THE EFFECTS OF SYNTHETIC SEX HORMONES ON COGNITIVE ABILITIES, AFFECTIVE STATE, THE GUT-BRAIN AXIS, AND ACUTE IMMUNE AND STRESS REACTIVITY DURING PUBERTY AND ADULTHOOD

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Abbreviations

COCs	Combined oral contraceptives
EE	Ethinyl Estradiol
P4	Progesterone
GnRH	Gonadotropin-releasing hormones
HPG	Hypothalamic-pituitary-gonadal axis
ER-a	Estrogen receptor alpha
ER-b	Estrogen receptor beta
PRα	Progesterone receptor alpha
PRβ	Progesterone receptor beta
AR	Androgen receptor
LH	Luteinizing hormone
FSH	Follicle-stimulating hormone
HPA	Hypothalamic-pituitary-adrenal axis
PVN	Paraventricular nucleus of the hypothalamus
CRH	Corticotropin-releasing hormone
ACTH	Adrenocorticotropic hormone
GCs	Glucocorticoids
CORT	Corticosterone
MRs	Mineralocorticoid receptors
GRs	Glucocorticoid receptors
TNFα	Tumor Necrosis Factor Alpha

IL-1	Interleukin-1
IL-6	Interleukin-6
NK	Natural killer
LPS	Lipopolysaccharides
IL-10	Interleukin-10
IL-12	Interleukin-12
IFNγ	Interferon Gamma (γ)
GABA	Gamma(γ)-aminobutyric acid
OC	Oral contraceptives
NC	Naturally cycling
DHT	Dihydrotestosterone
EE/LNG	Ethinyl estradiol/Levonorgestrel
EE/DRSP	Ethinyl estradiol/Drospirenone
BDNF	Brain-derived neurotrophic factor
PSD-95	Post-synaptic density protein 95
HIPP	Hippocampus
mPFC	Medial prefrontal cortex
DMSO	Dimethyl sulfoxide
PFA	Paraformaldehyde
E2	17β-estradiol
ELISA	Enzyme-linked immunosorbent assay (ELISA)
rRNA	Ribosomal ribonucleic acid
DNA	Deoxyribonucleic acid

IHC	Immunohistochemistry
TBS	Tris-buffered saline
PBS	Phosphate-buffer saline
ABC	Avidin-biotin complex
DAB	Diaminobenzidine
DA	Dopamine
NAc	Nucleus accumbens
VTA	Ventral tegmental area
NMDA	N-methyl-d-aspartate
TPH	Tryptophan hydroxylase
SCFAs	Short-chain fatty acids
FST	Forced swim test
EPM	Elevated plus maze
TST	Tail suspension test
OFT	Open field test
5HT-1A	Serotonin 1A receptor
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
mRNA	Messenger ribonucleic acid
IL-1β	Interleukin-1 beta
IL-12p70	Interleukin-12
HYP	Hypothalamus
PFC	Prefrontal cortex
NF-κB	Nuclear factor kappa B

Abstract

Combined oral contraceptives (COCs) are one of the most widely used forms of hormonal contraception by both adolescent and adult human females. While they are very effective at preventing pregnancy, they may also have effects on the central nervous system, cognition, and behaviour. However, there are a lot of inconsistencies reported on these effects. One possible explanation for these discrepancies could be that the effects of COCs on the brain are age-dependent and may be more pronounced during puberty and adolescence. The mechanisms underlying the effects of COCs on the brain and behaviour also remain unclear. The current thesis was designed to address these knowledge gaps and to investigate possible mechanisms through which COCs could impact the brain, cognitive abilities, and gut microbiome composition in an age-dependent manner in a mouse model (Article 1). Subsequently, we examined the effects of pubertal COC exposure on mental health outcomes such as depression-like and anxiety-like behaviors (Article 2). Lastly, we investigated whether COCs alter immune and stress responses in pubertal and adult CD-1 mice (Article 3). Together, the studies included in this dissertation furthers our understanding of the impact of combined oral contraceptives use during puberty and adulthood on women's health including the brain, behaviour, gut microbiome as well as stress and immune systems.

Résumé

Les contraceptifs oraux combinés (COCs) représentent l'une des méthodes de contraception hormonale les plus fréquemment utilisées par les adolescentes et les femmes adultes. Bien qu'ils soient hautement efficaces pour prévenir la grossesse, ils peuvent également induire des effets sur le système nerveux central, la cognition et le comportement. Cependant, il demeure une grande variabilité et plusieurs contradictions dans les résultats rapportés dans la littérature. Une avenue possible qui permettrait d'expliquer ces différences serait que les effets des COCs sur le cerveau varient en fonction de l'âge et pourraient être plus marqués durant la puberté et l'adolescence. Par ailleurs, les mécanismes qui sous-tendent ces effets des COCs particulièrement durant la puberté et l'adolescence demeurent incompris. Ainsi, la présente thèse a pour objectif, en utilisant un modèle de souris, d'explorer les mécanismes par lesquels les COCs pourraient affecter, selon l'âge, le cerveau, les fonctions cognitives et la composition du microbiote intestinal (Article 1). Ensuite, nous avons examiné les effets de l'exposition aux COCs durant la puberté sur les comportements de type dépressif ou anxieux chez la souris (Article 2). Enfin, nous avons investigué si les COCs modifient les réponses immunitaires et au stress chez les souris CD-1 pubères et adultes (Article 3). En somme, les études incluses dans cette thèse examinent l'impact de l'utilisation des COCs pendant la puberté et l'âge adulte sur la santé des femmes, notamment sur le cerveau, le comportement, le microbiote intestinal, ainsi que sur les systèmes de stress et immunitaire.

CHAPTER 1: THEORETICAL BACKGROUND

This chapter is adapted from:

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Combined oral contraceptives (COCs) were first introduced in the 1960s and are now used by over 150 million females worldwide (Christin-Maitre, 2013). COCs commonly contain a combination of synthetic hormones such as ethinyl estradiol (EE), a synthetic form of estradiol, and different types of synthetic progesterone (P4) (e.g., cyproterone acetate, levonorgestrel, drospirenone, desogestrel, norgestimate) (Kaplan, 1995; Raudrant & Rabe, 2003; Sitruk-Ware, 2006). Regardless of their formula, COCs effectively prevent pregnancy by suppressing the production and release of gonadotropin-releasing hormone (GnRH), inhibiting ovulation (Cooper & Mahdy, 2021), and thickening the cervical mucus, making it challenging for the spermatozoids to reach the fallopian tubes (Baird & Glasier, 1993).

These synthetic hormones bind to ovarian steroid hormone receptors in the brain, downregulate GnRH production and inhibit the hypothalamic-pituitary-gonadal (HPG) axis. More specifically, ethinyl estradiol binds to estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β) with twice the binding affinity of endogenous estradiol for ER-α (Blair et al., 2000; Oettel & Schillinger, 2012). These receptors are widely present in brain regions involved in cognitive processes (Shughrue et al., 1997), including the hippocampus and the prefrontal cortex (Almey et al., 2015). Synthetic progestins (e.g., cyproterone acetate, levonorgestrel, drospirenone, norgestimate) bind to progestin receptor-α (PRα) and progestin receptor-β (PRβ) (Schindler et al., 2008), which are also present in numerous brain regions involved in cognitive processing, including the hippocampus, frontal cortex and amygdala (Giatti et al., 2016; González-Orozco & Camacho-Arroyo, 2019; Wagner, 2008). Many synthetic progestins, like levonorgestrel, norgestrel, norethindrone, desogestrel, are derived from testosterone, have affinity to androgen receptors (AR) (i.e., androgenic activity) (Rapkin & Winer, 2007) and are located in several brain regions, including the nucleus accumbens, amygdala, preoptic area as

well as the hypothalamus (Davey & Grossmann, 2016). Variations in the pharmacological profile of progestins, particularly their interactions with steroid brain receptors in the brain are suspected to be key mediators of COCs effects on cognition and behaviour (Pletzer et al., 2023). Given that the synthetic sex hormones present in COCs also bind to these same receptors that are expressed in brain regions involved in cognitive processing, exposure to COCs could induce structural changes in these regions and alter various aspects of cognitive function, like executive function, memory consolidation, and emotion regulation (McEwen & Milner, 2017); (Berenbaum & Beltz, 2011).

COCs have changed over time and now contain lower concentrations of synthetic hormones to reduce the risk of cardiovascular disease, such as venous thromboembolism (Cerel-Suhl & Yeager, 1999; De Leo et al., 2016; Golobof & Kiley, 2016). While COCs are most commonly used to prevent pregnancy, they are also often prescribed for off-label regulatory purposes to treat premenstrual symptoms, androgen-related alopecia, hormonal acne, heavy menstrual bleeding, and dysmenorrhea (Bernardi et al., 2017; Davis & Westhoff, 2001; Haamid et al., 2017; Løkkegaard & Nielsen, 2014; Raudrant & Rabe, 2003; Trivedi et al., 2017; Wichianpitaya & Taneepanichskul, 2013), especially during adolescence.

1.0 Puberty : A sensitive period of development

Adolescence is defined as the transitional life stage that occurs between childhood and adulthood, beginning with the onset of puberty and involves physical maturation, as well as psychological, emotional, and social development (National Academies of Sciences et al., 2019). Puberty is a critical period of development marked by physiological changes during which the HPG axis becomes active and undergoes maturation, causing an increase in the production and release of gonadal steroid hormones, and leading to the development of secondary sexual

characteristics and sexual maturation (Limesand & Davis, 2018). The timing and onset of puberty is modulated by the neuropeptide kisspeptin which is encoded by the *kiss1* gene and regulates reproductive function. Kisspeptin binds to the G protein-coupled receptor (GPR54) and stimulates GnRH neurons in the hypothalamus to release GnRH (Padda et al., 2021; Xie et al., 2022). The pulsatile release of GnRH from the hypothalamus stimulates the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary into the bloodstream. LH and FSH then bind to receptors located on the gonads (i.e., ovaries and testes), which stimulates the release of sex steroid hormones (i.e., estrogens, progesterone, testosterone) (Dreher, 2015). In females, FSH promotes the maturation of ovarian follicles, whereas LH supports ovulation and the formation of the corpus luteum. In males, FSH promotes spermatogenesis, while testosterone production by Leydig cells is stimulated by LH (Dwyer, 1974; Gore, 2013). During this period, the brain also undergoes extensive remodeling and reorganizing leading to structural and functional changes that are driven by puberty and fluctuations in endogenous sex hormones (Schulz & Sisk, 2016; Vigil et al., 2016). Whether exposure to synthetic sex hormones during puberty interferes with brain development and maturation has yet to be investigated thoroughly.

1.1 Maturation of the hypothalamic-pituitary-adrenal axis

During puberty, the hypothalamic-pituitary-adrenal (HPA) axis also undergoes maturation which increases vulnerability to environmental factors and stress (Holder & Blaustein, 2014). The HPA axis is an essential neuroendocrine system responsible for inducing an adaptive response when facing a psychological or physiological stressor (Sapolsky et al., 2000). It regulates homeostasis as well as circadian rhythms, gut function, immune function, metabolism, and mood (Chrousos, 2009; Tsigos et al., 2000). Following detection of a stressor,

neurons in the paraventricular nucleus of the hypothalamus (PVN) release corticotropin-releasing hormone (CRH), which stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary, resulting in the production of glucocorticoids (GCs) (i.e., cortisol in humans or corticosterone (CORT) in rodents) from the adrenal glands (Howland et al., 2017; R. D. Romeo, 2010). Cortisol binds to mineralocorticoid receptors (MRs) to which they have high-affinity, as well as glucocorticoid receptors (GRs) to which they have lower affinity (Deuter et al., 2024; Howland et al., 2017; Russell D. Romeo, 2010; Smith & Vale, 2006). Both MRs and GRs are expressed in the hypothalamus, hippocampus, and pituitary gland, and are essential to the negative feedback regulation of the HPA axis. Particularly, at low basal cortisol levels, MRs are predominantly activated, whereas rising cortisol levels activate GRs, which in turn inhibit HPA axis activity by reducing GCs production from the adrenal glands (Stephens & Wand, 2012). Dysregulation of the HPA axis is associated with multiple conditions including depression, post-traumatic-stress-disorder, chronic fatigue syndrome, and metabolic syndrome (Lawrence & Scofield, 2024; Lei et al., 2025; Ring, 2025; Tsigos et al., 2000; Zhang & Wang, 2024). In addition, long term stress exposure can have damaging effects on the central nervous, reproductive, and immune systems (McEwen, 2007).

While all stressors activate the HPA axis, there are age and sex differences in HPA axis activity and in the response to stress. Adolescents display increased ACTH and cortisol levels when facing a stressor compared to both children and adults (Russell D. Romeo, 2010; Romeo, 2013). Both prepubertal male and female rats show higher ACTH and corticosterone in the pituitary and adrenal glands, respectively, compared to adults (Kann & Romeo, 2022). Similarly, prepubertal male and female rats display higher and longer ACTH and corticosterone responses

following a stressor compared to adult males and females, despite similar basal levels across developmental phases (Minhas et al., 2016).

Sex differences in HPA axis function also emerge during puberty as a result of HPG axis activation. For example, pubertal female rats displayed adult like corticosterone levels by P45 whereas pubertal males display increased corticosterone levels compared to adults following an acute stressor (Burke et al., 2026). These sex differences in the developmental trajectory of the HPA axis could lead to sex-specific vulnerability to stress-induced illnesses (Burke et al., 2026). In male rats, the shift from prepubertal (P30) to post-pubertal (P60) age was associated with increased CRH mRNA expression in the amygdala as well as a reduced c-Fos protein expression in the PVN following a stressor, whereas females showed an age-related increase in CRH mRNA expression in the PVN (Viau et al., 2005). These differences might be partially driven by the fluctuations in sex steroid hormones. Estradiol and ERα agonists have been associated with increased CORT and ACTH levels (Heck & Handa, 2019b; Weiser & Handa, 2009). Ovariectomized female rats also show reductions in ACTH and CORT concentrations compared to their gonadally intact counterparts – an effect that is reversed following estradiol treatment (Seale, Wood, Atkinson, Bate, et al., 2004; Seale, Wood, Atkinson, Harbuz, et al., 2004). Moreover, testosterone and progesterone have been associated with dampened HPA axis activity (Stephens et al., 2016). Gonadectomized male rats show heightened levels of ACTH and CORT which were then reduced after testosterone administration (Handa et al., 2013; Heck & Handa, 2019b; Viau et al., 2003). Interestingly, in post-pubertal adolescent rats (P45), testosterone treatment was associated with increased CORT following an acute stressor (Green et al., 2019). This increase in concentration could be explained by developmental differences in testosterone metabolism. During puberty, there is increased aromatization of testosterone into estradiol,

whereas in adult males 5α-reductase converts more testosterone in DHT (Green et al., 2019; Lund et al., 2006). Taken altogether, these findings suggest that sex steroid hormones are central in shaping sex differences in HPA axis function. Nevertheless, genetic differences are also essential in shaping the interaction between puberty, sex, and HPA function. Prepubertal C57BL/6 and BALB/c inbred male mice showed increased and longer CORT concentrations following a stressor compared to adult males whereas Swiss Webster outbred mice showed the opposite pattern with adult females displaying increased CORT levels compared to pubertal females (Romeo et al., 2013).

1.2 Maturation of the immune system

The immune system is a network comprised of proteins, cells and organs that also undergoes drastic changes and maturation during puberty (Holder & Blaustein, 2014). Main functions of immune system include recognition and defense against pathogens, toxins or disease (Marshall et al., 2018; Parkin & Cohen, 2001). It is categorized in two specific subtypes which include the innate and the adaptive immune system. The innate immune system serves as the primary, immediate, and non-specific defense of the body against microorganisms and relies on multiple elements and processes (Sabir & Jan, 2026). This defense system includes physical and anatomical barriers, antimicrobial enzymes (e.g., lysozyme) and proteins, phagocytosis, inflammation, and pattern-recognition receptors (e.g., Toll-like receptors) that detect pathogens and initiate an immune response (Marshall et al., 2018). When activated, the innate immune system relies on immune cells that release cytokines (e.g., Tumor Necrosis Factor Alpha (TNFα), Interleukin-1(IL-1), Interleukin-6 (IL-6) and mobilizes effector cells (i.e., neutrophils, macrophages, dendritic cells, mast cells, and natural killer (NK) cells) to eliminate pathogens, promote inflammation, and clear cellular debris (Marshall et al., 2018; Thompson, 2015). The

adaptive immune system is the second component of the immune system and provides a more specific and slower immune response. It is characterized by antigen specificity and recognition as well as immunological memory which provides protection upon re-exposure (Ahuja, 2008). Adaptive immunity is acquired throughout life and is mediated by B lymphocytes, which produce antibodies as part of humoral immunity, and T lymphocytes, which support cell-mediated immunity by regulating immune responses and destroying infectious cells (Marshall et al., 2024; Sabir & Jan, 2026).

