ACUTE AND ENDURING SEX-DEPENDENT EFFECTS OF PUBERTAL ANTIMICROBIAL AND LIPOPOLYSACCHARIDE TREATMENTS ON CELLULAR MECHANISMS AND BEHAVIOURS ASSOCIATED WITH NEURODEGENERATION

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Thesis submitted to the University of Ottawa

in partial fulfillment of the requirements for the

Doctorate of Philosophy with specialization in Experimental Psychology

School of Psychology

Faculty of Social Sciences

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Table of Contents

Acknowledgments

First and foremost, my deepest appreciation goes to my thesis supervisor, Dr. Nafissa Ismail. Your constant encouragement and exceptional mentorship have been beyond anything I could have asked for. You have guided me with patience and wisdom, and your belief in my capabilities has been a source of constant motivation. Thank you for being the best mentor I could ever wish for.

I would also like to express my deepest gratitude to my committee members, Dr. Melanie Sekeres, Dr. Hélène Plamondon, Dr. Vanessa Taler, and Dr. Shawn Hayley. Your expertise, support, and invaluable suggestions have been instrumental in shaping this dissertation into its final form. Your guidance has not only improved the quality of my work but has also enriched my academic journey immensely.

A heartfelt thank you to Jacky Liang, whose unwavering support has been a cornerstone of my thesis work. Your encouragement and assistance have been vital in producing the quality work presented here. I am profoundly grateful for your presence throughout this journey.

I would like to extend my sincere thanks to Julie Tremblay, who was always there to support me with animal work. Your expertise and willingness to help whenever you could were an invaluable resources during my research. Your dedication and support have significantly contributed to the success of my work, and for that, I am truly grateful.

I would also like to thank my fellow graduate students, Sarah Kheloui, Kevin Smith, and Michael Murack. Your support have been invaluable and I will cherish the memories and experiences we shared forever. You made the challenging moments of this journey bearable and the successes even more joyous.

To my undergraduate students, Michelle Gandelman and Cloudia Rodriquez, thank you for your dedication and hard work. Your contributions were critical to the success of my thesis, and your friendship has meant a great deal to me. I am grateful for the time we spent working together and the bond we have formed.

I owe a significant debt of gratitude to my early mentor, Dr. Uri Shalev. Your introduction to the fascinating world of neuroscience sparked a lifelong passion within me. Your continuous support throughout these years has been a pillar of strength and inspiration. Thank you for believing in me and nurturing my growth in this field.

I am profoundly thankful to my family, my mom, dad, sisters, and my wife. Your unwavering support and encouragement have been the bedrock upon which I have built my academic career. You have always believed in me and inspired me to pursue my dreams, and for that, I am eternally grateful.

Lastly, I would like to thank my best friends for helping me disconnect when needed. Your support and companionship have made this journey much more enjoyable and manageable. Without you, my Ph.D. experience would have been significantly harder.

Abbreviations

AAAs Aromatic Amino Acids

ACTH Adrenocorticotropic Hormone

Aβ Amyloid Beta

ACC Anterior Cingulate Cortex

AD Alzheimer's Disease

ALS Amyotrophic lateral sclerosis

ANOVA Analysis of Variance

BBB Blood-Brain-Barrier

BCA Bicinchoninic Acid Assay

BCAAs Branched-Chain Amino Acids

C3 Complement 3

CA1 Cornu Ammonis 1

CA2 Cornu Ammonis 2

CA3 Cornu Ammonis 3

CNS Central Nervous System

CORT Corticosterone

COX-1 Cyclooxygenase 1

CP Caudate-Putamen

CXCL2 Chemokine Ligand 2

CV Coefficient of Variation

DAB Diaminobenzidine

DG Dentate Gyrus

DPM	Disintegrations Per Minute
ELISA	Enzyme-Linked Immunosorbent Assay
FABP2	Fatty-Acid-Binding Protein 2
GC	Glucocorticoids
GDNF	Glial Cell Line-Derived Neurotrophic Factor
GFRA1	Glial Cell Line-Derived Neurotrophic Factor Alpha 1
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMV	Grey Matter Volume
GNRH	Gonadotropin-Releasing-Hormone
GRs	Glucocorticoid Receptors
HIPP	Hippocampus
HPA	Hypothalamic-Pituitary-Adrenal Axis
IF	Infralimbic Cortex
IFNγ	Interferon Gamma
IHC	Immunohistochemistry
IP	Intraperitoneal
IL1β	Interleukin 1 Beta
IL1	Interleukin 1
IL2	Interleukin 2
IL4	Interleukin 4
IL6	Interleukin 6
IL9	Interleukin 9
IL10	Interleukin 10

IL12	Interleukin 12
IL17A	Interleukin 17A
IL23	Interleukin 23
LPS	Lipopolysaccharide
LRRK2	Leucine-Rich Repeat Kinase 2
mPFC	Medial Prefrontal Cortex
M1	Primary Motor Cortex
M2	Secondary Motor Cortex
MRs	Mineralocorticoid Receptors
MS	Multiple Sclerosis
NDs	Neurodegenerative Disorders
NF- κB	Nuclear Factor Kapa B
NGS	Normal Goat Serum
NLRP3	Nod-like receptor family pyrin domain containing 3
PBS	Phosphate Buffered Saline
PD	Parkinson's Disease
PFA	Paraformaldehyde
PFC	Prefrontal Cortex
PLC	Prelimbic Cortex
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
SCFAs	Short Chain Fatty Amino Acids
S1R	Sigma-1 Receptor
SN	Substansia Nigra

SNCA Alpha-Synuclein

SOD-1 Superoxide Dismutase Type 1

TBS Tris-Buffered Saline

TDP-43 Transactivation Response DNA Binding Protein 43

TH Tyrosine Hydroxylase

TLR4 Toll-Like Receptor 4

TNFα Tumor Necrosis Factor Alpha

List of Figures and Tables

Article 1

Article 2

Article 3

Abstract

Puberty is a critical period of development accompanied by the maturation of various fundamental systems such as the central nervous system (CNS), immune system, and hypothalamic-pituitary-adrenal axis. The maturation of these systems renders puberty particularly sensitive to stressors, potentially increasing susceptibility to neurodegenerative disorders later in life. The gut microbiome may be a possible mechanism through which pubertal stress exposure could increase vulnerability to neurodegeneration. The gut microbiota communicates with the brain via enteric and autonomic neuroimmune and neuroendocrine pathways referred to as the "gut-brain" axis. Alterations to gut microbiota result in 'gut dysbiosis' and may negatively affect this bidirectional communication between the gut and the CNS, potentially influencing the development of neurodegenerative disorders. As such, we hypothesized that altering microbial composition through pubertal lipopolysaccharide (LPS) and antimicrobial treatments could influence behaviours and cellular mechanisms associated with neurodegeneration. The current thesis examined the sex-specific effects of pubertal LPS and antimicrobial treatment on acute cellular mechanisms associated with neurodegeneration (Article 1). Next, we examined the enduring sex-specific effects of pubertal LPS and antimicrobial treatments on behaviours and cellular mechanisms associated with neurodegeneration (Article 2). Lastly, we examined the sex-specific effects of pubertal LPS and antimicrobial treatment on the blood-brain barrier and intestinal permeability, a potential mechanism underlying the effects of the gut on the CNS and the development of neurodegenerative disorders. We also further examined the effects of pubertal LPS and antimicrobial treatment on gross motor coordination, heart rate, and core body temperature through the use of the G2 HR E-Mitter telemetry system (Article 3). Taken together, the current dissertation addresses whether pubertal LPS and

antimicrobial treatments can increase vulnerability to neurodegeneration later in life, in a sexually dimorphic manner.

Chapter 1: Conceptual and Theoretical Background

1.0 Puberty

1.1 Timing and sex differences

Puberty is a critical period of development marked by sexual maturation, the development of secondary sexual characteristics, the activation of hypothalamic-pituitary-gonadal axis, and the production of gonadal steroid hormones (Sisk & Foster, 2004). The hypothalamic-pituitary-gonadal axis initiates puberty by increasing the pulsatile gonadotropin releasing hormone (GnRH) secretion in the hypothalamus (Herbison, 2016). A key mechanism mediating the stimulation of GnRH neurons is the neuropeptide kisspeptin. Kisspeptin is a product of the *kiss-1* gene and has been shown to directly stimulate GnRH release by binding to GPR54, a G protein-coupled receptor that is located on GnRH neurons (Clarkson et al., 2010). Metabolic (i.e., leptin, body fat), photoperiodic (i.e., melatonin), and environmental factors (i.e., stress) also contribute to the activation of GnRH neurons and the timing of pubertal onset (Ebling, 2005; Moffitt et al., 1992; Murcia García et al., 2002). The release of GnRH stimulates the production of luteinizing hormone and follicle stimulating hormone from the anterior pituitary into the bloodstream. Luteinizing hormone and follicle stimulating hormone then stimulate the gonads to initiate the maturation of gametes (i.e., gametogenesis) and the production of gonadal steroid hormones such as estradiol, progesterone, and androgens (Marques et al., 2000; Raju et al., 2013).

Increased activation of the hypothalamic-pituitary-gonadal axis and circulating gonadal steroid hormones play a central role in the development of secondary sexual characteristics (i.e., enlarged breasts and pubic hair in females and testicular enlargement and pubic hair in males) and the ability of an organism to reproduce (i.e., menarche in females and spermarche in males)

(Abreu & Kaiser, 2016; Huang et al., 2012; Karapanou & Papadimitriou, 2010; Nielsen et al., 1986). In humans, this period of maturation typically begins around the ages of 8-13 in females and 9-14 in males (Wolf & Long, 2016) The timing of puberty in animals can vary depending on the housing conditions and the strain of the animal. A non-invasive approach to identify pubertal onset in mice is vaginal opening in females and preputial descent (i.e., separation of the prepuce to the glans penis) in males (Gaytan et al., 2017; Korenbrot et al., 1977). It is estimated that CD1 and C57B1/6 female mice housed in single sex rooms demonstrate vaginal opening approximately 30 days following birth and have their first estrous cycle 20 days post vaginal-opening (Holder & Blaustein, 2014; Ismail and Blaustein, unpublished observations). Identifying pubertal onset through preputial separation in male mice is more difficult, however, measurements of scrotum width in six-week-old male CD1 mice indicate that the scrotum does not reach adult size until they are eight-weeks-old (Murray, Butcher, Kearns, Lamba, Stinzi & Ismail, in preparation).

1.2 Brain reorganizing and remodeling

Puberty is also a period during which the brain undergoes significant reorganizing and remodeling (Sisk & Foster, 2004). More specifically, the central nervous system (CNS) undergoes significant functional and structural remodeling during this critical period of development including changes in both grey and white matter volumes (Blakemore et al., 2010). In humans and animals, alterations in grey and white matter volumes vary by sex and are associated with the onset of gonadarche, suggesting that circulating gonadal steroid hormones play a role in brain development (Giedd et al., 1999). In humans, grey matter volume (GMV) follows an inverted U-shape trajectory, with peak GMV being attained at the age of 11 for girls and 12 for boys in the frontal, temporal, and parietal lobes (Giedd et al., 1999). GMV increases

in childhood and reaches its peak in adolescence due to dendritic arborization and synaptogenesis. GMV then steadily decreases into adulthood due to synaptic pruning (Giedd et al., 1999).

Human males typically have greater GMV then human females while human females have greater gray matter density then human males, an effect that is primarily driven by differences in circulating gonadal steroid hormones (Gennatas et al., 2017). Testosterone is associated with increases in global GMV, while estradiol is associated with decreases in global GMV (Peper et al., 2009). The influence of circulating gonadal steroid hormones on GMV could also be region-specific. Testosterone has been associated with increases in GMV in the amygdala and decreases of GMV in the hippocampus, while estradiol has been associated with increases in limbic GMV, in humans (Neufang et al., 2009). Moreover, only human males show increases in GMV in the amygdala during puberty while only human females show increases in GMV in the hippocampus and striatum during puberty (Neufang et al., 2009). In contrast to GMV, white matter volume typically follows a linear trajectory from childhood to adolescence and then steadily stabilizes into adulthood in humans (Tamnes et al., 2010). Human males have greater global white matter volume then human females during puberty due to steeper age-related increases in axonal calibre (Perrin et al., 2008, 2009). Moreover, testosterone is associated with increases in white matter volume, while estradiol has been associated with either having no effect or a negative effect on white matter volume (Herting et al., 2012; Juraska & Markham, 2004). As such, circulating gonadal steroid hormones during puberty can have long-term effects on neurological changes and neural functioning into adulthood.

1.3 Maturation of the HPA axis and vulnerability to stress

A critical neuroendocrine system that develops and matures during puberty is the hypothalamic-pituitary-adrenal (HPA) axis (Romeo, 2010). The HPA axis is the body's primary stress processing system that has numerous adaptive physiological processes aimed at regulating allostatic load and maintaining homeostasis in response to a stressor (Roberts & Lopez-Duran, 2019). These processes include redirecting energy resources, increasing vasoconstriction, cognition and metabolism, and supressing immune and reproductive functions (Papadimitriou & Priftis, 2009; Silverman et al., 2005). When initially exposed to stressful stimuli, sympathetic nerves and the adrenal medulla are activated and rapidly release catecholamines (i.e., epinephrine, norepinephrine), resulting in increased heart rate and blood pressure along with decreased intestinal motility and bronchiolar dilatation (Chu et al., 2022; Goldstein, 2010). This rapid stress response is followed by a slower stress response mediated by the HPA axis.

In both humans and animals, the HPA axis response begins with excitatory signals from the prefrontal cortex (PFC) and the amygdala to the paraventricular nucleus of the hypothalamus (Bains et al., 2015; Smith & Vale, 2006). Stimulation of the paraventricular nucleus of the hypothalamus induces the release of corticotrophin-releasing hormone and arginine vasopressin, which bind to receptors in the anterior pituitary gland resulting in the release of adrenocorticotropic hormone (ACTH) (Stephens & Wand, 2012). ACTH then binds to receptors in the adrenal cortex, resulting in the synthesis of glucocorticoids (GC) (i.e., cortisol in humans and corticosterone in mice and rats) (Stephens & Wand, 2012). GC levels increase rapidly in the bloodstream following exposure to stress with peak levels being attained 20-40 minutes following initial stress exposure (Dickerson & Kemeny, 2004). However, peak latency can vary depending on various factors such as sex, age, psychosocial factors, and previous exposure to a stressor (Eller et al., 2006; Gustafsson et al., 2006; Kudielka et al., 2004; Reschke-Hernández et

al., 2017). GC have the ability to regulate their own production through a negative feedback mechanism. Mineralocorticoid (MRs) and glucocorticoid receptors (GRs) throughout the hypothalamus, pituitary, medial prefrontal cortex, and hippocampus play critical roles in the negative feedback mechanism (McCormick & Mathews, 2010). MRs demonstrate a high affinity for GC and are activated once GC levels are low (i.e., basal levels) (Herman et al., 2012). However, when GC levels increase, MRs become saturated resulting in the activation of GRs which have a lower affinity for GC. Once GRs are activated, signals are sent to the hypothalamus and pituitary to inhibit the production of GC (Herman et al., 2012). This process is critical as it permits the rapid downregulation of GC synthesis, allowing the body to return to homeostasis following exposure to a stressor.

There are age and sex differences in HPA axis reactivity which are dependent on the strain and species of the animal being analyzed. For example, inbred prepubertal male mice exposed to acute restraint stress show greater HPA axis reactivity, as shown through either a more prolonged (i.e., C57BL/6) or greater (i.e., BALB/c) corticosterone (CORT) response in comparison to their adult counterparts (Romeo et al., 2013). Inbred prepubertal female mice show similar HPA axis reactivity in comparison to their adult counterparts. Conversely, alterations to HPA axis reactivity in outbred mice (i.e., Swiss Webster) are only observed in adult female mice with these mice demonstrating a greater CORT response in comparison to their prepubertal counterparts (Romeo et al., 2013). Prepubertal male rats exposed to acute stressors (i.e., hypoxia, restraint, foot shock) show a more prolonged ACTH and CORT response in comparison to their adult counterparts (Goldman et al., 1973; Romeo et al., 2004, 2006; Vázquez & Akil, 1993). When exposed to chronic restraint stress, prepubertal male rats demonstrate a greater stress response which is followed by a quicker return to baseline in

comparison to their adult counterparts (Romeo et al., 2006). Adult male rats exposed to a homotypic stressor show a habituated stress response that is not observed in adolescent male rats (Girotti et al., 2006; Harris et al., 2004; Romeo & Sciortino, 2021). Furthermore, adult female rats demonstrate a greater stress response in comparison to their adult male counterparts (Heck & Handa, 2019). Taken together, these results demonstrate that HPA axis reactivity is highly dependent on the sex (i.e., male and female), species (i.e., mice and rats), and strain (i.e., inbred and outbred) of the subjects being analyzed.

Interestingly, age and sex differences in HPA axis reactivity emerge during puberty, suggesting a potential role of circulating gonadal steroid hormones (i.e., estradiol, testosterone, progesterone). In animals, testosterone appears to decrease HPA axis reactivity while estradiol appears to increase it (Goel et al., 2014). For example, gonadectomized female rats exposed to restraint stress display a decrease in ACTH and CORT concentrations in comparison to intact females, an effect that is reversed following estradiol treatment (Kalil et al., 2013; Lunga & Herbert, 2004; McCormick et al., 2002; Weiser & Handa, 2009). Conversely, gonadectomized male rats display an increase of ACTH and CORT concentrations following exposure to a stressor, an effect that is reversed following androgen treatment (Seale, Wood, Atkinson, Bate, et al., 2004; Seale, Wood, Atkinson, Harbuz, et al., 2004; Viau et al., 2003; Viau & Meaney, 2004). Thus, circulating gonadal steroid hormones play a critical role in HPA axis function and the increase of these hormones during puberty is essential for the development of adult-like patterns of HPA axis reactivity (Romeo, 2013).

In humans, maturation of the HPA axis during puberty is associated with a sex-dependent increase in vulnerability to stress-related disorders. Mental illnesses such as anxiety, depression, psychosis, eating disorders, bipolar disorder, substance abuse, and personality disorders,

predominantly emerge during adolescence and puberty (Gomes et al., 2016; Kessler et al., 2005; Paus et al., 2008). Moreover, the incidence of these mental illnesses are sex-dependent, with human females showing a higher prevalence of eating disorders, anxiety and depression while human males show a higher prevalence of psychosis and substance abuse (Albert, 2015; McHugh et al., 2018; McLean et al., 2011; Ochoa et al., 2012; Striegel-Moore et al., 2009). Sex differences for many of these disorders also emerge during puberty with pubertal status (Tanner Stage III) being a better predictor of these sex differences then chronological age (Angold et al., 2003; Hayward & Sanborn, 2002; Patton et al., 1996).

Factors contributing to the increased incidence of mental illness during puberty remain unclear, however, it is believed that exposure to stressors may interfere with the development of the HPA axis (Guerry & Hastings, 2011). An atypical development of the HPA axis could reduce an individual's ability to cope with stressors which may, in turn, increase vulnerability to stress-related disorders (Roberts & Lopez-Duran, 2019). For example, repeated exposure to stressors during puberty in humans can result in the sensitization of the HPA axis and the overproduction of GC (Bevans et al., 2008; Teicher et al., 2003). The overproduction of GC could then damage key brain regions (i.e., hippocampus, amygdala, PFC) responsible for the regulation of the HPA axis (Conrad, 2008; McCormick & Mathews, 2010; Mcewen et al., 1968). Dysregulation of the HPA axis could then result in the chronic overproduction of GC which could increase susceptibility to mental illness associated with chronic stress (i.e., depression, anxiety, and substance abuse) (Eiland & McEwen, 2012; Sinha, 2008; L. Yang et al., 2015). Alternatively, it is possible that exposure to stressors during puberty can result in the blunting of the HPA axis (Trickett et al., 2010). Pubertal stress exposure in humans may increase the expression of GRs which could facilitate the downregulation of GC and the blunting of the HPA axis (Susman,

2006). Consequently, the blunting of the HPA axis could increase susceptibility to disorders such as post-traumatic stress disorder and personality disorders (i.e., antisocial and borderline personality disorder) (Cohen et al., 2006; Drews et al., 2019; Fairchild et al., 2018).

1.4 Maturation of the immune system and enduring effects of lipopolysaccharide (LPS)

The immune system is a complex system of cells and proteins responsible for protecting an organism from viruses, bacteria, and other pathogens (Brenhouse & Schwarz, 2016). Like the HPA axis, the immune system also undergoes significant maturation during puberty (Holder & Blaustein, 2014). The immune system of vertebrates is made up of two parts, the innate and adaptive immune systems (Brenhouse & Schwarz, 2016). The innate immune system is the body's first line of defence which has a non-specific response to pathogens that are evolutionarily conserved such as, bacterial, fungal, viral, or foreign proteins. Innate immune responses to a pathogen include inflammation, phagocytosis, and lysis (Janeway & Medzhitov, 2002; Medzhitov & Janeway, 2000). The adaptive immune system develops throughout the lifespan and utilizes B and T cells to recognize and remember foreign pathogens (Marshall et al., 2018). Adaptive immune responses destroy invading pathogens either indirectly through the secretion of antibodies by B cells (i.e., antibody response) or directly by T cells (i.e., cell mediated immune response) (Alberts et al., 2002). An essential component of both the innate and adaptive immune systems is the production of cytokines. Cytokine is a general term used for a family of small proteins which include chemokines, interferons, interleukins, lymphokines, and tumour necrosis factors. Cytokines play critical roles in cell signaling and in the regulation of inflammation in response to invading pathogens (J.-M. Zhang & An, 2007).

A common method to examine the immune system in animals is through the administration of lipopolysaccharide (LPS). LPS is a bacterial endotoxin located on the outer

membranes of gram-negative bacteria (i.e., *Escherichia coli*) and is a potent stimulator of the innate immune system (Page et al., 2022). In the periphery, LPS binds to toll like receptor 4 which is predominantly expressed on immune cells (Chow, et al., 1999). LPS can also influence the CNS by crossing the blood brain barrier (BBB) and binding to toll like receptor 4 residing on microglia (i.e., primary innate immune cells of the brain) (McGeer et al., 2000; Mrak & Griffin, 2005; Page et al., 2022; Sharma et al., 2018). The stimulation of toll like receptor 4 induces a cascade of intracellular events that results in the stimulation of nuclear factor kapa B (NF- κB) (Page et al., 2022). The stimulation of NF- κB then results in the synthesis of prostaglandins (i.e., prostaglandin E2), cyclooxygenase, nitric oxide, and pro- (i.e., TNFα, IL1β, IL12) and anti-inflammatory cytokines (i.e., IL10, IL4, IL9) (McGeer et al., 2000; Mrak & Griffin, 2005; Sharma et al., 2018). Moreover, LPS administration in animals has been shown to induce anxiety, depression, neurodegeneration, and neurodevelopmental disorders (Kirsten et al., 2015; Murray et al., 2019; Zhao et al., 2019). Thus, LPS is an ideal candidate to examine the role that the immune system plays in various disorders.

LPS administration during puberty has enduring effects on brain functioning and behaviours. Previous research from our laboratory and others has shown that pubertal LPS treatment, in mice, induces cognitive deficits, supresses sexual receptivity in females, and increases anxiety-like behaviours in males and depression-like behaviours in females, in adulthood (Ismail et al., 2011; Kolmogorova et al., 2019; Laroche et al., 2009; Murray et al., 2019). Moreover, pubertal LPS has been shown to decrease estrogen receptor-α and increase c-fos expression in adulthood (Girard-Joyal et al., 2015; Ismail et al., 2011). Pubertal LPS treatment also has programming effects on immune and HPA axis reactivity. For example, pubertal LPS treatment permanently decreases GR expression in the paraventricular nucleus of

the hypothalamus of adult male mice (Smith et al., 2021). Furthermore, LPS treatment during puberty followed by a secondary immune challenge in adulthood results in an attenuated immune response as shown through decreases in peripheral IL6 and IFNγ concentrations and decreases in IL1β, TNFα, and IL6 mRNA expression in the PFC (Sharma et al., 2019).

There are also age and sex differences in immune responsivity following LPS treatment, due in part to the immune-enhancing effects of estrogens and the immune-suppressing effects of androgens and progesterone (Gubbels Bupp & Jorgensen, 2018; Khan & Ansar Ahmed, 2016; Taneja, 2018). In general, pubertal LPS treatment in mice induces a hypo-responsive immune response when compared to adult mice. For example, adult mice display greater peripheral pro-inflammatory cytokine concentrations compared to pubertal mice, while pubertal mice display greater peripheral anti-inflammatory cytokine concentrations compared to adult mice 10 hours following LPS treatment (Cai et al., 2016). However, pubertal male mice display greater IL1β, TNFα, and IL6 mRNA expression in the PFC compared to adult male mice 2 hours following LPS treatment (Sharma et al., 2018), and adult male mice display greater cytokine mRNA expression compared to pubertal male mice 8 hours following LPS treatment (Sharma et al., 2018). These age and sex differences in cytokine expression are also associated with greater sickness behaviours and hypothermia in adult male mice compared to their pubertal counterparts (Cai et al., 2016). Therefore, behavioural and physiological responses to an immune challenge are highly dependent on age and sex.

2.0 Neurodegeneration

Neurodegenerative disorders (NDs) such as Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and Huntington's disease are a common cause of morbidity and mortality in the elderly population (Erkkinen et al.,

2018). Aging is the primary risk factor for NDs and with increased life expectancy worldwide, the prevalence of these disorders is increasing (Azam et al., 2021; Fujii, 2021; Wyss-Coray, 2016). Neurodegeneration is commonly defined as the progressive loss of neuronal function in the CNS, resulting in impairments related to 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). The progressive loss of neuronal function in NDs is typically caused by abnormal protein aggregations (i.e., amyloidosis, tauopathies, synucleinopathies, and transactivation response DNA binding protein 43 proteinopathies), which can induce oxidative stress, excitotoxicity, mitochondrial dysfunction, and neuroinflammation, ultimately resulting in apoptosis (Amor et al., 2010; X. Chen et al., 2012; Dong et al., 2009; Dugger & Dickson, 2017; Hashimoto et al., 2003; Mattson, 2000).

The pathology of NDs is influenced by various factors. Abnormal protein aggregations are a hallmark of NDs with each ND being characterized by the aggregation of specific proteins. Examples of abnormal protein aggregations include amyloid beta (Aβ) in AD, tau in Pick's disease, alpha-synuclein in PD, and transactivation response DNA binding protein 43 (TDP-43) in ALS (Dugger & Dickson, 2017). Although NDs differ in their clinical presentations and histopathological features, the aggregation of pathological proteins induces similar neurodegenerative processes. For example, Aβ, tau, alpha-synuclein, and TDP-43 are known to localize on the mitochondrial membrane and prevent neurons from functioning normally by causing mitochondrial damage, disrupting the election transport chain, increasing the production of reactive oxygen species and inducing persistent neuroinflammation and glutamate excitotoxicity (Cheng & Bai, 2018; Pallo et al., 2016; Park et al., 2020; Reddy & Beal, 2008; Wang et al., 2020; Wang et al., 2019). Interestingly, the knockdown of these proteins in animals

does not cure or ameliorate neurodegeneration but rather results in severe motor (i.e., motor neuron degeneration), cognitive (i.e., spatial and long-term memory), and behavioural abnormalities (i.e., anxiety-like behaviour) (Gabriele et al., 2022; Gonçalves et al., 2020; Iguchi et al., 2013; Kokhan et al., 2012). These deficits reflect the essential role these proteins play in regulating the morphology and physiology of neurons (i.e., neural growth and repair, cytoskeleton scaffolding, regulation of gene expression, and neurotransmitter release) (Avila et al., 2004; Bendor et al., 2013; Brothers et al., 2018; Yang et al., 2014). While abnormal protein aggregations are fundamental to the pathology of NDs, other mechanisms may also be involved.

NDs differ in their symptoms due in part to differences in the cellular and neuroanatomical distribution of the proteins implicated in these disorders (Dugger & Dickson, 2017). For example, the early stages of AD in humans is typically characterized by cell loss and neurofibrillary tangles in the neocortex, hippocampus, entorhinal cortex, amygdala, and basal nucleus of Meynert (Wenk, 2003). Atrophy in these brain regions could result in memory loss, praxis, visuospatial impairments, and executive dysfunction; symptoms that are commonly observed in AD patients (Zvěřová, 2019). Conversely, the early stages of PD in humans is typically characterized by the loss of dopamine producing cells in the basal ganglia (i.e., caudate nucleus, putamen, globus pallidus, subthalamic nucleus, and substantia nigra), which causes severe motor dysfunction (i.e., tremors, bradykinesia, muscular rigidity) in patients suffering from PD (Caligiore et al., 2016). However, as NDs progress, a network of brain regions are affected resulting in significant overlap between NDs in their clinical features. As such, it is not uncommon for NDs to have comorbidities with other NDs and psychiatric issues (Dugger & Dickson, 2017). For example, AD and PD often have comorbidities with dementia with Lewy

Bodies, progressive supranuclear palsy, vascular dementia, cerebral amyloid angiopathy, and depression (DeTure & Dickson, 2019; Dugger et al., 2014).

2.1 Sex differences in neurodegeneration

There are definite sex differences in the prevalence and clinical presentations of NDs. For example, AD is more prevalent in women (2:1) while PD more prevalent in men (2:1) (Baldereschi et al., 2000; Elbaz et al., 2002; Plassman et al., 2011). Men suffering from AD tend to show more aggressive behaviours, have more comorbidities, and higher mortality rates than women, while women tend to show more affective symptoms (i.e., apathy, depression, irritability, anxiety), cognitive deterioration and higher survival rates than men (Irvine et al., 2012; Sinforiani et al., 2010). In terms of PD, females typically show a slower rate of decline, fewer symptoms, and a delayed onset of PD, while males tend to display more aggressive motor dysfunction (i.e., postural instability, falling, gait disturbances) and impairments in executive function and reduced processing speed (Haaxma et al., 2007; Reekes et al., 2020). There are also sex differences in the prevalence and clinical presentations of other NDs such as ALS, MS, frontotemporal dementia, and Huntington's disease (Hanamsagar & Bilbo, 2016; Hentosh et al., 2021; Illán-Gala et al., 2021). Therefore, sex is a critical factor in the pathology of NDs and should be taken into account when examining the etiology of NDs.

It is well established that immune dysfunction plays a critical role in the etiology of NDs and sex differences related to the immune system may mediate sex differences observed in NDs. Of particular interest is sex differences in the number and morphology of microglia. Research in rats has shown that males have more microglia than females at postnatal day 4 (P4) in the parietal cortex, hippocampus, and amygdala (Schwarz et al., 2012). However, this effect is reversed at adolescence (P30), with females displaying a greater number of activated microglia

then males (Schwarz et al., 2012). Sex differences in microglia number and morphology suggests that there may be sex-specific periods in development (i.e., puberty) where the over-activation of microglia can have enduring effects on microglial and neuronal function (Hanamsagar & Bilbo, 2016). Moreover, males tend to have a higher incidence of NDs earlier in life (i.e., ALS, PD, schizophrenia) whereas females tend to have a higher incidence of NDs later in life (i.e., AD, schizophrenia), supporting the notion that perturbations of microglial function during critical periods of development may influence the development of NDs later in life (McCombe & Henderson, 2010; Pinares-Garcia et al., 2018; Podcasy & Epperson, 2016; Yanguas-Casás, 2017). Thus, higher number of activated microglia during critical periods of development could be harmful and may influence the development of NDs in males and females.

