this timepoint. Thirdly, the estrous cycle was not examined in this study, therefore, we cannot be certain of whether differences in the stage of estrous cycle between our mice influenced the results. Lastly, microbial composition was not analyzed in this study, therefore, we cannot be certain that our treatment model (i.e., LPS and antimicrobials) is inducing alterations to the gut microbiome. Future research should examine the effects of LPS and antimicrobial treatment across multiple systems including metabolic function (i.e., glucose metabolism), sexual development (i.e., estrous cycle) and endocrine function (i.e., hypothalamic–pituitary–adrenal axis). Examining these systems would provide a more holistic picture of the influence of LPS and antimicrobial treatment across various systems. Future research should also examine the long-term effects of pubertal LPS and antimicrobial treatment on brain functioning and behaviours related to neurodegeneration. It would also be interesting to examine the effects of LPS and antimicrobial treatment on microbial composition to confirm whether our treatment model induces dysbiosis.

5. Conclusion

In conclusion, this study shows that pubertal LPS and antimicrobial treatment induces sex-dependent changes in acute cellular mechanisms associated with neurodegeneration. Overall, these findings suggest that pubertal female mice may have a more adaptive response to LPS and antimicrobial treatment in comparison to pubertal male mice, indicating that males may be more susceptible to the effects of LPS and antimicrobial treatment on neurodegenerative mechanisms. The current study is one of the first to examine the acute effects of pubertal LPS and antimicrobial treatment on cellular mechanisms associated with neurodegeneration in male and female mice. The results further our understanding of how the gut microbiome can influence the pathogenesis of neurodegenerative disorders during a critical period of development. This may allow for the development of therapeutic strategies that target the gut microbiome (i.e., probiotics) during the early stages of life, which can potentially prevent or prolong the development of neurodegenerative disorders.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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