During puberty, the rising levels of sex hormones directly bind to specific receptors expressed on immune cells resulting in alterations of immune function (Hoffmann et al., 2023). Pubertal development is associated with maturation of the immune response, including increased T helper 1 (Th1), Th17 and T follicular helper activity, and increased production of IgG, but also sex hormones effects on cytokine production which may increase the risk for autoimmune disorders in a sex specific manner (Kane & Ismail, 2017; Ucciferri & Dunn, 2022; Yang et al., 2023).

Lipopolysaccharide (LPS) is a bacterial toxin part of the outer cell wall of gram negative bacteria (e.g., *E.coli*, *Pseudomonas*) (Bertani & Ruiz, 2018; Farhana & Khan, 2026) and that is widely used to model immune activation. LPS binds to Toll-like receptor 4 (TLR4) complex on immune cells (e.g., dendritic cells , macrophages, B cells) which stimulates the secretion of proinflammatory cytokines, and nitric oxide (Farhana & Khan, 2026). LPS-induced immune activation has also been associated with increased risk for mood and neurological disorders (Kalyan et al., 2022; Murray et al., 2019), which highlights the role of immune activation in health and disease. Immune activation following LPS treatment has also been shown to be age- and sex-dependent. For instance, pubertal mice treated with LPS displayed increased anti-

inflammatory cytokine levels than adults (Cai et al., 2016). In addition, pubertal males display faster increase and quicker recovery in cytokine levels (i.e., IL-1, IL-6) levels following an immune challenge, whereas adult females show a more sustained and diverse cytokine response (i.e., TNFα, IL-10, IL-12, IL-1, IFNγ, IL-6) (Sharma et al., 2018). Similarly, prepubertal male rats display faster recovery from LPS-induced CORT and IL-6 increase as well as activation of PVN neurons than their adult counterparts (Goble et al., 2011).

1.3 Puberty and vulnerability for mental illness

As of puberty, females are two times more likely to develop mental disorders, like depression and anxiety, compared to males (Farhane-Medina et al., 2022; Kuehner, 2017; Salk et al., 2017; Zender & Olshansky, 2009). Depression and anxiety are some of the most commonly experienced mental illnesses worldwide and have high co-occurrence, with approximately 50% comorbidity (Chen, 2022; Kalin, 2020; Kessler et al., 2015; ter Meulen et al., 2021). Despite both being classified as internalizing affective disorders, they have significant clinical differences (Kalin, 2020). Depression is characterized by symptoms that impair daily functioning including persistent sadness, loss of interest/pleasure, suicidal thoughts, as well as altered cognition, and sleep (Chand & Arif, 2026). In contrast, anxiety is characterized by feelings of excessive worry and/or fear, restlessness, and physical symptoms such as hyperventilation, dizziness, heart palpitations (Craske et al., 2017). Both illnesses often emerge during puberty and are more prevalent among adolescent girls (Hayward & Sanborn, 2002; Salk et al., 2017) which could be explained by fluctuations in ovarian hormones. Although it remains unclear whether there is a causal effect of COCs use during puberty on incidence of mental illness, it is however plausible that COCs might have disruptive effects on the HPG axis resulting in increased vulnerability for both disorders. Some evidence has shown that adolescent COCs users have a

higher rate of depression and anti-depressant use compared to non-users (de Wit et al., 2020; Skovlund et al., 2016). Recent findings show that pubertal COCs use may also predict future vulnerability to depression in adulthood (Anderl et al., 2021; Anderl et al., 2020). Using a propensity model and controlling for covariates that may impact the association between COCs use and depression (e.g., age at menarche and sexual debut, education, marital status, endometriosis diagnosis, previous use of hormonal therapy, previous pregnancy, smoking habits, current COCs use, sex of sexual partners), recent findings show that pubertal COCs use may increase the vulnerability to depression in adulthood (Anderl et al., 2021; Anderl et al., 2020). Collectively, these findings suggest that COC use during sensitive developmental periods could result in altered trajectory of brain development resulting in increased vulnerability to mood disorders. However, the mechanisms underlying these enduring consequences remain unclear and have yet to be investigated.

2.0 COCs and the brain: Evidence from animal research

Examining the effects of synthetic hormones on the brain in animal models provides important insights into the neurobiological mechanisms through which COCs may alter brain structure and function. For example, rodent studies have shown that treatment with synthetic hormones reduces plasma endogenous estradiol and progesterone concentrations, decreases the volumes of the hippocampus and the cerebral cortex, and increases the expression of type A gamma-aminobutyric acid receptors compared to untreated females (Follesa et al., 2002; Porcu et al., 2012). Gamma-aminobutyric acid (GABA) is a neurotransmitter that plays a role in cognitive functions as well as in psychiatric disorders (Möhler, 2009; Porcu et al., 2019). Moreover, treatment with ethinyl estradiol and levonorgestrel decreases brain-derived neurotrophic factor expression in the hippocampus (Simone et al., 2015), which has been associated with decreased

neuroplasticity and impaired cognitive function (Autry & Monteggia, 2012; Bekinschtein et al., 2014). Exposure to synthetic hormones also alters spatial memory and navigation strategies. For example, ovariectomized rats treated with high doses of ethinyl estradiol tend to use place memory strategy while ovariectomized rats treated with low doses of ethinyl estradiol tend to use response memory strategy in a T-Maze spatial task (Lacasse, Patel, et al., 2022). Interestingly, the bias towards place memory strategy in ovariectomized female rats treated with high doses of ethinyl estradiol is inverted when synthetic progesterone is injected a few hours (e.g., 1 – 4 hours) prior to behaviour testing (Lacasse, Patel, et al., 2022). This suggests that progestins may have opposite effects to ethinyl estradiol on brain regions that involved in spatial abilities (e.g., hippocampus) (Lacasse, Patel, et al., 2022). Taken together, these results indicate strong modulating effects of synthetic hormones on brain function in adult humans and non-human animals. However, the effects of these hormones on the adolescent brain remain unclear.

3.0 COCs and the brain : Evidence from human studies

3.1 Structural brain changes

Recent work suggests that COCs may change brain structure. For example, compared to naturally cycling (NC) females, COC users display reduced cortical thickness in the orbitofrontal and posterior cingulate cortices, right putamen, left amygdala, and anterior parahippocampal gyrus (Lisofsky et al., 2016; Petersen et al., 2021; Petersen et al., 2015; Sharma, Smith, et al., 2020). COC users also show increased gray matter volume in the prefrontal cortex, and in the parahippocampal and fusiform gyri compared to naturally cycling females (Pletzer et al., 2010). COC use has also been associated with larger gray matter volumes in the hippocampus and in the basal ganglia but these effects were transient as they were not present when COC use was discontinued (Pletzer et al., 2019). COC users also display both decreased grey matter volume in

the right putamen and increased white matter volumes in the right putamen, right amygdala, right rectus as well as in the left hippocampus and parahippocampal gyrus compared to NC females (Sharma, Smith, et al., 2020).

3.2 Functional brain changes

Several functional changes have also been found in the brains of COC users. For example, compared to NC females, COC users display reduced activity in the left insula, left middle, and bilateral inferior frontal gyri when presented with either an emotional matching face stimulus or a neutral stimulus (e.g., geometrical shape) (Gingnell et al., 2013). COC users also show greater activation in the right fusiform area following the presentation of faces compared to NC females (Marecková et al., 2014). COCs users who began contraceptive use during puberty also display increased activity in the frontal and motor regions, the insula, the fusiform and lingual gyri in the left fusiform gyrus, right cuneus, bilateral lingual and left mid-temporal gyri as well as the right insula and pre-central gyrus while completing the positive block of *n*-back task compared to females who began taking COCs in adulthood (Sharma, Smith, et al., 2020). In contrast, females who began taking COCs in adulthood demonstrated increased brain activity in the left lingual and right fusiform gyri as well as in both the right putamen and right inferior frontal gyrus when exposed to the *n*-back task with negative stimuli, compared to females who began taking COCs in adolescence (Sharma, Smith, et al., 2020). Interestingly, COC users also display greater resting state functional connectivity in the reward, central executive, cortico-limbic, and salience networks compared to naturally cycling females (Sharma, Fang, et al., 2020). Moreover, females who began taking COCs during early adolescence display greater functional connectivity in the salience network compared to adult-onset COC users (Sharma, Fang, et al., 2020).

3.3 Cognitive brain changes

COC usage also seems to alter cognitive function (Bianchini et al., 2018; Monciunskaite et al., 2019). A systematic review showed that verbal and spatial memory is often impaired in COC users (Warren et al., 2014). However, further experimental studies would be required to better understand the effects of COCs on cognitive function as current studies are mainly observational and the methodological differences between them make it difficult to reconcile the data and draw conclusions (Warren et al., 2014). Nevertheless, COC users also show reduced perseverance compared to NC females when facing both simple (i.e., note the difference) and challenging (i.e., quantitative reasoning problems) tasks, which then results in lower cognitive performance (Bradshaw et al., 2020). However, no effect of oral contraception was observed on emotional recognition (Shirazi et al., 2020). COCs containing androgenic progestins (i.e., high affinity to androgen receptors) seem to improve visuospatial and face discrimination abilities compared to those with anti-androgenic progestins (Gurvich et al., 2020). Users of anti-androgenic COCs use different mental rotation strategies leading to reduced accuracy in their performance compared to naturally cycling females (Griksiene et al., 2018). Moreover, progestins with higher androgenic activity induced greater mood alterations compared to progestins with anti-androgenic properties (e.g., drospirenone) (Poromaa & Segebladh, 2012). Taken together, the structural, functional, and cognitive changes reported in COC users suggest complex and task and region-specific effects that are also modulated by factors such as age of onset, composition, and COC use duration. Although few research has directly attributed behaviour changes and increased risk for mental illness to COC use, a causal effect remains plausible considering that many of the altered brain regions play a role in regulating cognitive function an behaviour.

4.0 The gut microbiome

One possible mechanism mediating the effects of COCs on brain structure and function may be the gut microbiome, which is a complex and dynamic system comprised of trillions of microorganisms (e.g., viruses, bacteria and microorganisms) present in the digestive tract (Gill et al., 2006; Qin et al., 2010). The gut microbiota is responsible for the prevention of diseases via the regulation of a number of immune and metabolic functions (Prakash et al., 2011). Gut dysbiosis is defined as a disruption of the gut flora, and has been linked to several metabolic (Muscogiuri et al., 2019), gastro-intestinal (Mayer et al., 2014), and inflammatory diseases (Wang et al., 2020). Several environmental factors such as diet, stress, sleep disruption and antibiotics can alter the gut microbiota and induce gut dysbiosis (Martinez et al., 2021). In addition, the gut microbiome and the brain communicate bidirectionally through the autonomic and enteric nervous systems, and neuroimmune and neuroendocrine pathways forming what is commonly known as the gut-brain axis (Browning & Travagli, 2014; Cryan et al., 2019; Foster et al., 2017; Grenham et al., 2011; Osadchiy et al., 2019).

4.1 Sex hormones and the gut-brain axis

Beyond environmentally induced changes to the gut microbiota, there are multiple sex differences in gut microbial composition (Kim et al., 2020; Valeri & Endres, 2021). Interestingly, while some sex differences in gut microbial composition are present in infancy, they significantly increase during puberty, as the levels of endogenous sex hormones also increase (Yuan et al., 2020). In addition, growing evidence shows that this sex difference is modulated by endogenous sex hormones thus leading to sex-specific immune profiles and vulnerabilities for diseases (e.g., irritable bowel syndrome) (Mulak et al., 2014; Vemuri et al., 2019; Yoon & Kim, 2021). Men display greater abundance of *Acinetobacter*, *Dorea*,

Ruminococcus and *Megamonas* compared to females, while females display greater concentration of *Bacteroidetes* and lower concentration of *Firmicutes* compared to men (Shin et al., 2019). The estrobolome, a collection of enteric bacterial genes, plays a crucial role in estrogen metabolism through the regulation of β-glucuronidase enzymes activity (Chen & Madak-Erdogan, 2016; Lephart & Naftolin, 2022). However, gut dysbiosis may alter β-glucuronidase enzymatic activity leading to either increased circulatory levels of estrogens or decreased levels of deconjugated estrogens – both scenarios are involved in the etiology of several diseases related to estrogens (e.g., endometriosis, breast cancer, obesity, cognitive impairment) (Baker et al., 2017; Flores et al., 2012; Kwa et al., 2016; Valeri & Endres, 2021). Estrogens also target the functioning of organs and skeletal, cardiovascular and central nervous systems by binding to ER-α and ER-β receptors (Paterni et al., 2013; Paterni et al., 2014). The gut microbiota also impacts the metabolism of androgens (Colldén et al., 2019). For example, male mice with normal gut composition have higher concentrations of unconjugated testosterone and dihydrotestosterone (DHT) compared to germ free mice (Colldén et al., 2019). It has also been shown that high DHT levels in female rats with polycystic ovary syndrome were associated with reduced abundance of bacteria but they do not alter α diversity (Zheng et al., 2021). Thus, there is a strong bidirectional relationship between endogenous sex hormones and gut microbiota composition, which may impact neurobiological and neuroendocrine functions in the host through the gut brain axis (Cussotto et al., 2018). Moreover, gut dysbiosis has been linked to various mental illnesses such as depression, bipolar disorder, anxiety, and autism spectrum disorder (Clapp et al., 2017; Holingue et al., 2020; Huang et al., 2019), suggesting that the effects of sex hormones are important to better understand the effects of the gut-brain axis on mental health.

4.2 Combined oral contraceptives and the gut-brain axis

Recently, exogenous sex hormones have been shown to reduce bacterial richness and diversity in the gut (Mihajlovic et al., 2021). COC users display reduced alpha diversity but not beta diversity when compared to non-users (Mihajlovic et al., 2021). COCs are also associated with greater biosynthesis of several amino acids, such as lysine and threonine, as well as polymers like peptidoglycan (Hua et al., 2022), suggesting that the gut microbiome could play a key role in mediating the effects of COCs on the brain structure and function, and on mental health.

Considering the associations between the gut and brain health (Huang et al., 2019; Wang & Wang, 2016), COC-induced gut dysbiosis may also affect brain function and predispose to mental illnesses (Cenit et al., 2017; Makris et al., 2021; Mihajlovic et al., 2021). While there has been some research investigating the effects of COC on brain structure and function, mood, and gut dysbiosis, the findings remain conflicted. More research including other variables (e.g., age of onset, duration of use) would shed light on certain gaps and discrepancies in the literature. More importantly, the gut microbiota also changes throughout the lifespan (O'Toole & Claesson, 2010) and is dependent on sex (Valeri & Endres, 2021). However, despite the clear association between endogenous sex hormones and gut composition, far too little is known regarding how synthetic sex hormones affect the gut microbiome (Valeri & Endres, 2021). Thus, investigating how puberty onset and external factors such as hormonal birth control use during adolescence might impact the gut microbiota and the brain is essential. Decades of research indicates that COCs may alter brain structure, function, and cognition, but also that pubertal onset may interfere with brain development and increase risk for mood disorders. A growing body of evidence suggests that the gut microbiome may play a pivotal role in shaping COCs effects on

the brain via its influence on neuroinflammation, neurotransmitter synthesis, and short-chain fatty acid production. Given that gut microbiome undergoes significant, sex-specific changes during puberty, it is plausible that COC-induced dysbiosis during this period could disrupt the gut–brain axis.

Studies and Objectives

These studies aimed to determine the effects of COCs administration during puberty on the brain, gut microbiome, and cognitive abilities (Chapter 2), on mental health (e.g., depression- and anxiety-like behaviors) (Chapter 3), and on immune and stress reactivity (Chapter 4). We hypothesized that COCs will alter brain and behaviour as well as gut microbial composition in pubertal mice compared to adult mice. However, we also hypothesized that the androgenicity of progestins will modulate the effects of COCs. We also expected that pubertal mice would display increased depression- and anxiety-like behaviors along with increased expression of biomarkers associated with depression and anxiety. Finally, we hypothesized greater immune and stress responses in pubertal mice treated with COCs compared to both adult COC treated and control mice. Thus, this dissertation investigated the effects of COCs during key developmental stages, addressed gaps in the current scientific literature and provided insights into the clinical implications of synthetic sex hormones for women's health.