Sex differences in the transcriptome of microglia in adulthood could also explain sex differences observed in NDs (Hanamsagar et al., 2017; Thion et al., 2018). Profiling of microglia in 3-, 12-, and 24-month old male and female mice revealed sex differences in the transcriptome of microglia at all time points with the greatest sex differences observed in 24-month old mice (S. S. Kang et al., 2018). Moreover, single-nuclei RNA sequencing in the prefrontal cortex revealed large microglial transcriptomic differences between AD and control human brains (Mathys et al., 2019). Interestingly, a greater number of microglia associated with AD was observed in human female brains while a greater number of microglia not associated with AD was observed in human male brains (Mathys et al., 2019). These results suggest that the transcriptome of microglia becomes sexually divergent with age and that these transcriptomic sex differences could influence the development of NDs. Another factor that has been strongly linked to the development of NDs is the gut microbiome.

3.0 Gut Microbiome

The gut microbiome hosts trillions of microorganisms including bacteria, archaea, viruses and eukaryotic microbes**.** These microorganisms reside along the intestinal tract (i.e., esophagus, stomach, and intestine) and have various functions aimed at maintaining physiological homeostasis. Functions include vitamin and nutrient synthesis, carbohydrate fermentation, regulating immune function, and protecting against pathogens (Huttenhower et al., 2012; Shreiner et al., 2015). The understanding of the function and structure of the gut microbiome in health and disease has greatly increased over the years, due primarily to technological advancements (i.e., 16s RNA sequencing) in the analysis of microbial composition (Weersma et al., 2020). In healthy human individuals, the gut microbiome predominately consists of bacterial species from the *Bacteroidetes* and *Firmicutes* phyla. There are also less abundant amounts of bacterial species from the *Proteobacteria*, *Actinobacteria*, *Verrucomicrobia*, and *Fusobacteria* phyla (Hall et al., 2017). There are also varying amounts of bacteria depending on the region of the intestinal tract being examined. For example, the colon has a high density of bacteria from the *Bacteroidaceae*, *Prevotellaceae*, *Rikenellaceae*, *Lachnospiraceae* and *Ruminococcaceae* families, while the small intestine has a high density of *Lactobacillaceae* and *Enterobacteriaceae* families (Donaldson et al., 2016; Johansson et al., 2008). Various factors can influence the composition of the gut microbiome such as sex, genetics, antibiotics, probiotics, ethnicity, diet, and bacterial infections (Gaulke & Sharpton, 2018; Kim et al., 2020; Langdon et al., 2016; Xu & Knight, 2015; Yatsunenko et al., 2012; Y.-J. Zhang et al., 2015). An imbalance in the gut microbial community (i.e., dysbiosis) can have harmful effects on brain functioning and behaviour and is associated with various disorders such as autism, depression, anxiety, AD, and PD (Baizabal-Carvallo & Alonso-Juarez, 2020; Clapp et al., 2017; Fattorusso et al., 2019; S. Liu et al., 2020).

The bidirectional communication between microbiota and the brain is referred to as the 'gut-brain axis' (Rhee et al., 2009). The gut-brain axis is composed of multiple pathways including the CNS, autonomic nervous system, and enteric nervous system (Cryan et al., 2019). The autonomic system includes sympathetic and parasympathetic branches consisting of afferent and efferent fibers responsible for various involuntary physiological processes (i.e., heart rate, blood pressure, digestion) (Waxenbaum et al., 2022). Afferent signals begin in the intestinal lumen and travel to the CNS through spinal, vagal, and enteric pathways, while efferent signals begin in the CNS and travel to the intestinal wall (Carabotti et al., 2015). The gut microbiota can also influence the CNS through the production of several bioactive molecules such as cytokines, prostaglandins, and microbial antigens (i.e., LPS) (Farzi et al., 2018). These molecules can cross the BBB and directly influence the functioning of the CNS (Schächtle & Rosshart, 2021). Thus, both humoral and neural pathways are involved in the gut-brain axis and can have a profound influence on brain functioning and behaviour.

3.1 Role of microbiota in neurodevelopment

The gut microbiota plays a vital role in neurodevelopmental processes such as neurogenesis, myelination, maturation of microglia, and BBB formation (Cerdó et al., 2020; Erny et al., 2015; Hoban et al., 2016; Parker et al., 2020). For example, germ free (i.e., mice raised without microbiota) and antibiotic-treated mice display reduced expression of the tight junction proteins occludin and claudin-5 in the hippocampus, suggesting increased BBB permeability (Braniste et al., 2014a; Fröhlich et al., 2016). Furthermore, antibiotic treatment in mice results in a decrease of bromodeoxyuridine in the hippocampus, indicating a reduction in hippocampal neurogenesis (Möhle et al., 2016). Similarly, the absence or alteration of the gut microbiota can result in abnormal myelination and microglial function. For instance, germ free

mice display a high number of immature microglia in several brain regions (i.e., cortex, corpus collosum, hippocampus, cerebellum), an effect that is replicated in antibiotic-treated mice (Erny et al., 2015). Moreover, germ free mice display abnormal myelination of axons within the PFC (Gacias et al., 2016; Hoban et al., 2016). Normal functioning of these neurodevelopmental processes can be restored through the administration of microbial metabolites (short chain fatty amino acids; SCFA) and probiotics (Braniste et al., 2014a; Erny et al., 2015; Keogh et al., 2021; Möhle et al., 2016). Therefore, the gut microbiota plays a significant role in the regulation of neurodevelopment and can either have protective or harmful effects on the CNS during development.

Alterations to the gut microbiome is associated with neurodevelopmental disorders such as autism spectrum disorder and schizophrenia (Mehra et al., 2022; Munawar et al., 2021). Gut dysbiosis has been reported in autism spectrum disorder patients with these patients having elevated levels of *Proteobacteria*, *Lactobacillus*, *Bacteroides*, *Desulfovibrio,* and *Clostridium* along with decreased levels of *Bifidobacterium*, *Blautia, Dialister*, *Prevotella, Veillonell*a and *Turicibacter*, in comparison to controls (F. Liu et al., 2019). Similarly, schizophrenic patients have elevated levels of *Succinivibrio, Megasphaera*, *Collinsella, Clostridium, Klebsiella,* and *Methanobrevibacter* along with decreased levels of *Coprococcus, Roseburia*, and *Blautia,* in comparison to controls (Shen et al., 2018). Moreover, these neurodevelopmental disorders are often accompanied with gastrointestinal disorders including constipation, diarrhea, abdominal pain, celiac disease, and irritable bowel syndrome (Fadgyas-Stanculete et al., 2014; Hsiao, 2014; Kalaydjian et al., 2006). Providing treatments that target restoring gut microbiota homeostasis (i.e., probiotics) has also been shown to ameliorate the symptoms of autism spectrum disorder and schizophrenia, in humans(Abdellatif et al., 2020; Dickerson et al., 2014; Navarro et al.,

2016; Szeligowski et al., 2020). Taken together, these findings demonstrate the impact that the microbiota can have on brain functioning, and possibly mediate the onset and progression of neurodevelopmental disorders. However, the influence of the gut microbiota on the neurodevelopment during puberty and adolescence remains largely uninvestigated.

3.2 Microbiota and stress

Growing evidence suggests that the gut microbiome can influence the maturation and reactivity of the HPA axis (Farzi et al., 2018). Germ free mice exposed to 1 hour of restraint stress display greater levels of plasma ACTH and CORT in comparison to specific pathogen free mice (Sudo et al., 2004). Moreover, reconstitution with commensal bacteria at 3 weeks of age reverses the elevated HPA axis response observed in germ free mice. However, reconstitution at a later stage (i.e., before 6 weeks of age) has no effect on the HPA axis response (Sudo et al., 2004). The effects of the gut microbiome on HPA axis reactivity are not limited to blood markers. Germ free mice also display elevated levels of several glucocorticoid receptor pathway genes (i.e., Slc22a5, Aqp1, Stat5a, Ampd3, Plekhf1, and Cyb561) in the hippocampus (Luo et al., 2018). Although the mechanisms underlying the effects of microbiota on HPA axis responsiveness are not fully understood, it is believed that gut dysbiosis (i.e., induced by exposure to stressors) increases the production of bioactive molecules that can directly influence the HPA axis (Foster et al., 2017). For instance, gut dysbiosis upregulates cytokines (i.e., TNFα, IL1β, IL6) and prostaglandins (i.e., prostaglandin E2) which can subsequently cross the BBB and activate the HPA axis (Banks, 2005; Elias-Oliveira et al., 2020; Gądek-Michalska et al., 2013; Zimomra et al., 2011). Moreover, both LPS and peptidoglycan (i.e., components of the cell wall of gram-negative bacteria) are upregulated in response to gut dysbiosis and have been shown to be potent activators of the HPA axis (Arentsen et al., 2017; Sonali et al., 2022; Vakharia &

Hinson, 2005). As such, the gut microbiota can influence HPA axis reactivity and play a crucial role in the programming of the HPA axis.

Multiple lines of research have also demonstrated an association between microbiota and stress-related disorders such as anxiety and depression. Both anxiety and depression have high comorbidities with gastrointestinal disorders such as irritable bowel syndrome, Crohn's disease, celiac disease, and ulcerative colitis, in humans (Byrne et al., 2017; Jackson et al., 2012; Tap et al., 2017). Moreover, research with germ free mice has shown that the absence of microbiota results in a decrease of anxiety-like behaviours while germ free rats display an increase in anxiety-like behaviours (Crumeyrolle-Arias et al., 2014; Luczynski et al., 2016). Interestingly, colonizing germ free swiss webster mice with microbiota from Balb/C mice increases anxiety-like behaviours, while colonizing germ free Balb/C mice with microbiota from swiss webster mice decreases anxiety-like behaviours (Bercik et al., 2011). Similar findings are observed when examining the effects of gut microbiota on depression-like behaviours. For example, gut dysbiosis induced by chronic unpredictable mild stress in mice increases depression-like behaviours. Moreover, transferring microbiota from stressed mice to naïve mice results in an increase of depression-like behaviours in the naïve mice (Chevalier et al., 2020).

3.3 Microbiota and neurodegeneration

Gut dysbiosis has been implicated in various neurodegenerative processes including the production of amyloid proteins, inflammation, oxidative stress, impaired SCFA synthesis, and increased intestinal and BBB permeability (Braniste et al., 2014a; Luca et al., 2019; Roy Sarkar & Banerjee, 2019; Silva et al., 2020; Sochocka et al., 2019). Recent research has shown that AD patients suffer from dysbiosis as demonstrated by increased levels of *Ruminococcaceae*, *Enterococcaceae*, and *Lactobacillaceae* along with decreased levels of *Bacteroidaceae*,

Veillonellaceae, and *Lachnospiraceae*, in comparison to controls (Zhuang et al., 2018). Furthermore, antibiotic-induced dysbiosis in a mouse model of AD has been shown to reduce neuroinflammation and amyloidosis, implicating microbiota in the pathogenesis of AD (Minter et al., 2016). Similarly, PD patients have been reported to suffer from dysbiosis with increased levels of *Lactobacillus*, *Akkermansia*, and *Bifidobacterium* and decreased levels of *Lachnospiraceae* and *Faecalibacterium*, in comparison to controls (Romano et al., 2021). Moreover, in a rotenone-induced mouse model of PD, wild-type mice display greater motor deficits (i.e., motor strength and coordination) in comparison to germ free mice, further supporting the role of microbiota in the development of PD (Bhattarai et al., 2021).

It is theorized that proteins involved in NDs, such as Aβ and alpha-synuclein, can aggregate and spread throughout the brain in a prion-like manner (i.e., neuron-to-neuron propagation) (Desplats et al., 2009; Frost & Diamond, 2010). However, there is continuous debate regarding the site of origin for the pathological aggregation of these proteins. Both Aβ and alpha-synuclein aggregations have been observed in the gut of mice prior to spreading to the CNS, suggesting that both AD and PD pathology may originate in the gut (Angot & Brundin, 2009; Kowalski & Mulak, 2019). Aggregates of alpha-synuclein can be found within enteroendocrine cells (i.e., sensory cells of the gut) which synapse with enteric nerves. From enteric nerves, alpha-synuclein aggregates can enter the vagus nerve allowing for transportation to the brain (Liddle, 2018). Similarly, Aβ aggregates have been observed in the vagus nerve of mice, suggesting that a similar pathway may be used for the spreading of Aβ to the CNS (Sun et al., 2020). Research in mice has also shown that vagotomy reduces the pathologic spreading of Aβ and alpha-synuclein in the CNS, further supporting the role of the vagus nerve as a potential pathway involved in NDs (C. Chen et al., 2021; Uemura et al., 2018). As such, the gut

microbiome may not only influence the pathogenesis of NDs, but could also be the site of origin for NDs.

Current Studies and Objectives

The following studies examined the sex-dependent effects of pubertal LPS and antimicrobial treatments on acute cellular mechanisms associated with neurodegeneration (Chapter 2), the enduring effects of pubertal LPS and antimicrobial treatments on cellular mechanisms and behaviours associated with neurodegeneration (Chapter 3), and the effects of pubertal LPS and antimicrobial treatments on intestinal and BBB permeability along with examining gross motor coordination, heart rate, and core body temperature (Chapter 4). We hypothesized that pubertal LPS and antimicrobial treatments would have sex-dependent effects on acute and enduring cellular mechanisms and behaviours associated with neurodegeneration. We also expected that pubertal LPS and antimicrobial treatments would increase intestinal and BBB permeability along with decreasing gross motor coordination, heart rate, and core body temperature, in a sexually dimorphic manner. The goal of these experiments was to further elucidate the relationship between the gut microbiome and neurodegenerative disorders along with determining whether exposure to stressors during puberty can increase susceptibility to neurodegenerative disorders later in life.

CHAPTER 2: THE ACUTE EFFECTS OF ANTIMICROBIALS AND LIPOPOLYSACCHARIDE ON THE CELLULAR MECHANSIMS ASSOCIATED WITH NEURODEGENERATION IN PUBERTAL MALE AND FEMALE CD1

Published

Esposito, P., Gandelman, M., Rodriguez, C., Liang, J., & Ismail, N. (2022). The acute effects of antimicrobials and lipopolysaccharide on the cellular mechanisms associated with neurodegeneration in pubertal male and female CD1 mice. *Brain, Behavior, & Immunity - Health, 26*, 100543. https://doi.org/10.1016/j.bbih.2022.100543

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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 200μL 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 eight hours 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 behavior, 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.

Keywords: Puberty, Neurodegeneration, Dysbiosis, Inflammation, Sex, Immune system, Lipopolysaccharide, Antimicrobials

1.0 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 & 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 & 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 (K. B. Smith et al., 2021a). 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 & 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 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 & 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; Z. 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 signaling 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.

2.0 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. 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 200sev of mixed broad-spectrum antimicrobial solution or water through gavage twice daily for seven days as described in

(Esposito, Kearns, 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. 480125GM, 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 hours and 1800 hours, 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 hours (Cai et al., 2016).

2.4 Sickness monitoring

Sickness monitoring was conducted at 2, 4, 6, and 8 hours 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.

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 anti-coagulant, EDTA. Tubes were kept at 4°C until plasma extraction. Within three hours of blood collection, samples were centrifuged at 1,000 x g at 20°C for 15 minutes 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 300μM, 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, 0.78gm NaH2PO4•H2O, 24gm NaCl, 0.6gm 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 10μL 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 90-110%. 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 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 & 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; 20mM Tris base, 137mM 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 x 10-min washes with TBST and were then incubated for one hour 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 x10-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 hours) 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 x 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 $p < .05$.

3.0 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, $p < 0.01$; Figure 2A and B). Mice treated with LPS displayed significantly less sickness behaviours 2 hours 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 hours (*MD* = 1.22, *SE* = 0.09, $p < 0.01$; Figure 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; Figure 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; Figure 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 ($F_{(1, 72)} = 4.08$, $p = 0.05$, $\eta_p^2 = 0.05$), 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; Figure 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; Figure 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; Figure 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; Figure 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, $p < 0.01$; Figure 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 (Figure 5A and B). Furthermore, no significant difference in TH expression was found in the SN (Figure 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; Figure 5C).

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; Figure 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 ($F_{(1, 48)} = 7.33$, $p = .001$, $\eta_p^2 = 0.13$; $F_{(1, 48)} = 9.23$, $p < .001$, $\eta_p^2 = 0.16$; 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).

4.0 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 neurodegeneration in adult male subjects (Batista et al., 2019; Brandscheid et al., 2017; Fröhlich et al., 2016; S. 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; J. 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 & 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 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 & 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 & 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 & 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 & 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 eight

hours 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.

4.2 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.

Funding: This work was supported by the National Sciences and Engineering Research Council of Canada (2020-04302) to NI.

Acknowledgments: The authors would like to thank all the members of the NISE Lab and the ACVS staff at the University of Ottawa for their assistance with this project.

Declaration of Interests: None

Figures, Tables and Captions

Table 1.

Table. 1 Summary of Primer Sequences

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

Figure 1.

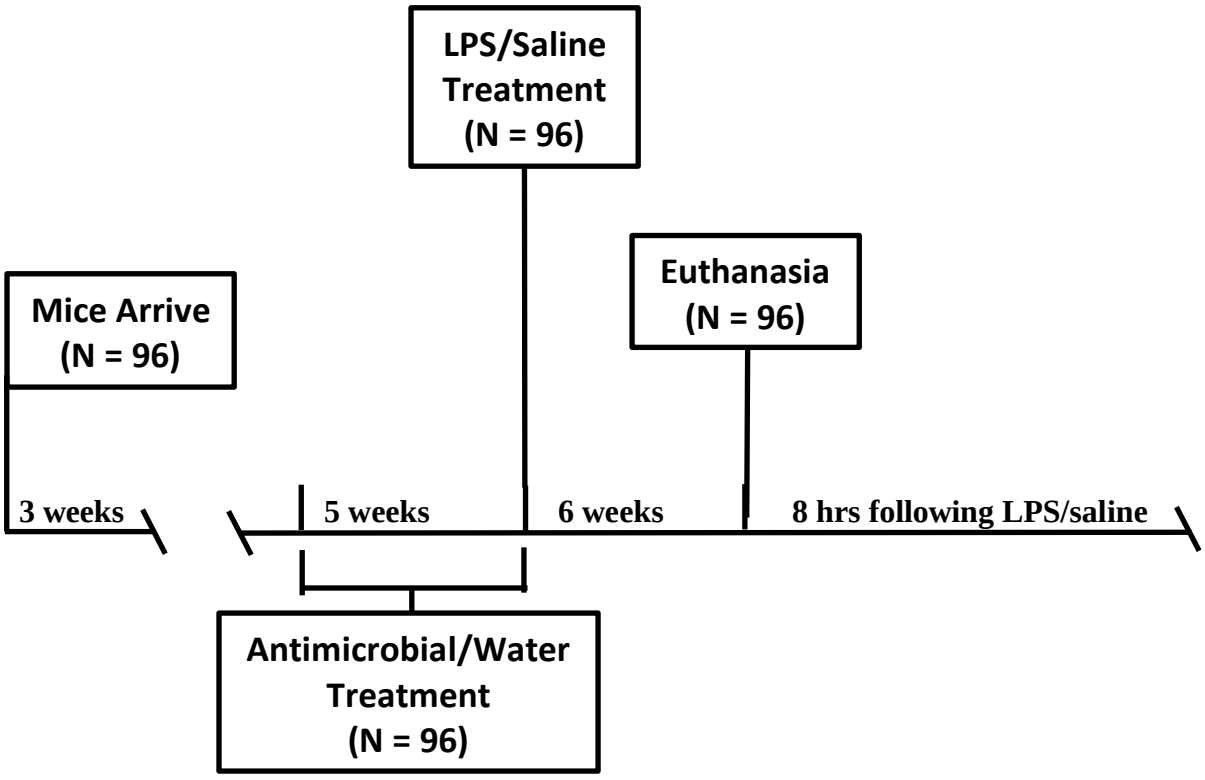
LPS/Saline Treatment (N = 96)
Mice Arrive (N = 96)
Euthanasia (N = 96)
3 weeks
5 weeks
6 weeks
8 hrs following LPS/saline
Antimicrobial/Water Treatment (N = 96)

Figure 2.

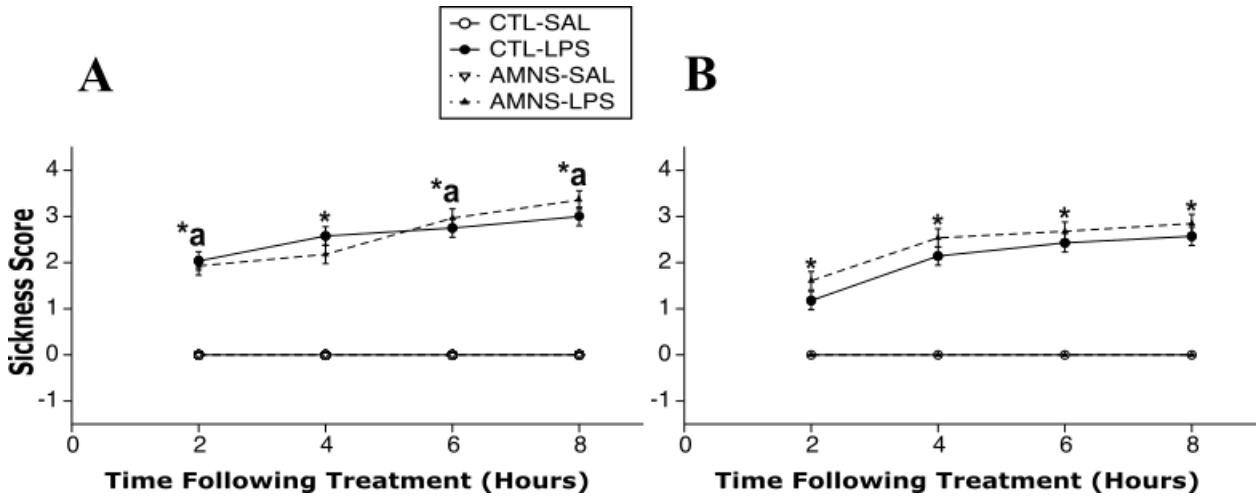
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
A
B
Sickness Score
Time Following Treatment (Hours)
Time Following Treatment (Hours)

Figure 3.

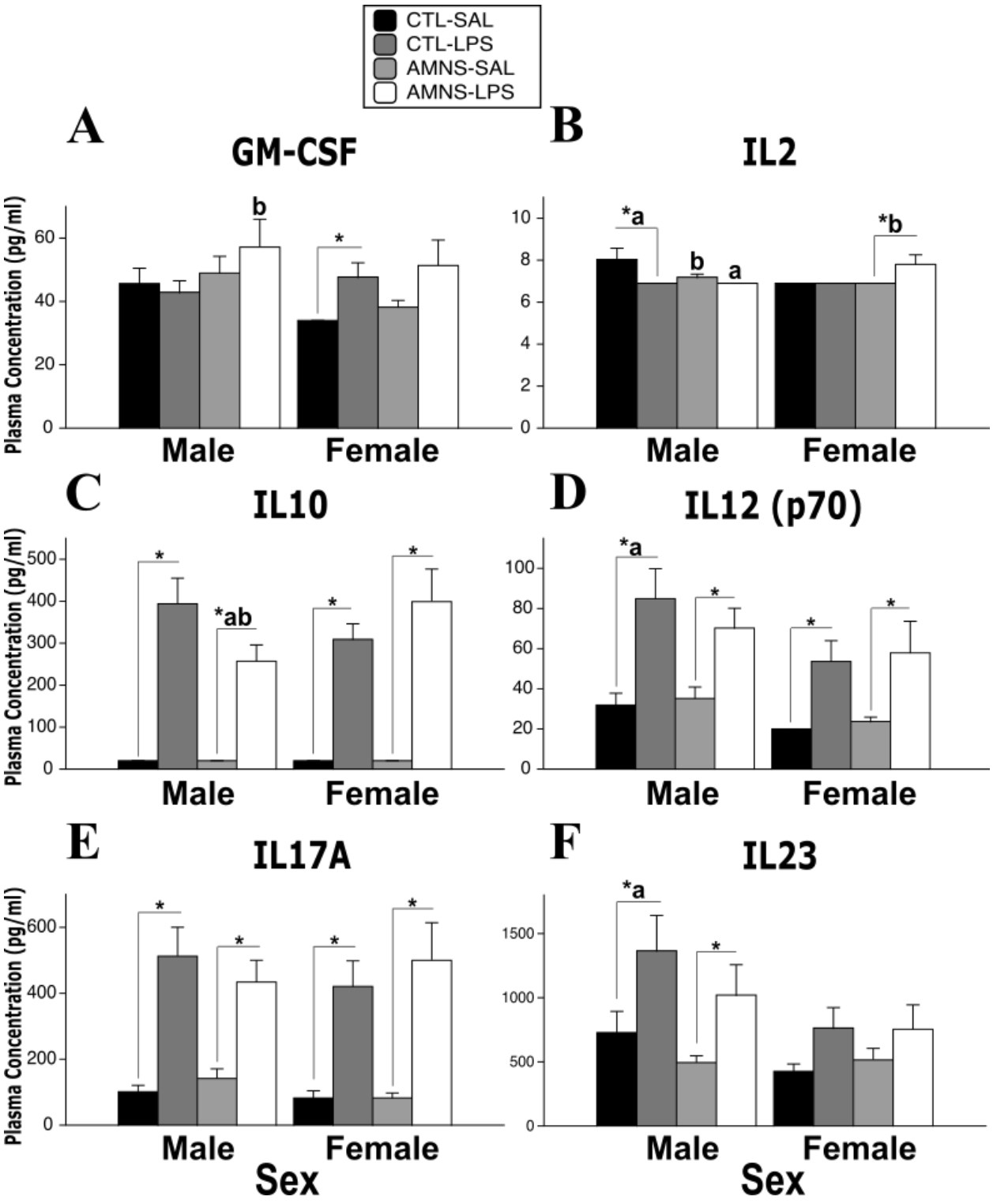
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
A
GM-CSF
B
IL2
C
IL10
D
IL12 (p70)
E
IL17A
F
IL23
Plasma Concentration (pg/ml)
Male
Female
Sex
b
*
*a
a
*b
*ab

Figure 4.

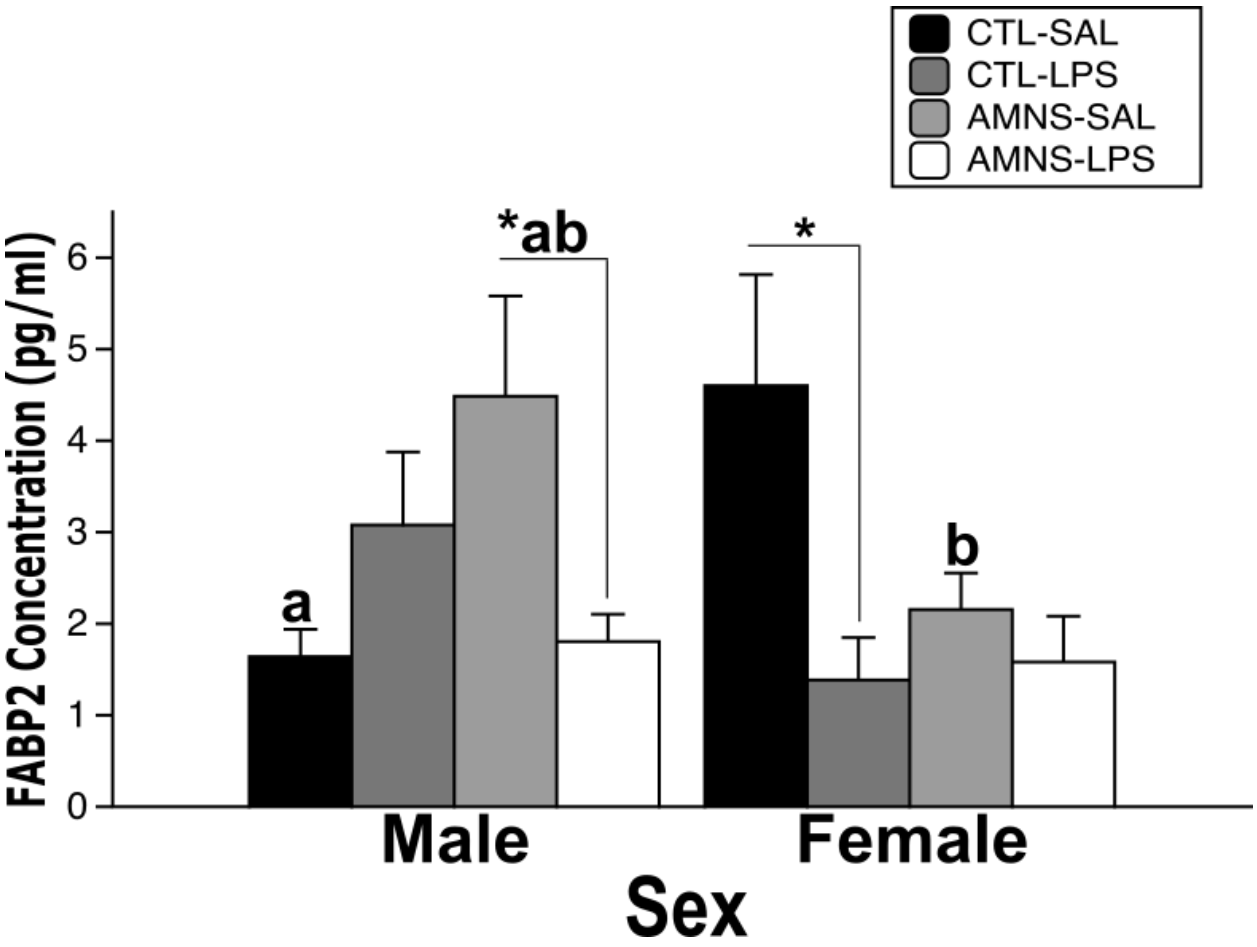
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
FABP2 Concentration (pg/ml)
0
1
2
3
4
5
6
a
*ab
*
b
Male
Female
Sex

Figure 5.

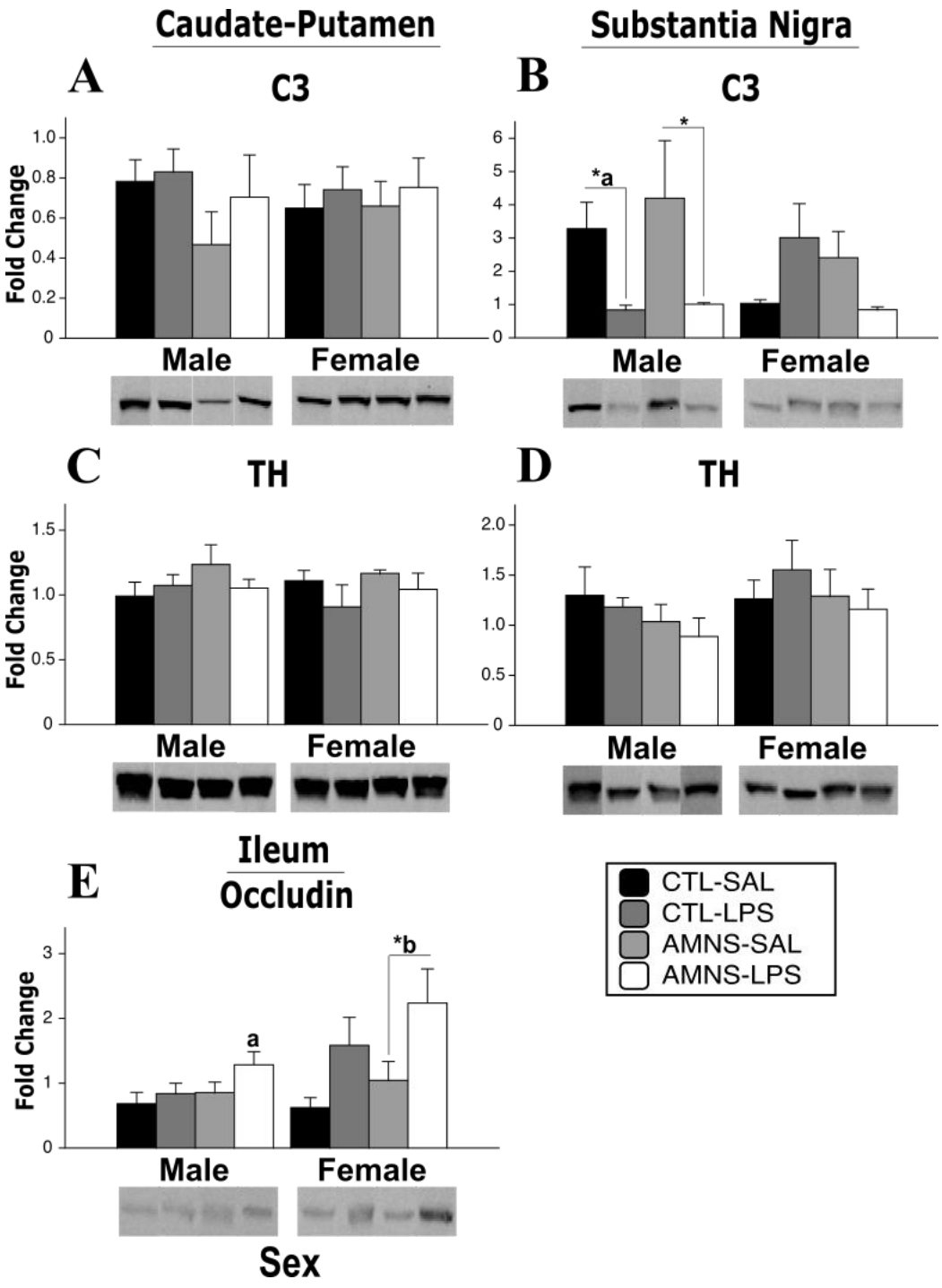
Caudate-Putamen
Substantia Nigra
A
C3
B
C3
Fold Change
1.0
0.8
0.6
0.4
0.2
0
6
5
4
3
2
1
0
*a
*
Male
Female
Male
Female
C
TH
D
TH
Fold Change
1.5
1.0
0.5
0
2.0
1.5
1.0
0.5
0
Male
Female
Male
Female
E
Ileum
Occludin
Fold Change
3
2
1
0
a
*b
Male
Female
Sex
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS

Figure 6.

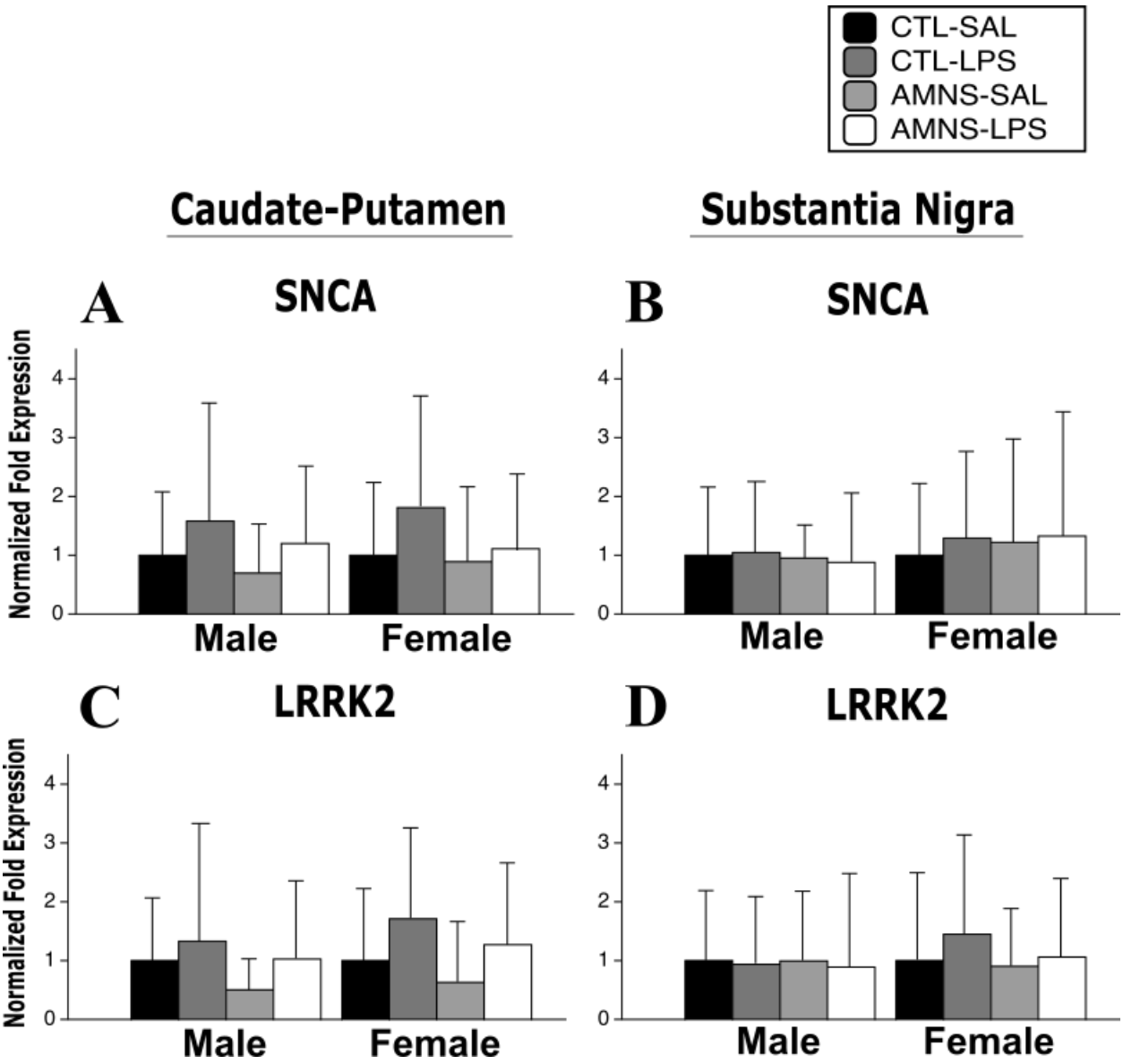
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
Caudate-Putamen
Substantia Nigra
A
SNCA
B
SNCA
C
LRRK2
D
LRRK2
Normalized Fold Expression
Normalized Fold Expression
0
1
2
3
4
Male
Female

Figure Captions

Figure 1.

Title: Experimental timeline

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

Figure 2.

Title: Sickness behavior symptoms

Caption: Mean (±SEM) sickness score 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 ($p < 0.05$).

Figure 3.

Title: Cytokine concentrations

Caption: 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 ($p < 0.05$).

Figure 4.

Title: FABP2 concentration

Caption: 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 ($p < 0.05$).

Figure 5.

Title: C3, TH, and occludin protein expression

Caption: 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 ($p < 0.05$).

Figure 6.

Title: SNCA and LRRK2 mRNA expression

Caption: 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 ($p < 0.05$).

CHAPTER 3: ENDURING EFFECTS OF PUBERTAL ANTIMICROBIALS AND LIPOPOLYSACCHARIDE TREATMENTS ON BEHAVIOURS AND CELLULAR MECHANISMS ASSOCIATED WITH NEURODEGENERATION IN MALE AND FEMALE CD1 MICE

Published

Esposito, P., Dubé-Zinatelli, E., Gandelman, M., Liu, E., Cappelletti, L., Liang, J., & Ismail, N. (2024). The Enduring Effects of Antimicrobials and Lipopolysaccharide on the Cellular Mechanisms and Behaviours Associated with Neurodegeneration in Pubertal Male and Female CD1 Mice. Neuroscience, 557, 67-80. https://doi.org/10.1016/j.neuroscience.2024.08.007

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Abstract

Puberty is a sensitive developmental period during which stressors can cause lasting brain and behavioural deficits. While the acute effects of pubertal lipopolysaccharide (LPS) and antimicrobial (AMNS) treatments are known, their enduring impacts on neurodegeneration-related mechanisms and behaviours remain unclear. This study examined these effects in male and female mice. At five weeks old, mice received 200ul of either broad-spectrum antimicrobials or water through oral gavage twice daily for seven days. At six weeks of age, they received an intraperitoneal injection of either saline or LPS. Four weeks later, adult mice underwent neurodegeneration-related behavioural tests, including the rotarod, forepaw stride length, reversed grid hang, open field, and buried pellet tests. Two days after the final test, brain and ileal samples were collected. Results showed that female mice treated with both AMNS and LPS exhibited deficits in neuromuscular strength, while males treated with LPS alone showed increased anxiety-like behaviours. Males treated with AMNS alone had decreased sigma-1 receptor (S1R) expression in the cornu ammonis 1 (CA1) and dentate gyrus (DG), while females treated with both AMNS and LPS had decreased S1R expression. Additionally, males treated with either LPS or AMNS had lower glial-derived neurotrophic factor receptor alpha-1 (GFRA1) expression in the primary motor cortex (M1) than females. Mice treated with LPS alone had decreased GFRA1 expression in the DG and decreased S1R expression in the secondary motor cortex (M2). These findings suggest that pubertal AMNS and LPS treatments may lead to enduring changes in biomarkers and behaviours related to neurodegeneration.

Keywords: Puberty, Neurodegeneration, Sex, Dysbiosis, Lipopolysaccharide, Antimicrobials

1.0 Introduction

Puberty is a critical period of development marked by physical changes (i.e., vaginal opening in females and preputial descent in males) and a transition from a non-reproductive to a reproductive state (Abreu & Kaiser, 2016; Huang et al., 2012; Sisk & Foster, 2004). The central nervous system (CNS) undergoes significant reorganizing and remodeling during puberty, rendering this period of development particularly sensitive to stressors (Esposito, Kearns, et al., 2022; Ismail et al., 2011; Murack et al., 2021; Murray et al., 2020; Sisk & Foster, 2004). For example, pubertal exposure to the bacterial endotoxin lipopolysaccharide (LPS) increases anxiety-like behaviours in adult male mice and depression-like behaviours in adult female mice (Murray et al., 2019). Pubertal exposure to LPS also increases Parkinson-like behaviours in male mice, along with inducing enduring deficits in cognitive functioning (i.e., spatial and learning memory) in both male and female mice (Dinel et al., 2014; Girard-Joyal & Ismail, 2017; Kolmogorova et al., 2019). Moreover, male mice treated with LPS during puberty demonstrate apoptosis in the medial prefrontal cortex (mPFC) along with increased expression of postsynaptic density protein 95 and microglia in the hippocampus (HIPP) in adulthood. Conversely, female mice treated with LPS during puberty demonstrate increased microglia expression in the paraventricular nucleus of the hypothalamus, along with a more “activated” microglial state in the mPFC and HIPP in adulthood (Kolmogorova et al., 2021; Kolmogorova & Ismail, 2021). These findings demonstrate that pubertal exposure to stressors induces enduring sex-dependent behavioural and neurochemical changes, potentially increasing susceptibility to psychiatric disorders later in life in a sexually dimorphic manner. Due to the increased sensitivity to stressors during puberty, it is possible that pubertal exposure to stressors can also increase susceptibility to neurodegenerative disorders later in life.

Neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD) are characterized by the progressive loss of neuronal function and apoptosis throughout the CNS (Dugger & Dickson, 2017). The progressive atrophy of the CNS during neurodegenerative diseases results in deficits related to motor skills (i.e., gait, ataxia), cognitive functioning (i.e., memory, executive functions), emotions (i.e., anxiety, dysphoric and euphoric mood) and behaviours (i.e., disinhibition, apathy) (Esposito & Ismail, 2022; Haack et al., 2016; Levenson et al., 2014; Wirth et al., 2013). A key factor in the progressive loss of neuronal function in neurodegenerative diseases is abnormal protein aggregations such as beta-amyloid in AD and alpha-synuclein in PD (LaFerla et al., 2007; Stefanis, 2012). In addition to these abnormal protein aggregations, several biomarkers associated with regulating neurodegenerative processes (i.e., excitotoxicity, mitochondrial dysfunction, neuroinflammation, oxidative stress, and cell death) are altered during neurodegenerative diseases (Amor et al., 2010; Andreone et al., 2020; Chen et al., 2012; Dong et al., 2009; Dugger & Dickson, 2017; Hashimoto et al., 2003). For example, early-onset AD patients show decreased sigma-1 receptor (S1R) expression, a protein that regulates calcium homeostasis, glutamate activity, mitochondrial function, endoplasmic reticulum function, reactive oxygen species production, and neuroinflammation (Mishina et al., 2008; Nguyen et al., 2015). Moreover, patients with multiple systems atrophy display decreased glial-derived neurotrophic factor (GDNF) expression, a protein that plays a critical role in the survival of dopaminergic, noradrenergic, and motor neurons (Allen et al., 2013; Ubhi et al., 2010). However, the mechanisms underlying the modulation of these biomarkers remain unknown. Growing evidence suggests that the gut microbiome may play a role in the pathogenesis of neurodegenerative disorders.

The gut microbiome is a complex and dynamic system that hosts trillions of microorganisms (Qin et al., 2010). These microbes are responsible for regulating physiological homeostasis through various functions such as carbohydrate fermentation, vitamin, and nutrient synthesis, regulating immune function and protecting against pathogens (Esposito & Ismail, 2022; Huttenhower et al., 2012; Shreiner et al., 2015). Alterations to the gut microbiome (i.e., dysbiosis) have been linked with the development of neurodegenerative disorders such as AD and PD. For example, increased amyloid concentrations in AD patients are associated with gut dysbiosis, decreased anti-inflammatory cytokine concentrations (i.e., IL10) and increased blood LPS and pro-inflammatory cytokine (IL1β, IL6, NLRP3, CXCL2) concentrations (Marizzoni et al., 2020). Moreover, germ-free mice overexpressing alpha-synuclein display reduced neuroinflammation, alpha-synuclein inclusions, and motor deficits compared to wild-type mice. However, colonization of germ-free mice with microbially produced short-chain fatty amino acids increases neuroinflammation and motor deficits, implicating the gut microbiome in the pathogenesis of PD (Sampson et al., 2016). Moreover, PD patients display increased concentrations of toll-like receptor 4 in the colon, and TLR4 KO mice treated with rotenone (i.e., broad-spectrum insecticide, piscicide, or pesticide) display less intestinal inflammation, neuroinflammation, motor dysfunction, and neurodegeneration compared to wild-type mice (Perez-Pardo et al., 2019). As such, TLR4-mediated inflammation in the intestine and brain could play a role in the pathogenesis of neurodegenerative disorders. Taken together, these findings demonstrate that the gut microbiome modulates several neurodegenerative processes and could play a central role in the development of neurodegenerative disorders.

Recent research from our laboratory has demonstrated that pubertal LPS and antimicrobial (AMNS) treatments induce sex-dependent changes in acute cellular mechanisms

associated with neurodegeneration (Esposito et al., 2022). Furthermore, AMNS-induced alterations to the gut microbiome have a significant influence on the host immune response during LPS-induced inflammation and can induce neurodevelopmental deficits (Esposito, Kearns, et al., 2022; Jacobs et al., 2020; Tejkalová et al., 2023). However, the enduring effects of pubertal LPS and AMNS treatments on brain functioning and behaviours related to neurodegeneration remain unknown. Moreover, more information is needed regarding how neurodegenerative processes could affect males and females differently. Therefore, the objective of this study was to examine the enduring effects of pubertal LPS and AMNS treatments on behaviours and brain functioning related to neurodegeneration in both male and female mice. We hypothesized that pubertal LPS and AMNS treatments would induce deficits in neurodegenerative-related behaviours (i.e., muscular strength, gait, olfactory function, anxiety), decrease the expression of biomarkers involved in the regulation of neurodegenerative processes (i.e., S1R, GDNF receptor alpha 1; GFRA1), and increase the expression of ileal TLR4 expression, in a sexually dimorphic manner.

2.0 Experimental procedures

2.1 Animals

80 male and female CD-1 mice were delivered from Charles River Laboratories (Saint-Constant, Québec, Canada) at three weeks of age. Upon arrival, mice were pair-housed in polycarbonate Lexan housing cages (17 cm wide × 28 long × 12 cm high) in either all-male or all-female rooms, which 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). Cages 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 behavioural tests were completed during the dark phase under dim red light. Under our housing conditions, six-week-old CD-1 female mice do not display estrous cycling, and scrotal width of six-week-old male mice has not reached adult size (Murray et al., 2023). As such, 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 200μL of mixed broad-spectrum antimicrobial solution or water through gavage twice daily for seven days as described in Esposito et al., (2022). The treatments were administered at 0600 hours and 1800 hours, respectively. The AMNS solution contained 15 mg/mL of ampicillin (No. BP1760-5, Fisher Scientific, Geel, Belgium), neomycin (No. 480125GM, 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 and was made fresh daily. This dosage and treatment regimen has been shown to sufficiently suppress total microbial content (Zarrinpar et al., 2018).

2.3 Lipopolysaccharide administration

Male and female six-week-old CD-1 mice received an intraperitoneal (ip) injection of either 1.5 mg/kg of LPS (Escherichia coli serotype 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 induces sickness behaviours for approximately 24-48 hours in both male and female mice (Cai et al., 2016).

2.4 Sickness monitoring

Sickness monitoring was conducted 2, 4, 6, 8, 12, 24, and 48 hours following the LPS/saline injection. A non-invasive and unbiased approach where two raters blind to the experimental conditions was used to assess the progression of sickness behaviours (as described in Kolmogorova et al., 2017). Mice were visually assessed by the raters for symptoms of lethargy (reduced locomotion), huddling (curled body posture), ptosis (drooping eyelids), and piloerection (erection of fur). The total number of symptoms displayed by each mouse (one symptom = 1, two symptoms = 2, three symptoms = 3, four symptoms = 4) was scored by the raters at each time point. Sickness scores from the two raters at each time point were averaged and used in statistical analyses.

2.5 Behavioural testing

One month following LPS/saline injections, when all mice reached adulthood, they underwent behavioural testing (i.e., rotarod, forepaw stride length, open field, reversed grid

hang, and olfactory tests). Behavioural tests were conducted over two weeks with a two-day rest between each test to avoid any carry-over effects.

2.4.1 Forepaw stride length test

The forepaw stride length test was used to assess whether mice displayed a Parkinsonian motor phenotype. The paws of the mice were dipped in non-toxic paint and were subsequently placed to walk on a strip of paper attached to a plank (60 x 5.5 cm) which was elevated 20 centimeters from a mat. The strides of the mice were then measured with a ruler. Each measurement started from the toe of the first step to the heel of the second step. The mean length of the three longest forepaw strides was calculated and used in the analysis. A longer stride indicated better motor coordination.

2.4.2 Reversed grid hang test

The reversed grid hang test was used to assess neuromuscular strength. The mice were put on a grid with holes measuring 1 x 1 cm (length x height), slowly reversing the grid and waiting for the mice to fall for a maximum of 60 seconds. There were three trials, and the mice were given a 60-second break between each trial. The best performance of each animal was considered in the analysis. The longer the mice stayed on the grid, the better their neuromuscular strength was.

2.4.3 Open field test

The open field test was used to assess locomotor activity and anxiety-like behaviours. Each mouse was put in a 100 x 100 cm square arena for five minutes. The peripheral (20 cm from each wall) and central zones (30 cm x 30 cm) were divided into equal areas. Greater time spent in the peripheral zones is associated with a higher level of anxiety. Panasonic video cameras were used

to observe and measure their movement and the amount of time spent in the different zones. The EthoVision (1.7) software was used to record and analyze the locomotor activity.

2.4.4 Rotarod test

The Rotarod test (Columbus Instruments, Ohio, USA) was used to measure motor coordination and balance. The mice were placed on a rotating rod (3.18 cm diameter) for a maximum of 5 minutes. This rod was set to turn at a speed of 1 rotation per minute with an acceleration of 0.1 rotations per minute, with a maximum of 30 rotations per minute. There were 3 trials, with a 1-minute rest between each of the trials. The best performance of each animal was considered in the analysis. A longer time without falling off the rod indicated better motor balance and coordination.

2.4.5 Buried pellet test

The buried pellet test was used to assess olfactory function. The mice were housed individually for 48 hours before the commencement of the test with *ad libitum* access to food and water. The test was run over 3 days following the 48 hours. On the first day, the mice were exposed to two Fruit Loops (Kellogg's, ON, Canada). On the second day, consumption of the Fruit Loops from day 1 was verified to ensure the mice's interest and all mice were given an additional two Fruit Loops. On the third day, mice were placed individually in clean cages and were deprived of food for 6 hours. Following the food deprivation, mice were removed from their cages, and a Fruit Loop was hidden randomly in a corner of the cage (1.5cm deep, under the bedding). The time taken to find the pellet and eat it was measured with a maximum time of 15 minutes. A longer time to find and consume the Fruit Loop indicated a reduction in olfactory function.

2.5 Tissue collection for Immunohistochemistry

Two days following the final behavioural test (at 12 weeks of age), the mice were euthanized with Euthanyl (Sodium pentobarbital; 500 mg/kg, ip). Intracardial perfusion was performed with 20 ml of 0.9% saline, followed by 20 ml of 4% paraformaldehyde (PFA). After perfusion, brains were extracted and placed in vials containing 10 ml of 4% PFA for 2 hrs at 4 °C. Then, the PFA solution was replaced with 10 ml of 30% sucrose for 24 hrs. The brains were then transferred to another vial containing 10 ml of 30% sucrose and stored at 4 °C until processing. The brains were sliced with a Leica VT1200 S automated vibrating blade microtome at a thickness of 30 μm. Sections were stored in tubes containing cryoprotectant at −20 °C until immunostaining.

2.6 Tissue extraction for Enzyme-linked immunosorbent assay (ELISA)

Following brain collection, the distal 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, 0.78gm NaH2PO4•H2O, 24gm NaCl, 0.6gm KCl, 3L dH2O). Following the washing step, the luminal surface of the ileum was scraped off and placed in tubes. These ileum samples were then 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 ileum homogenates were incubated on ice for 10 min, then centrifuged 3 times at 4 °C at 21,000 g for 10 minutes. The supernatants were then collected to assay total protein concentrations using the Pierce™ Bicinchoninic Acid Assay (BCA) protein assay kit (Thermo Fisher Scientific).

2.7 Immunohistochemistry (IHC)

Brain sections were rinsed three times with tris-buffered saline (TBS) (pH 7.6) for five minutes. Sections were incubated for 30 minutes in an antigen retrieval solution containing

0.05M sodium citrate tribasic dihydrate, followed by rinsing for 3 x 5 minutes in TBS and a 30-minute incubation in a 0.1M glycine solution. The sections were then rinsed for 3 x 5 minutes in TBS and incubated for 30 minutes in a concentrated blocking solution containing TBS, 20% normal goat serum (NGS), 0.1% Tween 20, and 1% hydrogen peroxide. Sections were transferred to a solution (TBS, 2% NGS, 0.1% Tween 20) containing a diluted primary antibody specific to either S1R (1/300; Abcam; cat: ab53852) or GFRα1 (1/250; Abcam; cat: ab233444) and incubated overnight at room temperature. Sections were then washed in a diluted blocking solution (TBS, 1% NGS, 0.1% Tween 20) three times for five minutes. Sections were incubated for 60 minutes at room temperature in a solution (TBS, 2% NGS, 0.1% Tween 20) containing a diluted secondary antibody (1/500; Vector, BA-1000, Biotinylated Goat anti-rabbit IgG; PC38 Millipore). Sections then underwent 3 x 10-minute washes with 0.1% Tween 20 and PBS, followed by a 1 hr incubation in the ABC detection system (1/100; Vector, PK-6100, ABC-Elite standard; Vector Laboratories, Burlingame, CA, USA). The sections again underwent 3 x 10-minute washes in TBS and were then incubated in freshly prepared diaminobenzidine (DAB) solution (DAB Peroxidase Substrate kit, SK-4100 Vector Laboratories, Burlingame, CA, USA), followed by 3 x 5-minute rinses in cold TBS. Lastly, sections were mounted on Fisherbrand Superfrost Plus microscope slides and coverslipped with Fisherbrand coverslips after applying Permount solution.

2.8 Image analysis and cell counting

The identification of the mPFC (Bregma +1.54 mm), HIPP (Bregma - 1.94 mm), and motor cortex (Bregma + 0.62 mm) was based on *The Mouse Brain Atlas in Stereotaxic Coordinates* (Paxinos & Franklin, 2019). Specific regions in the mPFC that were examined include the prelimbic cortex (PLC), anterior cingulate cortex (ACC), and infralimbic cortex (IF).

Hippocampal regions that were examined include the cornu ammonis 1 (CA1), cornu ammonis 2 (CA2), cornu ammonis 3 (CA3), and the dentate gyrus (DG). Lastly, the primary motor cortex (M1) and secondary motor cortex (M2) were examined in the motor cortex. Best-matched images of S1R and GFRA1 immunoreactive cells were imaged at 20x on an Olympus BX51 light microscope connected to a Jenoptik ProRes, MF scanning camera. For S1R, one slice was counted for each animal by two raters blind to the experimental conditions using Image J's cell counter. A threshold of 20% coefficient of variation (CV) was applied to ensure consistency in the cell counts between the raters. S1R-expressing cells have a unique ring-like structure (see Figure 4F), and manual counting was utilized to ensure the accurate counting of these cells alone (Liu et al., 2022). Cell counting for GFRA1 was done through automated counting on Image J. Binary output images were generated by applying an automatic intensity threshold, watershed segmentation (specifically for hippocampal regions) and background subtraction. Furthermore, binary objects that were abnormally small were removed from the output images by applying a defined minimum circularity and pixel size for each region. The accuracy of Image J's auto-counting was confirmed by ensuring the CV did not exceed 5% between automated and manual counts across five different brain sections for each region. The accuracy of each image was further verified by visually examining the outline of counted cells in the output image.

2.9 Enzyme-linked immunosorbent assay (ELISA)

TLR4 protein concentration in the ileum was measured with ELISA. The ELISA kit (No. CSB-E14275M; Cusabio) was used according to the manufacturer's instructions and ileal samples were measured in duplicates. Each plate contained one pooled sample to monitor inter-assay variation. All plates were read with Biotek Powerwave XS2 and analyzed with the Gen 5 V2.0 software.

2.10 Statistical analysis

Boxplots were used to identify outliers, and all cases that exceeded the 1.5 interquartile range were considered statistical outliers and were winsorized to the next extreme data point in the group (Anscombe, 1973). 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, 8, 12, 24, and 48 hours) 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., behavioural tests, IHC, ELISA), a 2 x 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 $p < .05$.

3.0 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 ($F_{(4.03,318.39)} = 83.64$, $p < 0.01$, $\eta p2 = 0.53$) along with time x LPS ($F_{(4.3,318.39)} = 59.42$, $p < 0.01$, $\eta p2 = 0.45$) and time x LPS x AMNS interactions ($F_{(4.3,318.39)} = 2.49$, $p < 0.05$, $\eta p2 = 0.03$). The three-way mixed ANOVA also found a significant between subjects' main effects of sex ($F_{(1, 74)} = 29.45$, $p < 0.01$, $\eta p2 = 0.10$) and LPS ($F_{(1, 74)} = 517.93$, $p < 0.01$, $\eta p2 = 0.66$) along with a significant sex x AMNS interaction ($F_{(1, 74)} = 14.75$, $p < 0.05$, $\eta p2 = 0.05$). Pairwise comparisons showed that regardless of sex and AMNS treatment, LPS-treated mice displayed significantly greater sickness behaviours in comparison to their saline-treated counterparts (*MD* = 1.90, *SE* = 0.16, $p < 0.01$). Moreover, male mice displayed significantly greater sickness behaviours than female mice (*MD* = 0.45, *SE* = 0.16, $p < 0.01$). AMNS-treated male mice displayed significantly greater sickness behaviours than their AMNS-treated female counterparts (*MD* = 0.78, *SE* = 0.23, $p < 0.01$; Figure 1A and B). AMNS-treated male mice displayed significantly more sickness behaviours than their water-treated counterparts (*MD* = 0.54, *SE* = 0.23, $p < 0.05$). Furthermore, LPS-treated mice displayed significantly less sickness behaviours 2 hours following treatment in comparison to 4 (*MD* = -0.52, *SE* = 0.09, $p < 0.01$), 6 (*MD* = -0.98, *SE* = 0.11, $p < 0.01$), 8 (*MD* = -1.03, *SE* = 0.10, $p < 0.01$), and 12 (*MD* = -0.86, *SE* = 0.10, $p < 0.01$; Figure 1A and B) hours following treatment. Lastly, AMNS-treated males displayed significantly more sickness behaviours then AMNS-treated females at 2 (*MD* = 0.85, *SE* = 0.38, $p < 0.05$), 4 (*MD* = 1.00, *SE* = 0.36, $p < 0.01$), 6 (*MD* = 1.3, *SE* = 0.38, $p < 0.01$), 8 (*MD* = 1.40, *SE* = 0.47, $p < 0.01$), 12 (*MD* = 1.10, *SE* = 0.49, $p < 0.05$), 24 (*MD* = 0.80,

SE = 0.36, *p* = 0.03), and 48 (*MD* = 0.40, *SE* = 0.18, *p* < 0.05; Figure 1A and B) hours following treatment. Water and LPS-treated male and female mice did not display any significant difference in sickness behaviours in comparison to their AMNS and LPS-treated counterparts.

3.2 Forepaw stride length, rotarod, and buried pellet tests

The ANOVA did not find any significant main effects or interactions for the forepaw stride length, rotarod, and buried pellet tests (data not shown).

3.3 Reversed grid hang test

The ANOVA found a significant main effect of sex ($F_{(1, 72)} = 14.52$, $p < 0.01$, $\eta p2 = 0.17$) along with significant sex x LPS ($F_{(1, 72)} = 4.47$, $p < 0.05$, $\eta p2 = 0.06$), AMNS x LPS ($F_{(1, 72)} = 4.30$, $p < 0.05$, $\eta p2 = 0.06$), and sex x AMNS x LPS interactions ($F_{(1, 72)} = 5.76$, $p < 0.05$, $\eta p2 = 0.07$). Pairwise comparisons revealed that regardless of LPS and AMNS treatments, male mice hung on the grid for a significantly shorter time in comparison to female mice (*MD* = 22.25, *SE* = 5.84, *p* < 0.01). Furthermore, water and saline-treated male mice hung on the grid for a significantly shorter time in comparison to their water and saline-treated female counterparts (*MD* = -47.71, *SE* = 11.68, *p* < 0.01). AMNS and saline-treated mice hung on the grid for a significantly shorter time in comparison to their water and saline-treated counterparts (MD = -18.960, SE = 8.26, *p* < 0.05). AMNS and LPS-treated male mice hung on the grid for a significantly shorter time than AMNS and LPS-treated female mice (*MD* = -24.83, *SE* = 11.68, *p* < 0.05). AMNS and saline-treated female mice hung on the grid for a significantly shorter time than their water and saline-treated counterparts (*MD* = -32.07, *SE* = 11.68, *p* < 0.01). Lastly, water and LPS-treated female mice hung on the grid for a significantly shorter time in comparison to their water and saline-treated counterparts (*MD* = -36.80, *SE* = 11.68, *p* < 0.01; Figure 2B).