CHAPTER 2 : THE EFFECTS OF COMBINED ORAL CONTRACEPTIVES ON THE GUT MICROBIOME, BRAIN, AND COGNITIVE ABILITIES IN ADULT AND PUBERTAL CD-1 MICE

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Abstract

Combined oral contraceptives (COCs) are used daily by over 150 million females globally. Recent findings suggest that COCs alter brain structure and function, including cognitive abilities. However, the mechanism underlying the effects of COCs on brain and cognition remains unclear, especially during puberty. One possibility may be that the effects of COCs on the brain cognition are mediated by the gut-brain axis, as recent evidence showed changes to the gut microbiota can impact the brain and cognitive function. This study examined whether COCs alter the brain, cognition, and the gut microbiome of pubertal and adult CD-1 mice. At five (pubertal) or 10 (adult) weeks of age, female mice were treated daily with one of two COC formula containing either a combination of ethinyl estradiol and levonorgestrel (EE/LNG) or ethinyl estradiol and drospirenone (EE/DRSP) through oral gavage for seven weeks. Adult female and male control mice were administered 240μL of sterile water via oral gavage daily for seven weeks. Fecal samples were collected at three timepoints (i.e., week 1, week 3, and week 7). Spatial, social, and object memories were assessed using the Barnes maze, social recognition, and novel object recognition tests. Then, mice were euthanized, and brains, intestines, and blood samples were collected. EE/LNG- and EE/DRSP-treated pubertal females displayed increased chemo-investigation and altered spatial learning and BDNF and PSD-95 expressions in the hippocampus and medial prefrontal cortex. Microbiota composition was altered in an age- and treatment-dependent manner and was associated with neuroplasticity measures. These findings suggest that COCs use may play a role in shaping brain neuroplasticity and cognitive function through the gut-brain axis, but these effects are dependent on age and COC formulation.

Keywords: Combined oral contraceptives; Puberty; Cognition; Gut microbiome; Brain; Hormonal contraception; Sex differences

1. Introduction

Combined oral contraceptives (COCs), which contain both synthetic estradiol (i.e., ethinyl estradiol) and synthetic progesterone (i.e., progestin) (Cooper & Mahdy, 2021), are the most commonly used form of reversible hormonal contraception by females worldwide (Black et al., 2009). To prevent pregnancy, these synthetic hormones bind to endogenous sex hormones receptors, downregulate the hypothalamic-pituitary-gonadal axis (HPG), and inhibit ovulation, thicken the cervical mucus, and thin the endometrium (Rivera et al., 1999). Various types of COCs are available on the market and they differ based on when they were introduced in the market (i.e., generation), their composition (e.g., type of progestin), dose of synthetic hormone, and the cycle phases (Hall & Trussell, 2012).

Progestins differ based on their pharmacological profiles and possess different binding affinities to androgen receptors, which can induce distinct side effects (Breech & Braverman, 2010; Sitruk-Ware, 2004, 2006). Second generation progestins (e.g., levonorgestrel, norgestrel) have a high binding affinity to androgen receptors (i.e., high androgenicity) and have been associated with undesirable side effects like acne, bloating and hair growth (Darney, 1995; Sitruk-Ware, 2004). These negative effects are reduced in the third (e.g., desogestrel, norgestimate) and fourth (e.g drospirenone, nestorone) generation progestins, which have low binding affinity to androgen receptors and act by blocking the androgen receptors (anti-androgenic activity) (Darney, 1995; Sitruk-Ware, 2004).

COC usage has been associated with structural and neurochemical changes in the brain. For example, adult COC users display greater white matter volume (De Bondt et al., 2013; Sharma, Fang, et al., 2020; Sharma, Smith, et al., 2020) and less grey matter volume (Chen et al., 2021; Sharma, Fang, et al., 2020; Sharma, Smith, et al., 2020) in specific brain regions compared

to naturally cycling (NC) females. Animal studies suggest that progesterone and some progestins might induce similar effects, via their action on progesterone receptors, although ovarian status, progestins androgenicity, and ethinyl estradiol dose might mediate these effects (Bencker et al., 2025; Hampson, 2023; Lacasse et al., 2024; Pletzer et al., 2023). According to a recent systematic review, progesterone treatment increases myelination and dendritic spine density in regions like the hippocampus, prefrontal cortex and the amygdala of rodents (Pletzer et al., 2023). It also modulates neurotransmitter systems such as γ-aminobutyric acid (GABA) signaling via its metabolite allopregnanolone and serotonin neurotransmission in the hippocampus. Progestins found in COCs induce similar effects by increasing synaptogenesis, neuronal proliferation and modulating neurotransmitters (e.g., GABA, dopamine, serotonin and glutamate) but the effects vary and are more pronounced with older generations progestins (e.g., medroxyprogesterone) (Pletzer et al., 2023). Long-term COC treatment also reduces BDNF mRNA and protein levels in the hippocampus of female rats (Simone et al., 2015), and alters GABA-A receptor subunit expression in brain regions involved in cognitive function (e.g., hippocampus) (Follesa et al., 2002; Porcu et al., 2012; Porcu et al., 2019).

In addition to impacting brain structure, COCs have been shown to alter cognitive functions in both humans and animal models (Hampson, 2023; Kheloui & Ismail, 2024; Lacasse, Gomez-Perales, et al., 2022; Lacasse et al., 2024; Lacasse et al., 2025; Sharma, Fang, et al., 2020; Sharma, Smith, et al., 2020). For example, COC users display better spatial abilities than NC females during a mental rotation task (Beltz et al., 2015), but these effects are dependent on the androgenicity of progestins (Gurvich et al., 2020; Wharton et al., 2008) and the phase of the COCs pill (active/inactive) (Hampson et al., 2022). Both the inactive phase of the pill and androgenic progestins are associated with increased accuracy in mental rotation and in negative

emotion recognition (e.g., sadness, anger, disgust) (Hampson et al., 2022; Hamstra et al., 2014). Furthermore, rats exhibit alterations in cognitive functioning appear to be dependent on the dose of ethinyl estradiol. In rats treated with high doses of ethinyl estradiol, response is based on place memory, whereas the response of rats treated with low doses of ethinyl estradiol depends on response strategy when solving a T-maze (Lacasse, Patel, et al., 2022). Interestingly, the preference for place memory shifts towards response strategy with high doses of ethinyl estradiol if progestin is administered 1-4 hours before testing (Lacasse, Patel, et al., 2022). Despite evidence indicating alterations in cognitive function associated with COC use, current literature remains limited and inconsistent due to methodological differences (e.g., COC duration, dosage, composition), highlighting the need for systematic or more controlled research (Hampson, 2023; Pletzer & Kerschbaum, 2014; Porcu et al., 2019).

The age of onset of COC use is another factor that should be accounted for as it may also contribute to inconsistent findings. COCs are commonly prescribed to pubertal and adolescent females to manage medical conditions like hormonal acne, dysmenorrhea, and premenstrual symptoms (Bahamondes et al., 2015). However, puberty is a critical developmental period marked by the activation of the HPG axis and the production and release of endogenous sex steroid hormones, which will then guide reproductive maturation, and the remodeling and reorganizing of the brain (Schulz & Sisk, 2016; Sisk & Zehr, 2005; Vigil et al., 2016; Vigil et al., 2011). Since COCs inhibit the HPG axis and the production of endogenous sex steroid hormones (Hilz, 2022; Stomati et al., 1998), it is possible that the synthetic hormones in COCs could impact the brain remodeling and reorganizing that occur during puberty (Kheloui et al., 2023), which could lead to enduring changes in brain structure and function. Indeed, females that began COC use during puberty show increased white matter volume in the right fusiform gyrus

compared to NC females (Sharma et al., 2020a). Moreover, females that began COC use during puberty show increased BOLD response in the left fusiform gyrus, right cuneus, bilateral lingual, left mid-temporal gyri, right insula, and pre-central gyrus during an emotional working memory task compared to adult COC users (Sharma, Smith, et al., 2020). Pubertal COC users also show increased functional connectivity in the salience network compared to adult COC users (Sharma, Fang, et al., 2020). These findings suggest that COC affects brain structure and function especially during puberty (Cahill, 2018; Hilz, 2022; Lacasse, Gomez-Perales, et al., 2022; Pletzer et al., 2023).

Given that COCs are consumed orally, the gut microbiome may mediate their effects on the brain and behaviour. The gastrointestinal tract hosts billions of microorganisms that maintain homeostasis and regulate metabolism and immunity (Fung et al., 2017; Shreiner et al., 2015). Environmental factors (e.g., antibiotics, diet, stress) can alter the gut microbiota, causing gut dysbiosis, which can increase gut permeability and contribute to the onset of immune, metabolic, neurological and psychiatric diseases (Das & Nair, 2019; Kamada et al., 2013; Mitrea et al., 2022; Rosendo-Silva et al., 2023; Stolfi et al., 2022; Zeng et al., 2017). Since there is bidirectional communication between the gut and the brain via the gut-brain axis (Forsythe et al., 2014; Foster et al., 2017; Ma et al., 2019; Makris et al., 2021), changes to the gut microbiota can alter brain function. During puberty, rising sex hormones concentrations also shapes the gut microbiome, causing sex differences in gut microbial composition, which could contribute to disparities in physical and mental health conditions between males and females (Cussotto et al., 2018; d'Afflitto et al., 2022; He et al., 2021; Santos-Marcos et al., 2023). Gonadectomy alters the gut microbiome in a sex-specific manner (Org et al., 2016). While it is clear that endogenous sex hormones impact the gut microbiota, the effects of the synthetic hormones present in COCs on

the gut microbial composition remain unclear due to contradictory findings. Although one study found a small reduction in bacterial alpha diversity between COC users and naturally cycling females (Mihajlovic et al., 2021), subsequent research found no such difference (Hua et al., 2022).

Therefore, our study aimed to assess the effects of two types of COC formulae (i.e., EE/LNG and EE/DRSP) administered during puberty and in adulthood on cognitive function, as well as on markers of synaptic plasticity, including the brain-derived neurotrophic factor (BDNF) and the postsynaptic density-95 protein (PSD-95; a marker of synaptic density), in the hippocampus (HIPP) and medial prefrontal cortex (mPFC). We also explored possible effects on gut permeability and on gut microbial composition. According to our hypotheses, mice treated with COCs would display reduced expression of BDNF and PSD-95 in the HIPP and mPFC and impaired cognitive function compared to naturally cycling females. Secondly, we hypothesized that mice treated with EE/LNG during puberty would show greater cognitive impairment and greatest reductions in BDNF and PSD-95 in the HIPP and mPFC than those treated in adulthood or mice treated with EE/DRSP. Finally, we hypothesized that females exposed to COCs would display increased intestinal permeability, and reduced microbial diversity in the gut compared to naturally cycling females, with increased alterations in females exposed to COCs since puberty.

2. Methods

2.1. Animals

A total of 70 male and female CD-1 mice were shipped from Charles River Laboratories Inc. (Saint-Constant, Québec, Canada) at 3 weeks of age. Mice were housed in same-sex pairs in polycarbonate Lexan housing cages (17 × 28 × 12 cm [width x length x height]) bedded with Teklad Corn Cob (Harlan Laboratories, Inc., Madison, WI, USA, .25 in. diameter). Two square

pieces of nestlet (Ancare Corp., Bellmore, NY, USA) and a cardboard refuge hut (Ketchum Manufacturing, Inc., Brockville, ON, Canada) were added to the cages for environmental enrichment. Mice had *ad libitum* access to food (Harlan Laboratories, Inc., Madison, WI, US, T2018 – Global 18% rodent) and water. The housing rooms were kept at constant temperature (24 ± 2 °C) and relative humidity (40 ± 5 %), and on a reversed light cycle (14h:10h light/dark) with lights off at 10 A.M. All procedures were approved by the Animal Care Committee of the University of Ottawa.

2.2. Combined oral contraceptives treatment

Mice were divided into six groups based on sex and experimental conditions. Two control groups consisted of adult males (n = 10) and naturally cycling (NC) females (n = 20) which received 1.6 µL dimethyl sulfoxide (DMSO) diluted in 238.6 µL water, for a total volume of 240.2 µL per mouse. Four experimental groups consisted of pubertal (n = 20) and adult (n = 20) females who received either ethinyl estradiol with levonorgestrel (EE/LNG) or ethinyl estradiol with drospirenone (EE/DRSP). Mice in the EE/LNG group received 0.22 µg ethinyl estradiol and 0.92 µg levonorgestrel dissolved in DMSO, while those in the EE/DRSP group received 0.22 µg ethinyl estradiol and 18.45 µg drospirenone dissolved in DMSO. For each mouse, the synthetic hormones were dissolved in a 240.2 µl solution containing 1.6 µl of soluble DMSO and 238.6 µl of water via oral gavage. Dosage of synthetic hormones was chosen based on the typical doses found in COCs (i.e., 0,035 mg EE; 0,15 mg LNG; 3 mg DRSP) and a mouse equivalent dose was calculated using an Animal Equivalent Dose (AED) conversion factor (Nair & Jacob, 2016) and then multiplied by the average weight of CD-1 mice (30g).

2.3. Vaginal cytology

Estrus cycle was determined through daily vaginal lavages (McLean et al., 2012) (see Figure 1). Vaginal cells were collected by gently flushing approximately 25-50 µl of 0.9% saline at the opening of the vaginal canal using a transfer pipette. Cells were then placed on Fisherbrand Superfrost Plus microscope slides and covered with Fisherbrand coverslips. Cycle staging was determined with a 10X magnification objective lens using a Leica DAS microscope, attached to a SONY DXC-390P digital camera and Northern Eclipse 8.0 software (Empix Imaging, Mississauga, Ontario, Canada).

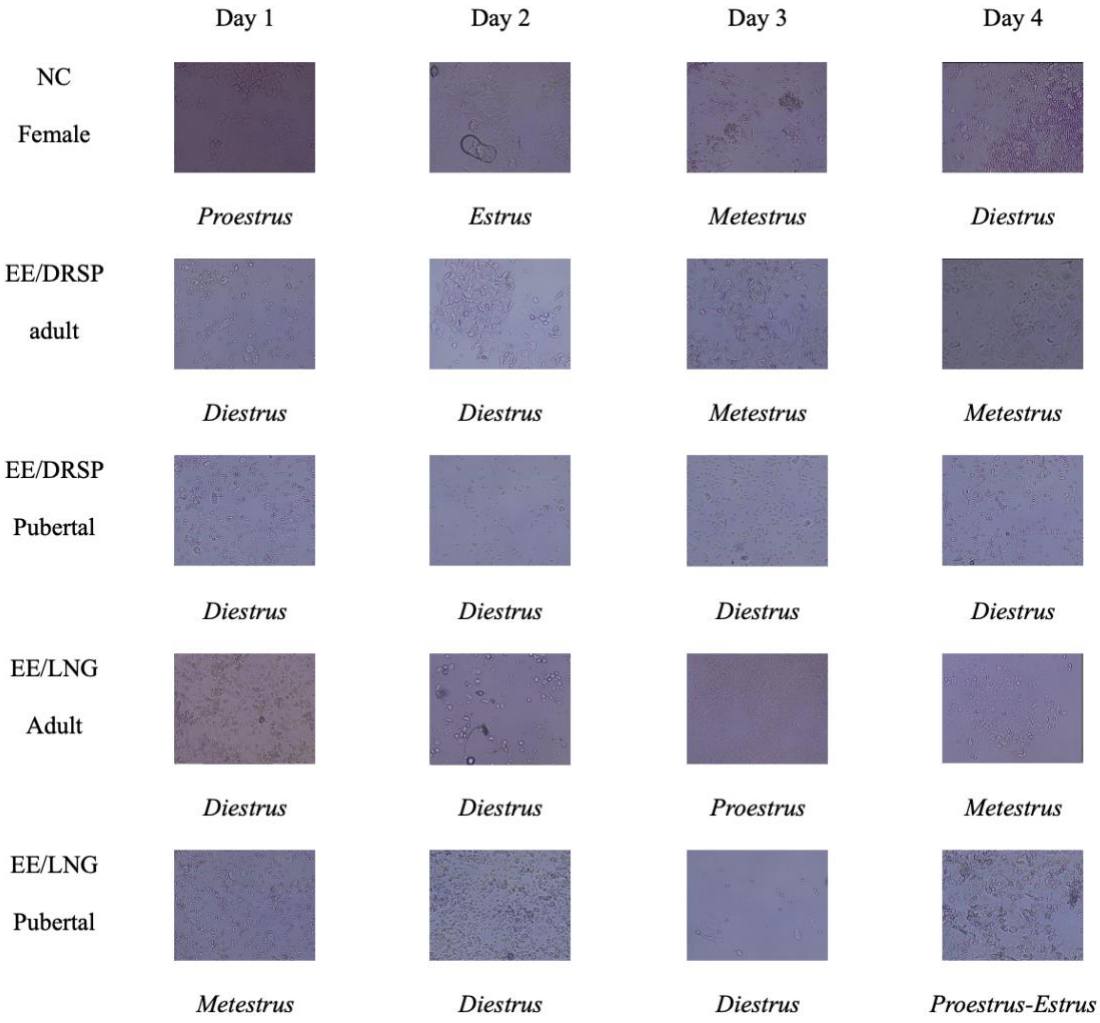

Figure 1. Estrus cycle staging in CD-1 female mice

Note. Images of vaginal sample cytology collected over four consecutive days from each mouse in each group. Staging was performed according to (Ajayi & Akhigbe, 2020). The top row shows samples from a naturally cycling female mouse. The natural estrous cycle in female mice lasts 4 to 5 days and is generally divided into three stages: proestrus, estrus, and diestrus (further subdivided into metestrus and diestrus). The first image in row 1 represents proestrus, characterized by predominantly nucleated epithelial cells. The second image shows estrus, identified by mainly cornified squamous epithelial cells. The third image depicts metestrus, marked by a mix of cornified squamous epithelial cells, nucleated epithelial cells, and leukocytes. The fourth image illustrates diestrus, featuring mostly leukocytes along with some nucleated epithelial cells. Rows 2 and 3 show vaginal cytology images from an adult and a pubertal mouse treated with EE/DRSP, respectively. The images consistently display varying numbers of leukocytes, indicating that mice given EE/DRSP remained in a constant state of diestrus-metestrus. In the final bottom rows, adult (row 4) and pubertal (row 5) mice treated with EE/LNG are represented. In row 4, the diestrus/metestrus phase persisted for two days, with proestrus observed in the third picture; however, estrus phase was skipped, as evidenced by the presence of leukocytes, cornified squamous, and nucleated epithelial cells. Additionally, row 5, displaying pubertal mice treated with EE/LNG, indicates that the diestrus/metestrus phase lasted for three consecutive days, with the last day resembling a mixture of proestrus and estrus, characterized by a mix of nucleated and cornified cells.

2.4 Behavioural testing.

2.4.1 Barnes Maze

Spatial learning and memory were evaluated using the Barnes Maze as previously described in Kolmogorova et al. (2019). The maze consisted of an elevated circular arena (121

cm in diameter) containing 16 evenly spaced holes (9 cm in diameter) and one escape box. Three unaltered and distal visual cues (22 x 28 cm) were placed around the platform. The behavioral examination included three phases, the habituation, the data acquisition and the probe trial (Kolmogorova et al., 2019). One day prior to data acquisition, mice were exposed to the maze. Following the habituation phase, mice were exposed to the maze during the spatial acquisition phase (Days 1-4). During this phase, mice were placed at the center of the maze in an opaque container. Following a 10s delay, the container was lifted and the mice were exposed to two aversive stimuli, an 85-95 dB white noise and a 27 W bright light, to motivate them to find the escape box. The spatial acquisition phase was carried out over four consecutive days with four trials per day (15 minutes inter-trial interval). Each trial of the spatial acquisition phase had a maximum duration of 5 minutes but was terminated upon entry of the mouse into the escape box. After the acquisition phase, two *probe* trials were administered in order to assess short-term (Day 5) or long-term (Day 12) spatial memory. Each trial ended either once the mouse located the target hole or following a maximum duration of 1 minute. All trials were recorded by EthoVision XT software. Analyses included measures of crossed distance (cm), speed (cm/s), latency to target hole (s), primary errors (i.e., number of nose pokes to the target hole), and secondary errors (i.e., number of nose pokes to any hole).

2.4.2. Social Recognition Test (SRT)

Juvenile CD-1 male and female mice aged between 3 and 7 weeks were used as stimuli for this test. All test mice were exposed to a same-sex juvenile mouse for three consecutive 5-minute trials in the home cage of the testing mouse. Following that, mice were exposed to a novel stimulus same-sex juvenile mouse for 5 minutes. There was a 15-minute inter-trial interval for all trials. Total chemo-investigation and approach durations and frequencies were recorded

during each trial by an overhead video system. Chemo-investigation involved the act of sniffing the juvenile's head, torso, or anogenital region, whereas approach behaviour was defined as the deliberate movement towards the stimulus mouse, wherein the subject mouse follows the stimulus without engaging in any physical contact. This procedure was adapted from Engelmann et al. (1995).

2.4.3.Novel object recognition (NORT)

The NORT is considered a valid test to evaluate non-spatial object recognition memory and is based on the rodents' innate preference for novelty (Lueptow, 2017). Mice were placed at the center of an open arena (60 cm × 60 cm × 32 cm) for a 5-minute habituation period. Immediately after, two identical objects were placed at opposite corners of the arena and mice were allowed to explore the objects for a total of 3 minutes (Sample phase). Mice showed no preference for either object or location within the arena, spending equal exploration time regardless of object positioning. After a 30-minute delay, mice were reintroduced to the open arena for another 5-minute period (Choice phase). In this phase, they-were-exposed to one of the familiar objects and a novel one, located at opposite corners (same as in the sample phase). The selected objects were of sufficient weight to prevent any displacement by the mice and pilot data indicated that the mice had no preference for either object. Investigation time (i.e., head oriented towards the object, touching, sniffing and nose pokes) for each object was recorded using the EthoVision XT software. Prior to analyses the preference ratio for the novel object was computed by dividing the time spent investigating the novel object by the total time spent exploring both the novel and familiar object and multiplying by 100 (Lueptow, 2017)

2.5. Plasma extraction

Blood was collected by cardiac puncture and placed into Microvette CB 300 K2E tubes (Sarstedt AG & Co, Nümbrecht, Germany) that were coated with an anti-coagulant, EDTA. Tubes were kept at 4°C until centrifugation at 1100 x g at 20°C for 15 minutes within four hours of blood collection, The resulting plasma was aliquoted and stored at -80°C.

2.6. Intestine tissue extraction

The intestine was extracted and stored at -80°C until processing. The intestinal tissue was dissected open longitudinally on ice and washed with 10 mM phosphate-buffered saline (PBS). Luminal surface of the intestine was then scraped off and was stored at -80°C until protein extraction.

2.7. Brain tissue extraction

Upon euthanasia, 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 post-fixed with 10 ml of 4% PFA for 2 hours at 4 °C. Then, the PFA solution was replaced with 10 ml of 30% sucrose for 24 hours. The brains were then transferred to fresh 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.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Plasma concentrations of 17β-estradiol (E2) and progesterone were measured in duplicate with ELISA(E2: No. 501890; progesterone: No. 582601; Cayman Chem.) according to the manufacturer's instructions. A pooled sample was used to monitor inter-assay variation. Plates were read with a Biotek Powerwave XS2 reader and analyzed with the Gen 5 V2.0 software.

Intra-assay variability was assessed and samples with a coefficient of variation (CV) greater than 20% were excluded from the analysis. Inter-assay variability was calculated using pooled samples across plates (Standard deviation/Mean). All inter-assay CVs were below 20%.

2.9. Quantitative western blot

Dissected intestinal tissues were first homogenized in Tissue Protein Extraction Reagent (T-PER; Thermo Scientific, cat: 78510) containing the protease inhibitors Roche PhosSTOP™ (Millipore Sigma; cat: 04001) and cOmplete™ ULTRA EDTA-free (Millipore Sigma; cat: 05001). Homogenates were incubated on ice for 10 minutes, then centrifuged at 21,000 x g for 10 minutes at 4 °C. Supernatants were then collected to determine the total protein concentrations using the Pierce™ Bicinchoninic Acid Assay (BCA) protein assay kit (Thermo Fisher Scientific). 20 μg of protein samples were mixed with Laemmli sample loading buffer containing fresh 2-Mercaptoethnol and then heated to 95 ºC for 5 minutes. Samples were electrophoresed on 12 % polyacrylamide gels using Mini-PROTEAN® Tetra system (Bio-Rad). A pooled protein sample was loaded to each gel to account for inter-gel variation (Eaton et al., 2013; Fosang & Colbran, 2015). Separated proteins were transferred to 0.2 μm nitrocellulose membranes with a Mini Trans-Blot® Electrophoretic Transfer Cell system (Bio-Rad). Membranes were treated with Revert 700 Total Protein Stain (LiCor Biosciences, Cat: 926-11016) according to the manufacturer's instructions and imaged in the 700 nm channel using an Odyssey Scanner (LiCor Biosciences). These total protein staining images were used for loading normalization in place of a housekeeping protein (Eaton et al., 2013; Fosang & Colbran, 2015). Membranes were blocked for 1 hour at room temperature in 5% skim milk in PBS. Following blocking, membranes were 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-occludin primary antibody (1/ 250; Abcam; cat: 40-4700). Membranes were washed three times for 10 minutes each with TBST and then incubated with a goat anti-rabbit IRDye® 800CW secondary antibodies (LiCor Biosciences; cat: 92632211) at room temperature in a solution of 5% skim milk / TBST. After another three washes of 10 minutes each with TBST, the protein signal intensity was measured using Odyssey® Scanner and software (LiCor Biosciences). Signal intensity was normalized to the total protein measurement. To account for inter-gel differences, the normalized measurement of each sample was calculated as a ratio using the pooled sample within the same gel. The ratio is reported as a fold change (mean of duplicate ± SEM), facilitating comparison of relative protein abundance between groups.

2.10. 16S rRNA Sequencing

Bacterial DNA was extracted from the fecal samples using a FastDNA Spin Kit (MP Biomedicals, Cat.: 116540600) with modification to the sample homogenization step. Briefly, the fecal samples were mixed with the kit's cell lysis solution CLS-TC in the Lysing Matrix A tubes and homogenized with two cycles of mechanical lysis using a Mixer Mill MM 400 (Retsch GmbH) at 25 Hz for 40 seconds, with 5-minute incubation on ice between cycles. Negative controls were included in the extraction and subsequent PCR reactions. The remaining DNA isolation and purification steps were performed according to the manufacturer's instructions. Extracted DNA was quantified using a Qubit Fluorometer (Thermo Fisher Scientific) and the Qubit HS DNA Assay Kit (Thermo Fisher Scientific, Cat.: Q33230) and stored at -80 °C until further processing.

16S rRNA gene amplicons and library preparation were performed according to Illumina recommended instructions. Amplification of the 16S rRNA gene was performed using the

forward primer B969F (5′-ACGCGHNRAACCTTACC-3′) and the reverse primer BA1406R (5′-ACGGGCRGTGWGTRCAA-3′) (Le Guern et al., 2023). Amplicon indexing was performed using Illumina DNA/RNA UD Indexes Sets, and libraries were sequenced on a NextSeq 2000 system using a NextSeq 1000/2000 P1 XLEAP-SBS Reagent Kit (Illumina, Inc.).

Bioinformatic processing was performed using the nf-core/ampliseq pipeline (v2.12.0). Briefly, raw sequencing reads were quality-filtered, trimmed, and denoised to generate amplicon sequence variants (ASVs) using DADA2. Taxonomic classification was assigned to the resulting ASVs using the SILVA v138 reference database.

2.11. Immunohistochemistry (IHC)

Brain sections were rinsed 3 x 5-minute with tris-buffered saline (TBS; pH 7.6). Sections were incubated for 30 minutes in an antigen retrieval solution (0.05M sodium citrate tribasic dihydrate), followed by rinsing 3 x 5-minute in TBS and a 30-minute incubation in a 0.1M glycine solution. The sections were then rinsed 3 x 5 minute in TBS and incubated for 30 minutes in a concentrated blocking solution containing TBS, 20% normal goat serum (NGS), 0.1% Triton X-100, and 1% hydrogen peroxide. Sections were transferred to a primary antibody solution (TBS, 2% NGS, 0.1% Triton X-100) containing either anti-BDNF (1/600; Alomone; new cat: ANT-010-GP) or anti-PSD-95 (1/200; Alomone; cat: APZ-009) and incubated overnight at room temperature. Sections were then washed 3 x 5-minute in a diluted blocking solution (TBS, 1% NGS, 0.1% Triton X-100). Sections were incubated for 60 minutes at room temperature in a secondary antibody solution (TBS, 2% NGS, 0.1% Triton X-100) contained either biotinylated goat anti-guinea pig IgG (1/500; Vector Laboratories; cat: BA-7000) for BDNF, or biotinylated goat anti-rabbit IgG (1/500; Vector Laboratories; cat: BA-1000) for PSD-95. Sections then underwent 3 x 10-minute washes in TBS with 0.1% Triton X-100, followed by

a 1-hour incubation in the VECTASTAIN® Elite ABC-HRP Kit (Vector Laboratories; cat: PK-6100). The sections again underwent 3 x 10-minute washes in TBS and were then incubated in freshly prepared 3,3'-diaminobenzidine (DAB) solution (Vector Laboratories; cat: SK-4100), followed by 3 x 5-minute rinses in cold TBS. Lastly, sections were mounted on Fisherbrand Superfrost Plus microscope slides and mounted with Permount mounting medium.

2.11.1 Image analysis and cell counting

The identification of the medial prefrontal cortex (mPFC ; Bregma +1.69 mm) and the hippocampus (HIPP ; Bregma - 1.94 mm) were based on *The Mouse Brain Atlas in Stereotaxic Coordinates* (Paxinos & Franklin, 2019). Best-matched images of BDNF and PSD-95 immunoreactive cells were imaged at 10x on an Olympus BX51 light microscope connected to a Jenoptik ProRes, MF scanning camera. Cell quantification was performed using the software Fiji (i.e., Image J) (Schindelin et al., 2012).

2.12. Experimental design

Mice were administered either a COC (EE/LNG or EE/DRSP) or a vehicle daily for seven weeks as of either 5 weeks of age (pubertal groups) or 10 weeks of age (adult groups). Fecal samples were collected from mice at three different time points. First sample was collected as a baseline before COC or vehicle treatment (T1), second sample was collected two weeks before behavioural testing (T2), and final sample was collected prior to euthanasia (T3). Upon extraction, all fecal samples were placed in 2ml tubes and stored at -80°C until processing. After five weeks of synthetic sex hormones treatment, all groups were submitted to behavioural testing for 2 weeks to examine social recognition, spatial learning and memory and novel object recognition. All behaviour testing was conducted during the dark phase of the light:dark cycle between 12 PM and 5PM in order to minimize diurnal cortisol variations. On each day of

behaviour testing, the estrus cycle was assessed. Upon completion of the experiment, all mice were euthanized with Euthanyl (Sodium pentobarbital; 500 mg/kg, intraperitoneal (i.p.)). They were intracardially perfused, and blood, intestines, and brains were extracted. Procedures and timeline experiment are depicted in Figure 2.

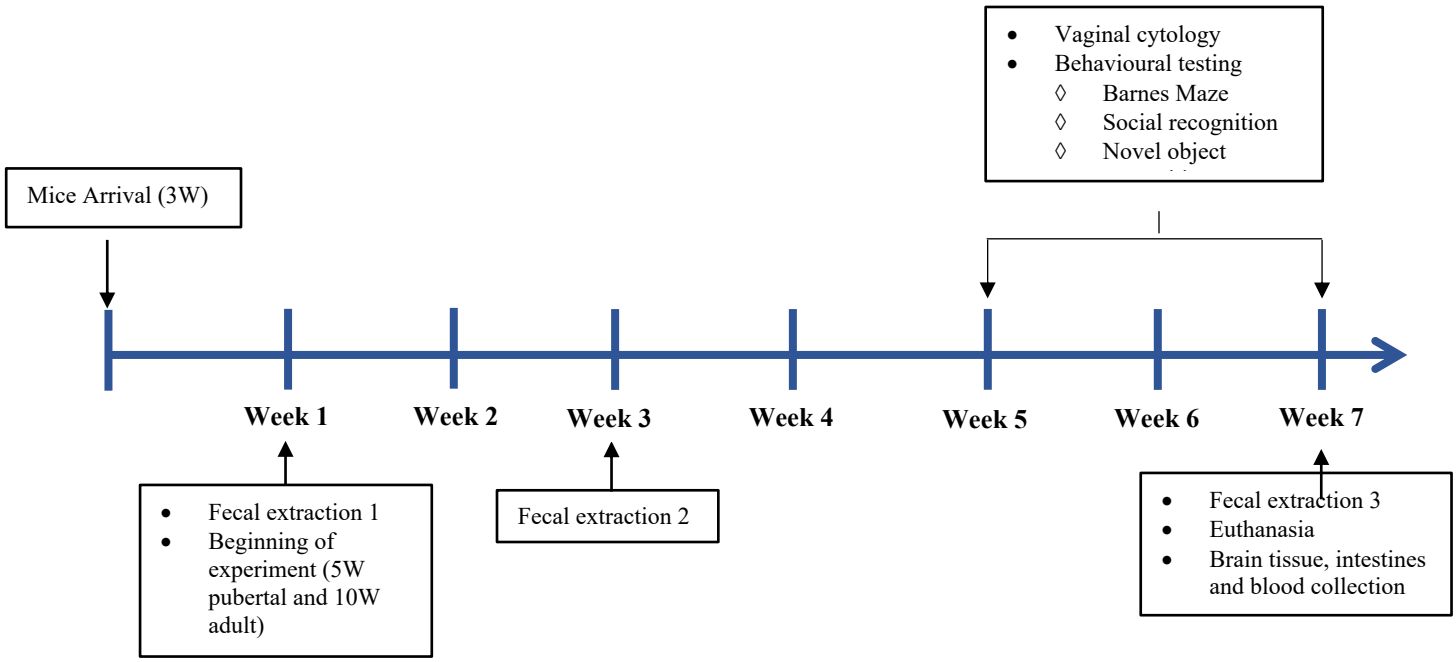

Figure 2. Procedures and timeline experiment

2.13. Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics software, version 28. Prior to the analyses, data were screened for outliers using boxplots. Outliers were winsorized to the next outer-most score within the 1.5 interquartile range (Anscombe, 1973). On average, one data point per group per analysis was winsorized. One-way repeated measures analyses of variance (ANOVAs) were used to analyze the results of the spatial acquisition phase in the Barnes Maze and the results of the social recognition test to determine the effects of days x group condition. Greenhouse-Geisser corrections were applied for F-values that violated Mauchly's sphericity test with an epsilon (ε) less than .75, while Huynh-Feldt correction was used when ε exceeded .75

(Girden, 1992). Results from the short-term and long-term memory trials, NORT test, enzyme-linked immunoassays and IHC were assessed via one-way ANOVAs. Significant interactions were followed by pairwise comparisons with Fisher's Least Significant Difference (LSD). Effect sizes were estimated using partial eta-squared (η_p^2) for all significant main effects and interactions. Statistical significance was set to $p < .05$.