3.4 Open field test

The ANOVA found a significant sex x AMNS interaction ($F_{(1, 72)} = 4.67$, $p < 0.05$, $\eta p2 = 0.06$) for total distance travelled. A significant main effect of sex ($F_{(1, 72)} = 10.83$, $p < 0.01$, $\eta p2 = 0.13$) was found for duration spent in the inner zone. There were also trends towards a significant main effect of LPS ($F_{(1, 72)} = 3.12$, $p = 0.08$, $\eta p2 = 0.04$) and a significant sex x LPS interaction ($F_{(1, 72)} = 2.88$, $p = 0.09$ $\eta p2 = 0.04$) for duration spent in the inner zone. Pairwise comparisons revealed that regardless of LPS and AMNS treatments, males spent a significantly shorter time in the inner zone in comparison to female mice (*MD* = -3.00, *SE* = 0.91, $p < 0.01$). Furthermore, LPS-treated mice spent a shorter time in the inner zone in comparison to saline-treated mice (*MD* = -1.61, *SE* = 0.91, $p = 0.08$). LPS-treated female mice spent a significantly longer time in the inner zone in comparison to LPS-treated male mice (*MD* = 4.54, *SE* = 1.29, $p < 0.01$). LPS-treated male mice spent a significantly shorter time in the inner zone in comparison to their saline-treated counterparts (*MD* = -3.15, *SE* = 1.29, $p < 0.05$; Figure 2A). Interestingly, no significant difference in time spent in the inner zone was observed in AMNS and LPS-treated male mice.

3.5 GFRA1 expression in the PLC, ACC, IF, CA1, CA2, and CA3

The ANOVA did not find any significant main effects or interactions for GFRA1 expression in the PLC ACC, IF, CA1, CA2, and CA3 regions (data not shown).

3.6 GFRA1 expression in the DG

The ANOVA found a significant sex x LPS interaction ($F_{(1, 72)} = 4.74$, $p < 0.05$, $\eta p2 = 0.06$). Pairwise comparisons revealed that LPS-treated male mice displayed significantly less GFRA1 expression in comparison to their saline-treated counterparts in the DG (*MD* = 118.30, *SE* = 51.16, $p < 0.05$; Figure 3A and D).

3.7 GFRA1 expression in the M1

The ANOVA found a significant main effect of sex ($F_{(1, 72)} = 4.17$, $p < 0.05$, $\eta p2 = 0.06$) and a significant sex x AMNS x LPS interaction ($F_{(1, 72)} = 4.30$, $p < 0.05$, $\eta p2 = 0.06$). Pairwise comparisons revealed that regardless of AMNS and LPS treatments, female mice displayed significantly more GFRA1 expression than male mice in the M1 (*MD* = 193.38, *SE* = 95.64, $p < 0.05$). Water and LPS-treated male mice displayed significantly less GFRA1 expression in comparison to their water and LPS-treated female counterparts in the M1 (*MD* = -403.80, *SE* = 191.28, $p < 0.05$). AMNS and saline-treated male mice displayed significantly less GFRA1 expression than their AMNS and saline-treated female counterparts in the M1 (*MD* = -385.00, *SE* = 191.28, $p < 0.05$). Lastly, AMNS and LPS-treated male mice displayed significantly greater GFRA1 expression than their water and LPS-treated counterparts in the M1 (*MD* = 413.60, *SE* = 191.28, $p < 0.05$; Figure 3B and E).

3.8 GFRA1 expression in the M2

The ANOVA found a significant main of sex ($F_{(1, 72)} = 5.78$, $p < 0.05$, $\eta p2 = 0.07$). Pairwise comparisons revealed that regardless of AMNS and LPS treatments, male mice displayed significantly less GFRA1 expression in comparison to female mice in the M2 (*MD* = -142.50, *SE* = 59.27, $p < 0.05$; Figure 3C and F).

3.9 S1R expression in the ACC, IF, CA3, and M1

The ANOVA did not find any significant main effect or interaction for SIR expression in the ACC, IF, CA3, and M1 regions (data not shown).

3.10 S1R expression in the PLC

The ANOVA found a significant sex x AMNS interaction ($F_{(1, 72)} = 4.88$, $p < 0.05$, $\eta p2 = 0.06$). Pairwise comparisons revealed that water-treated male mice displayed significantly less S1R expression than water-treated female mice in the PLC (*MD* = -106.35, *SE* = 41.51, $p <$

0.05). Furthermore, AMNS-treated male mice displayed significantly greater S1R expression than their water-treated counterparts in the PLC (*MD* = 90.72, *SE* = 41.51, $p < 0.05$; Figure 4A and G). No significant difference was observed in LPS-treated or AMNS and LPS-treated mice.

3.11 S1R expression in the CA1

The ANOVA found a significant main effect of sex ($F_{(1, 72)} = 12.42$, $p < 0.01$, $\eta p2 = 0.15$) and AMNS ($F_{(1, 72)} = 8.81$, $p < 0.01$, $\eta p2 = 0.11$). There was also a trend towards a significant sex x AMNS x LPS interaction ($F_{(1, 72)} = 3.72$, $p = 0.06$, $\eta p2 = 0.05$). Pairwise comparisons revealed that regardless of LPS and AMNS treatment, male mice displayed significantly greater S1R expression than female mice in the CA1 (*MD* = 104.15, *SE* = 29.56, $p < 0.01$). AMNS-treated mice displayed significantly less S1R expression than water-treated mice in the CA1 (*MD* = -87.70, *SE* = 29.56, $p < 0.01$). Furthermore, AMNS and LPS-treated male mice displayed significantly greater S1R expression in comparison to AMNS and LPS-treated female mice in the CA1 (*MD* = 211.35, *SE* = 59.11, $p < 0.01$). AMNS and saline-treated male mice displayed significantly less S1R expression than their water and saline-treated counterparts in the CA1 (*MD* = -166.85, *SE* = 59.11, $p < 0.01$). AMNS and LPS-treated female mice displayed significantly less S1R expression in comparison to their water and LPS-treated counterparts in the CA1 (*MD* = -122.50, *SE* = 59.11, $p < 0.05$). Lastly, AMNS and LPS-treated male mice displayed significantly greater S1R expression in comparison to their AMNS and saline-treated counterparts in the CA1 (*MD* = 123.25, *SE* = 59.11, $p < 0.05$; Figure 4B and H). Water and LPS-treated male mice did not display any significant difference in S1R expression in the CA1 in comparison to their water and saline-treated counterparts.

3.12 S1R expression in the CA2

The ANOVA found a significant main effect of AMNS ($F_{(1, 72)} = 4.63$, $p < 0.05$, $\eta p2 = 0.06$). Pairwise comparisons revealed that regardless of sex and LPS treatment, AMNS-treated mice displayed significantly less S1R expression than water-treated mice in the CA2 (*MD* = -6.15, *SE* = 2.86, $p < 0.05$; Figure 4C and I). Water and LPS-treated mice did not display any significant difference in S1R expression in the CA1 in comparison to their water and saline-treated counterparts.

3.13 S1R expression in the DG

The ANOVA found significant main effects of sex ($F_{(1, 72)} = 9.59$, $p < 0.01$, $\eta p2 = 0.12$) and AMNS ($F_{(1, 72)} = 11.35$, $p < 0.01$, $\eta p2 = 0.14$) along with a significant sex x AMNS x LPS interaction ($F_{(1, 72)} = 8.04$, $p < 0.01$, $\eta p2 = 0.10$). Pairwise comparisons revealed that AMNS-treated mice displayed significantly less S1R expression than water-treated mice in the DG (*MD* = -109.66, *SE* = 32.56, $p < 0.01$). Furthermore, water and saline-treated male mice displayed significantly greater S1R expression than their female counterparts in the DG (*MD* = 178.05, *SE* = 65.11, $p < 0.01$). AMNS and LPS-treated male mice displayed significantly greater S1R expression than AMNS and LPS-treated female mice in the DG (*MD* = 208.20, *SE* = 65.11, $p < 0.01$). AMNS and saline-treated male mice displayed significantly less S1R expression in comparison to their water and saline-treated counterparts in the DG (*MD* = -218.45, *SE* = 65.11, $p < 0.01$). Lastly, AMNS and LPS-treated female mice displayed significantly less S1R expression in comparison to their water and LPS-treated counterparts in the DG (*MD* = -185.45, *SE* = 65.11, $p < 0.01$; Figure 4D and J).

3.14 S1R expression in the M2

The ANOVA found a significant main effect of sex ($F_{(1, 72)} = 7.88$, $p < 0.01$, $\eta p2 = 0.10$) and LPS ($F_{(1, 72)} = 6.69$, $p < 0.05$, $\eta p2 = 0.09$) along with a significant AMNS x LPS interaction ($F_{(1, 72)} = 6.31$, $p < 0.05$, $\eta p2 = 0.08$). Pairwise comparisons revealed that regardless of AMNS

and LPS treatments, male mice displayed significantly less S1R expression than female mice in the M2 (*MD* = -118.99, *SE* = 42.40, $p < 0.01$). LPS-treated mice displayed significantly less S1R expression than saline-treated mice in the M2 (*MD* = -109.64, *SE* = 42.40, $p < 0.05$). Furthermore, AMNS and LPS-treated mice displayed significantly greater S1R expression than water and LPS-treated mice in the M2 (*MD* = 149.40, *SE* = 59.96, $p < 0.05$). Water and LPS-treated mice displayed significantly less S1R expression than water and saline-treated mice in the M2 (*MD* = -216.15, *SE* = 59.96, $p < 0.01$; Figure 4E and K).

3.15 TLR4 expression in the ileum

The ANOVA did not find any significant main effects or interactions for TLR4 expression in the ileum (data not shown).

4.0 Discussion

Pubertal exposure to LPS and AMNS has been shown to influence acute cellular mechanisms associated with neurodegeneration in a sex-dependent manner (Esposito, Gandelman, et al., 2022). However, the enduring effects of these treatments on neurodegenerative processes remain unknown. As such, this study was designed to examine the enduring effects of pubertal LPS and AMNS treatments on behaviours and cellular mechanisms associated with neurodegeneration. Our results demonstrated that pubertal LPS and AMNS treatments were associated with sex-dependent alterations in acute sickness behaviours, and enduring neurodegenerative-related behaviours (i.e., neuromuscular strength, anxiety), and biomarkers associated with the regulation of neurodegenerative processes (i.e., S1R, GFRA1).

Our results showed that water and saline-treated male mice demonstrated greater neuromuscular deficits than their water and saline-treated female counterparts. This finding suggests that female mice inherently possess greater neuromuscular strength than male mice. This aligns with previous research demonstrating that female mice have a higher endurance exercise capacity due to their superior fatty acid utilization (Holcomb et al., 2022). Furthermore, our results showed that pubertal AMNS and LPS treatments induced enduring deficits in neuromuscular strength, specifically in female mice. Specifically, AMNS and LPS-treated male mice displayed greater neuromuscular deficits than their male counterparts. Furthermore, female mice treated with either LPS or AMNS displayed greater neuromuscular deficits than their control (i.e., water or saline treated) treated counterparts. These findings support our hypothesis and are partially consistent with previous research linking pubertal LPS treatment with enduring deficits in Parkinson's-like behaviours (Girard-Joyal & Ismail, 2017). The sex-dependent effects of pubertal AMNS and LPS treatments on neuromuscular strength seen in our study may be due

differences in circulating gonadal hormones between males and female. Specifically, estradiol has been shown to enhance neuromuscular strength, while decreased estradiol levels have been associated with impairments in neuromuscular strength (Chidi-Ogbolu & Baar, 2019; Willoughby et al., 2024). Moreover, AMNS and LPS treatments have been shown to have a significant impact on the expression of estradiol (Dickson et al., 2022; X. Guo et al., 2022; Ismail et al., 2011). As such, pubertal AMNS and LPS treatments in our current study may have induced enduring deficits related to the synthesis of estradiol, causing long-lasting neuromuscular impairments in our female mice.

Our results also showed that pubertal LPS treatment increased anxiety-like behaviours in adult male mice. This finding supports our hypothesis and is consistent with previous research demonstrating the sex-dependent, enduring effects of pubertal LPS treatment on anxiety-like behaviours (Girard-Joyal & Ismail, 2017; Murray et al., 2020). Notably, up to 40% of individuals suffering from PD also suffer from anxiety-related disorders with males being twice more likely to develop PD than females (Cerri et al., n.d.; Dissanayaka et al., 2010; Lutz et al., 2016; Pontone et al., 2009). Moreover, anxiety disorders often precede the development of motor symptoms observed in PD (Radulovic et al., 2022). The increase in anxiety-like behaviours observed in our male mice is likely due to the enduring effects of LPS treatment on testosterone levels. Testosterone has anxiolytic effects, and LPS treatment has been shown to decrease the expression of testosterone (Shen et al., 2022; Tong et al., 2019). As such the increased anxiety-like behaviour observed in our male mice may be mediated by the enduring effects of pubertal LPS treatment on the concentration of circulating testosterone. However, concentrations of gonadal hormones were not examined in this study and further research is required to determine

whether LPS treatment modulates sex hormones concentration to better understand the effect of LPS treatment on anxiety-like behaviour.

Interestingly, the increase of anxiety-like behaviours observed in our LPS-treated male mice was negated when AMNS treatment was introduced. This finding coincided with a significant increase of S1R in the PLC of male mice following AMNS treatment. S1R has been shown to have anxiolytic effects, while the PLC plays an essential role in the modulation of anxiety-like behaviours (Ji et al., 2016; Li et al., 2021). For example, administration of the anxiolytic drug fabomotizole, in BALB/c mice, results in an increase of time spent in the open-arm on the elevated plus-maze, indicating a decrease in anxiety-like behaviours. However, this effect is reversed when the S1R antagonist, BD-1047, is administered to the mice (Voronin et al., 2021). Furthermore, lesions in the PLC cortex of Wistar rats significantly decreases time spent in the inner zone of an open field and decreases time spent in the open-arms of the elevated plus maze, indicating an increase in anxiety-like behaviours (Jinks & McGregor, 1997). AMNS treatment during adolescence has also been reported to decrease anxiety-like behaviours. AMNS treatment in adolescent C57BL/6 mice administered with Aβ to induce AD, significantly decreases anxiety-like behaviours on the open-field, light-dark-box, and zero maze tests (Mosaferi et al., 2021). As such, our results further support the potential protective effects of AMNS treatment on the development of anxiety-like behaviours. We suggest that this protective effect may be mediated by AMNS-induced increases of S1R expression in the PLC.

Pubertal AMNS treatment also decreased the expression of S1R in the CA1 of male mice, while AMNS and LPS treatments had a similar effect in female mice. Furthermore, pubertal AMNS treatment decreased S1R expression in the CA2. Interestingly, although our results demonstrated an increase in sickness behaviours following LPS treatment in both males and

females, AMNS treatment specifically increased sickness behaviours in male mice, suggesting that pubertal AMNS treatment seems to have a greater influence on male mice than in female mice. These findings support our hypothesis and are consistent with previous research linking AMNS and LPS treatments with alterations in hippocampal neuronal activity and sickness behaviours (Çalışkan et al., 2022; Esposito, Kearns, et al., 2022; Valero et al., 2014). Both the CA1 and CA2 regions contain pyramidal neurons that play essential roles in learning and memory (Danielson et al., 2016; Dudek et al., 2016). Furthermore, S1R has been shown to have neuroprotective effects on hippocampal pyramidal neurons (Martina et al., 2007). As such, the loss of S1R in the CA1 and CA2 observed in our study suggests that pubertal AMNS and LPS treatments may influence hippocampal pyramidal cell functioning, in a sex-dependent manner.

Pubertal AMNS treatment also decreased S1R expression in the DG of male mice, while AMNS and LPS treatments had similar effects in female mice. Furthermore, pubertal LPS treatment was shown to decrease the expression of GFRA1 in the DG. These findings are in line with our hypothesis and with previous research linking the effects of AMNS and LPS treatments on hippocampal neurogenesis (Möhle et al., 2016; Perez-Dominguez et al., 2019). The DG plays an essential role in neurogenesis, while both S1R and GFRA1 contribute to the formation of new neurons (Bonafina et al., 2019; Piatti et al., 2013; Ryskamp et al., 2019). As such, it is possible that decreased S1R and GFRA1 expressions following pubertal AMNS and LPS treatments observed in our study influenced neurogenesis in the DG. However, neurogenesis was not directly examined in this study and further research is needed to determine whether decreased S1R and GFRA1 expression affects the formation of new neurons in the brain region.

Our results also showed that pubertal LPS treatment decreases the expression of S1R in the M2. However, AMNS and LPS-treated mice did not display decreased S1R expression in the

M1 nor the M2 regions. Moreover, pubertal AMNS and LPS treatments were associated with sex-dependent changes in GFRA1 expression in the M1. Specifically, male mice treated with either AMNS or LPS displayed decreased expression of GFRA1 in the M1 in comparison to their female counterparts. Furthermore, AMNS and LPS-treated male mice displayed decreased GFRA1 expression in comparison to their water and LPS-treated counterparts in the M1. Notably, male mice treated with only LPS did not display any significant difference in GFRA1 expression in the M1 in comparison to their water-treated counterparts. These findings are consistent with our hypothesis and are in line with previous research examining the interactions between both S1R and GFRA1 with LPS (Luo et al., 2023; Wang & Zhao, 2019). S1R is highly expressed in motor neurons and decreased S1R expression has been implicated in the pathogenesis of the motor neuron disease, amyotrophic lateral sclerosis (ALS) (Herrando-Grabulosa et al., 2021). Furthermore, GFRA1 is a receptor for GDNF which plays a crucial role in the survival of motor neurons (Suzuki et al., 2007). As such, decreased S1R and GFRA1 expression following pubertal AMNS and LPS treatments in our current study may suggest increased susceptibility to motor neuron degeneration. Our results also suggest that males may be more susceptible to these deficits, particularly in the M1 region, a finding that is in line with males being 2-3 times more susceptible to develop ALS than women (Pape & Grose, 2020). However, further research is required to determine whether reduced S1R and GFRA1 expression in the motor cortex directly influences neurodegenerative mechanisms.

No enduring changes in intestinal TLR4 expression were observed following pubertal AMNS and LPS treatments. This finding is not in line with our hypothesis and with previous research examining the effects of AMNS and LPS treatments on intestinal TLR4 expression (S. Guo et al., 2015; Zhang & Chen, 2019). Research with C57BL/10 mice showed that seven days

of AMNS treatment significantly increases the expression of TLR4 in the ileum and colon (Grasa et al., 2015). Similarly, five days of LPS treatment causes a significant increase in TLR4 expression in Caco-2 cells (S. Guo et al., 2015). Our laboratory has also shown that a single injection of LPS during puberty results in an enduring increase of TLR4 expression in the paraventricular nucleus of the hypothalamus in male CD1 mice (Murray et al., 2019). These discrepancies with our findings are likely due to differences in the time points of when TLR4 expression was analyzed. The majority of these studies examined intestinal TLR4 expression immediately following AMNS or LPS treatment whereas our study examined TLR4 expression multiple weeks following AMNS and LPS treatments. As such, AMNS and LPS treatments may have acute effects on intestinal TLR4 expression, however, it seems that normal levels of TLR4 expression are restored over time. Moreover, although LPS treatment has enduring effects on TLR4 expression in the brain, this finding does not seem to extend to intestinal TLR4 expression.

4.1 Limitations and future directions

Our treatment regimen of AMNS and LPS influences the functioning of various systems such as brain function, metabolic function, sexual development, and immune function (Clarke et al., 2014; Dinan & Cryan, 2017; Kennedy et al., 2018). As such, unravelling the precise mechanisms involved in the effects observed in this study is challenging. To provide a more holistic picture of the effects of pubertal AMNS and LPS treatments, future research should analyze the effects of these treatments across various systems. Secondly, our gavage treatment may have introduced chronic stress effects in our mice. However, whether gavage treatment is a significant stressor remains unclear. Some studies have reported that gavage increases plasma corticosterone levels in rats while other studies have reported that gavage does not affect plasma

corticosterone levels in mice (Brown et al., 2000; Jones et al., 2016). To provide greater clarity on the effects of gavage treatment on the rodent stress response, future research should consider utilizing a control non-gavage group to confirm whether gavage has stress-inducing effects. Future research could also consider utilizing an alternative method to administer the AMNS treatment such as through drinking water. Lastly, given that we did not analyze the microbial composition of our mice, we cannot be certain of whether our treatment model (i.e., AMNS and LPS treatments) induced enduring changes in microbial composition. Future research should consider examining the effects of pubertal AMNS and LPS treatments on microbial composition to confirm whether gut dysbiosis plays a role in the effects observed in this study.

4.2 Conclusion

In conclusion, this study shows that pubertal LPS and AMNS treatments induce long-lasting sex-dependent changes in cellular mechanisms and behaviours associated with neurodegeneration. Our findings suggest that female mice are more susceptible to developing neuromuscular deficits following pubertal AMNS and LPS treatments while male mice are more susceptible to developing anxiety-like behaviours following pubertal LPS treatment. Furthermore, pubertal LPS and AMNS treatments cause enduring alterations in biomarkers (i.e., S1R and GFRA1) associated with cell survival and neurogenesis in the hippocampus and motor cortex, with male mice tending to demonstrate greater deficits than female mice. The current study is one of the first to examine the enduring effects of pubertal LPS and AMNS treatment on cellular mechanisms and behaviours associated with neurodegeneration. The results further our understanding of how alterations to the gut microbiome during puberty can increase susceptibility to neurodegeneration later in life, in a sex-dependent manner.

Funding: This work was supported by a Discovery grant from the Natural Sciences and Engineering Research Council of Canada (2020-04302) to NI.

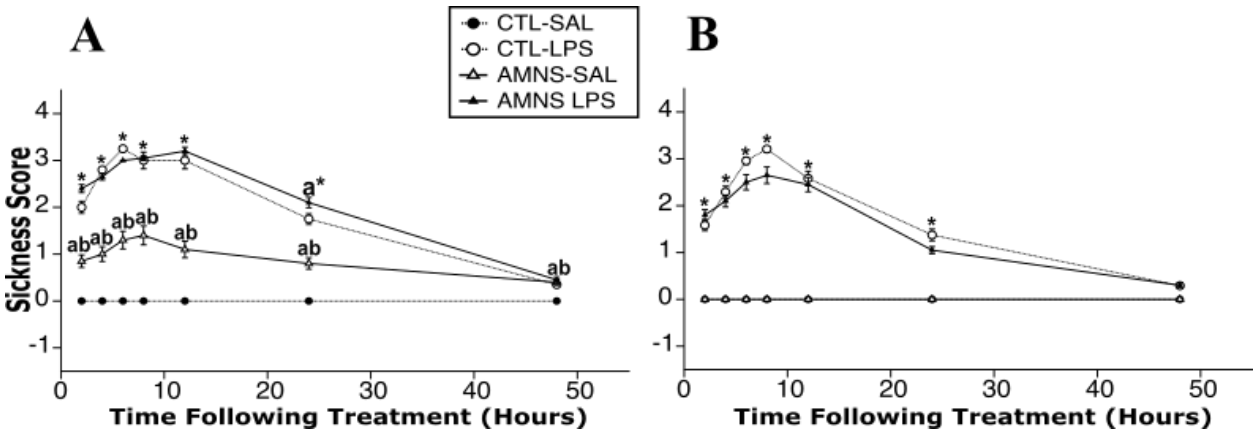
Author Contributions: **Pasquale Esposito:** Conceptualization, Methodology, Investigation, Writing – Original Draft, Visualization, Project administration, Formal analysis. **Eleni Dubé-Zinatelli:** Investigation. **Michelle Gandelman:** Investigation. **Ella Liu:** Investigation. **Luna Cappelletti:** Investigation. **Jacky Liang:** Investigation, Resources. **Nafissa Ismail:** Writing – Reviewing and Editing, Supervision, Funding acquisition.

Acknowledgments: The authors would like to thank all the members of the NISE Lab and the ACVS staff at the University of Ottawa for their assistance with this project.

Declaration of Interests: None

Figures, Tables and Captions

Figure 1.



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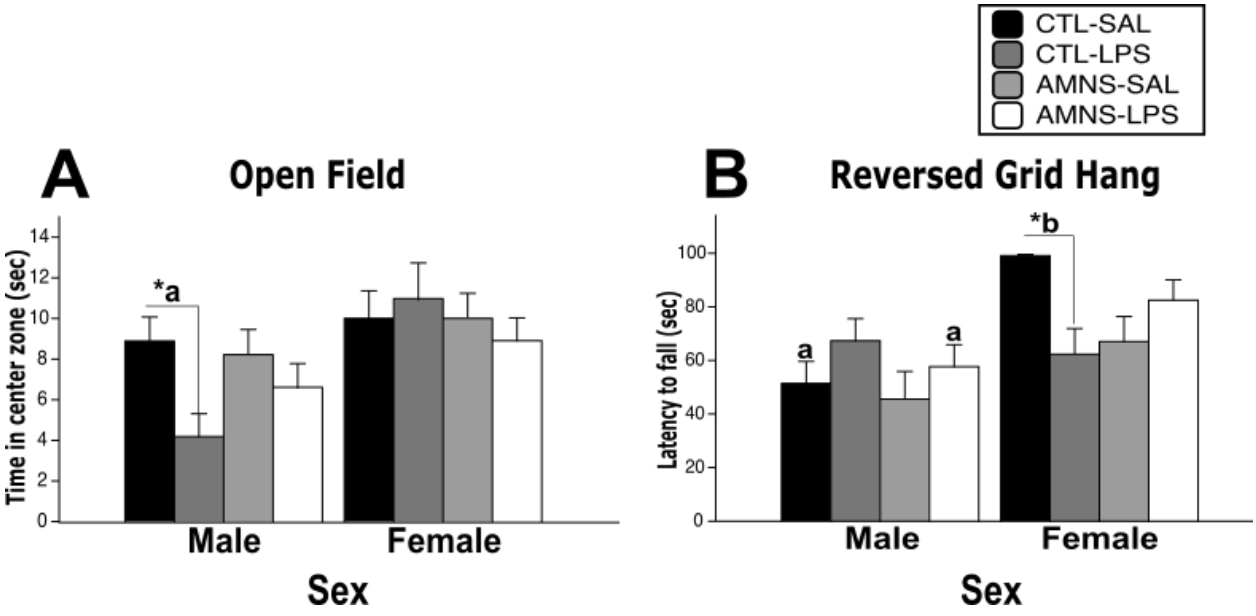

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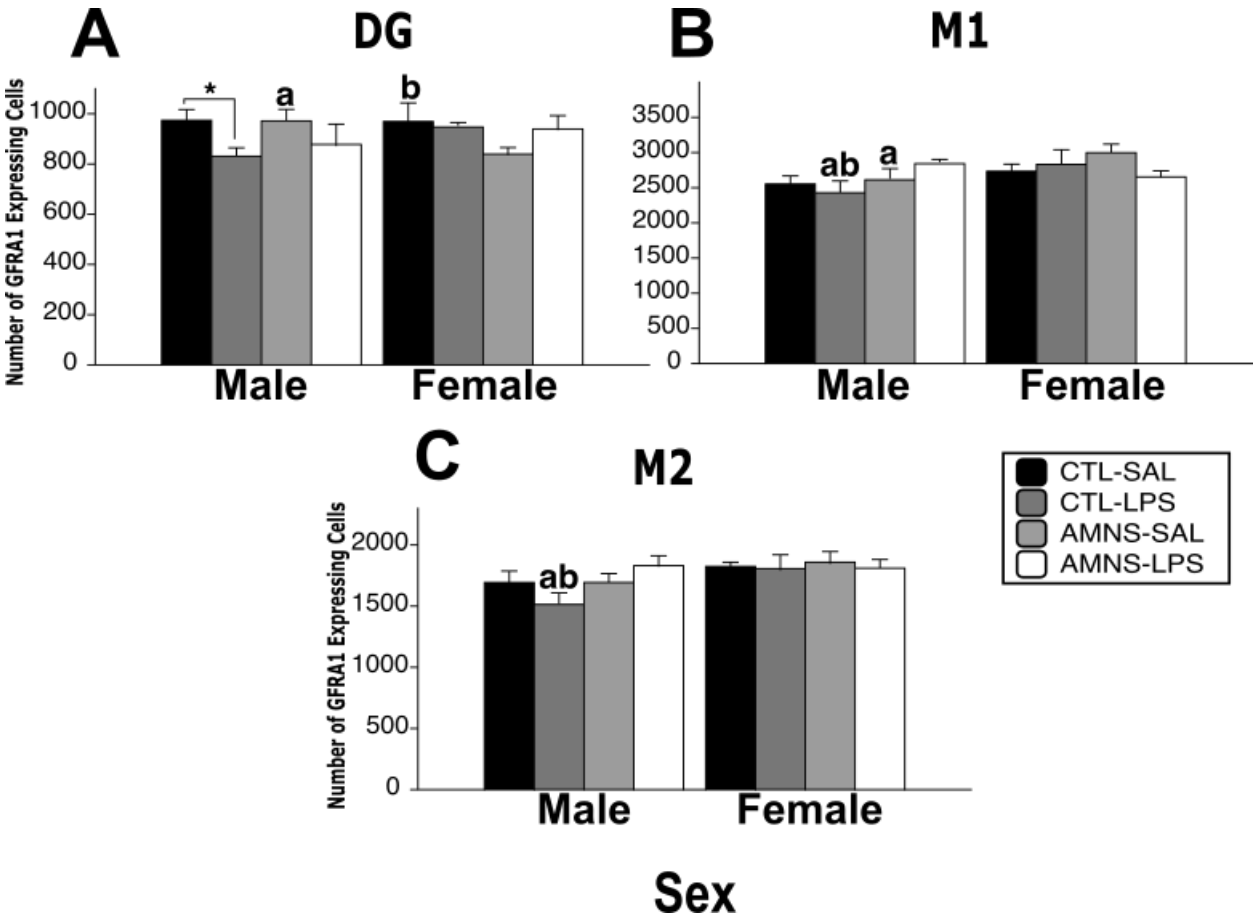
A
DG
B
M1
C
M2
Number of GFRA1 Expressing Cells
1000
800
600
400
200
0
*
a
b
3500
3000
2500
2000
1500
1000
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ab
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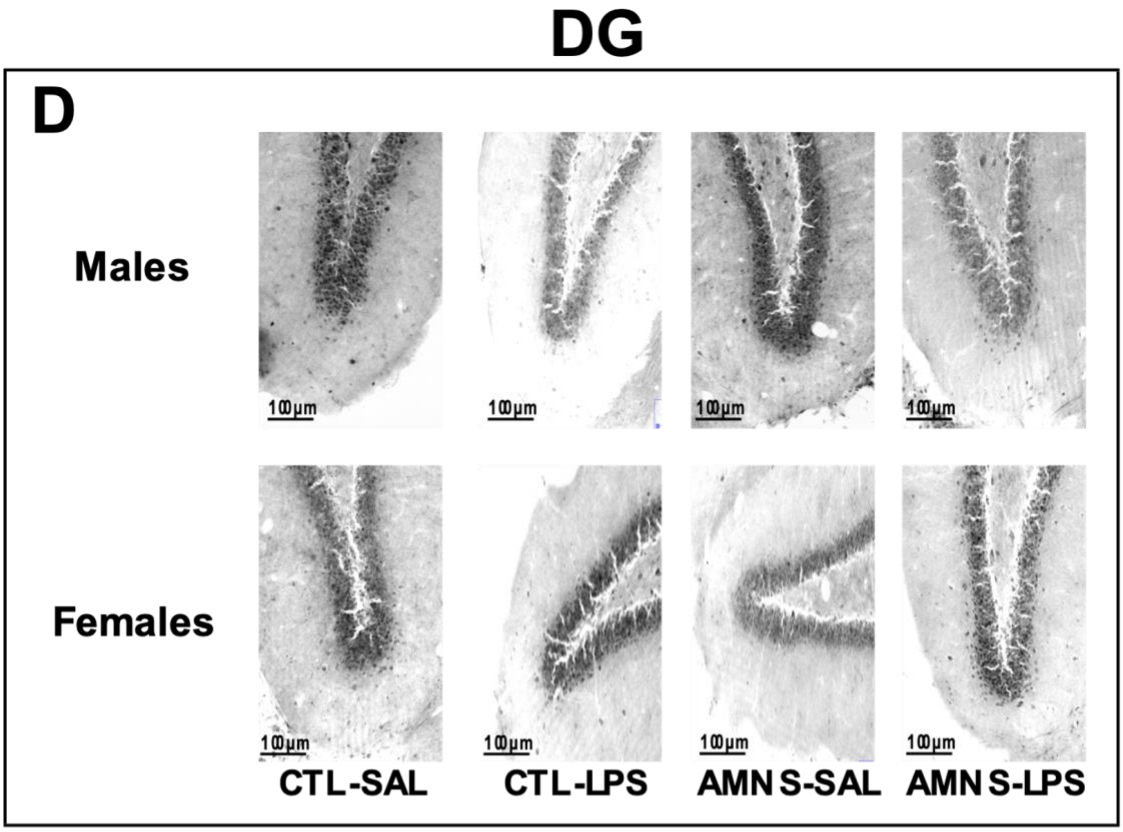
2000
1500
1000
500
Male
Female
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
Sex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Males
Females
100µm
CTL-SAL
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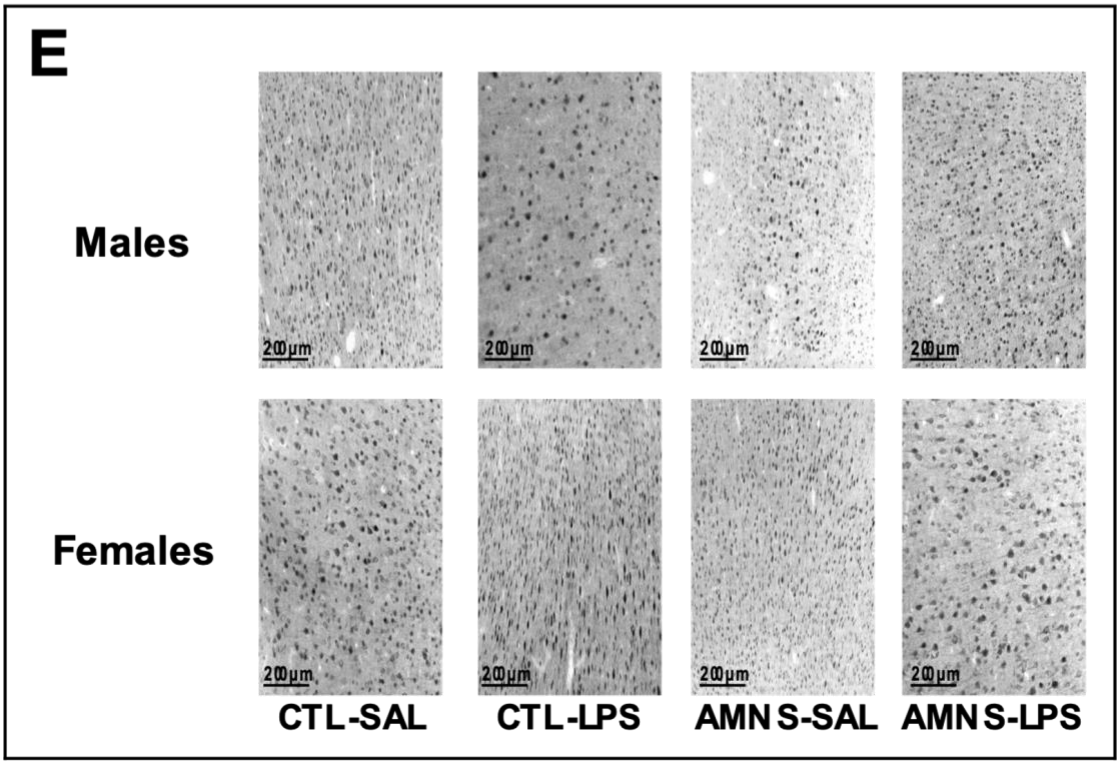
AMNS-SAL
AMNS-LPS