We analyzed 16S amplicon data for microbiome diversity and relative abundance. Sequencing data was processed into a phyloseq object (phyloseq 1.48.0) and analyzed in R v4.4.1. Alpha diversity (Shannon, Simpson, Chao1) was calculated using phyloseq and compared across experimental groups (Age X Treatment X Sex) with Kruskal–Wallis tests followed by Wilcoxon rank-sum tests adjusted for multiple testing with the Benjamini–Hochberg method (rstatix 0.7.2). Beta diversity was assessed using Bray–Curtis dissimilarities with PCoA ordination (vegan 2.6.8), and group-level differences were tested by PERMANOVA (adonis2) and dispersion tests (betadisper). Cage effects were controlled by applying ComBat batch correction (sva 3.52.0). Differential abundance testing was performed at Family and Genus levels using DESeq2 (1.44.0), and taxa with FDR-adjusted $p < 0.05$ were retained. Results were visualized as group–taxon networks using igraph (2.0.3) and ggraph (2.2.1).

Microbiome data were CLR-transformed (microbiome 1.26.0), ComBat-corrected (sva 3.52.0), and integrated with host molecular and behavioural measures. At T3 fecal sample collection, microbial abundances were correlated with PSD-95 and BDNF immunohistochemistry, occludin isoforms (25 kDa, 54 kDa), and plasma estradiol and progesterone data using Spearman correlations. Associations were variance-filtered, restricted to features with sufficient prevalence, and summarized as dot plots (ggplot2 3.5.2), correlation networks (igraph 2.0.3; ggraph 2.2.1) and heatmaps (pheatmap 1.0.12).

Behavioural outcomes were analyzed in parallel using linear mixed-effects models (lme4 1.1.37; lmerTest 3.1.3), with housing cage included as a random effect to control for non-independence. For the Social Recognition Test (SRT), models evaluated both within-time point associations and Δchanges (microbiome T3–T2 vs. behaviour T3–T1), requiring matched cage-mates for paired comparisons. The NORT was modeled by relating the discrimination ratio (RatioD) to microbial features, reporting marginal and conditional R^2 values (MuMIn 1.48.11; performance 0.10.8). Barnes Maze performance (Day 1–3 of spatial acquisition at T2; Day 12 probe at T3) was analyzed similarly, testing genus- and family-level microbial associations with escape latency, distance traveled, and error counts. Across all association analyses, taxa were prevalence- and variance-filtered, CLR-transformed, and results corrected for multiple testing using the Benjamini–Hochberg method.

3. Results

3.1. Barnes Maze

3.1.1. Spatial learning : Frequency of primary errors

There was a main effect of Days ($F_{(1.385,88.667)} = 12.684$, $p < .001$, $\eta p^2 = .17$) and a significant Days x Group interaction ($F_{(6.927,88.667)} = 4.994$, $p < .001$ $\eta p^2 = .28$) on the frequency of primary errors (i.e., number of nose pokes to the target hole). Pairwise comparisons revealed that on Day 1 of the spatial acquisition phase, the pubertal EE/LNG group made significantly more primary errors than males (*MD* = 6.06, *SE* = 1.53, $p < .001$), NC females (*MD* = 4.51, *SE* = 1.33, $p = .001$), adult EE/LNG (*MD* = 5.73, *SE* = 1.53, $p < .001$), adult EE/DRSP (*MD* = 3.88, *SE* = 1.53, $p = .01$), and pubertal EE/DRSP (*MD* = 5.53, *SE* = 1.53, $p < .001$). On Day 3, pubertal EE/LNG mice committed more primary errors than males (*MD* = 1.35, *SE* = .61, $p = .03$), NC females (*MD* = 1.18, *SE* = .52, $p = .03$), adult EE/LNG (*MD* = 1.70, *SE* = .61, $p = .01$) and adult

EE/DRSP (*MD* = 1.88, *SE* = .605, *p* = .003) groups. Moreover, pubertal EE/DRSP mice made more primary errors on Day 3 than adult EE/LNG (*MD* = 1.50, *SE* = .61, *p* = .02) and adult EE/DRSP (*MD* = 1.68, *SE* = .61, *p* = .01) mice. On Day 4, males made significantly more primary errors than NC females (*MD* = .66, *SE* = .33, *p* = .05) (Figure 3).

3.1.2. Spatial learning : Frequency of secondary errors

There was a main effect of Days ($F_{(2.531,161.998)} = 17.484$, $p < .001$, $\eta p^2 = .22$). and a significant Days x Group interaction ($F_{(12.656,161.998)} = 3.027$, $p < .001$, $\eta p^2 = .19$) on the frequency of secondary errors (i.e., number of nose pokes to any hole). Pairwise comparisons showed that pubertal EE/LNG mice made significantly more secondary errors on Day 1 than adult EE/LNG (*MD* = 15.58, *SE* = 4.57, *p* = .01), pubertal EE/DRSP mice (*MD* = 13.58, *SE* = 4.57, *p* = .004), control males (*MD* = 9.48, *SE* = 4.57, *p* = .04), and NC females (*MD* = 11.92, *SE* = 3.96, *p* = .004). On Day 2, pubertal EE/LNG mice made significantly more secondary errors than pubertal EE/DRSP mice (*MD* = 11.53, *SE* = 3.34, *p* = .001), adult EE/LNG (*MD* = 7.98, *SE* = 3.34, *p* = .02) and adult EE/DRSP (*MD* = 8.70, *SE* = 3.34, *p* = .01) mice. Moreover, pubertal EE/DRSP mice performed significantly fewer secondary errors than NC females (*MD* = -5.98, *SE* = 2.87, *p* = .04). However, on Day 3, pubertal EE/DRSP mice made significantly more secondary errors than NC (*MD* = 7.08, *SE* = 2.79, *p* = .01), adult EE/LNG (*MD* = 8.53, *SE* = 3.23, *p* = .01), and adult EE/DRSP females (*MD* = 9.03, *SE* = 3.23, *p* = .01). In addition, males made significantly more secondary errors than adult EE/DRSP mice (*MD* = 6.80, *SE* = 3.23, *p* = .04). On Day 4, males made more secondary errors than NC (*MD* = 7.33, *SE* = 2.97, *p* = .02), adult EE/LNG (*MD* = 9.13, *SE* = 3.43, *p* = .01), and adult EE/DRSP (*MD* = 7.60, *SE* = 3.43, *p* = .03) females (Figure 3).

3.1.3. Spatial learning : Velocity

Results revealed a main effect of Days ($F_{(2.762,176.794)} = 32.302$, $p < .001$, $\eta p^2 = .34$) and a significant Days x Group interaction ($F_{(13.812,176.794)} = 4.261$, $p < .001$, $\eta p^2 = .25$) on speed of travel. Pairwise comparisons revealed that on Day 1, males moved significantly faster than NC (*MD* = 1.57, *SE* = .48, *p* = .002), adult EE/DRSP (*MD* = 1.85, *SE* = .56, *p* = .001), adult EE/LNG (*MD* = 1.27, *SE* = .56, *p* = .03), pubertal EE/DRSP (*MD* = 1.21, *SE* = .56, *p* = .03), and pubertal EE/LNG females (*MD* = 2.23, *SE* = .56, *p* < .001). However, on Day 3, pubertal EE/LNG females were significantly faster than males (*MD* = 3.12, *SE* = .99, *p* = .002), and NC (*MD* = 2.20, *SE* = .86, *p* = .01), adult EE/LNG (*MD* = 2.17, *SE* = .99, *p* = .03), and adult EE/DRSP (*MD* = 2.35, *SE* = .99, *p* = .02) females. On Day 4, pubertal EE/LNG females were significantly faster than males (*MD* = 3.66, *SE* = 1.25, *p* = .01), and NC (*MD* = 2.74, *SE* = 1.08, *p* = .01) and adult EE/DRSP (*MD* = 2.99, *SE* = 1.25, *p* = .02) females. Moreover, adult EE/LNG were faster than males (*MD* = 3.00, *SE* = 1.25, *p* = .02) (Figure 3).

3.1.4. Spatial learning : Distance

There was a main effect of Days ($F_{(2.842,181.908)} = 22.238$, $p < 0.001$, $\eta p^2 = .26$) and a significant Days x Groups interaction ($F_{(14.212,181.908)} = 3.142$, $p < 0.001$, $\eta p^2 = .20$). Pairwise comparisons showed that pubertal EE/LNG females travelled significantly longer distance on Day 1 than males (*MD* = 355.99, *SE* = 137.17, *p* = .01), NC females (*MD* = 382.94, *SE* = 118.79, *p* = .002), adult EE/LNG (*MD* = 442.20, *SE* = 137.17, *p* = .002), adult EE/DRSP (*MD* = 298.82, *SE* = 1237.17, *p* = .03), and pubertal EE/DRSP (*MD* = 464.50, *SE* = 137.17, *p* = .001) females before reaching the target hole. On Day 2, pubertal EE/LNG travelled a significantly longer distance than adult EE/DRSP (*MD* = 198.61, *SE* = 85.21, *p* = .02), and pubertal EE/DRSP females (*MD* = 227.83, *SE* = 85.21, *p* = .01). On Day 3, pubertal EE/LNG females travelled

greater distance before reaching the target hole than NC (*MD* = 241.14, *SE* = 104.19, *p* = .02), adult EE/LNG (*MD* = 254.72, *SE* = 120.30, *p* = .04), and adult EE/DRSP (*MD* = 259.98, *SE* = 120.30, *p* = .03) females. Moreover, pubertal EE/DRSP females travelled greater distance than NC (*MD* = 294.96, *SE* = 104.19, *p* = .01), adult EE/LNG (*MD* = 308.53, *SE* = 120.30, *p* = .01), and adult EE/DRSP (*MD* = 313.79, *SE* = 120.30, *p* = .01) females. Interestingly, on Day 4, males travelled greater distance than NC (*MD* = 214.65, *SE* = 82.72, *p* = .01), and adult EE/DRSP (*MD* = 194.05, *SE* = 95.52, *p* = .046) females (Figure 3).

3.1.5. Spatial learning : Latency

There was a main effect of Days ($F_{(2.070,132.487)} = 30.933$, $p < .001$, $\eta p^2 = .33$) and a significant Days x Group interaction ($F_{(10.351,132.487)} = 5.613$, $p < .001$, $\eta p^2 = .31$) on latency to find the escape box. Pairwise comparisons showed that on Day 1, pubertal EE/LNG females displayed longer latency to locate the escape box than males (*MD* = 79.85, *SE* = 21.44, *p* < .001), NC females (*MD* = 69.16, *SE* = 18.57, *p* < .001), adult EE/LNG (*MD* = 82.43, *SE* = 21.44, *p* < .001), adult EE/DRSP (*MD* = 62.96, *SE* = 21.44, *p* = .01) and pubertal EE/DRSP (*MD* = 85.03, *SE* = 21.44, *p* < .001) females. Interestingly, on Day 2, pubertal EE/DRSP females displayed shorter latency to find the escape box than NC females (*MD* = -17.64, *SE* = 8.61, *p* = .04). On Day 3, pubertal EE/DRSP females displayed longer latency to locate the escape box than NC (*MD* = 29.51, *SE* = 11.69, *p* = .01), adult EE/LNG (*MD* = 34.83, *SE* = 13.50, *p* = .01), adult EE/DRSP females (*MD* = 35.00, *SE* = 13.50, *p* = .01). On Day 4, males displayed longer latency to find the escape box than NC (*MD* = 34.56, *SE* = 11.33, *p* = .003), adult EE/LNG (*MD* = 35.51, *SE* = 13.09, *p* = .01), adult EE/DRSP (*MD* = 29.44, *SE* = 13.09, *p* = .03), pubertal EE/LNG (*MD* = 33.52, *SE* = 13.09, *p* = .01), and pubertal EE/DRSP (*MD* = 27.99, *SE* = 13.09, *p* = .04) females (Figure 3).

3.1.6. Short-term memory

There was a significant group difference for distance travelled during short-term memory assessment ($F_{(5,64)} = 2.405$, $p = .046$, $\eta^2 = .16$). Post-hoc tests indicated that pubertal EE/LNG females travelled greater distance than adult EE/LNG females (*MD* = 153.14, *SE* = 73.29, $p = .04$). Moreover, pubertal EE/DRSP group travelled significantly more distance than both adult EE/LNG females (*MD* = 232.78, *SE* = 73.29, $p = .002$) and adult EE/DRSP females (*MD* = 178.38, *SE* = 73.29, $p = .02$). There was also a significant group difference for the number of primary errors during short-term memory assessment ($F_{(5,64)} = 6.316$, $p < .001$, $\eta^2 = .33$). Pairwise comparisons revealed that control males made significantly more primary errors than NC control females (*MD* = .95, *SE* = .23, $p < .001$), adult EE/LNG (*MD* = 1.2, *SE* = .27, $p < .001$), adult EE/DRSP (*MD* = 1.2, *SE* = .27, $p < .001$), pubertal EE/LNG (*MD* = 1.2, *SE* = .27, $p < .001$), and EE/DRSP pubescent mice (*MD* = 1.2, *SE* = .27, $p < .001$) (Figure 4).

3.1.7. Long-term Memory

The ANOVA revealed that there was a significant difference in the number of primary errors during long-term memory assessment ($F_{(5,64)} = 2.844$, $p = .022$, $\eta^2 = .18$). Post-hoc tests showed that NC females made more primary errors than males (*MD* = .45, *SE* = .19, $p = .02$), EE/LNG adults (*MD* = .45, *SE* = .19, $p = .02$) and EE/DRSP pubertal group (*MD* = .45, *SE* = .19, $p = .02$). In addition, pubertal EE/LNG females made significantly more mistakes than males (*MD* = .50, *SE* = .22, $p = .03$), adult EE/LNG (*MD* = .50, *SE* = .22, $p = .03$), and EE/DRSP pubescent mice (*MD* = .50, *SE* = .22, $p = .03$) (Figure 4).