M1
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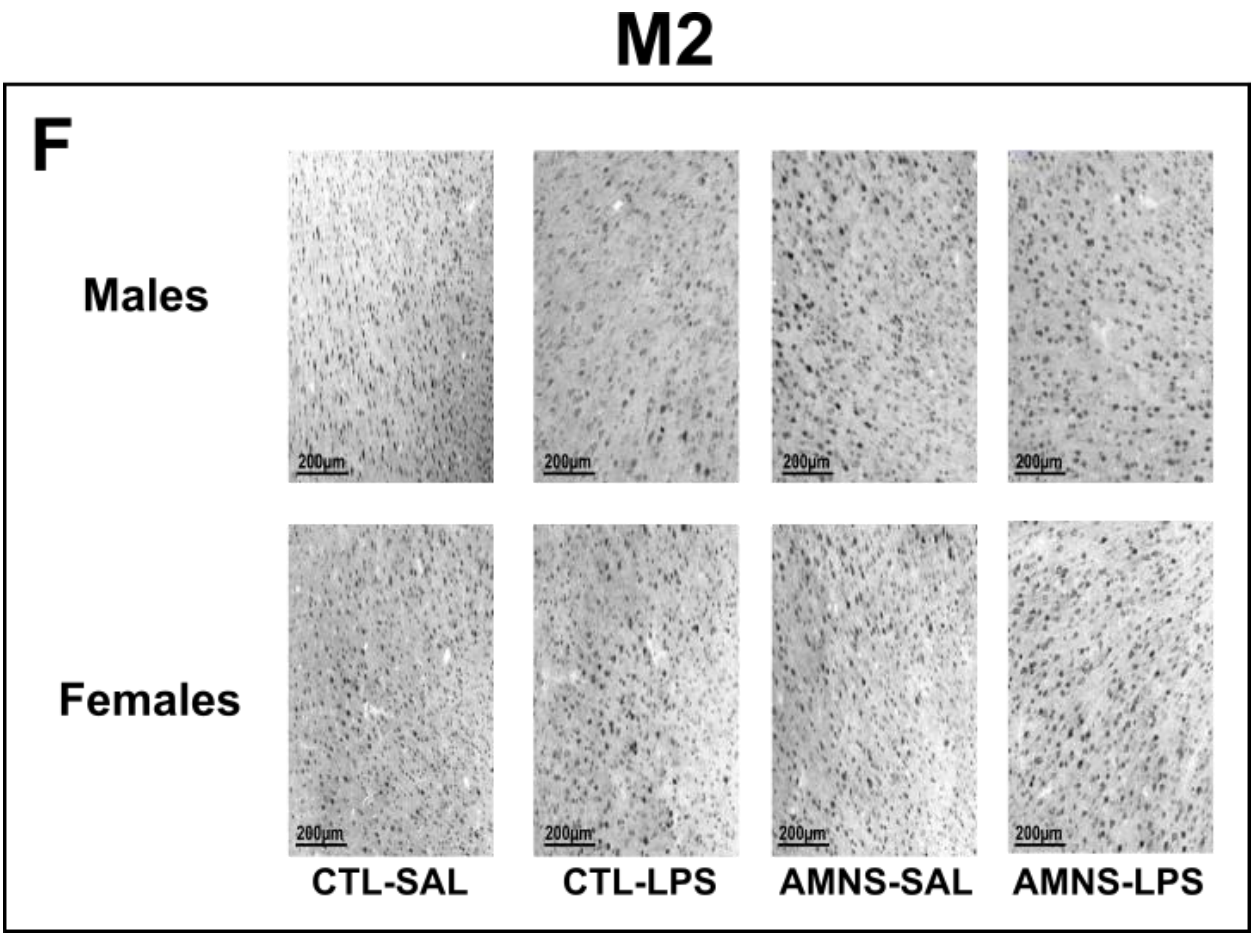
Males
Females
200µm
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS


M2
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Males
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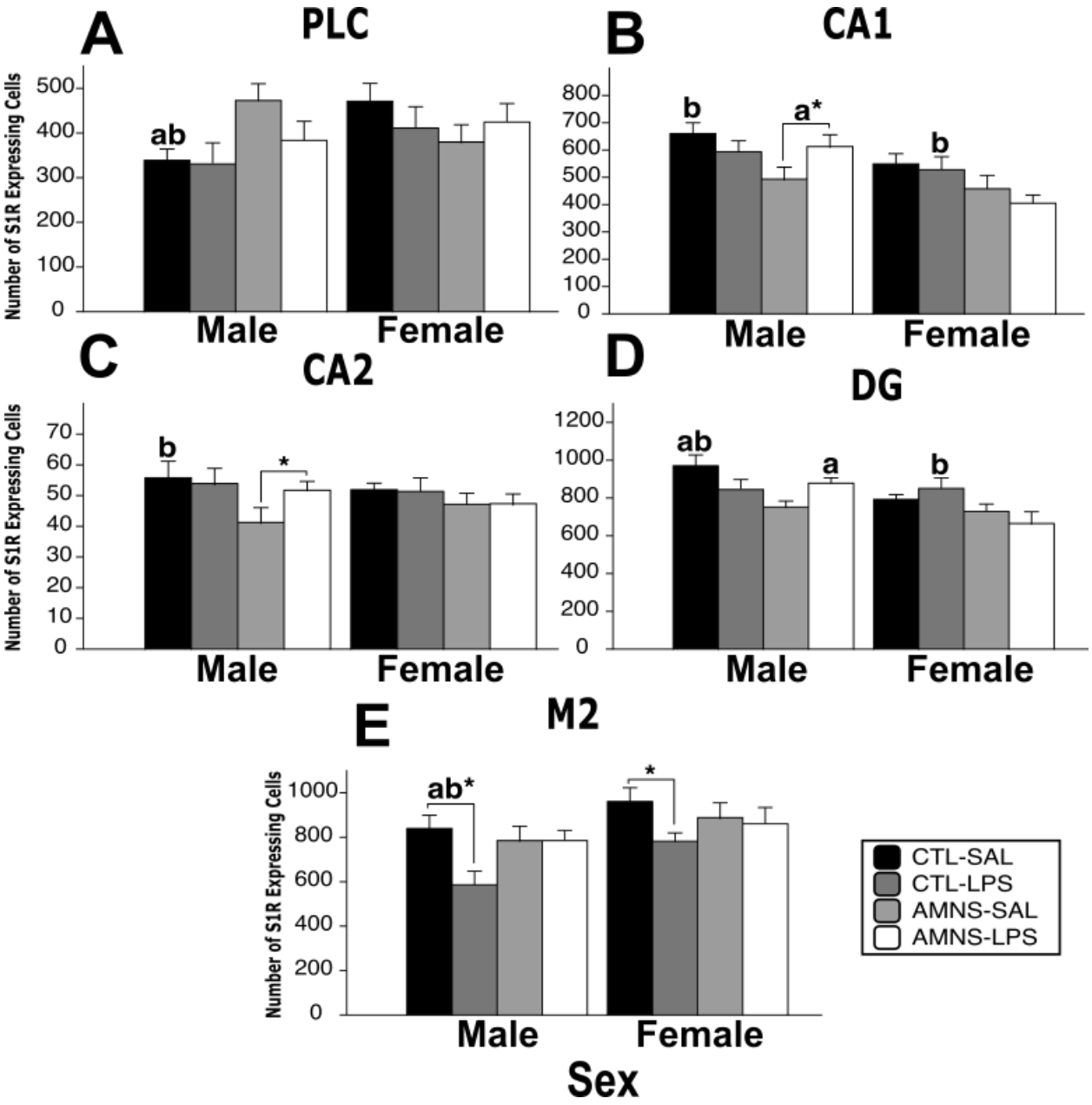
200µm
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS

Figure 4.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PLC
Number of S1R Expressing Cells
ab
Male
Female
B
CA1
b
a*
b
C
CA2
b
*
D
DG
ab
a
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E
M2
ab*
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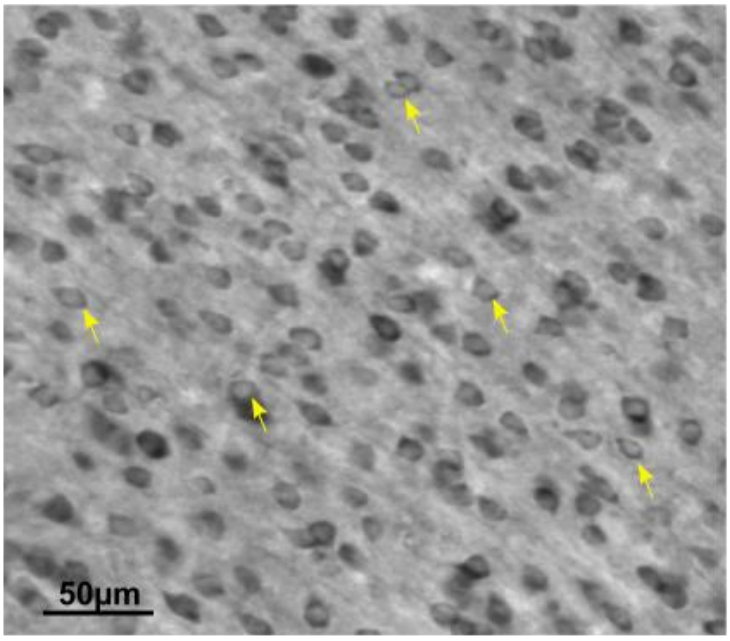
Sex
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS

F



PLC

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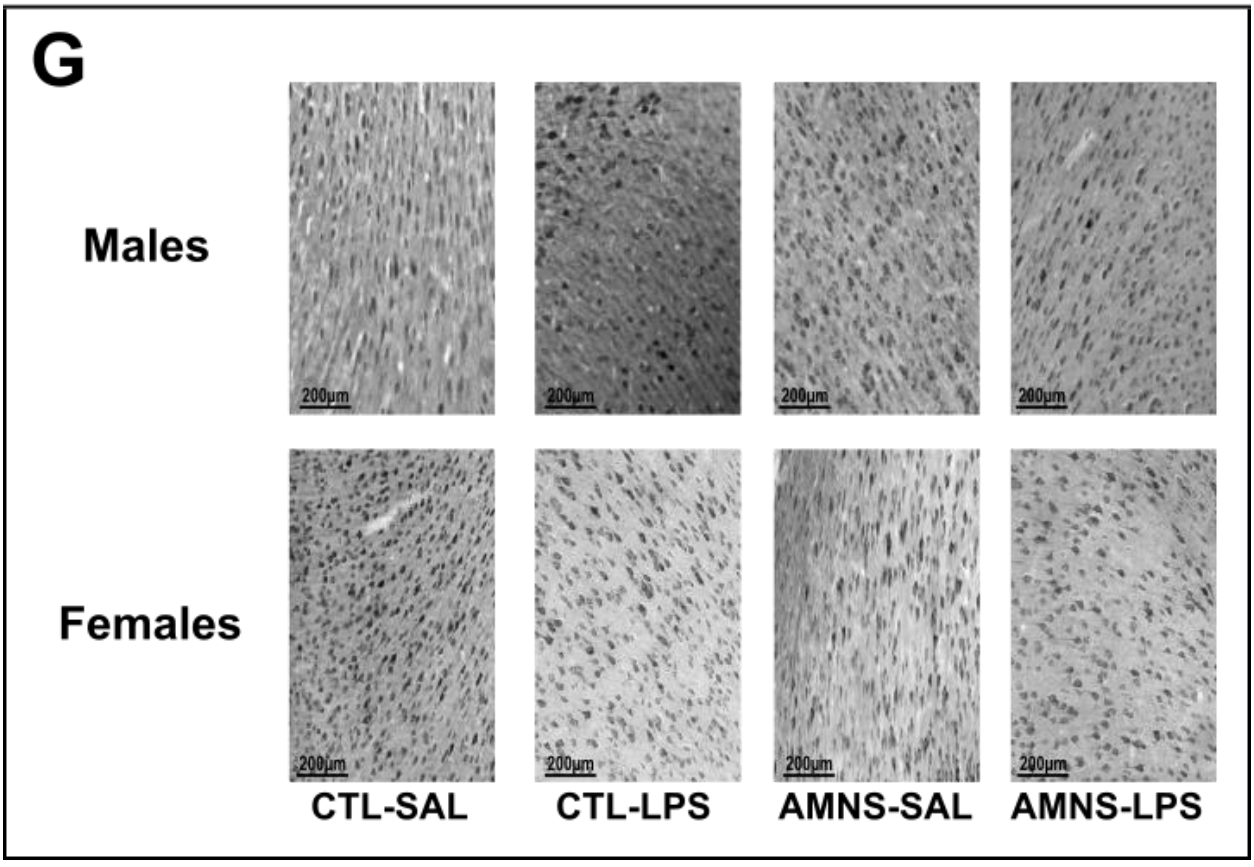
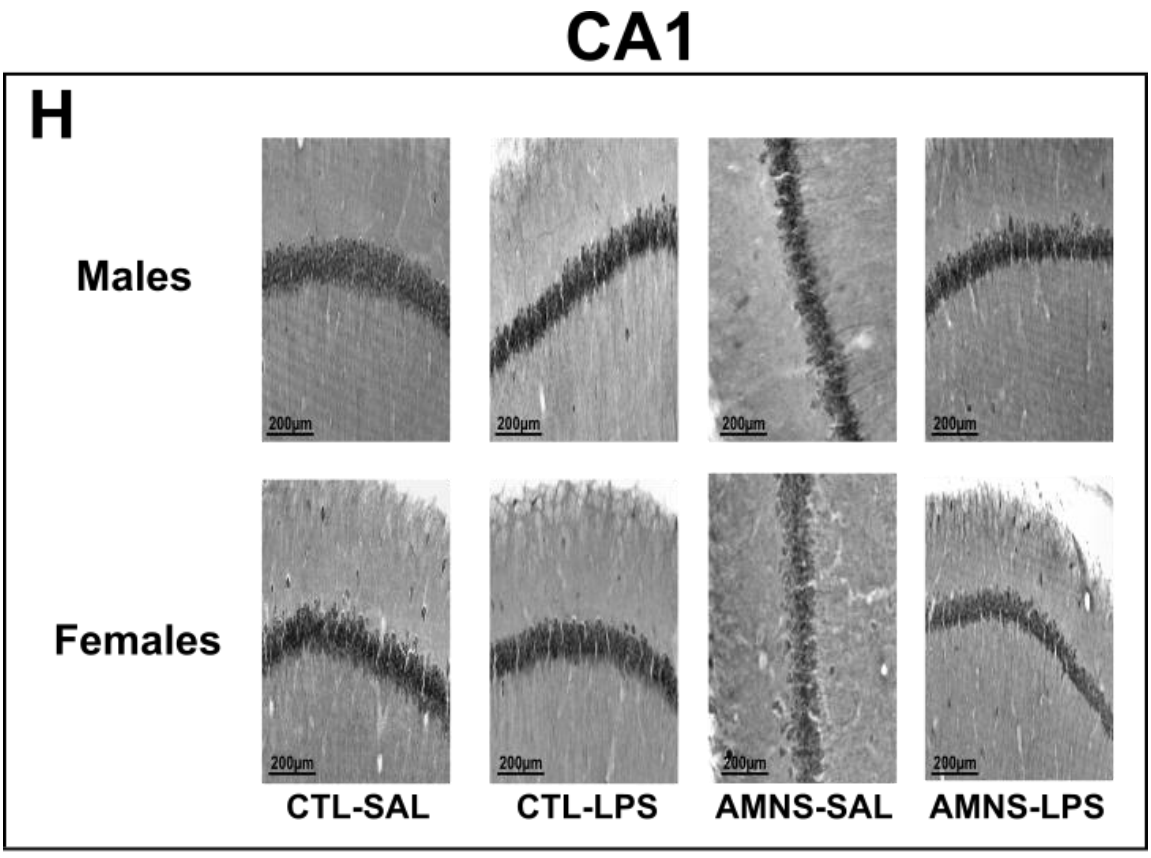
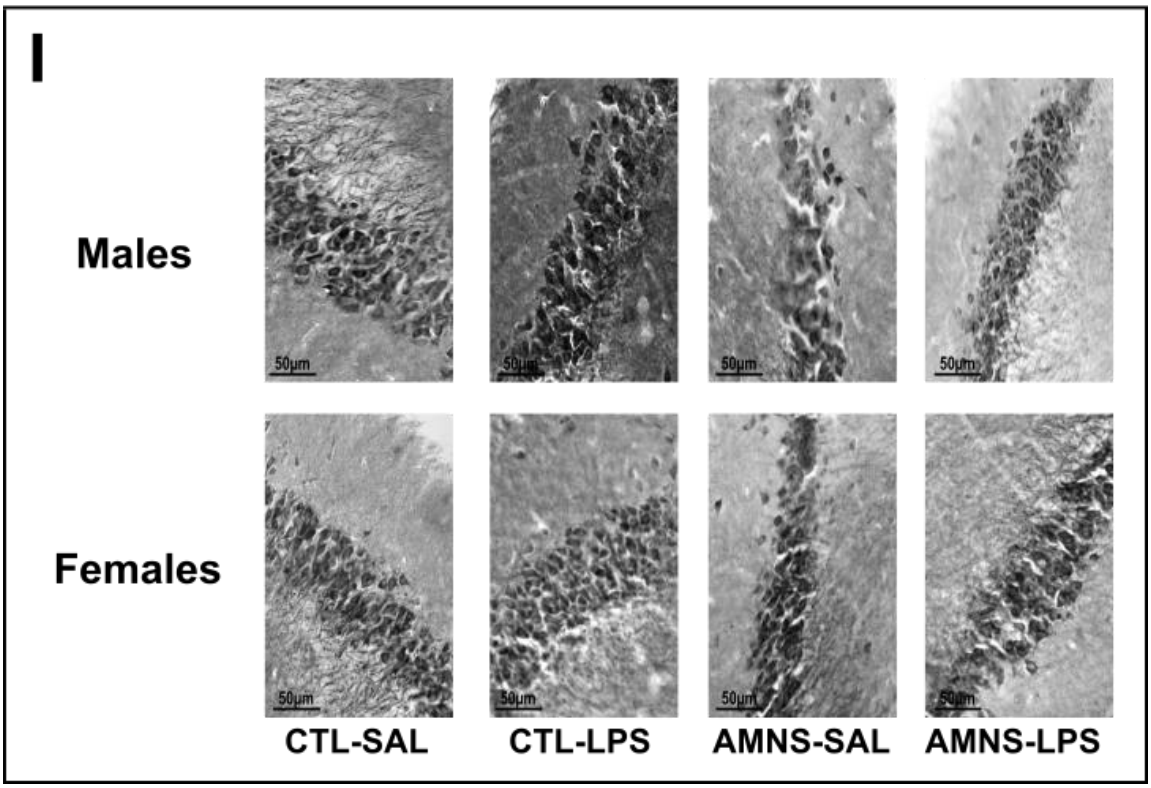
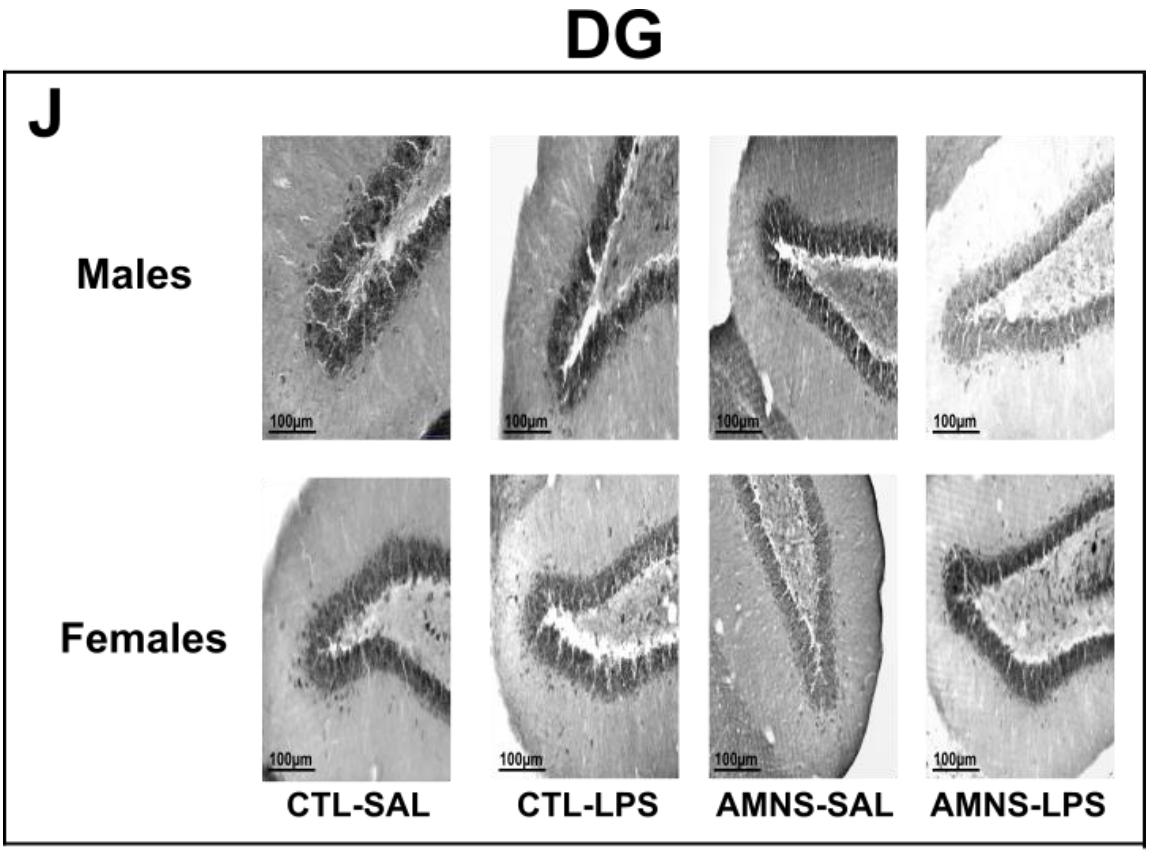



CA1
H
Males
Females
200µm
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS


CA2
I
Males
Females
50µm
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS


DG
J
Males
Females
100µm
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS

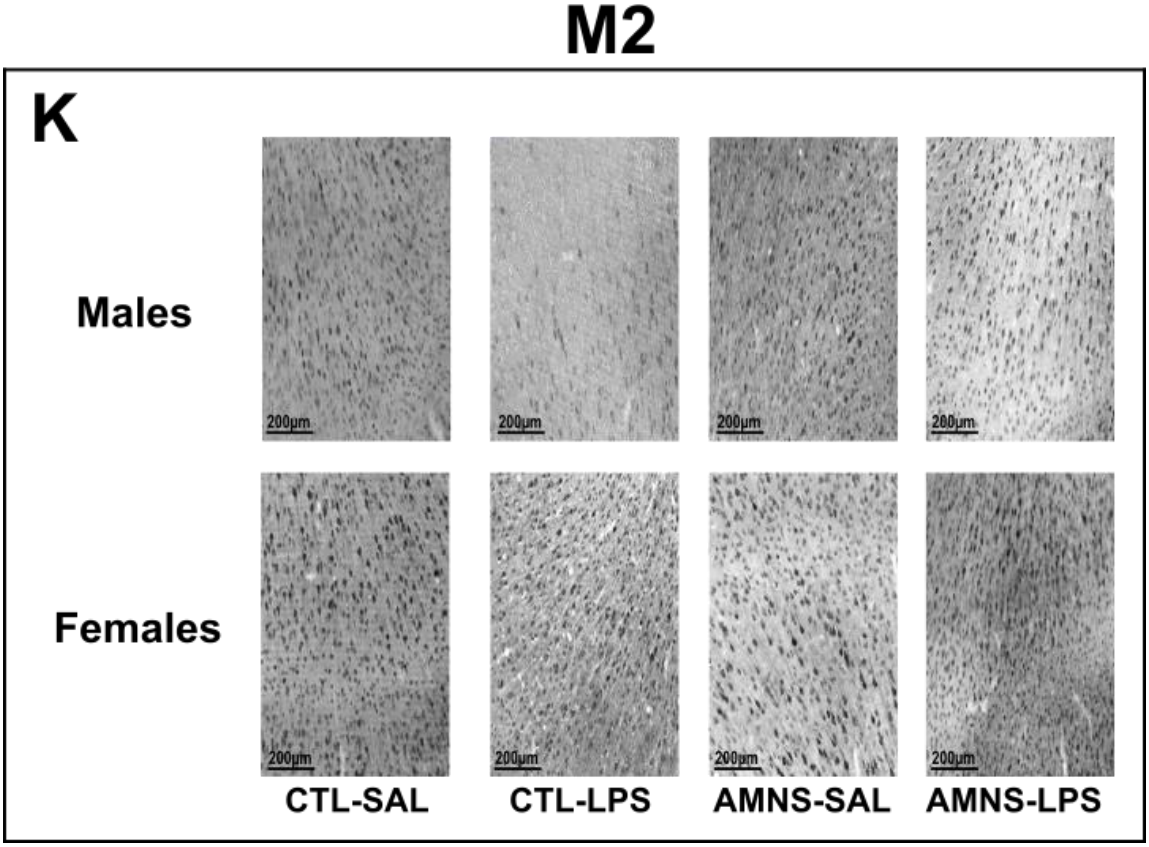

M2
K
Males
Females
200µm
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS

Figure Captions

Figure 1.

Title: Sickness behaviour symptoms

Caption: Mean (±SEM) sickness score 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 ($p < 0.05$), and (b) denotes a significant difference between water and antimicrobial treatments ($p < 0.05$).

Figure 2.

Title: Reversed grid hang and open field tests results

Caption: Mean (±SEM) (A) duration in the inner zone and (B) latency to fall from grid of male and 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 ($p < 0.05$), and (b) denotes a significant difference between water and antimicrobial treatments ($p < 0.05$).

Figure 3.

Title: GFRA1 expressing cells in the DG, M1, and M2

Caption: Mean (±SEM) number of GFRA1 expressing cells in the (A) DG, (B) M1, and (C) M2 regions of male and 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 ($p < 0.05$), and (b) denotes a significant difference between water and antimicrobial treatments ($p < 0.05$). Representative photomicrographs of GFRA1 expression in the (D) DG, (E) M1, and (F) M2 regions of male and female mice treated with either saline (SAL) or lipopolysaccharide (LPS), and with either water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS).

Figure 4.

Title: S1R expressing cells in the PLC, CA1, CA2, DG, and M2

Caption: Mean (±SEM) number of S1R expressing cells in the (A) PLC, (B) CA1, (C) CA2, (D) (E) DG, and (F) M2 regions of male and 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 ($p < 0.05$), and (b) denotes a significant difference between water and antimicrobial treatments ($p < 0.05$). (J) Photomicrograph demonstrating the unique ring-like structure of S1R expressing cells. Representative photomicrographs of GFRA1 expression in the (G) PLC, (H) CA1, (I) CA2, (K) DG, and (L) M2 regions of male and female mice treated with either saline (SAL) or lipopolysaccharide (LPS), and with either water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS).

Figure 5.

Title: Ileal TLR4 concentrations

Caption: Mean (±SEM) TLR4 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.