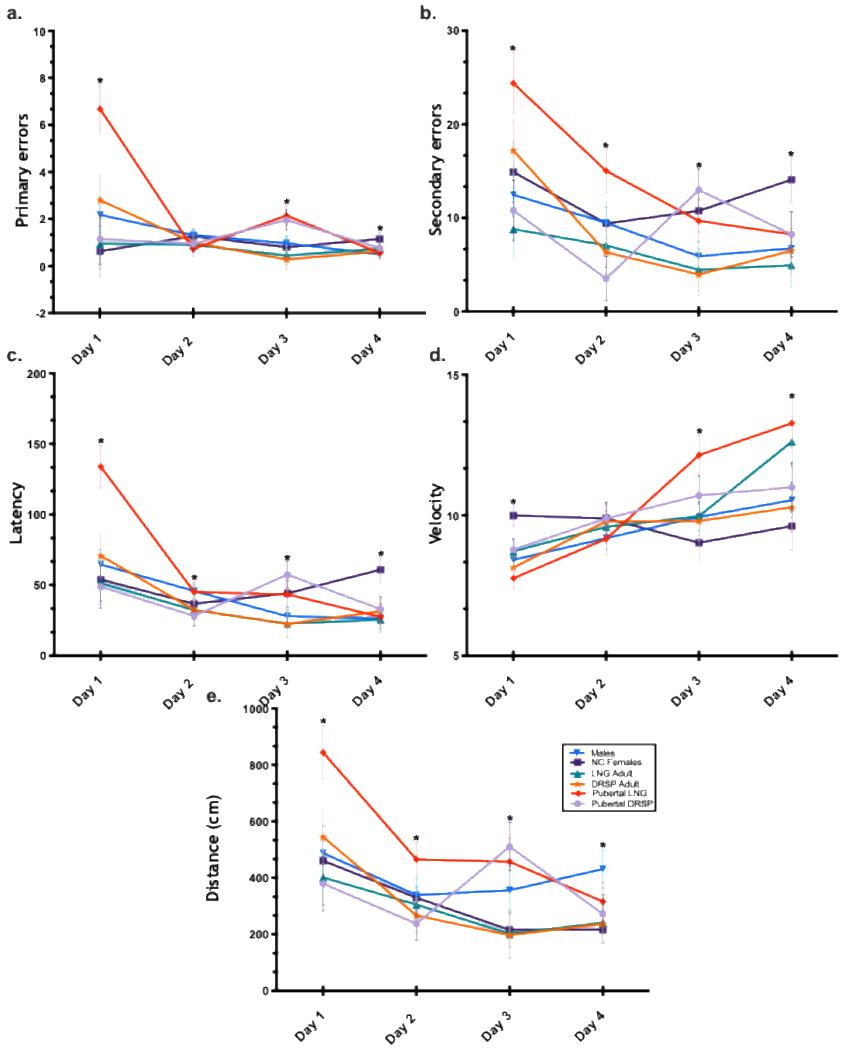

Figure 3. Barnes Maze performance for spatial acquisition

Note. Group means (±*SEM*) in one-way repeated measures ANOVA for spatial acquisition; (a.) Primary errors, (b.) Secondary errors, (c.) Latency, (d.) Speed, and (e.) Distance (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

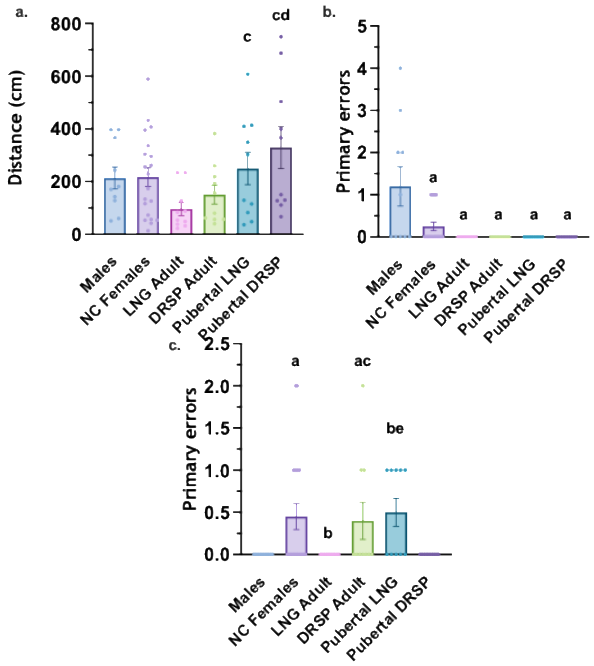

Figure 4. Barnes Maze short-term and long-term memory trials

Note. Group means (±*SEM*) for Day 5, i.e., short-term memory and Day 12, i.e., long-term memory. Data presented as (a.) Distance on Day 5 (Short-term memory trial), (b.) Primary errors on Day 5 (Short-term memory trial), and (c.) Primary errors on Day 12 (Long-term memory trial). (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

The following labels represent pairwise comparisons between groups in the study:

a. Males vs. NC females, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **b.** NC females vs. males, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **c.** LNG Adult vs. males, NC females, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **d.** DRSP Adult vs. males, NC females, LNG Adult, LNG Pubertal, and DRSP Pubertal; **e.** LNG Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and DRSP Pubertal; **f.** DRSP Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and LNG Pubertal.

3.2. Social Recognition

There was a significant main effect of Trials ($F_{(3,186)} = 22.594$, $p < .001$, $\eta p^2 = .27$) and a significant Trials x Group interaction ($F_{(15,186)} = 2.815$, $p < .001$, $\eta p^2 = .19$) for approach behaviour. Pairwise comparisons demonstrated that, when exposed to the novel stimulus mouse (Trial 4), pubertal EE/LNG displayed significantly more approach behaviour than NC (*MD* = 3.68, *SE* = 1.15, $p = .002$), adult EE/LNG (*MD* = 2.97, *SE* = 1.31, $p = .03$), and adult EE/DRSP (*MD* = 3.85, *SE* = 1.31, $p = .01$) females. Pubertal EE/DRSP mice also exhibited more approach behaviour than NC females (*MD* = 2.37, *SE* = 1.12, $p = .04$) during Trial 4.

There was also a significant main effect of both Trials ($F_{(2.869,177.894)} = 20.259$, $p < .001$, $\eta p^2 = .25$) and Groups ($F_{(5,62)} = 3.517$, $p = .007$, $\eta p^2 = .22$) on the duration of chemo-investigation. Moreover, there was a significant interaction on the duration of chemo-investigation ($F_{(14.346,177.894)} = 2.601$, $p < .001$, $\eta p^2 = .17$). Pairwise comparisons indicated that on Trial 1 pubertal EE/LNG displayed significantly greater chemo-investigation duration than NC (*MD* = 17.51, *SE* = 8.76, $p = .05$) and adult EE/DRSP (*MD* = 20.46, *SE* = 9.94, $p = .04$) females. On Trial 2, males displayed greater chemo-investigation duration than NC (*MD* = 18.91, *SE* = 6.48, $p = .005$), adult EE/LNG (*MD* = 18.76, *SE* = 7.42, $p = .01$), adult EE/DRSP (*MD* = 15.43, *SE* = 7.42, $p = .04$), and pubertal EE/LNG (*MD* = 22.25, *SE* = 7.62, $p = .005$) females. On Trial 3, males displayed greater chemo-investigation duration than NC (*MD* = 26.10, *SE* = 6.31, $p < .001$), adult EE/LNG (*MD* = 24.63, *SE* = 7.22, $p = .001$), adult EE/DRSP (*MD* = 22.36, *SE* = 7.22, $p = .003$), and pubertal EE/LNG (*MD* = 30.21, *SE* = 7.42, $p < .001$) females. In addition, Trial 3 also revealed that pubertal EE/DRSP displayed greater chemo-investigation duration than NC (*MD* = 16.79, *SE* = 6.31, $p = .01$), adult EE/LNG (*MD* = 15.33, *SE* = 7.22, $p = .04$), and pubertal EE/LNG (*MD* = 20.90, *SE* = 7.42, $p = .006$) females. Moreover, on Trial 4, males

exhibited significantly more chemo-investigation time than NC females (*MD* = 25.99, *SE* = 7.51, $p < .001$), EE/LNG adults (*MD* = 18.81, *SE* = 8.60, $p = .03$), and adult EE/DRSP (*MD* = 29.53, *SE* = 8.60, $p = .001$). Pubertal EE/DRSP mice also displayed significantly greater chemo-investigation duration than both NC females (*MD* = 22.66, *SE* = 7.51, $p = .004$) and adult EE/DRSP (*MD* = 26.20, *SE* = 8.60, $p = .003$). Finally, pubertal EE/LNG also displayed higher investigation time than adult EE/DRSP (*MD* = 18.16, *SE* = 8.84, $p = .04$) (Figure 5).

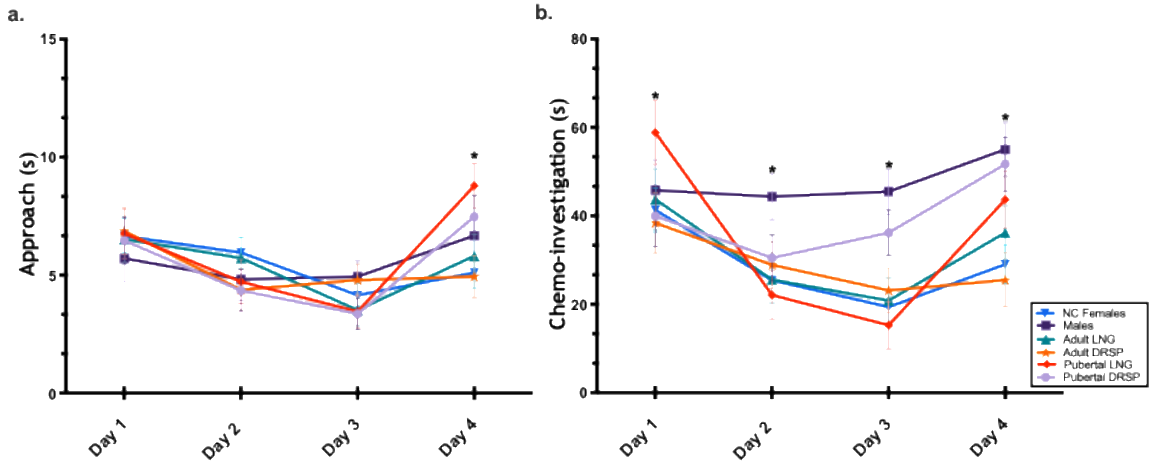

Figure 5. Approach and chemo-investigation time for social recognition

Note. Group means (±*SEM*) in one-way repeated measures ANOVA; (1.) Approach, (2.) Chemo-investigation (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

3.3. NORT

Novel object recognition analyses demonstrated no statistically significant group mean differences ($F_{(5,64)} = 2.039$, $p = .085$, $\eta p^2 = .14$). Groups means (±*SEM*) are shown in Figure 6.

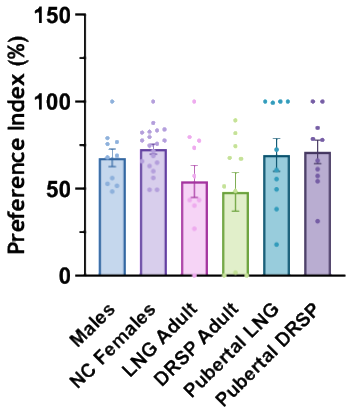

Figure 6. Preference index ratio of the novel object

Note. Means (±*SEM*) of the preference index ~~ratio~~. It represents the ratio of the time each animal spent exploring one object compared to the total time spent on both objects. A preference index above 50% indicates a preference for the novel object, below 50% indicates a preference for the familiar object, and 50% indicates no preference

3.4. Immunohistochemistry

3.4.1 BDNF cell expression

There were significant group mean differences ($F_{(5,58)} = 3.083$, $p = .016$, $\eta^2 = .21$) in BDNF expression in the CA1 region of the hippocampus. Post-hoc analyses showed that pubertal EE/LNG females had a significantly greater BDNF expression compared to males (*MD* = 392.13, *SE* = 109.22, $p < .001$), NC (*MD* = 306.37, *SE* = 92.07, $p = .002$), adult EE/DRSP (*MD* = 214.40, *SE* = 103.62, $p = .04$), adult EE/LNG (*MD* = 236.40, *SE* = 103.62, $p = .03$), and pubertal EE/DRSP (*MD* = 236.67, *SE* = 106.15, $p = .03$) females.

There were significant group mean differences ($F_{(5,58)} = 3.627$, $p = .006$, $\eta^2 = .24$) in BDNF expression in the CA3 region of the hippocampus. Post-hoc analyses revealed that pubertal EE/DRSP had significantly higher BDNF expression compared to adult EE/DRSP

females (*MD* = 234.74, *SE* = 75.40, *p* = .003), control males (*MD* = 264.44, *SE* = 79.74, *p* = .002) and NC females (*MD* = 222.81, *SE* = 66.41, *p* = .001). Post-hoc analyses also indicated that pubertal EE/LNG females had a significantly greater BDNF expression compared to males (*MD* = 173.13, *SE* = 82.06, *p* = .04). No significant group mean difference in BDNF expression in the DG region of the hippocampus was found ($F_{(5,57)} = 1.426$, *p* = .23, $\eta^2 = .11$) (Figure 7).

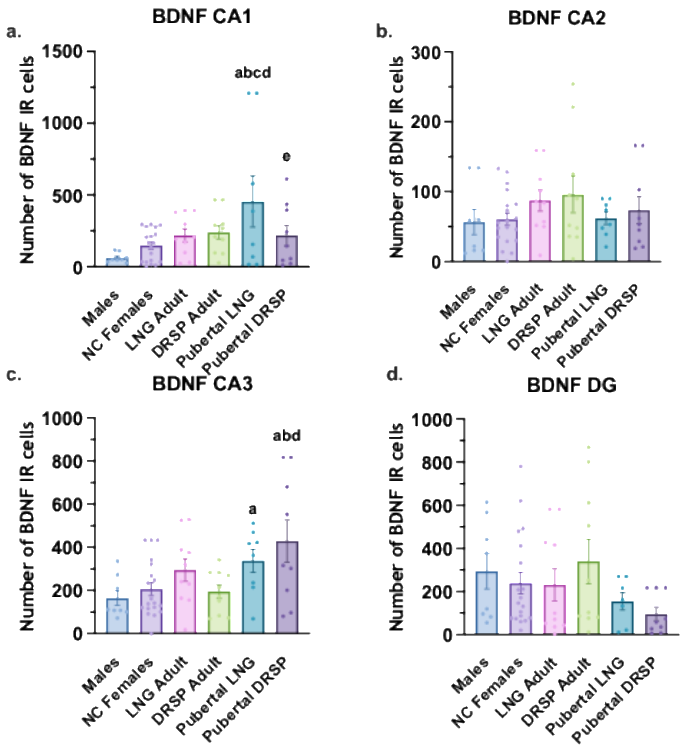

Figure 7. Hippocampal BDNF immunoreactive (IR) cell expression

Note. Group means (±*SEM*) in one-way ANOVA for IHC. Data presented as (a.) CA1, (b.) CA2, (c.) CA3, and (d.) Dentate gyrus ($p < .05$).

The following labels represent pairwise comparisons between groups in the study:

a. Males vs. NC females, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **b.** NC females vs. males, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **c.** LNG Adult

vs. males, NC females, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **d.** DRSP Adult vs. males, NC females, LNG Adult, LNG Pubertal, and DRSP Pubertal; **e.** LNG Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and DRSP Pubertal; **f.** DRSP Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and LNG Pubertal.

There were significant group mean differences ($F_{(5,53)} = 8.788$, $p < .001$, $\eta^2 = .45$) in BDNF expression in the anterior cingulate dorsal (ACAd) region of the medial prefrontal cortex (mPFC). Post-hoc analyses showed that adult EE/LNG females had significantly greater BDNF expression than males (*MD* = 448.11, *SE* = 89.54, $p < .001$), NC females (*MD* = 452.25, *SE* = 79.79, $p < .001$), adult EE/DRSP (*MD* = 436, *SE* = 92.13, $p < .001$), pubertal EE/LNG (*MD* = 412.89, *SE* = 89.54, $p < .001$), and pubertal EE/DRSP (*MD* = 524, *SE* = 89.54, $p < .001$) females. There were significant mean differences ($F_{(5,53)} = 2.917$, $p = .02$, $\eta^2 = .22$) in BDNF expression in the prelimbic (PrL) region of the mPFC. Post-hoc analyses indicated that adult EE/LNG mice displayed significantly greater BDNF expression than males (*MD* = 505.64, *SE* = 192.44, $p = .01$), NC females (*MD* = 402.19, *SE* = 171.49, $p = .02$), adult EE/DRSP (*MD* = 524, *SE* = 198.02, $p = .01$), pubertal EE/LNG (*MD* = 693.75, *SE* = 192.44, $p < .001$), as well as pubertal EE/DRSP (*MD* = 507.64, *SE* = 192.44, $p = .01$) females. There was no statistically significant mean difference ($F_{(5,50)} = .618$, $p = .69$, $\eta^2 = .06$) in BDNF expression in the infralimbic (ILA) region of the mPFC (Figure 8).

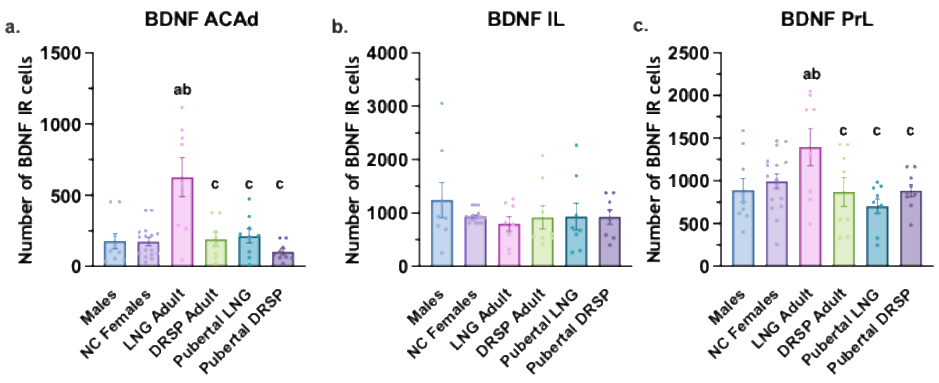

Figure 8. Medial prefrontal cortex BDNF IR cell expression

Note. Group means (±*SEM*) in one-way ANOVA for IHC. Data presented as (a.) ACAd, (b.) IL, and (c.) PrL ($p < .05$).

The following labels represent pairwise comparisons between groups in the study:

a. Males vs. NC females, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **b.** NC females vs. males, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **c.** LNG Adult vs. males, NC females, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **d.** DRSP Adult vs. males, NC females, LNG Adult, LNG Pubertal, and DRSP Pubertal; **e.** LNG Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and DRSP Pubertal; **f.** DRSP Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and LNG Pubertal.

3.4.2. PSD-95 cell expression

There were significant mean differences ($F_{(5,58)} = 4.624$, $p = .001$, $\eta^2 = .29$) in PSD-95 expression in the CA1 region of the hippocampus. Post-hoc analyses demonstrated that pubertal EE/DRSP mice displayed significantly greater PSD-95 expression compared to males (*MD* = 3300.17, *SE* = 752.56, $p < .001$), NC females (*MD* = 2734.37, *SE* = 670.63, $p < .001$), adult EE/LNG (*MD* = 2767.67, *SE* = 752.56 $p < .001$), adult EE/DRSP (*MD* = 2912.07, *SE* = 790.35, $p < .001$), and pubertal EE/LNG (*MD* = 2804.02, *SE* = 769.59, $p < .001$). There was no significant

mean difference in PSD-95 expression in the CA2 ($F_{(5,58)} = .796$, $p = .56$, $\eta^2 = .06$), CA3 ($F_{(5,56)} = 1.649$, $p = .16$, $\eta^2 = .13$), or DG ($F_{(5,54)} = 1.809$, $p = .13$, $\eta^2 = .14$) regions of the hippocampus (Figure 9).

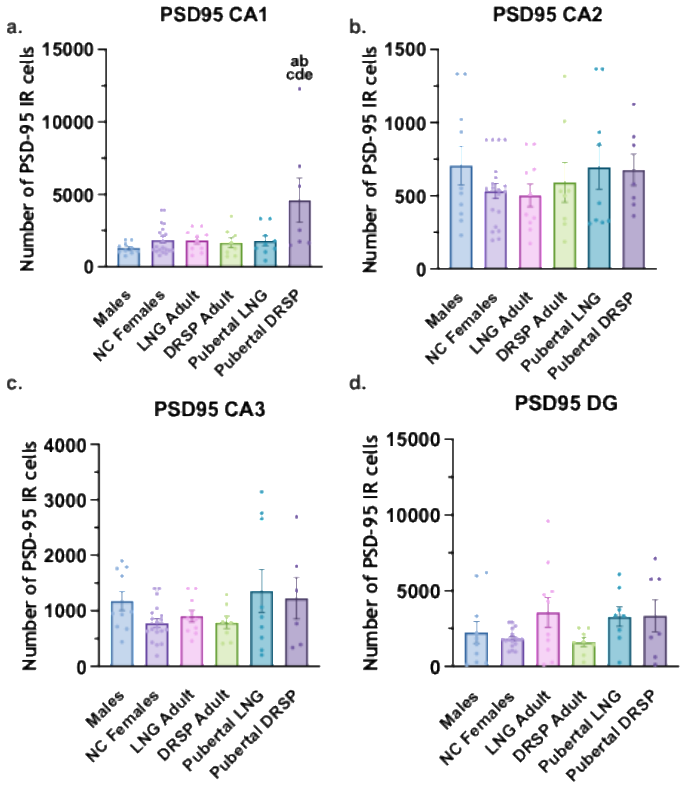

Figure 9. Hippocampal PSD-95 IR cell expression

Note. Group means (±*SEM*) in one-way ANOVA for IHC. Data presented as (a.) CA1, (b.) CA2, (c.) CA3, and (d.) Dentate gyrus ($p < .05$).

The following labels represent pairwise comparisons between groups in the study:

a. Males vs. NC females, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **b.** NC females vs. males, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **c.** LNG Adult vs. males, NC females, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **d.** DRSP Adult vs. males, NC females, LNG Adult, LNG Pubertal, and DRSP Pubertal; **e.** LNG Pubertal vs. males,

NC females, LNG Adult, DRSP Adult, and DRSP Pubertal; **f.** DRSP Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and LNG Pubertal.

There was no significant mean difference ($F_{(5,56)} = 1.450$, $p = .22$, $\eta^2 = .16$) in PSD-95 expression in the ACAd region of the mPFC. Similarly, there was no significant difference for the PRL region ($F_{(5,56)} = 1.238$, $p = .30$, $\eta^2 = .10$). However, there were significant group differences in PSD-95 expression in the ILA region ($F_{(5,56)} = 2.683$, $p = .03$, $\eta^2 = .19$). Post-hoc analyses revealed that pubertal EE/LNG had significantly lower PSD-95 expression than NC females (*MD* = -529.22, *SE* = 195.77, $p = .01$), adult EE/LNG (*MD* = -529.33, *SE* = 226.06, $p = .01$), and pubertal EE/DRSP mice (*MD* = -567.44, *SE* = 226.06, $p = .02$) (Figure 10).

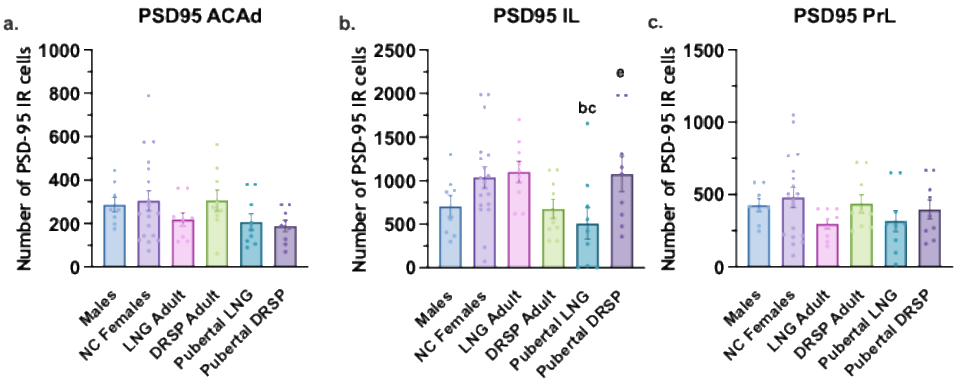

Figure 10. Medial prefrontal cortex PSD-95 IR cell expression

Note. Group means (±*SEM*) in one-way ANOVA for IHC. Data presented as (a.) ACAd, (b.) IL, and (c.) PrL ($p < .05$).

The following labels represent pairwise comparisons between groups in the study:

a. Males vs. NC females, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **b.** NC females vs. males, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **c.** LNG Adult vs. males, NC females, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **d.** DRSP Adult vs.

males, NC females, LNG Adult, LNG Pubertal, and DRSP Pubertal; **e.** LNG Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and DRSP Pubertal; **f.** DRSP Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and LNG Pubertal.

3.5. ELISA

There were significant group differences in progesterone concentrations in the plasma ($F_{(5,61)} = 6.426$, $p < .001$, $\eta^2 = .35$). Post-hoc analyses revealed that NC females had significantly higher progesterone concentrations than males (*MD* = 28002.10, *SE* = 6317.38, $p < .001$), adult EE/LNG (*MD* = 20351.25, *SE* = 6317.38, $p = .002$), adult EE/DRSP (*MD* = 15809.25, *SE* = 6317.38, $p = .02$), and pubertal EE/DRSP mice (*MD* = 19847.05, *SE* = 6317.38, $p = .003$). In addition, pubertal EE/LNG females displayed significantly higher concentrations of progesterone than males (*MD* = 28727.99, *SE* = 7089.17, $p < .001$), adult EE/LNG (*MD* = 21077.15, *SE* = 7089.17, $p = .004$), adult EE/DRSP (*MD* = 16535.15, *SE* = 7089.17, $p = .02$), and pubertal EE/DRSP (*MD* = 20572.95, *SE* = 7089.17, $p = .01$) females.

There were also significant group differences ($F_{(5,58)} = 2.428$, $p = .046$, $\eta^2 = .17$) for estradiol concentration. Post-hoc analyses revealed that adult EE/LNG females had significantly higher estradiol concentration than males (*MD* = 40.20, *SE* = 13.99, $p = .006$), NC (*MD* = 31.65, *SE* = 12.77, $p = .02$), adult EE/DRSP (*MD* = 34.53, *SE* = 13.99, $p = .02$), pubertal EE/LNG (*MD* = 33.37, *SE* = 14.38, $p = .02$), and pubertal EE/DRSP (*MD* = 42.28, *SE* = 13.99, $p = .004$) mice (Figure 11).

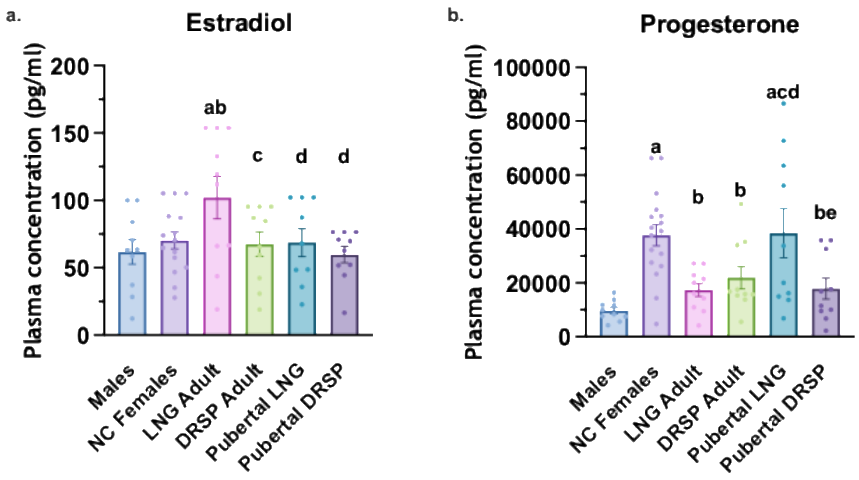

Figure 11. ELISA plasma concentrations (pg/ml)

Note. Group means (±*SEM*) in one-way ANOVA for IHC. Data presented as (a.) Estradiol, and (b.) Progesterone ($p < .05$).

The following labels represent pairwise comparisons between groups in the study:

a. Males vs. NC females, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **b.** NC females vs. males, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **c.** LNG Adult vs. males, NC females, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **d.** DRSP Adult vs. males, NC females, LNG Adult, LNG Pubertal, and DRSP Pubertal; **e.** LNG Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and DRSP Pubertal; **f.** DRSP Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and LNG Pubertal.

3.6. Western blot

There were no significant mean difference in occludin expression at 25kDa ($F_{(5,69)} = .596$, $p = .703$, $\eta^2 = .05$) (Figure 12). However, there were significant mean differences in occludin expression at 54kDa ($F_{(5,69)} = 2.414$, $p = .046$, $\eta^2 = .16$). Post-hoc analyses demonstrated that control males mice displayed significantly greater occludin expression compared to NC females ($MD = 2.93$, $SE = .99$, $p = .002$), adult EE/LNG ($MD = 2.59$, $SE = 1.07$, $p = .019$), pubertal

EE/LNG (*MD* = 2.88, *SE* = 1.07, *p* = .009), and pubertal EE/DRSP (*MD* = 2.91, *SE* = 1.07, *p* = .009) (Figure 12).

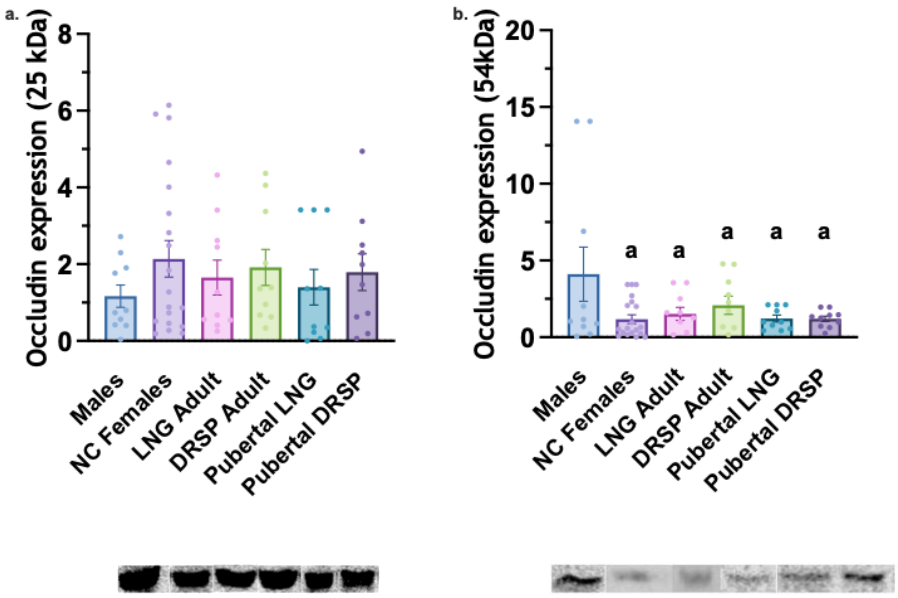

Figure 12. Occludin protein expression

Note. Group means (±*SEM*) in one-way ANOVA for IHC. Data presented as (a.) Occludin expression (25kDa), and (b.) Occludin expression (54kDa) ($p < .05$).

The following labels represent pairwise comparisons between groups in the study:

a. Males vs. NC females, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **b.** NC females vs. males, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **c.** LNG Adult vs. males, NC females, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **d.** DRSP Adult vs. males, NC females, LNG Adult, LNG Pubertal, and DRSP Pubertal; **e.** LNG Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and DRSP Pubertal; **f.** DRSP Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and LNG Pubertal.

Adult, DRSP Adult, and LNG Pubertal.

3.6. 16S rRNA sequencing

3.6.1 Alpha and beta diversity of gut microbiota across experimental groups

Alpha diversity was measured using Chao1 richness, Shannon diversity, and Simpson diversity indices at timepoint 1 and timepoint 3 (Figure 13 A–B). At baseline, groups displayed broadly similar distributions across all indices, and no significant group differences were detected (Kruskal–Wallis, $p > 0.7$ for all). By timepoint 3, variation between groups became more apparent. Adult water males exhibited the highest alpha diversity across all three indices, whereas adult EE/DRSP females showed comparatively lower values. Despite these trends, none of the overall group differences reached statistical significance (Kruskal–Wallis, Chao1 $p = 0.16$; Shannon $p = 0.18$; Simpson $p = 0.26$), and pairwise Wilcoxon tests did not identify any significant pairwise differences after multiple testing correction. Beta diversity was assessed using Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarities (Figure 13 C–D). At both timepoints, samples from different groups overlapped extensively in ordination space, and no clear clustering by age, treatment, or sex was observed.