CHAPTER 4: SEX-DEPENDENT EFFECTS OF PUBERTAL ANTIMICROBIAL AND LIPOPOLYSACCHARIDE TREATMENTS ON INTESTINAL AND BLOOD-BRAIN-BARRIER PERMEABILITY

Published
Esposito, P., Dubé-Zinatelli, E., Krnel, R., Cappelletti, L., Liang, J. and Ismail, N., 2024. Sex-dependent effects of antimicrobials and lipopolysaccharide on blood-brain-barrier permeability in pubertal male and female CD1 mice. *Hormones and Behavior*, 165, 105615. https://doi.org/10.1016/j.yhbeh.2024.105615

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Abstract

Exposure to stressors during puberty can disrupt normal development and possibly increase susceptibility to neurodegenerative disorders later in life. However, the mechanisms underlying the relationship between pubertal stress exposure and neurodegeneration remain unclear. As such, the current study was designed to examine the effects of pubertal antimicrobial (AMNS) and lipopolysaccharide (LPS) treatments on intestinal and blood-brain-barrier (BBB) permeability in male and female mice. Moreover, we also examined the sex-specific effects of pubertal AMNS and LPS treatments on gross motor activity, heart rate, and core body temperature. At four weeks of age, male and female CD1 mice were implanted with the G2 HR E-Mitter telemetry system. At five weeks of age, mice received 200μL of broad-spectrum antimicrobial or water, through oral gavage, twice daily for seven days. Mice received an intraperitoneal injection of either saline or LPS at six weeks of age. BBB and intestinal permeability were examined 24 hours, 72 hours, and one week post-LPS/saline treatment. Telemetric data was collected for 48 hours post-LPS/saline treatment. The results showed that pubertal AMNS and LPS treatments increased sickness behaviours and decreased body temperature and heart rate, in a sex-dependent manner. Furthermore, pubertal AMNS and LPS treatments resulted in sex-dependent regional increases in BBB permeability 24 hours and 72 hours post-LPS/saline treatment, while global increases in BBB permeability were only observed one week post-LPS/saline treatment. These results further our understanding of the combined effects of AMNS and LPS treatments on physiology and on the enduring negative changes observed following pubertal exposure to stressors.

Keywords: Puberty, Neurodegeneration, Microbiome, Blood-Brain-Barrier, Antimicrobials, Liposaccharide

1.0 Introduction

Neurodegenerative disorders are debilitating disorders that affect various vital functions such as cognition (i.e., memory, executive functions), motor skills (i.e., gait, resting tremor, rigidity, ataxia), emotions (i.e., anxiety, dysphoric and euphoric mood), and behaviours (i.e., disinhibition, apathy) (Esposito & Ismail, 2022; Haack et al., 2016; Levenson et al., 2014; Nuber et al., 2008; Wirth et al., 2013). There are a wide range of neurodegenerative disorders including, Alzheimer's disease, Parkinson's disease, multiple sclerosis, Huntington's disease, and amyotrophic lateral sclerosis. Despite decades of extensive research on the etiology of neurodegenerative disorders, the mechanisms that underlie these disorders remain unclear.

Puberty is a critical period of development that is vulnerable to exposure to stressors. Exposure to certain stressors can interfere with the maturation of several fundamental systems (i.e., hypothalamic-pituitary-adrenal axis, hypothalamic-pituitary-gonadal axis, central nervous system, immune system, etc.), possibly resulting in enduring negative changes that can increase susceptibility to neurodegeneration later in life (Esposito, et al., 2022; Holder & Blaustein, 2014; Murack et al., 2021; Murray et al., 2020; Smith et al., 2021a, Smith et al, 2021b). For example, pubertal exposure to antimicrobials (AMNS) and lipopolysaccharide (LPS) results in sex-specific acute changes in cellular mechanisms associated with neurodegeneration such as increased peripheral inflammation (i.e., IL10, IL23, IL17A, IL12) along with the increased expression of complement 3, alpha-synuclein, and leucine-rich repeat kinase 2 (Esposito, et al., 2022). Moreover, pubertal LPS treatment has been shown to cause enduring deficits in spatial memory and learning as well as increasing Parkinson 's-like behaviours in male, but not in female, mice (Dinel et al., 2014; Girard-Joyal & Ismail, 2017; Kolmogorova et al., 2019). The negative effects of pubertal LPS treatment have also been associated with sex-specific alterations in LPS-induced

hypothermia and sickness behaviours (Cai et al., 2016). Notably, circulating gonadal hormones play a critical role in the host immune response and thermoregulation, likely contributing to sex differences observed following LPS treatment (Cai et al., 2016). As such, the physiological response following exposure to an immune challenge (i.e., LPS) during puberty can result in enduring alterations in behaviour and brain function, potentially influencing the development of neurodegenerative disorders.

Alterations to the composition of the gut microbiome could mediate the effects of pubertal LPS and AMNS treatments on the development of neurodegenerative disorders. The gut microbiome hosts trillions of microorganisms which reside along the intestinal tract (i.e., esophagus, stomach, and intestines) (Van den Abbeele et al., 2011). These microorganisms have several vital functions including vitamin and nutrient synthesis, carbohydrate fermentation, regulating immune function, and protecting against pathogens (Huttenhower et al., 2012; Shreiner et al., 2015). Alterations to the composition of the gut microbiome (i.e., gut dysbiosis) have been strongly linked to the development of neurodegenerative disorders (Goyal et al., 2021; Hirschberg et al., 2019). For example, gut dysbiosis induced through oral administration of *Ecc1* (i.e., non-pathogenic enterobacteria) propagates the expression of immune hemocytes in the brain, resulting in neurodegeneration through tumor necrosis factor-Jun N-terminal kinase mediated apoptosis (Wu et al., 2017). Moreover, in a mouse model of Parkinson's disease, chronic rotenone (i.e., broad-spectrum insecticide, piscicide, and pesticide) administration results in dysbiosis and increased colonic inflammation and locomotor deficits (i.e., spontaneous locomotor activity, motor coordination) along with increased alpha-synuclein aggregates and tyrosine hydroxylase degeneration in the brain (Yang et al., 2018). Although there seems to be a

clear link between the gut microbiome and the progression of neurodegenerative disorders, the mechanisms underlying the effects of the gut microbiome on neurodegeneration remain unclear.

Gut dysbiosis-induced increases in intestinal and blood-brain-barrier (BBB) permeability could be a mechanism that influences the development of neurodegenerative disorders. Cytokines and chemokines that are produced as a response to gut dysbiosis have been shown to alter the expression of tight-junction proteins, resulting in alterations in intestinal and BBB function (Fukui, 2016; Kacimi et al., 2011; Leclercq et al., 2014). For example, mice fed with a high-fat diet demonstrate gut dysbiosis along with increased pro-inflammatory cytokine expression (i.e., TNFα, IL1β, IFNγ) and decreased expression of colonic intectin and occludin, markers associated with intestinal permeability. Interestingly, these adverse effects are reversed when mice are treated with the probiotic *Lactobacillus acidophilus* (Kang et al., 2022). Moreover, germ-free mice show a reduction in the expression of occludin and claudin-5 in the frontal cortex, striatum, and hippocampus (HIPP) when compared to specific-pathogen-free mice, indicating alterations in BBB function. However, when germ-free mice are given a single gavage of the fecal matter from specific-pathogen-free mice, a significant increase in the expression of zonula occludin 1, occludin, and claudin-5 is observed, 14 days following gavage (Braniste et al., 2014). Notably, while alterations in tight junction protein expression might suggest a potential impact on BBB permeability, it is essential to directly assess BBB function to draw definitive conclusions. Therefore, interpretations of such findings should be made cautiously. Nonetheless, these findings are significant as gut dysbiosis along with alterations in intestinal and BBB function have been observed in patients suffering from neurodegenerative disorders (Raimondi et al., 2020; Soni et al., 2022). As such, alterations in intestinal and BBB

function induced by gut dysbiosis may be an underlying mechanism influencing several neurodegenerative processes (i.e., neuroinflammation, apoptosis, excitotoxicity).

Alterations to the composition of the gut microbiome during puberty can influence intestinal and BBB permeability, resulting in enduring negative consequences on behaviours and brain functioning, potentially increasing susceptibility to neurodegeneration later in life (Esposito, Gandelman, et al., 2022; McCormick et al., 2020). However, our current understanding of how pubertal exposure to stress can contribute to these negative changes and the pathogenesis of neurodegenerative disorders is complex and unclear. Moreover, there are significant sex differences in the development of neurodegenerative disorders, which are likely mediated by differences in circulating gonadal hormones. As such, the objective of the current study was to examine the sex-dependent effects of pubertal AMNS and LPS treatments on gross locomotor activity, heart rate, body temperature, and intestinal and BBB permeability. We hypothesized that pubertal AMNS and LPS treatments would increase intestinal and BBB permeability along with decreasing body weight, gross motor activity, heart rate, and body temperature, in a sexually dimorphic manner.

2.0 Methods

2.1 Animals

280 male and female CD1 mice were shipped from Charles Rivers Laboratories (Saint-Constant, Québec, Canada) at three weeks of age. 240 of these mice were used for the analysis of intestinal and BBB permeability while 40 of these mice were used for the analysis of heart rate, gross motor activity, and core body temperature. For the analysis of intestinal and BBB permeability, mice were housed in either groups of three or four. For the analysis of heart rate, core body temperature, and gross motor activity, mice were pair-housed with only one of these mice undergoing the implantation of the telemetry system. Pair-housing was used in this case to avoid any unnecessary stress to the mice resulting from being single-housed. All mice were housed in sex-specific rooms that 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. Under our housing conditions, female CD1 mice do not display estrous cycling until 60 days following birth and scrotum width of six-week-old male CD1 mice has not yet reached adult size (Murray et al., 2023). Therefore, our six-week-old male and female CD1 mice are pubescents. All experiments were approved by the Animal Care Committee of the University of Ottawa.

2.2 Implantation and analysis of the G2 HR E-Mitter telemetry system

At four weeks of age, the G2 HR E-Mitter (Starr Life Sciences Corp., Oakmont, PA; USA) was implanted according the manufacturer's instructions in 40 mice. Briefly, mice were anesthetized using 1.5L/min oxygen and 3-4% isoflurane. Carprofen was administered subcutaneously (5mg/kg body weight) before the surgery and 24 and 48 hours following the surgery. A midline abdominal incision was made before the insertion of the telemetry system into the abdominal cavity. A small hole was made on the left and right side of the abdominal incision to divert the leads (negative lead on the animal's right and positive lead on the animal's left) and ensure the correct placement of the electrodes in a 45° to 60° angle. The abdominal incision was then closed with Vicryl sutures. The negative lead was then placed subcutaneously near the right clavicle and the positive lead was placed to the left of the xiphoid process and cranial to the last rib. Metal ferrules were then plugged on the tips of both the positive and negative leads. The negative lead was then attached near the *pectoralis superficialis* and the positive lead was attached near the *cutaneus trunci* with metal sutures. The remaining incisions were then closed with 34-gauge stainless steel suture material followed by topical application of Bupivacaine on incision sites. Heart rate (bpm), gross motor activity (counts), and core body temperature (°C) were assessed in freely moving mice with the use of the VitalView® Data Acquisition System software (Version 4.200.2; Starr Life Sciences Corp., Oakmont, PA; USA). Telemetric data was obtained for 48 hours following LPS/saline injections of six-week-old mice. Samples were obtained in 7-minute and 30-second intervals where 30-minute means were calculated for each animal and subsequently used for data analysis.

2.3 Antimicrobial treatment

Mice were administered 200μL of mixed broad-spectrum antimicrobial solution or water through gavage twice daily for seven days at five weeks of age as described in (Esposito, et al.,

2022). The AMNS was prepared daily and contained 15 mg/mL of ampicillin (No. BP1760-5, Fisher Scientific, Geel, Belgium), neomycin (No. 480125GM, 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 hours and 1800 hours, respectively. This dosage and treatment regimen has been shown to sufficiently suppress total microbial content (Zarrinpar et al., 2018).

2.4 Lipopolysaccharide administration

Six-week-old male and female mice received an intraperitoneal injection of either 1.5 mg/kg of LPS (Escherichia coli serotype 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, one week following AMNS treatment. This dose of LPS has been shown to induce sexually dimorphic sickness behaviours for approximately 24-48 hours (Cai et al., 2016).

2.5 Body weight analyses

Body weights were measured at baseline (immediately before LPS injection) and 24 hours post-LPS injection in the 240 mice who underwent examination of BBB permeability. Changes in body weights were examined as a percent change in body weight from baseline, where day 0 (immediately before LPS injection) was subtracted from day 1 (24 hours post-LPS injection) and converted to a percentage.

2.6 Sickness monitoring

Sickness monitoring was conducted at 2, 4, 6, 8, 12, 24, and 48 hours following LPS/saline injections with the 40 mice who underwent the implantation of the G2 HR E-Mitter. The progression of sickness behaviours was assessed using a non-invasive and unbiased approach

with two raters blind to the experimental conditions (as described in Kolmogorova et al., 2017). Briefly, the raters visually assessed the mice for symptoms of lethargy (reduced locomotion), huddling (curled body posture), ptosis (drooping eyelids), and piloerection (erection of fur). They scored the total number of symptoms displayed by each mouse (one symptom = 1, two symptoms = 2, three symptoms = 3, four symptoms = 4) at each time point. The sickness scores from both raters at each time point were averaged and used in statistical analyses. Inter-rater reliability was assessed with Cronbach's alpha. Over a total of 280 scores, Cronbach's alpha was $\alpha = .97$.

2.7 Radioactive measures of *in vivo* BBB permeability

In vivo BBB permeability was assessed 24 hours, 72 hours, and one week following LPS/saline injections. The whole brain and specific brain regions (i.e., prefrontal cortex; PFC, HIPP, cerebellum, and caudate putamen; CP) were assessed at each time point. Briefly, mice were anesthetized with an intraperitoneal injection of 40% urethane followed by a jugular injection of ^{14}C-sucrose (106 dpm in 0.2 mL of lactated Ringer's solution/1% BSA) (Banks et al., 2015). 10 minutes following the injection of ^{14}C-sucrose, blood was collected from the descending abdominal aorta. Following blood collection, an intracardial perfusion was performed with 20 ml of lactated Ringer's solution. Whole brain and brain regions were then extracted, weighed and solubilized. Collected blood was centrifuged for 5 minutes at 10,000x*g* and serum was collected. Radioactivity in the brain and serum was measured using a PerkinElmer Tri-Carb 2910 TR scintillation counter. Data is expressed as mean counts per minute (CPM) per brain sample (g) divided by the CPM per μL in the corresponding serum (μL/g).

2.8 Ileum tissue extraction

Following brain collection, the ileum was collected 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, 0.78gm NaH2PO4•H2O, 24gm NaCl, 0.6gm KCl, 3L dH2O). Following the washing step, the luminal surface of the ileum was scraped off and placed in tubes. The ileum tissue was then homogenized in PBS containing the protease inhibitors Roche PhosSTOP™ (Millipore Sigma; cat: 04001) and Roche cOmplete™ ULTRA Tablets EDTA-free (Millipore Sigma; cat: 05001). The ileum homogenates were incubated on ice for 10 min, and centrifuged 3 times at 4 °C at 21,000 g for 10 minutes. The supernatants were collected to assay total protein concentrations using the Pierce™ Bicinchoninic Acid Assay (BCA) protein assay kit (Thermo Fisher Scientific).

2.9 Enzyme-linked immunosorbent assay (ELISA)

Claudin-3 protein concentration in the ileum was measured with ELISA. The ELISA kit (No. MBS2884546; MyBioSource) was used according to the manufacturer's instructions and ileal samples were measured in duplicates. Each plate contained one pooled sample to monitor inter-assay variation. All plates were read with Biotek Powerwave XS2 and analyzed with the Gen 5 V2.0 software. An inter-assay coefficient of variation (CV) of 15% and an intra-assay CV of 10% was achieved to ensure the reliability of our results.

2.10 Statistical analysis

Boxplots were used to identify outliers. All cases that exceeded the 1.5 interquartile range were considered statistical outliers and were winsorized to the next extreme data point in the group (Anscombe, 1973). For each dependent variable, a maximum of two data points were winsorized per group per ANOVA. For sickness behaviours and telemetric measures, a four-way mixed analysis of variance (ANOVA) was used to examine the within-subject effects of time 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., body weight, ELISA, BBB disruption), a 2 x 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 ($\eta p2$). Statistical significance was set to $p < .05$.

3.0 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 ($F_{(4.30, 137.46)} = 43.14$, $p < 0.05$, $\eta_p^2 = 0.57$) along with significant time x sex ($F_{(4.30, 137.46)} = 3.15$, $p < 0.05$, $\eta_p^2 = 0.09$), time x LPS ($F_{(4.30, 137.46)} = 19.86$, $p < 0.05$, $\eta_p^2 = 0.38$) and time x sex x LPS interactions ($F_{(4.30, 137.46)} = 3.32$, $p < 0.05$, $\eta_p^2 = 0.09$). The ANOVA also found significant between-subjects main effects of sex ($F_{(1,32)} = 30.33$, $p < 0.05$, $\eta_p^2 = 0.49$) and LPS ($F_{(1,32)} = 596.20$, $p < 0.05$, $\eta_p^2 = 0.95$) along with a significant LPS x AMNS interaction ($F_{(1,32)} = 19.67$, $p < 0.05$, $\eta_p^2 = 0.38$). Pairwise comparisons showed that AMNS and saline-treated mice displayed greater sickness behaviours than saline and water-treated mice ($MD = 0.27$, $SE = 0.10$, $p < 0.05$). Similarly, AMNS and LPS-treated mice displayed greater sickness behaviours than LPS and water-treated mice ($MD = 0.38$, $SE = 0.10$, $p < 0.05$). LPS-treated mice displayed greater sickness behaviours than saline-treated mice at 2 ($MD = 1.80$, $SE = 0.24$, $p < 0.05$), 4($MD = 2.23$, $SE = 0.19$, $p < 0.05$), 6 ($MD = 1.98$, $SE = 0.22$, $p < 0.05$), 8 ($MD = 2.15$, $SE = 0.22$, $p < 0.05$), 12 ($MD = 2.35$, $SE = 0.15$, $p < 0.05$), and 24 ($MD = 2.03$, $SE = 0.15$, $p < 0.05$) hours following treatment. Lastly, LPS-treated male mice displayed greater sickness behaviours than LPS-treated female mice at 8 ($MD = 0.75$, $SE = 0.30$, $p < 0.05$) and 24 ($MD = 1.25$, $SE = 0.21$, $p < 0.05$) hours following treatment (Figure 1A and B).

3.2 Body weight change

The ANOVA found significant main effects of LPS ($F_{(1, 232)} = 579.84$, $p < 0.05$, $\eta_p^2 = 0.71$) and AMNS ($F_{(1, 232)} = 20.75$, $p < 0.05$, $\eta_p^2 = 0.08$) along with a significant AMNS x LPS interaction ($F_{(1, 232)} = 7.27$, $p < 0.05$, $\eta_p^2 = 0.03$). Pairwise comparisons showed that AMNS and

saline-treated mice displayed significantly lower body weights than saline and water-treated mice (*MD* = -0.65, *SE* = 0.13, $p < 0.05$). Furthermore, water and LPS-treated mice displayed significantly lower body weights in comparison to their water and saline-treated counterparts (*MD* = -2.40, *SE* = 0.13, $p < 0.05$). Similarly, AMNS and LPS-treated mice displayed significantly lower body weights in comparison to their AMNS and saline-treated counterparts (*MD* = -1.92, *SE* = 0.13, $p < 0.05$; Figure 2).

3.3 Body Temperature

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 ($F_{(7.82, 250.07)} = 7.68$, $p < 0.05$, $\eta_p^2 = 0.19$) along with significant time x sex ($F_{(7.82, 250.07)} = 2.42$, $p < 0.05$, $\eta_p^2 = 0.07$), time x LPS ($F_{(7.82, 250.07)} = 7.50$, $p < 0.05$, $\eta_p^2 = 0.19$), time x sex x LPS ($F_{(7.82, 250.07)} = 2.20$, $p < 0.05$, $\eta_p^2 = 0.06$) and time x sex x AMNS interactions ($F_{(7.82, 250.07)} = 2.00$, $p < 0.05$, $\eta_p^2 = 0.06$). The ANOVA also found a significant between-subjects main effect of sex ($F_{(1, 32)} = 2.35$, $p < 0.05$, $\eta_p^2 = 0.55$). Pairwise comparisons showed that LPS-treated mice displayed a sustained decrease in body temperature in comparison to saline-treated mice from 150 minutes to 330 minutes and 1260 minutes to 1470 minutes post-treatment. Body temperature of saline-treated male mice was significantly lower than saline-treated female mice from 510 minutes to 1530 minutes and from 1680 minutes to 2310 minutes post-treatment. Similarly, body temperature of LPS-treated male mice dropped significantly more than LPS-treated female mice from 150 minutes to 2310 minutes post-treatment (Figure 3A and B). Furthermore, AMNS-treated male mice displayed significantly lower body temperature than water-treated male mice from 1200 minutes to 1320 minutes (Figure 3A). AMNS-treated female mice displayed significantly larger body temperature drops

than water-treated female mice from 1530 minutes to 1650 minutes and 2130 minutes to 2220 minutes (Figure 3B).

3.4 Heart rate

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 ($F_{(7.46, 231.27)} = 14.64$, $p < 0.05$, $\eta_p^2 = 0.32$) along with a significant time x LPS interaction ($F_{(7.46, 231.27)} = 3.42$, $p < 0.05$, $\eta_p^2 = 0.10$). The ANOVA also found a between-subjects main effect of sex ($F_{(1, 31)} = 10.97$, $p < 0.05$, $\eta_p^2 = 0.26$). Pairwise comparisons revealed that regardless of AMNS and LPS treatments, male mice had significantly lower heart rates than female mice ($MD = -46.32$, $SE = 13.99$, $p < 0.05$). LPS-treated mice displayed a significant increase in heart rate in comparison to saline-treated mice from 30 minutes to 120 minutes post-treatment. Furthermore, LPS-treated mice displayed a sustained decrease in heart rate from 780 minutes to 1200 minutes and 1800 minutes to 2280 minutes. Lastly, LPS-treated mice displayed significantly lower heart rates in comparison to saline-treated mice from 870 minutes to 1110 minutes post-treatment (Figure 3C and D).

3.5 Gross motor activity

The four-way mixed ANOVA did not reveal any significant difference in gross motor activity (Figure 3 E and F).

3.6 Whole brain and regional BBB disruption 24 hours post-LPS/saline injection

The ANOVA did not show any significant difference in whole brain BBB permeability 24 hours post-LPS/saline injection (Figure 4E). However, the ANOVA did show a significant main effect of sex ($F_{(1, 32)} = 5.53$, $p < 0.05$, $\eta_p^2 = 0.15$) along with a significant main effect of LPS in the cerebellum ($F_{(1, 32)} = 4.26$, $p < 0.05$, $\eta_p^2 = 0.12$) and in the CP ($F_{(1, 32)} = 5.09$, $p <$

0.05, $\eta_p^2 = 0.14$). A significant AMNS x LPS interaction was also found in the cerebellum ($F_{(1, 32)} = 8.52$, $p < 0.05$, $\eta_p^2 = 0.21$), PFC ($F_{(1, 32)} = 8.94$, $p < 0.05$, $\eta_p^2 = 0.22$), CP ($F_{(1, 32)} = 8.45$, $p < 0.05$, $\eta_p^2 = 0.21$), and HIPP ($F_{(1, 32)} = 4.20$, $p < 0.05$, $\eta_p^2 = 0.12$). Pairwise comparisons revealed that regardless of AMNS and LPS treatments, male mice displayed significantly greater BBB permeability than female mice in the cerebellum (*MD* = 2.96, *SE* = 1.26, $p < 0.05$). LPS-treated mice displayed significantly greater BBB permeability than saline-treated mice in the cerebellum (*MD* = 2.60, *SE* = 1.26, $p < 0.05$) and CP (*MD* = 2.97, *SE* = 1.32, $p < 0.05$). Furthermore, AMNS and LPS-treated mice displayed significantly greater BBB permeability than water and LPS-treated mice in the cerebellum (*MD* = 4.64, *SE* = 1.78, $p < 0.05$), PFC (*MD* = 5.10, *SE* = 1.72, $p < 0.05$), CP (*MD* = 5.76, *SE* = 1.86, $p < 0.05$), and HIPP (*MD* = 6.07, *SE* = 2.89, $p < 0.05$). Lastly, AMNS and LPS-treated mice displayed significantly greater BBB permeability than AMNS and saline-treated mice in the cerebellum (*MD* = 6.26, *SE* = 1.78, $p < 0.05$; Figure 4A), PFC (*MD* = 5.62, *SE* = 1.72, $p < 0.05$; Figure 4D), CP (*MD* = 6.81, *SE* = 1.86, $p < 0.05$; Figure 4B), and HIPP (*MD* = 7.98, *SE* = 2.89, $p < 0.05$; Figure 4C).

3.7 Whole brain and regional BBB disruption 72 hours post-LPS/saline injection

The ANOVA did not show any significant difference in whole brain and cerebellum BBB permeability 72 hours post-LPS/saline injection (Figure 5A and E). However, the ANOVA revealed a significant main effect of sex in the CP ($F_{(1, 32)} = 5.42$, $p < 0.05$, $\eta_p^2 = 0.15$) and HIPP ($F_{(1, 32)} = 4.97$, $p < 0.05$, $\eta_p^2 = 0.13$) along with a significant sex x AMNS interaction in the CP ($F_{(1, 32)} = 4.05$, $p < 0.05$, $\eta_p^2 = 0.11$). There was also a trend towards a significant sex x AMNS interaction in the PFC ($F_{(1, 32)} = 3.56$, $p = 0.07$, $\eta_p^2 = 0.10$). Pairwise comparisons revealed that regardless of AMNS and LPS treatments, female mice displayed significantly greater BBB permeability than male mice in the CP (*MD* = 3.26, *SE* = 1.40, $p < 0.05$) and HIPP (*MD* = 3.72,

SE = 1.67, $p < 0.05$; Figure 5C). Furthermore, AMNS-treated female mice displayed significantly greater BBB permeability than their water-treated counterparts in the PFC (*MD* = 4.96, *SE* = 2.22, $p < 0.05$). AMNS-treated female mice also displayed significantly greater BBB permeability than AMNS-treated male mice in the PFC (*MD* = 5.70, *SE* = 2.22, $p < 0.05$; Figure 5D) and CP (*MD* = 6.08, *SE* = 1.98, $p < 0.05$; Figure 5B).

3.8 Whole brain and regional BBB disruption one week post-LPS/saline injection

The ANOVA did not show any significant difference in BBB permeability in the cerebellum (Figure 6A), PFC (Figure 6D), CP (Figure 6B) and HIPP (Figure 6C) one week post-LPS/saline treatment. However, the ANOVA revealed a significant main effect of AMNS ($F_{(1, 32)} = 19.47$, $p < 0.05$, $\eta_p^2 = 0.38$) along with significant sex x AMNS ($F_{(1, 32)} = 4.18$, $p < 0.05$, $\eta_p^2 = 0.12$), sex x LPS ($F_{(1, 32)} = 10.73$, $p < 0.05$, $\eta_p^2 = 0.25$), and AMNS x LPS ($F_{(1, 32)} = 10.17$, $p < 0.05$, $\eta_p^2 = 0.24$) interactions in whole-brain BBB permeability. Pairwise comparisons revealed that regardless of sex and LPS treatment, AMNS-treated mice displayed significantly greater whole-brain BBB permeability than water-treated mice (*MD* = 2.80, *SE* = 0.63, $p < 0.05$). AMNS-treated female mice displayed significantly greater whole-brain BBB permeability than their water-treated counterparts (*MD* = 4.10, *SE* = 0.90, $p < 0.05$). Furthermore, LPS-treated male mice displayed significantly greater whole-brain BBB permeability than their saline-treated counterparts (*MD* = 2.93, *SE* = 0.90, $p < 0.05$). AMNS- and saline-treated mice displayed significantly greater whole-brain BBB permeability than saline and water-treated mice (*MD* = 4.82, *SE* = 0.90, $p < 0.05$). Lastly, water and LPS-treated mice displayed significantly greater whole-brain BBB permeability than water and saline-treated mice (*MD* = 2.88, *SE* = 0.90, $p < 0.05$; Figure 6E).

3.9 Ileal claudin-3 concentrations 24 hours, 72 hours, and one week post-LPS/saline injection

The ANOVA did not show any significant difference in ileal claudin-3 concentration 24 hours, 72 hours, and one week post-LPS injection (Figure 7A, B, and C).

4.0 Discussion

Exposure to stressors in pubertal mice results in long-term physiological and behavioural consequences that are not observed when similar stressors are introduced at other developmental time points, highlighting the unique sensitivity and critical nature of this developmental period for long-term health outcomes (Blaustein & Ismail, 2013; Ismail et al., 2011; Smith et al., 2023). Furthermore, pubertal exposure to LPS and AMNS can have enduring consequences on behaviour and brain functioning, potentially increasing susceptibility to neurodegenerative disorders later in life. However, the mechanisms underlying these enduring effects are unknown. As such, the purpose of this study was to examine the sex-dependent effects of pubertal AMNS and LPS treatments on sickness behaviours, body weight, core body temperature, heart rate, and gross motor activity along with examining the effects of these treatments on intestinal and BBB permeability. Our results suggest that pubertal AMNS and LPS treatments may lead to sex-dependent changes in sickness behaviours, core body temperature, heart rate, and BBB permeability, based on preliminary evidence in mice.

AMNS and LPS treatments resulted in a significant drop in body temperature, but LPS-treated mice also demonstrated an initial increase in heart rate which was followed by a sustained decrease in heart rate. Alterations in body temperature and heart rate also coincided with an increase in sickness behaviours. These results are in line with our hypothesis and with previous research examining the effects of AMNS and LPS treatments on sickness behaviours, body temperature and heart rate (Cai et al., 2016; Kuo et al., 2019). Interestingly, we did not observe any significant changes in gross motor activity following AMNS and LPS treatments. The lack of significant changes in gross motor activity is likely due to unreliable recordings from our telemetry system. Visual observations following LPS treatment indicated minimal movement in

our LPS-treated mice, however, our telemetry system indicated high levels of movement, suggesting inaccurate recordings of gross motor activity. We do not believe that the heart rate and core body temperature measures were affected by this problem because the telemetry system does not record these measures in the same way as it does gross motor activity. Furthermore, whereas the variability was substantially high for gross motor activity, the same issue was not seen for heart rate and core body temperature.

AMNS and LPS-induced alterations in sickness behaviours, body temperature, and heart rate observed in our study are likely due to increased circulating cytokine levels. AMNS and LPS treatments stimulate the production and release of various cytokines such as TNFa, IL10, IL6, IL1B and COX-1 which have been shown to influence heart rate and produce a hypothermic response (Dogan et al., 2002; Harden et al., 2014; Mukherjee et al., 2010; L. Sun et al., 2019). Increased heart rate and decreased body temperature following LPS treatment is an adaptive response to effectively counteract the effects of systemic inflammation (Leon, 2004; Parrillo Joseph E., 1993). However, the subsequent sustained decrease in heart rate observed in our study may represent damage to cardiac cells. LPS treatment initiates a cascade of cellular events that can influence the functioning of cardiac myocytes by impairing intracellular calcium homeostasis, causing alterations in excitation-contraction coupling, and enhancing programmed cell death (Kuo et al., 2019). Ultimately, alterations in these processes can result in reduced contractility of cardiac cells, resulting in reduced heart rate (Tzimas et al., 2017; Vincent, 2008). Importantly, reduced functioning of cardiac cells has been associated with various neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease (Bartl et al., 2023; Critchley et al., 2018; Jefferson, 2010; Moore et al., 2021). As such, although pubertal LPS and AMNS treatments evoke adaptive hypothermic and tachycardic

responses initially, LPS treatment may have long-term effects on the functioning of cardiac cells, potentially increasing susceptibility to cardiovascular disorders and neurodegeneration later in life.

LPS-induced increase in sickness behaviours and decrease in body temperature were also more pronounced in males than in females. The sex-dependent effects of LPS treatment on sickness behavior and body temperature may be due to sex differences in the immune response. Male mice typically demonstrate a more pronounced immune response following pubertal LPS treatment than female mice. For example, male mice display greater concentrations of peripheral pro-inflammatory cytokines (i.e., IL12 (p70), IL23, IL17A, IL1B, IL6) and less peripheral anti-inflammatory cytokines (i.e., IL10) than female mice following pubertal LPS treatment (Esposito, Gandelman, et al., 2022; Sharma et al., 2018). These results also extend to central cytokine concentrations with male mice demonstrating greater mRNA expression of IL1B, TNFa, and IL6 in the PFC following pubertal LPS treatment (Sharma et al., 2018). Considering the vital role that cytokines play in regulating body temperature and sickness behaviours, it is likely that a greater immune response in the male mice contributed to the more pronounced alterations in body temperature and sickness behaviours following LPS treatment. Notably, our results also demonstrated that saline-treated male mice displayed lower body temperatures than saline-treated female mice, suggesting an inherent sex difference in body temperature. This finding is in line with previous research demonstrating that female mice have greater baseline body temperatures than male mice, an effect that is mediated by increased levels of estradiol and progesterone in females (Sanchez-Alavez et al., 2011). As such, sex differences in the immune response can contribute to sex differences in body temperature and sickness behaviours,

however, inherent sex differences in body temperature likely play a role in the effects observed in this study.

Our results also showed that pubertal AMNS and LPS treatments displayed regional increases in BBB permeability 24 hours following saline or LPS treatment. Specifically, AMNS and LPS treatments resulted in increased BBB permeability in the PFC, CP, cerebellum, and HIPP. Notably, LPS treatment on its own did not result in increased BBB permeability, suggesting that AMNS treatment potentiates the effects of LPS on BBB permeability. These results are in line with our hypothesis and with previous research examining the effects of LPS and AMNS treatments on BBB permeability (Haruwaka et al., 2019; Kolmogorova et al., 2021; L. Sun et al., 2019). The BBB is disrupted by LPS treatment through its effects on paracellular and transcellular transport routes (Banks et al., 2015; Erickson et al., 2018; Nishioku et al., 2009). Furthermore, LPS and AMNS-induced dysbiosis have been shown to disrupt the BBB, however, the mechanisms explaining these effects remain unclear (Livingston et al., 2023; N. Sun et al., 2021). Given that microbial dysbiosis can contribute to maladaptive immune responses, it is plausible that LPS and AMNS treatments in our current study induced microbial dysbiosis, potentiating the inflammatory response induced by LPS, resulting in increased BBB permeability (Galea, 2021; Livingston et al., 2023; Yoo et al., 2020). Consequently, increased BBB permeability results in the infiltration of peripheral immune cells (i.e., neutrophils, monocytes, T lymphocytes) into the brain, increased microglial activation, and the promotion of brain tissue damage (Greiner & Kipp, 2021; Patel & Frey, 2015; Qiu et al., 2021). Damage to the brain during puberty can influence neuronal development and connectivity, having long-term effects on neurodevelopment and brain functioning (Cao et al., 2021; Malave et al., 2022). Importantly, these effects are region-specific, suggesting that increases in BBB permeability 24

hours following saline or LPS treatment are localized to the brain regions examined in this study (i.e., PFC, CP, HIPP, cerebellum) rather than there being a global increase in BBB permeability. Therefore, the PFC, CP, HIPP, and cerebellum may be more vulnerable to inflammation-mediated damage 24 hours following saline or LPS treatment.

AMNS treatment also resulted in regional (i.e., CP and PFC) increases in BBB permeability 72 hours post-saline or LPS injection and global increases in BBB permeability one week post-saline or LPS injection in female mice. Furthermore, LPS treatment resulted in global increases in BBB permeability one week post-saline or LPS injection in male mice. These results are in line with our hypothesis and suggest that AMNS and LPS treatments can have long-term negative consequences on BBB function. Enduring increases in BBB permeability in AMNS-treated females, but not in males, can be attributed to the sex-dependent effects of AMNS treatment on microbial composition. For example, AMNS treatment significantly reduces the abundance and structural composition of *Firmicutes* along with decreasing the expression of branched-chain amino acids (BCAAs; leucine, isoleucine, and valine), short-chain fatty acids (SCFAs; acetate, butyrate, and propionate), and aromatic amino acids (AAAs; phenylalanine and tyrosine) in female but not male mice (Gao et al., 2019). Furthermore, altered expression of BCAAs, SCFAs, and AAAs can influence the BBB barrier and affect its permeability (Chi et al., 2023; Fernstrom, 2005; Fock & Parnova, 2023).

The enduring global increases in BBB permeability observed in LPS-treated male mice can be attributed to the enhanced inflammatory response that is often observed in pubertal male mice following LPS treatment (Esposito, Kearns, et al., 2022; Sharma et al., 2018). The BBB is heavily influenced by inflammatory markers (i.e., IL6, TNFa, IL1B) and increased expression of these markers can influence BBB function, potentially resulting in the enduring global

dysfunction of the BBB in our male mice (Galea, 2021). The lack of regional increases in BBB permeability one week post-saline or LPS treatment suggests that regional BBB disruption subsides at this time point. Furthermore, the observed global increases in BBB permeability at this time point are likely due to the cumulative impact of minor alterations in BBB permeability across various brain regions. As such, AMNS and LPS treatments can have long-term, sex-dependent effects on BBB permeability, increasing the likelihood of causing damage to the brain and potentially increasing susceptibility to various neurodevelopmental and neurodegenerative disorders.

Our results did not show any significant difference in intestinal permeability following LPS treatment. This result is not in line with our hypothesis and with previous research examining the effects of LPS and AMNS treatments on intestinal permeability (Ling et al., 2016; Ran et al., 2020). For example, LPS treatment significantly decreases the expression of claudin-3 in Caco-2 cells and increases the expression of ileal claudin-3 in Sprague-Dawley rats (Ling et al., 2016). Furthermore, vancomycin-treated mice display significant decreases in colonic mRNA claudin-3 expression (Ran et al., 2020). These discrepancies with our findings may be due to various methodological differences such as the length of AMNS and LPS treatments, the species that were used, the age of the animals, the tissue that was analyzed (i.e., colon vs ileum), and the antimicrobials that were utilized. Nevertheless, our current treatment model does suggest that the ileum is relatively resistant to alterations in claudin-3 expression following pubertal LPS and AMNS treatments in male and female CD-1 mice.

4.1 Limitations and future directions

This study focused on how pubertal LPS and AMNS-induced changes in BBB function, intestinal permeability, gross motor activity, heart rate, and body temperature may contribute to

enduring negative consequences related to neurodegeneration. However, neurodegenerative disorders are multifactorial, involving various genetic, environmental, and lifestyle factors that likely play a role in the development of these disorders. Future research should analyze the influence of these different factors to gain a more holistic insight into the development of neurodegenerative disorders. Secondly, all mice were euthanized over a relatively short period of time (i.e., 24 hours, 72 hours, and one week post-LPS or saline injection) which limits our ability to make inferences about physiological and brain functioning beyond these time points. Future research should examine the effects of pubertal LPS and AMNS treatments over a longer period of time to further our understanding of the long-term effects of these treatments on physiological and brain functioning. Lastly, although we believe that our treatment model did induce alterations in microbial composition, we cannot be certain that this is the case considering that microbial composition was not analyzed in this study. Future research utilizing our treatment model should analyze microbial composition to confirm whether this treatment model induces dysbiosis.

4.2 Conclusion

In conclusion, this study shows that pubertal AMNS and LPS treatments result in sex-dependent changes in physiological and brain functioning. Overall, these findings suggest that pubertal male mice are more susceptible to alterations in body temperature while both male and female mice display impaired cardiac function following pubertal LPS treatment. Furthermore, both male and female mice display increased BBB permeability 24 hours following saline or LPS treatment. However, AMNS treatment results in more enduring changes in BBB permeability in pubertal female mice while LPS treatment results in more enduring changes in BBB permeability in pubertal male mice. The current study is the first to examine the sex-

dependent effects of both LPS and AMNS treatments on physiological and BBB functioning during puberty. The results provide us with mechanistic insight into how pubertal AMNS and LPS treatments induce enduring negative changes in brain functioning, potentially increasing susceptibility to neurodegenerative disorders.

Funding: This work was supported by a Discovery grant from the Natural Sciences and Engineering Research Council of Canada (2020-04302) to NI.

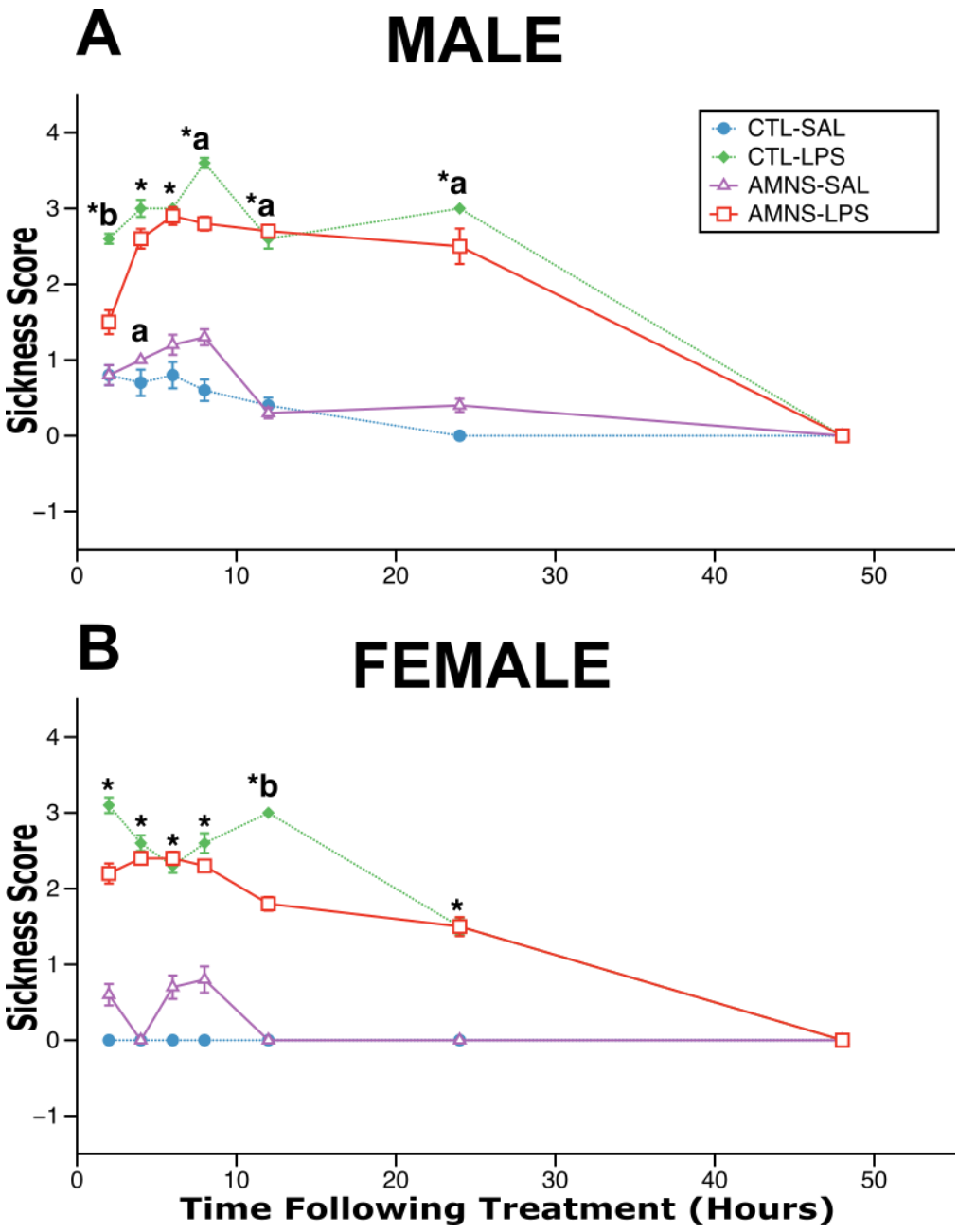
Author Contributions: **Pasquale Esposito:** Conceptualization, Methodology, Investigation, Writing – Original Draft, Visualization, Project administration, Formal analysis. **Eleni Dubé-Zinatelli:** Investigation. **Rebecca Krnel :** Investigation. **Luna Cappelletti:** Investigation. **Jacky Liang:** Investigation, Resources. **Nafissa Ismail:** Writing – Reviewing and Editing, Supervision, Funding acquisition.

Acknowledgments: The authors would like to thank all the members of the NISE Lab and the ACVS staff at the University of Ottawa for their assistance with this project.

Declaration of Interests: None

Figures, Tables, and Captions

Figure 1.



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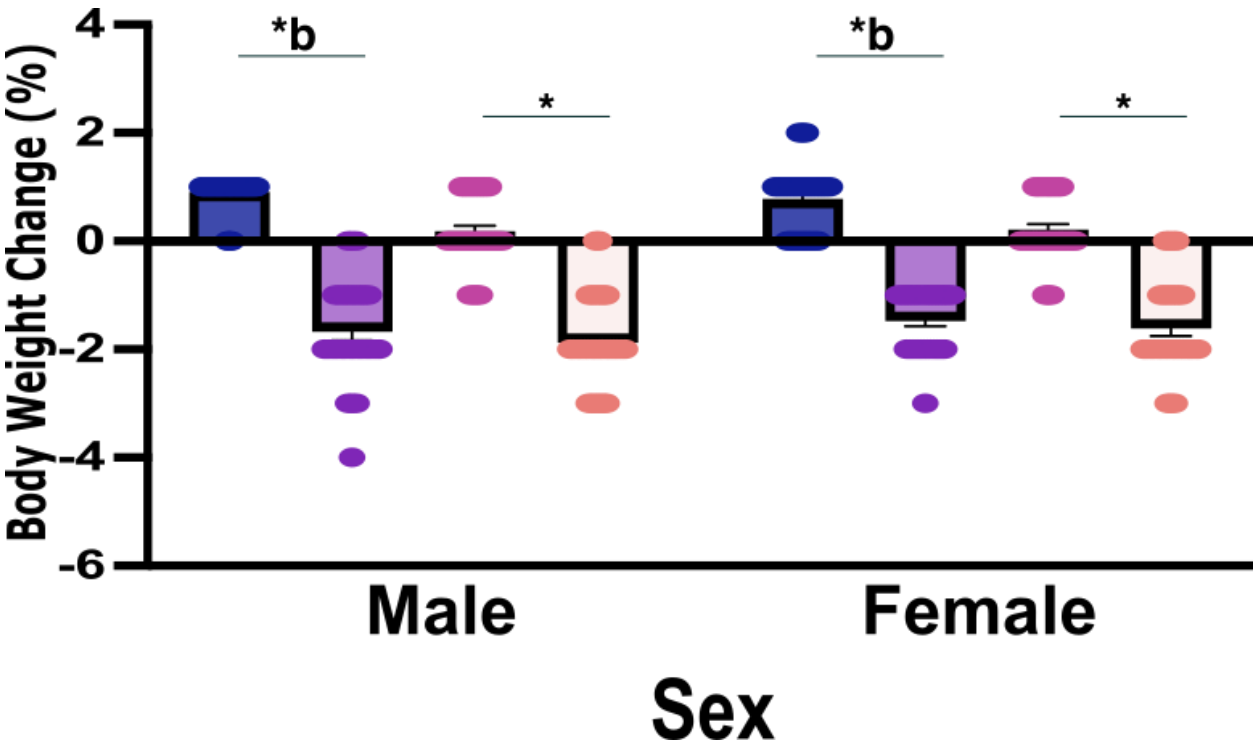

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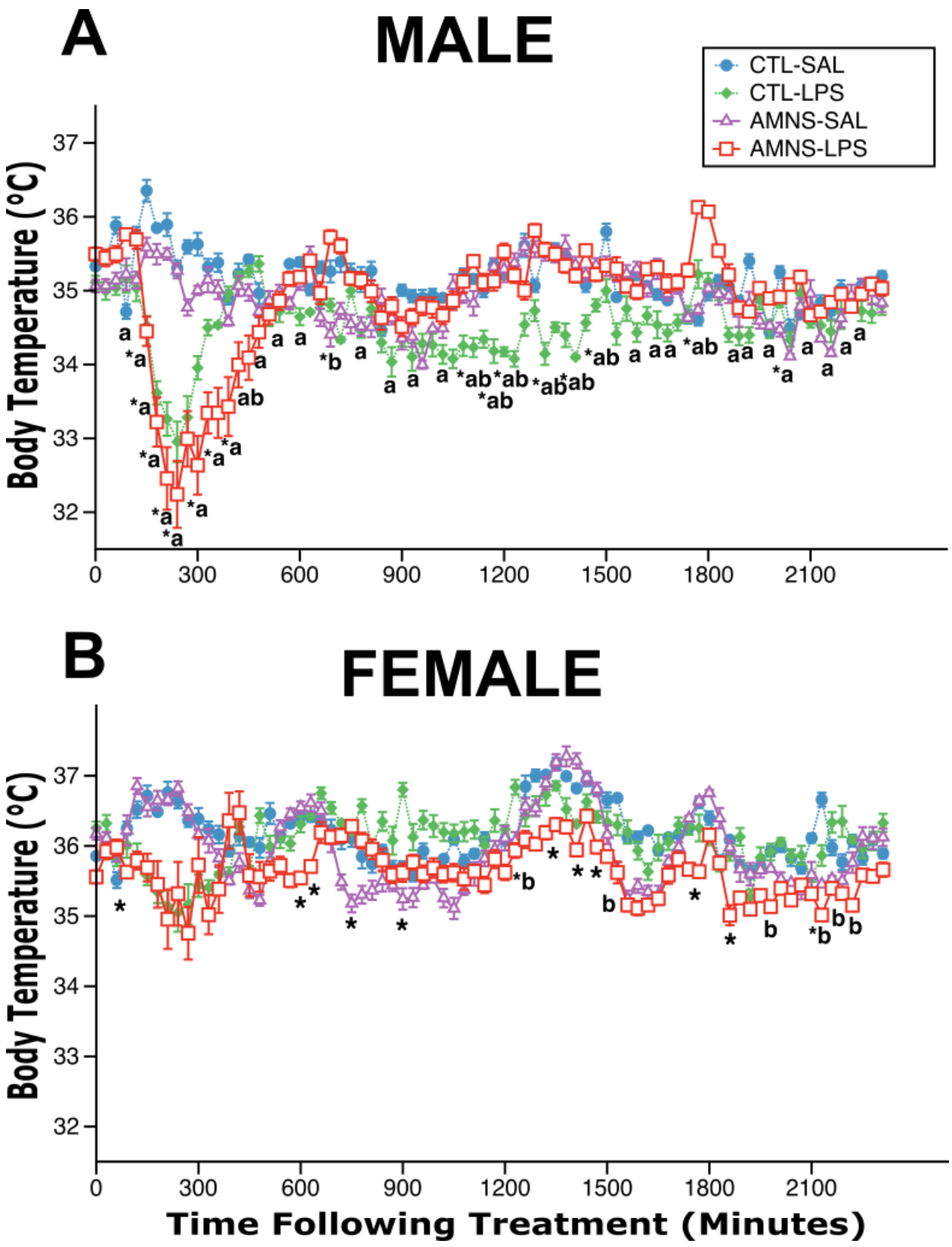
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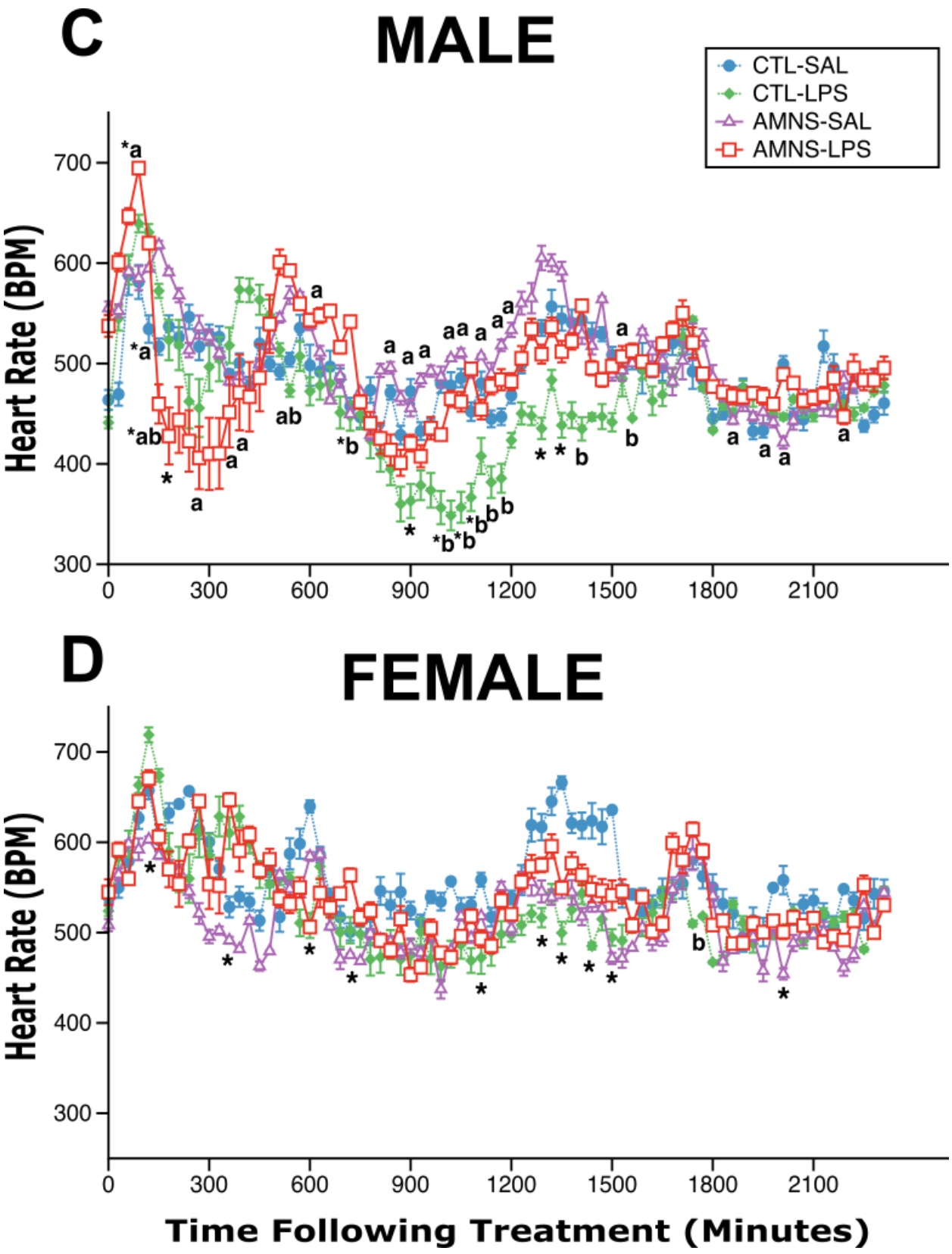
MALE
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
Body Temperature (°C)
37
36
35
34
33
32
0
300
600
900
1200
1500
1800
2100
B
FEMALE
Body Temperature (°C)
Time Following Treatment (Minutes)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CTL-SAL
CTL-LPS
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AMNS-LPS
Heart Rate (BPM)
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D
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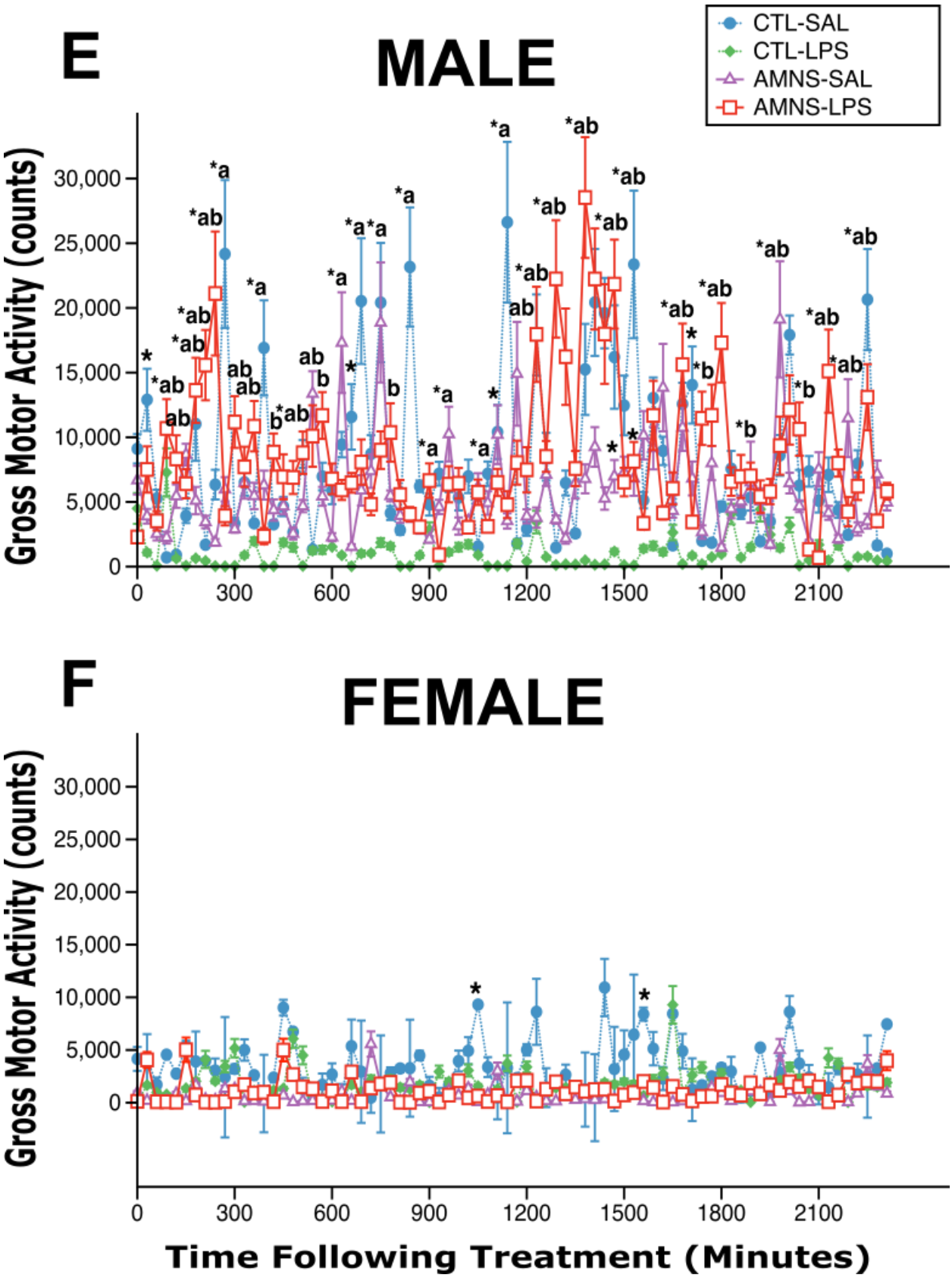
Heart Rate (BPM)
Time Following Treatment (Minutes)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AMNS-SAL
AMNS-LPS
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20,000
15,000
10,000
5,000
0
0
300
600
900
1200
1500
1800
2100
F
FEMALE
Gross Motor Activity (counts)
Time Following Treatment (Minutes)

Figure 4.

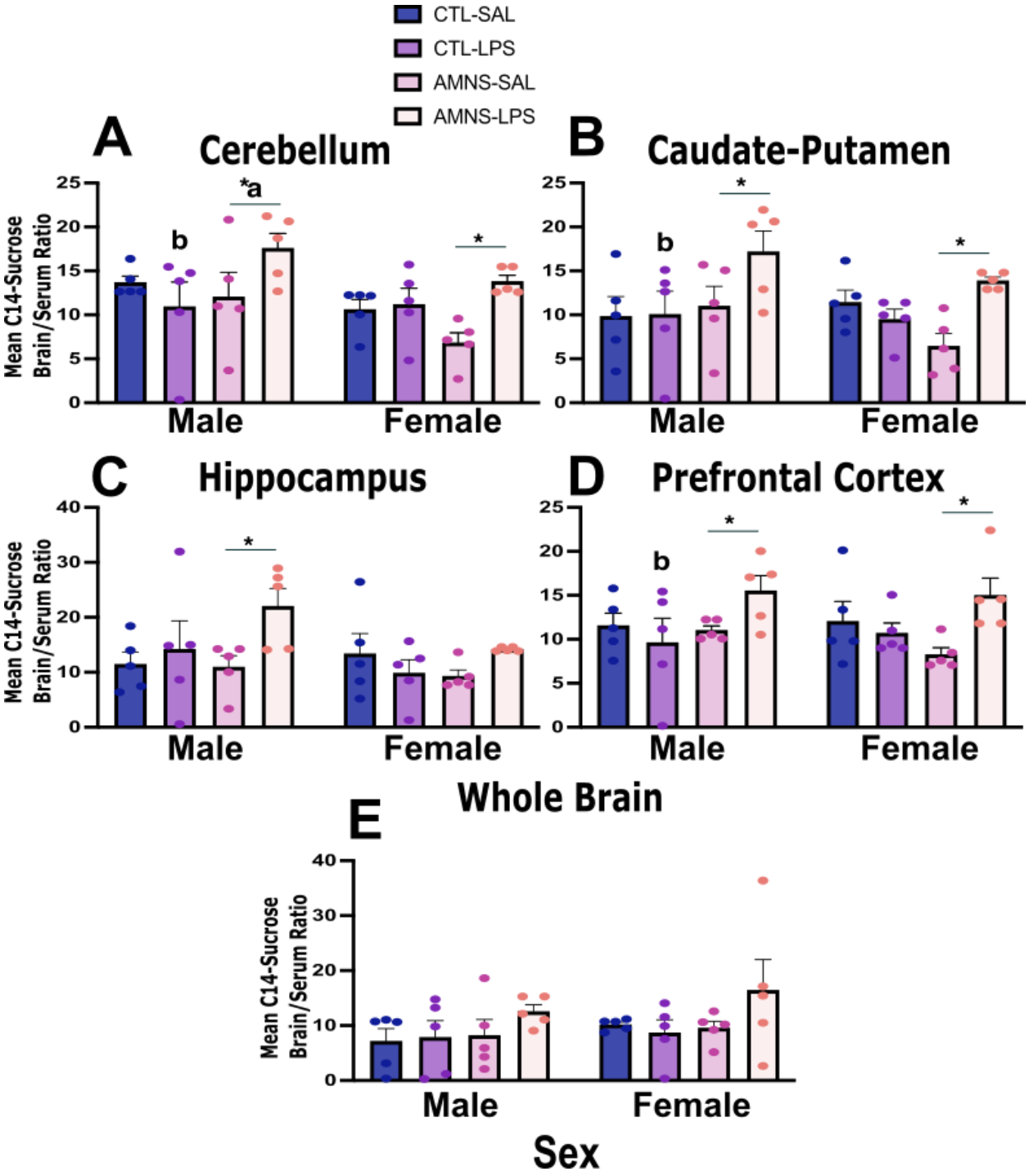

CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
A
Cerebellum
B
Caudate-Putamen
C
Hippocampus
D
Prefrontal Cortex
E
Whole Brain
Mean C14-Sucrose Brain/Serum Ratio
Male
Female
Sex
*a
b
*

Figure 5.

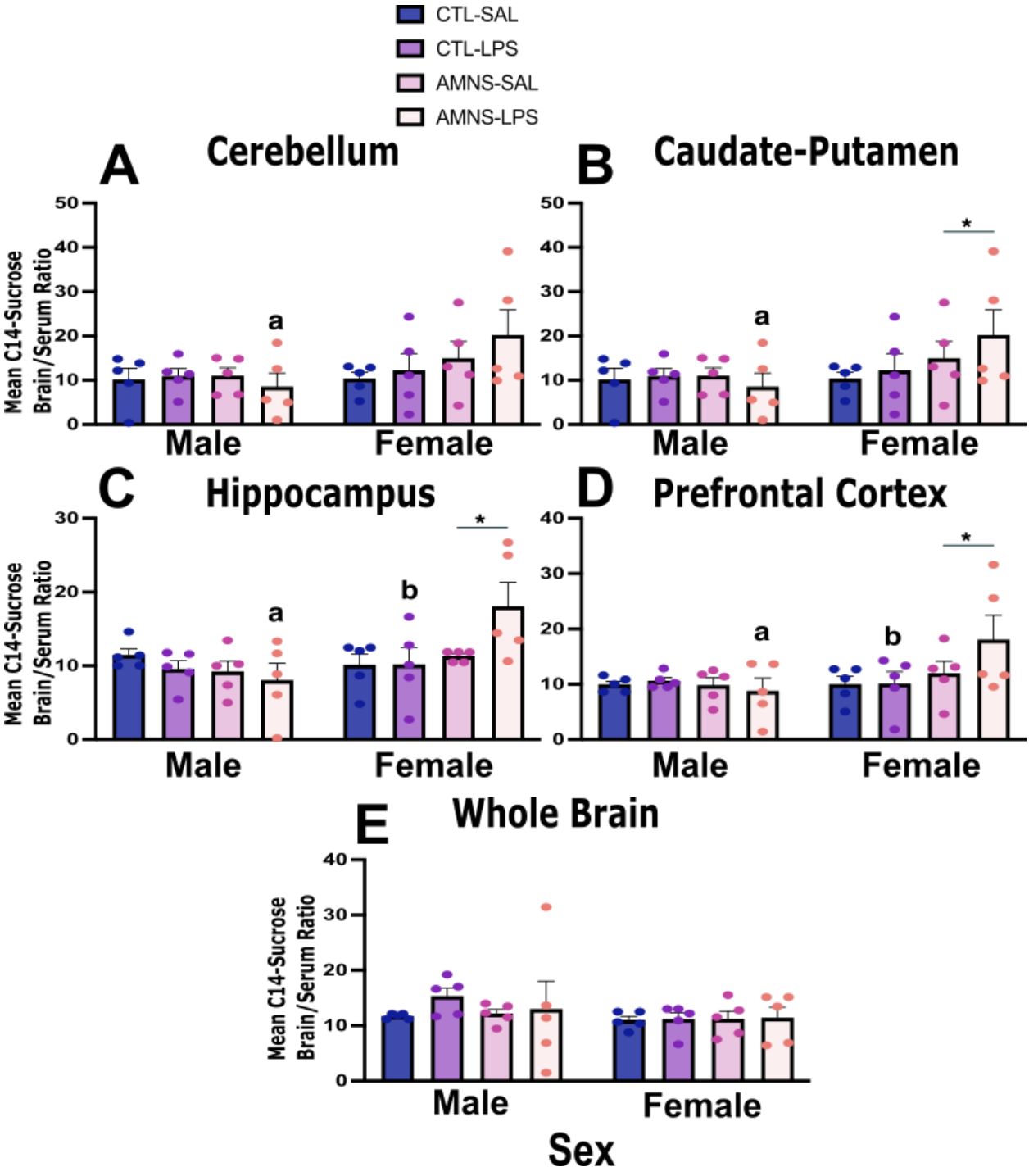
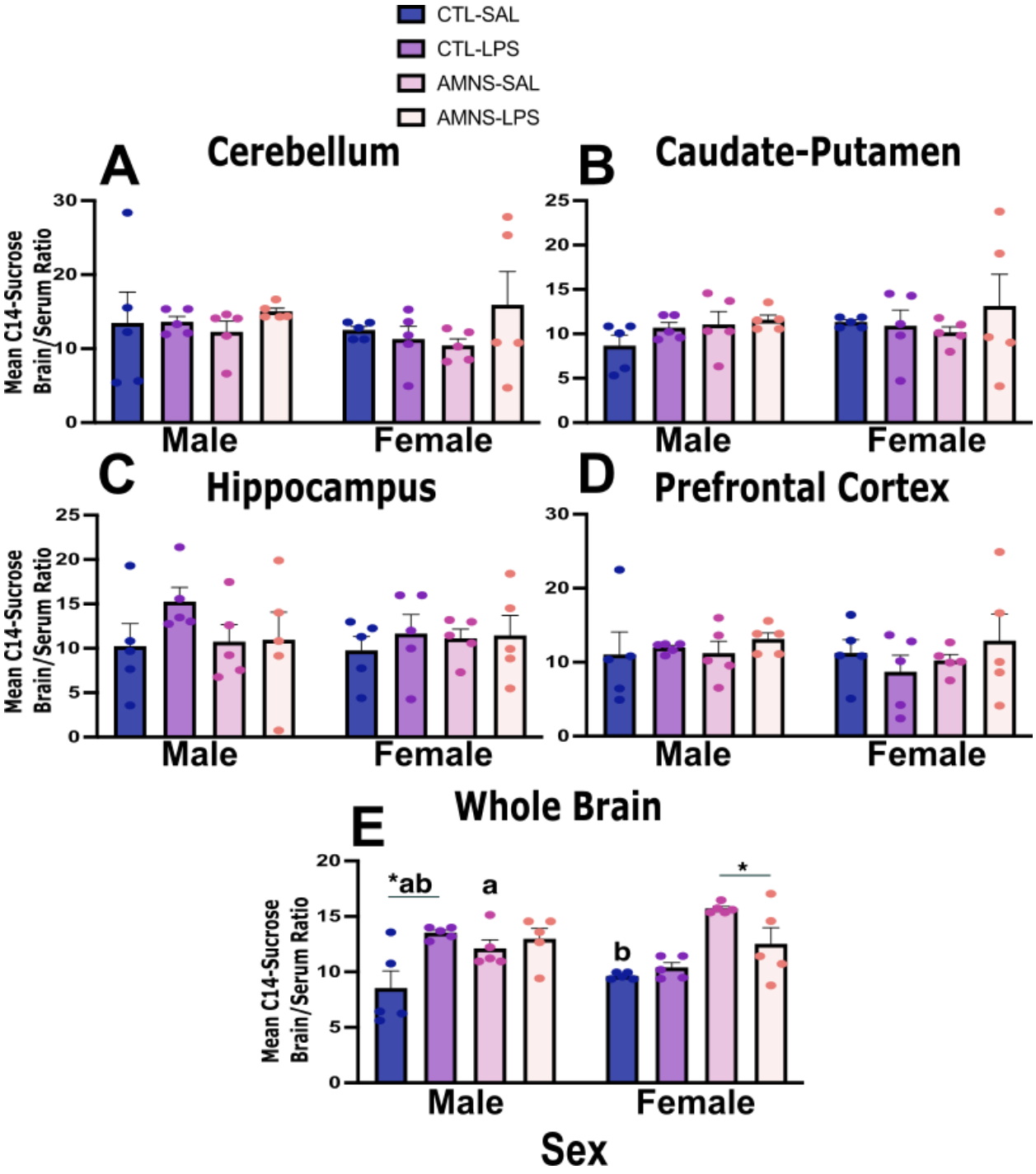

CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
A
Cerebellum
B
Caudate-Putamen
C
Hippocampus
D
Prefrontal Cortex
E
Whole Brain
Mean C14-Sucrose Brain/Serum Ratio
Male
Female
Sex
a
b
*

Figure 6.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CTL-LPS
AMNS-SAL
AMNS-LPS
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Cerebellum
B
Caudate-Putamen
C
Hippocampus
D
Prefrontal Cortex
E
Whole Brain
Mean C14-Sucrose Brain/Serum Ratio
Male
Female
*ab
a
b
*
Sex

Figure 7.

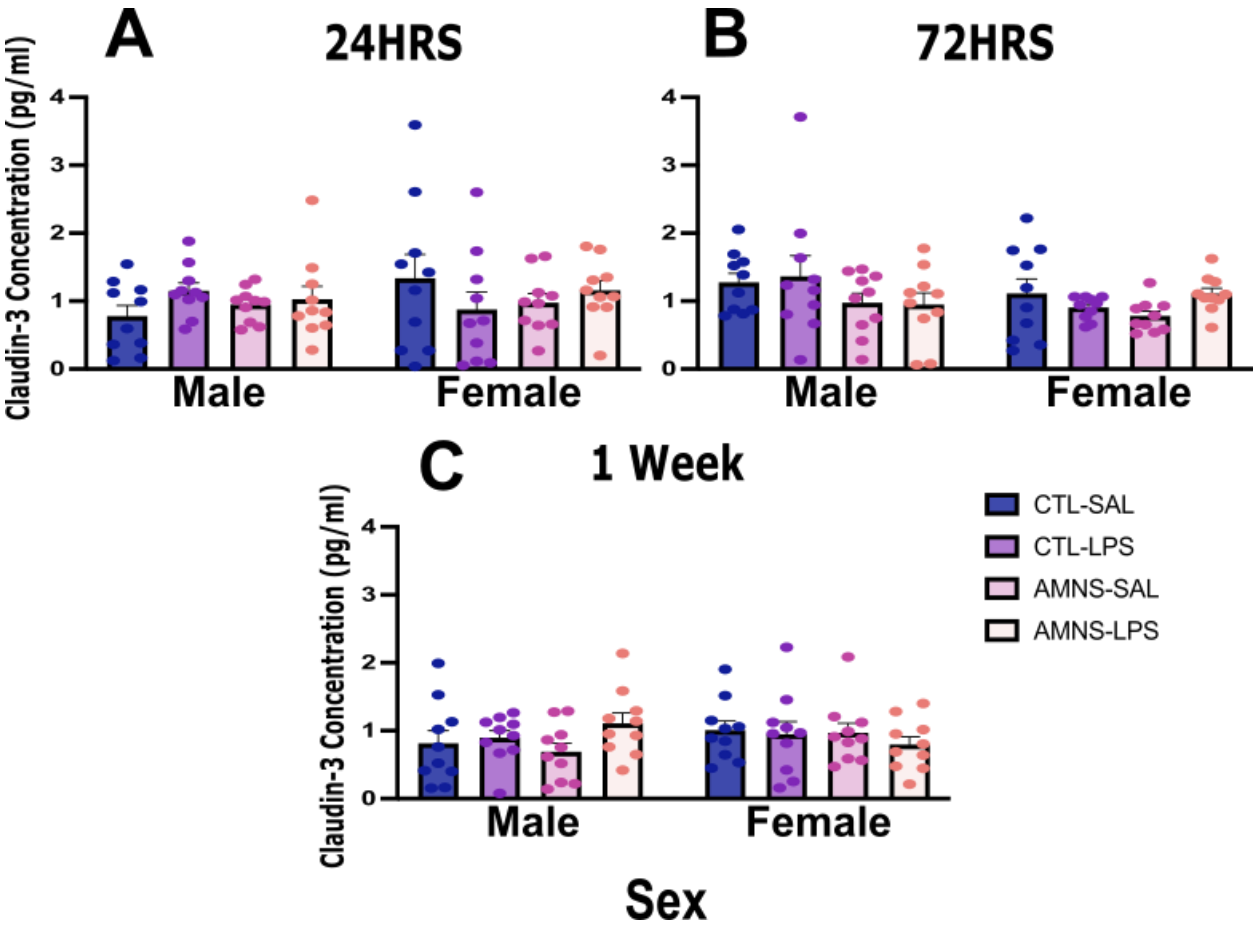
A
24HRS
B
72HRS
Claudin-3 Concentration (pg/ml)
0
1
2
3
4
Male
Female
C
1 Week
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
Sex

Figure Captions

Figure 1.

Title: Sickness behaviour symptoms

Caption: Mean (±SEM) sickness score 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 = 5/group. The asterisks (*) denotes a significant difference between saline- and LPS-treated 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 ($p < 0.05$).

Figure 2.

Title: Percent body weight change

Caption: Mean (±SEM) percent body weight change in 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 (*) denotes a significant difference between saline- and LPS-treated counterparts ($p < 0.05$), and (b) denotes a significant difference between water and antimicrobial treatments ($p < 0.05$).

Figure 3.

Title: Body temperature, heart rate, and gross motor activity changes

Caption: Mean (±SEM) of (A) male body temperature, (B) female body temperature, (C) male heart rate, (D) female heart rate, (E) male gross motor activity, and (F) female gross motor activity of mice treated with either saline (SAL) or lipopolysaccharide (LPS), and either water

(CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS), n = 5/group. The asterisks (*) denotes a significant difference between saline- and LPS-treated 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 ($p < 0.05$).

Figure 4.

Title: ^{14}C-sucrose brain/serum (μL/g) ratios in the cerebellum, CP, HIPP, PFC, and whole brain 24 hours post-LPS injection

Caption: Mean (±SEM) ^{14}C-sucrose brain/serum (μL/g) ratios in the (A) Cerebellum, (B) Caudate-Putamen, (C) Hippocampus, (D) prefrontal cortex, and (E) whole-brain 24 hours post-LPS injection of mice treated with either saline (SAL) or lipopolysaccharide (LPS), and either water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS), n = 5/group. The asterisks (*) denotes a significant difference between saline- and LPS-treated 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 ($p < 0.05$).

Figure 5.

Title: ^{14}C-sucrose brain/serum (μL/g) ratios in the cerebellum, CP, HIPP, PFC, and whole brain 72 hours post-LPS injection

Caption: Mean (±SEM) ^{14}C-sucrose brain/serum (μL/g) ratios in the (A) Cerebellum, (B) Caudate-Putamen, (C) Hippocampus, (D) prefrontal cortex, and (E) whole-brain 72 hours post-LPS/saline injection of mice treated with either saline (SAL) or lipopolysaccharide (LPS), and either water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS), n = 5/group.

The asterisks (*) denotes a significant difference between saline- and LPS-treated 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 ($p < 0.05$).

Figure 6.

Title: ^{14}C-sucrose brain/serum (μL/g) ratios in the cerebellum, CP, HIPP, PFC, and whole brain one week post-LPS injection

Caption: Mean (±SEM) ^{14}C-sucrose brain/serum (μL/g) ratios in the (A) Cerebellum, (B) Caudate-Putamen, (C) Hippocampus, (D) prefrontal cortex, and (E) whole-brain one week post-LPS/saline injection of mice treated with either saline (SAL) or lipopolysaccharide (LPS), and either water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS), n = 5/group. The asterisks (*) denotes a significant difference between saline- and LPS-treated 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 ($p < 0.05$).

Figure 7.

Title: Ileal claudin-3 concentration 24 hours, 72 hours, and one week post-LPS injection

Mean (±SEM) ileal claudin-3 concentrations (A) 24 hours, (B) 72 hours, and (C) one week post-LPS/saline injection of 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.

General Discussion

Neurodegenerative disorders are one of the leading causes of morbidity and mortality characterized by impairments related to cognition (i.e., memory, executive functions), motor skills (i.e., gait, resting tremor, rigidity, ataxia), emotions (i.e., anxiety, dysphoric and euphoric mood), and behaviour (i.e., disinhibition, apathy) (Erkkinen et al., 2018; Esposito & Ismail, 2022; Haack et al., 2016; Levenson et al., 2014; Nuber et al., 2008; Wirth et al., 2013). Exposure to certain stressors, like the bacterial endotoxin lipopolysaccharide (LPS), during puberty, in mice, can have sex-dependent effects on the development of various fundamental systems such as the hypothalamic-pituitary-adrenal axis, hypothalamic-pituitary-gonadal axis, central nervous system (CNS), and immune system, potentially increasing susceptibility to neurodegenerative disorders later in life (Esposito, Gandelman, et al., 2022; Holder & Blaustein, 2014; Murack et al., 2021; Murray et al., 2019; Smith, Murray, Chandrasegaram, et al., 2021; Smith, Murray, Gregory, et al., 2021). One promising explanation for the enduring effects of pubertal immune challenge may lie in how alterations to the composition of the gut microbiome influence brain and behavioural functioning.

The overarching goal of this doctoral thesis was to test the hypothesis that pubertal LPS and antimicrobial (AMNS) treatments have sex-dependent effects on cellular mechanisms and behaviours related to neurodegeneration in male and female CD1 mice. Furthermore, this thesis aimed to gain greater insight into the underlying mechanisms involved in the enduring effects of pubertal LPS and AMNS treatments on brain functioning and behaviours. This thesis first examined whether pubertal LPS and AMNS treatments acutely influences the cellular mechanisms related to neurodegeneration (see Study 1; (Esposito, Gandelman, et al., 2022). Secondly, this thesis examined whether pubertal LPS and AMNS treatments have enduring

effects on cellular mechanisms and behaviours associated with neurodegeneration (see Study 2). Lastly, this thesis examined how pubertal LPS and AMNS treatments influenced gross motor activity, core body temperature, and heart rate, along with examining the effects of these treatments on intestinal and blood-brain barrier (BBB) permeability.

Pubertal LPS and AMNS treatments influence neurodegeneration-related acute cellular mechanisms in a sex-dependent manner

Exposure to an immune challenge during puberty in mice has programming effects on the hypothalamic-pituitary-adrenal axis, influences immune responsivity, suppresses the hypothalamic-pituitary-gonadal axis, and increases anxiety and depression-like behaviours in a sex-dependent manner (Esposito, Kearns, et al., 2022; Murray et al., 2019; Smith, Murray, Chandrasegaram, et al., 2021; Smith, Murray, Gregory, et al., 2021). However, whether exposure to LPS and AMNS treatments during puberty influences acute cellular mechanisms related to neurodegeneration remains unknown.

The current thesis confirms that exposure to LPS and AMNS treatments during puberty has sex-dependent effects on acute cellular mechanisms related to neurodegeneration (see Study 1; (Esposito, Gandelman, et al., 2022). Specifically, LPS-treated mice demonstrated increased SNCA and leucine-rich repeat kinase 2 (LRRK2) expression in the caudate-putamen (CP). Both SNCA and LRRK2 regulate neuroinflammation and have been implicated in the pathogenesis of several neurodegeneration disorders observed in humans (i.e., Alzheimer's disease; AD, and Parkinson's disease; PD, dementia with Lewy bodies, and multiple system atrophy) (Meade et al., 2019; Santpere & Ferrer, 2009; Siddiqui et al., 2016). Increased expression of SNCA and LRRK2 following pubertal LPS treatment observed in this work suggests that these

neurodegeneration-related markers may mediate an LPS-induced enduring inflammatory response, potentially increasing susceptibility to neurodegeneration later in life.

Interestingly, this work showed that female mice demonstrated a more adaptive acute response to pubertal LPS and AMNS treatments than male mice (see Study 1; (Esposito, Gandelman, et al., 2022). Specifically, female mice demonstrated greater concentrations of peripheral anti-inflammatory cytokines (i.e., IL10) and lower concentrations of pro-inflammatory cytokines (i.e., IL12 p (70), Il17A, IL23) than male mice following pubertal LPS and AMNS treatments. Furthermore, LPS-treated female mice displayed increased concentrations of ileal occludin expression while LPS-treated male mice displayed decreased expression of C3 in the substantia nigra (SN). Increased ileal occludin expression in our female mice could be a protective response to the LPS treatment where intestinal permeability is decreased, resulting in decreased microbial translocation and a reduced inflammatory response (Fukui, 2016). Moreover, decreased C3 expression in our male mice may indicate deficits in synaptic pruning and a decreased ability to remove invading pathogens (Fagan et al., 2017; Magdalon et al., 2020; Maier et al., 2008). The sex differences observed in this work are likely due to the protective effects of estradiol and the inhibitory effects of testosterone on immune responsivity (Taneja, 2018). Overall, these findings demonstrate that male mice have a more detrimental acute response to pubertal LPS and AMNS treatments, indicating that they may be more susceptible to developing neurodegenerative disorders later in life.

Pubertal LPS and AMNS treatments cause enduring sex-dependent neural alterations

The current thesis demonstrates that exposure to LPS and AMNS treatments during puberty results in enduring sex-dependent neural alterations related to neurodegeneration (see Study 2). AMNS-treated adult male mice showed decreased sigma-1 receptor (S1R) expression

in the cornu ammonis 1 (CA1), and dentate gyrus (DG) while LPS and AMNS treatments had similar effects in adult female mice. Both male and female AMNS-treated mice showed decreased S1R expression in the secondary motor cortex (M2) in adulthood. Moreover, in the DG, LPS-treated male and female mice showed decreased glial-derived neurotrophic factor receptor alpha 1 (GFRA1) in adulthood. In contrast, LPS or AMNS-treated adult male mice showed decreased GFRA1 expression in the primary motor cortex (M1) compared to their female counterparts. Decreased S1R expression in the CA1 and CA2 is associated with the loss of pyramidal neurons, a phenomenon that is often observed in human patients suffering from AD (Jansen et al., 1993). Furthermore, both GFRA1 and S1R have neuroprotective effects on motor neurons and play an essential role in the formation of new neurons in the DG, in both humans and animals (Bonafina et al., 2019; Herrando-Grabulosa et al., 2021; Piatti et al., 2013; Ryskamp et al., 2019; Suzuki et al., 2007). As such, alterations in S1R and GFRA1 expression following pubertal LPS and AMNS treatments suggest that these treatments could have enduring effects on neurodegenerative processes by causing hippocampal and motor neuron degeneration along with hindering the formation of new neurons in the DG. Moreover, our male mice seem to be more susceptible to motor neuron degeneration following pubertal LPS and AMNS treatments, a finding that is in line with human males being more susceptible to developing motor neuron diseases than human females (Alonso et al., 2009; Logroscino et al., 2018).

The enduring neural alterations following pubertal LPS and AMNS treatments may arise in part from the effects of these treatments on acute cellular mechanisms associated with neurodegeneration (Esposito, Gandelman, et al., 2022). Exposure to stressors during puberty in mice can increase the production of cytokines and C-reactive proteins, resulting in alterations in brain functioning later in life (Bilbo & Schwarz, 2009; Danese et al., 2009). As such, it is

plausible that the increased production of peripheral cytokines along with the altered expression of neuroinflammatory mediators (i.e., C3, SNCA, LRRK2) following pubertal LPS and AMNS treatments (Esposito, Gandelman, et al., 2022) had long-term consequences on brain function, resulting in the enduring neural alterations observed in this thesis (see Study 2).

Pubertal LPS and AMNS treatments cause enduring behavioral alterations, in a sex-dependent manner

In addition to demonstrating enduring sex-dependent neural alterations, this thesis showed that pubertal LPS and AMNS treatments result in sex-dependent, enduring behavioural alterations related to neurodegeneration (see Study 2). Specifically, LPS and AMNS-treated female mice displayed deficits in neuromuscular strength while LPS-treated male mice displayed increased anxiety-like behaviours in adulthood. The sex-dependent effects of LPS and AMNS treatments on anxiety-like behaviours and neuromuscular strength are likely mediated by the effects of these treatments on circulating gonadal hormones. Estradiol enhances neuromuscular strength while testosterone has anxiolytic effects, effects that are observed in both humans and animals (Chidi-Ogbolu & Baar, 2019; Tong et al., 2019; Willoughby et al., 2024). Furthermore, LPS and AMNS treatments in mice decrease the expression of estradiol and testosterone (Guo et al., 2022; Ismail et al., 2011; Shen et al., 2022). As such, it is possible that LPS and AMNS treatments had enduring effects on the synthesis of estradiol and testosterone, resulting in neuromuscular deficits in adult female mice and increased anxiety-like behaviours in adult male mice.

The long-lasting effects of LPS and AMNS treatments on circulating gonadal hormones and neurodegeneration-related behaviours could be explained by the effects of these treatments on acute cellular mechanisms related to neurodegeneration (Esposito, Gandelman, et al., 2022).

Gonadal hormones are known to regulate cytokine production in both humans and animals, however, cytokines also play a vital role in the regulation of gonadal hormone synthesis through their effects on the hypothalamic-pituitary-gonadal axis (Barabás et al., 2020; Bouman et al., 2005; Foster et al., 2003). Furthermore, considering the role that C3, SNCA and LRRK2 play in immune function, it is possible that both these markers influence the synthesis of estrogens and testosterone (Tansey et al., 2022). As such, LPS and AMNS-induced alterations in cytokine, C3, SNCA, and LRRK2 production during puberty could have long-lasting effects on the synthesis of gonadal hormones which could have contributed to the enduring sex-dependent behavioural alterations observed in this thesis.

Sex-dependent BBB and physiological changes following pubertal LPS and AMNS treatment could contribute to enduring consequences.

Increased BBB permeability during puberty can allow peripheral immune cells and pathogens to enter the brain and cause long-lasting changes to brain functioning and neurodevelopment (Cao et al., 2021; Malave et al., 2022). Similarly, physiological responses (i.e., heart rate, body temperature) to an immune challenge (i.e., LPS) can dictate how effectively pathogens are handled and how much damage the CNS undergoes (Evans et al., 2015; Williams et al., 2019). This work showed that pubertal LPS and AMNS treatments result in increased BBB permeability in the prefrontal cortex (PFC), caudate-putamen (CP), hippocampus (HIPP), and cerebellum, 24 hours post-LPS/saline injection, in both male and female CD1 mice (see Study 3). However, only AMNS-treated female mice displayed increased BBB permeability in the CP and HIPP 72 hours post-LPS/saline injection and global increases in BBB permeability one week post-LPS/saline injection. LPS-treated male mice displayed global increases in BBB permeability one week post-LPS/saline injection (see Study 3).

Increased regional BBB permeability 24 hours post-LPS/saline injection can be attributed to the effects of LPS and AMNS treatments on the composition of the gut microbiome. LPS and AMNS treatments may have induced alterations to the composition of the gut microbiome which could have potentiated the immune response induced by LPS, resulting in increased BBB permeability (Galea, 2021; Livingston et al., 2023; Yoo et al., 2020). Regional and global increases in AMNS-treated female mice 72 hours and one week post-LPS/saline injection can be attributed to the sex-dependent effects of AMNS treatment on the gut microbiome. AMNS treatment selectively influences microbial composition (i.e., *Firmicutes)* and microbial metabolites (i.e., branched-chain amino acids, short-chain fatty acids, and aromatic amino acids) in female mice which likely contributed to the alterations in BBB function observed in our female mice (Gao et al., 2019). Furthermore, the BBB is also heavily influenced by inflammatory markers (i.e., IL6, TNFa, IL1B) (Galea, 2021). As such, global increases in BBB permeability in LPS-treated male mice one week post-LPS/saline injection may be due to the more pronounced immune response observed in pubertal male mice following LPS treatment (Esposito, Gandelman, et al., 2022).

This work also showed that pubertal LPS and AMNS treatments induced significant drops in body temperature, an effect that was more pronounced in male mice than in female mice (see Study 3). Furthermore, both male and female LPS-treated mice displayed an initial increase in heart rate followed by a sustained decrease in heart rate (see Study 3). Alterations in body temperature and heart rate following pubertal LPS and AMNS treatments are likely due to increased peripheral cytokine levels (Esposito, Gandelman, et al., 2022). Furthermore, the sex-dependent effects of LPS treatment on body temperature can be attributed to the more

pronounced immune response that is observed in pubertal male mice following pubertal LPS treatment (Esposito, Gandelman, et al., 2022).

Alterations in BBB function and the physiological response to pubertal LPS and AMNS treatments may also mediate the enduring effects of these treatments on brain functioning and behaviour (see Study 2). Increased BBB permeability may have caused the infiltration of peripheral cytokines (i.e., IL17A, IL23, IL12 (p70), IL10) into the CNS, causing alterations in biomarkers associated with neurodegeneration (i.e., C3, SNCA, LRRK2), and increasing microglial activation (Esposito, Gandelman, et al., 2022; Patel & Frey, 2015). These cellular processes may have caused brain tissue damage, resulting in the enduring neural and behavioural alterations observed in this thesis (see Study 2). Furthermore, the sustained decrease in heart rate following pubertal LPS treatment suggests that this treatment could impair cardiovascular function, possibly mediating the enduring effects observed in this work (see Studies 2 and 3). LPS treatment in animals can damage cardiac cells, resulting in reduced contractibility and functioning of cardiac cells (Firoz et al., 2015; K. Y. Liu et al., 2022; Moore et al., 2021). Ultimately, reduced functioning of cardiac cells can increase susceptibility to cardiovascular disorders and have enduring effects on cellular mechanisms and behaviours associated with neurodegeneration (Clarke et al., 2014; Dinan & Cryan, 2017; Kennedy et al., 2018).

Limitations and future directions

The current studies are not without their limitations. Firstly, LPS and AMNS treatments have effects on multiple systems such as brain function, immune function, metabolic function, and sexual development (Esposito, Gandelman, et al., 2022). As such, it is difficult to determine the precise mechanisms involved in the effects observed in these studies. Future research should consider analyzing the effects of LPS and AMNS treatments across various systems to gain a

more holistic picture of how these treatments influence brain functioning and behaviour. Secondly, these studies focused on how LPS and AMNS treatments during puberty can contribute to the development of neurodegenerative disorders later in life. However, various genetic, environmental, and lifestyle factors influence the development of neurodegenerative disorders which could have also played a role in the effects observed in these studies. Future research should analyze the effects of these different factors to better understand how exposure to stressors during puberty can increase susceptibility to neurodegeneration later in life. For example, it would be interesting to examine the effects of pubertal LPS and AMNS treatments on the epigenome which may be another mechanism involved in the development of neurodegenerative disorders. Lastly, the microbial composition of our mice was not analyzed in these studies. As such, we cannot be certain that our treatment model induced acute or enduring changes in the composition of the gut microbiome. Future research utilizing LPS and AMNS treatments should consider analyzing microbial composition to determine whether microbial dysbiosis plays a role in the effects observed in these studies.

Implications

Despite the limitations mentioned above, the studies included in this thesis have significant implications for our understanding of neurodegenerative disorders and the role of gut microbiome alterations, specifically during puberty, in influencing susceptibility to these disorders later in life. This dissertation highlights the critical role of the gut-brain axis in neurodegeneration, suggesting that alterations in the gut microbiome during puberty may significantly impact the development of neurodegenerative diseases later in life. This is a crucial insight, as it points to a potential period (puberty) where interventions could be particularly effective in preventing or mitigating neurodegenerative conditions. This research also

underscores significant sex-dependent differences in the response to LPS and AMNS treatments, with male and female mice showing distinct inflammatory responses, intestinal permeability, and neurodegenerative markers. The findings also suggest that male and female brains might have different susceptibilities and resilience mechanisms to inflammatory insults, which could inform sex-specific therapeutic strategies. Lastly, given that puberty appears to be a critical period for the impact of gut microbiome alterations on future neurodegeneration, early interventions (i.e., probiotics, diet modifications, anti-inflammatory treatments) during this period could potentially reduce the risk of developing neurodegenerative disorders later in life.

Summary

The current studies have furthered our understanding of how pubertal exposure to LPS and AMNS treatments influence neurodegeneration-related cellular mechanisms and behaviours in mice. This dissertation has shown that LPS and AMNS treatments during puberty result in sex-dependent changes in immune responsivity, intestinal permeability and neurodegeneration-related markers such as C3, SNCA and LRRK2, with male mice showing a more detrimental response than female mice (Esposito, Gandelman, et al., 2022). We also demonstrated that these treatments cause long-lasting changes in neural structures and functions, such as altering the expression of S1R and GFRA1 with male mice seeming particularly susceptible to the development of motor neuron degeneration (see Study 2). Furthermore, pubertal LPS and AMNS treatments lead to enduring sex-dependent behavioural alterations, including neuromuscular deficits in female mice and increased anxiety-like behaviours in male mice (see Study 2). Lastly, the findings from this thesis showed that pubertal LPS and AMNS treatments result in increased BBB permeability and alterations in body temperature and heart rate, in a sex-dependent manner. These results suggest that disruptions in BBB function and the physiological response to LPS

could mediate the enduring effects of pubertal LPS and AMNS treatments on brain functioning and behaviours (see Study 3). This thesis emphasizes the role of hormonal differences and the gut-brain axis in mediating these effects, suggesting the need for sex-specific therapeutic strategies and early interventions to mitigate the risk of neurodegenerative disorders. We must continue to examine how exposure to stressors during puberty can have long-term negative consequences and develop strategies to potentially protect the brain and prevent the onset of neurodegeneration later in life.

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