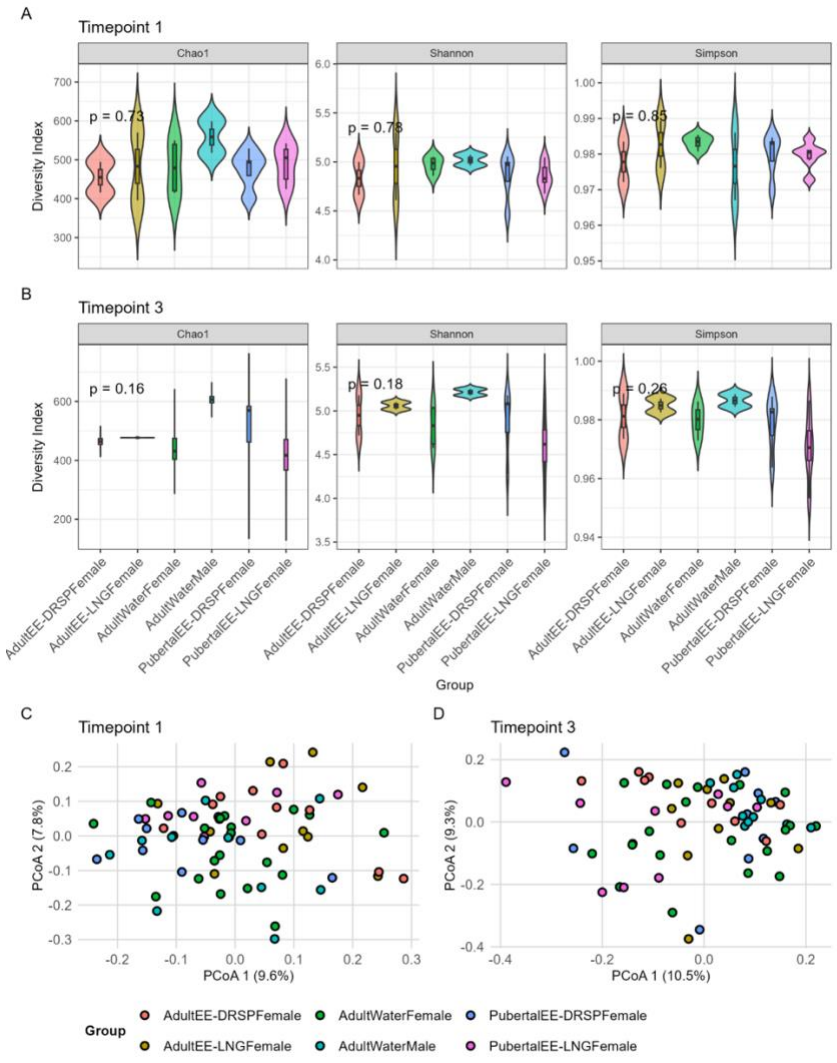

Figure 13. Alpha and beta diversity of gut microbiota across experimental groups and timepoints.

Note. (A–B) Alpha diversity at Timepoint 1 (A) and Timepoint 3 (B) measured by Chao1 richness, Shannon diversity, and Simpson diversity indices. Violin plots show the distribution of diversity values for each experimental group, with embedded boxplots indicating median and interquartile ranges. P-values represent Kruskal–Wallis tests for overall group differences within each diversity metric. Pairwise Wilcoxon rank-sum tests did not identify any significant differences between groups for any alpha diversity metric after multiple testing correction. (C–D)

Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarities for Timepoint 1 (C) and Timepoint 3 (D). Points represent individual samples, coloured by experimental group. Percentages in axis labels indicate the proportion of variation explained by each PCoA axis.

3.6.2. Gut microbiome composition differences across age, treatment, and sex

Although alpha diversity and PCoA ordination did not reveal significant differences between groups, more sensitive pairwise PERMANOVA and dispersion analyses identified consistent and reproducible shifts in microbial community composition. All comparisons comprised of pairwise analysis of all treatment combinations along with mice age to identify shifts in microbial community. Pairwise permanova analyses results are provided in Supplementary File S1. Across 198 total comparisons, nearly half (49%) showed no detectable difference in either metric, while 46% revealed significant composition differences without changes in dispersion. A small proportion of comparisons (4%) exhibited both composition and dispersion differences, and only 1% reflected differences in dispersion alone. These results indicate that most significant findings represented genuine compositional differences, with minimal evidence of confounding by unequal variability.

Effect sizes for significant PERMANOVA comparisons ranged from $R^2 = 0.050$ to 0.150. Several contrasts showed persistent and reproducible differences across multiple timepoints. The most consistent patterns were observed for adult water mice, which displayed distinct community composition across all three timepoints when compared with pubertal EE/DRSP mice (R^2 up to 0.11, $p.\ adj < 0.01$), adult EE/DRSP mice (R^2 up to 0.10, $p.\ adj < 0.05$), and adult EE/LNG mice (R^2 up to 0.10, $p.\ adj < 0.05$). Adult control male mice also differed from pubertal EE/LNG mice ($p.\ adj < 0.05$) and adult control females ($p.\ adj < 0.05$) in at least two timepoints. Additional robust differences were observed for pubertal EE/DRSP mice, which diverged

significantly from both adult EE/DRSP mice and pubertal EE/LNG mice (*p. adj* < 0.05). Likewise, adult EE/LNG mice remained distinct from adult control males across all timepoints (*p. adj* < 0.05) and from adult control females at later timepoints (*p. adj* < 0.05). These findings suggest that specific groups, particularly adult control males, pubertal EE/DRSP mice, and adult EE/LNG mice, harbor stable and divergent microbiome profiles that persist across the experimental timeline.

To further disentangle the relative contributions of natural maturation versus treatment effects, comparisons were classified as either pure age contrasts (pubertal versus adult) or treatment contrasts within the same age group. Pure age contrasts accounted for 54 significant comparisons (average $R^2 = 0.092$, *p. adj* < 0.05), while treatment-within-age contrasts accounted for 46 significant comparisons (average $R^2 = 0.088$, *p. adj* < 0.05). The similarity in both frequency and magnitude indicates that age-related and treatment-related influences were of comparable strength, suggesting that both processes independently contributed to shaping microbiome structures.

3.6.3. Differential abundance in pubertal groups and network analysis of group–taxa associations

Differential abundance analyses were limited to pubertal groups, as these showed the most consistent compositional shifts in PERMANOVA. Comparisons were performed between T1 and T3 within each group (Figure 14A–B). In pubertal EE/DRSP mice, significant enrichment at T3 was observed for *Turicibacter* ($\log_2$ fold change [Log2FC] = +23.5, p. adj < 1×10^{-13}), the family *Sutterellaceae* (Log2FC = +2.07, p. adj = 0.031), and GCA-900066575 (Log2FC = +1.67, *p. adj* = 0.004). Taxa significantly enriched at T1 included the NK4A214 group (Log2FC = –1.19, *p. adj* = 0.004) and *Escherichia–Shigella* (Log2FC = –2.59, *p. adj* =

0.010). The distribution of $-\log_{10}$ adjusted p-values was generally higher in EE/DRSP mice compared with EE/LNG mice, indicating stronger statistical support for differential abundance in this group. In pubertal EE/LNG mice, *Escherichia–Shigella* and members of the family *Enterobacteriaceae* were significantly more abundant at T1, while *Akkermansia, Acetivibrio ethanolgignens* group, and *Lachnospiraceae* UCG-010 were significantly more abundant at T3 ($p.\ adj < 0.05$ for all).

A co-occurrence network was generated using DESeq2 results restricted to T2 and T3, in order to focus on post-treatment differences (Figure 14C). Adult water mice showed the largest number of associations, followed by pubertal EE/LNG mice. Frequently shared taxa across groups included *Parabacteroides*, *Parasutterella, Lachnospiraceae* UCG-006, *Bacteroides*, and the families *Tannerellaceae, Rikenellaceae,* and *Bacteroidaceae.* Group-specific associations were observed for pubertal EE/DRSP mice with *Alistipes* and *Colidextribacter*, while *Turicibacter* was unique to this group.

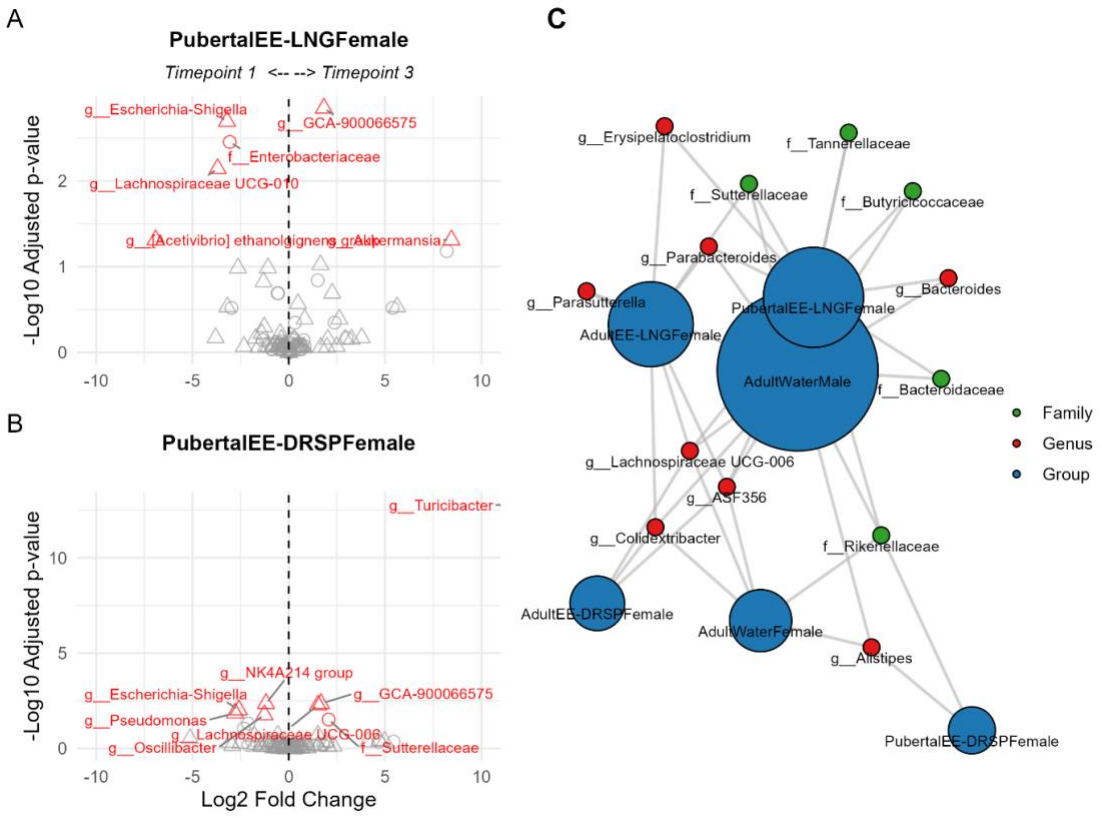

Figure 14. Differential abundance and network analysis of Pubertal EE/LNG female and Pubertal EE/DRSP female groups between T1 and T3.

Note. Differential abundance analyses were limited to pubertal groups, as these showed the most consistent compositional shifts in PERMANOVA. (A–B) Volcano plots showing $\log_2$ fold change (Log2FC) versus $-\log_{10}$ adjusted p-value for taxa at Family (circles) and Genus (triangles) levels, combined in each plot. Positive Log2FC indicates enrichment in timepoint 3, negative Log2FC indicates enrichment in timepoint 1. Taxa with *p. adj* < 0.05 are colored red and labeled; non-significant taxa are grey. The x-axis is truncated at ±10 for clarity, with most points falling within ±5. (C) Co-occurrence network showing significant taxa (Family = green, Genus = red) linked to experimental groups (blue nodes). Node sizes for groups are proportional to their number of significant associations, while taxa nodes are fixed size. Edges represent significant differential

abundance (*p. adj* < 0.05) relationships identified in DESeq2 analyses restricted to timepoints 2 and 3.

3.6.4. Microbial family associations with BDNF and PSD-95 markers

Correlation analyses ($|\rho| \geq 0.5$) revealed widespread associations between microbial families and BDNF and PSD-95, with EE/DRSP and EE/LNG groups showing two- to three-fold more strong correlations than controls (Figure 15). *Bacteroidaceae* showed strong signals mainly in adults, with positive associations with PSD-95 *ACC* and negative associations with BDNF *CA1*, while correlations were weak in pubertal mice and absent in controls. *Tannerellaceae* varied by treatment and age: in adults, EE/DRSP produced mixed associations, whereas EE/LNG females showed a single strong positive association with PSD-95 *CA3*; in pubertal mice, correlations were mostly positive in EE/DRSP and negative in EE/LNG. *Rikenellaceae* demonstrated consistently negative correlations across BDNF subregions and estradiol in nearly all groups, a pattern similarly observed for *Alistipes*. *Prevotellaceae* was repeatedly negatively associated with BDNF *ACC* and occludin 25 kDa in EE/LNG adults and pubertal groups, whereas these associations were minimal or absent in controls.

Among Firmicutes, the *Lachnospiraceae* family showed contrasting treatment patterns: negative associations with BDNF in cortical regions of EE/LNG adults, but positive associations in hippocampal subregions of EE/DRSP adults, with genus *Coprococcus* showing positive associations in EE/DRSP and *Roseburia* showing negative associations in EE/LNG treated mice. *Ruminococcaceae* was found to have positive associations to some regions in BDNF in adult EE/DRSP but were found to be negatively associated with regions in adult EE/LNG. This is largely mirrored in pubertal mice and no association was found in adult female controls. *Oscillospiraceae* was generally positively associated with BDNF across treatments and ages, but

negatively associated with PSD-95 *ILA* in pubertal EE/DRSP. *Erysipelotrichaceae* was repeatedly negatively associated with BDNF and PSD-95 across treatments. *Lactobacillaceae* showed strong positive correlations with PSD-95 *CA3* in EE/DRSP and EE/LNG adults but was absent from controls. *Butyricicoccaceae* was consistently negatively associated with PSD-95 *CA1* and Occludin 25 kDa. Other treatment-linked families included *Clostridiaceae* (negative association with BDNF *CA2* in pubertal EE/DRSP, positive association with PSD-95 *CA1* in EE/LNG), *Sutterellaceae* (negative association with BDNF *CA1* but positive association with PSD-95 *ACC* in EE/LNG), *Desulfovibrionaceae* (positive with PSD-95 *ILA* in EE/DRSP but negative with BDNF), *Eggerthellaceae* (positive association with BDNF *CA3*, negative association with Occludin 25 kDa), and *Christensenellaceae* (positive association with BDNF *CA3* in EE/DRSP adults).

3.6.5. Microbial family associations with Estradiol, Progesterone, and Occludin

Hormone-related associations were stronger and more frequent in COC groups, which showed about two-fold more signals than controls (Figure 15). *Bacteroidaceae* and *Lactobacillaceae* were positively correlated with estradiol in adults under both EE/DRSP and EE/LNG, while *Rikenellaceae*, *Alistipes*, and *Ruminococcaceae* were consistently negatively correlated with estradiol across groups, stronger in treated animals and sparse in controls. For progesterone, Family *XIII* UCG-001 was negatively correlated in both COC groups, while *Marvinbryantia* showed positive associations mainly in EE/LNG adults.

Occludin isoforms showed the clearest treatment-driven contrasts. *Prevotellaceae*, *Coprococcus*, *Butyricicoccaceae*, and UCG-003 were negatively correlated with occludin 25 kDa across EE/DRSP and EE/LNG groups, with only occasional weak signals in controls. *Oscillibacter* was positively associated with occludin 54 kDa in both COC groups, while *Turicibacter* was

negatively associated with occludin 54 kDa specifically in EE/LNG. *Lactobacillaceae* was negatively correlated with occludin 54 kDa in treated adult and pubertal groups but not in controls. Overall, COC groups showed broader estradiol-positive associations (*Bacteroidaceae*, *Lactobacillaceae*) and more consistent occludin 25 kDa negatives *(Prevotellaceae*, *Coprococcus*, *Butyricicoccaceae*), while controls exhibited markedly fewer strong associations.

3.6.6. Microbiome Associations with Social Recognition Test (SRT) Learning and Recall Performance

Linear mixed models identified 282 significant associations (FDR < 0.05) between Δmicrobiome (Timepoint 2→3) and ΔSRT (Timepoint 1→3) during the habituation phase of the social recognition test (Figure S1). In adults, COC treatments yielded dense, negative-leaning profiles. EE/DRSP females (45 associations) showed repeated negatives associations for *Tuzzerella*, *Tyzzerella*, and *Lachnospiraceae* with Approach behaviour, while EE/LNG females (54) were dominated by negatives associations involving *Bacteroides*, *Bacteroidaceae*, *Desulfovibrionaceae*, and *Marinifilaceae* across chemo-investigation and approach behaviours. In contrast, water controls showed more balanced profiles. Adult control males (39) had positives associations for *Bacteroides*, *Roseburia*, and *Ruminococcus* with Investigation, alongside negatives associations for *Tuzzerella* and *Marvinbryantia*. Adult control females (37) showed positives associations with *Rikenellaceae*, *Sutterellaceae*, and *Ruminococcus* for chemo-investigation. In pubertal groups, COC treatments again produced the strongest patterns. EE/DRSP females (59) showed negatives associations for *Desulfovibrionaceae*, *Eggerthellaceae*, and *Marinifilaceae* with chemo-investigation, with positives associations for *Roseburia*, *Rikenellaceae*, and *Sutterellaceae*. EE/LNG females (48) also showed predominantly negative

associations, especially for *Tuzzerella*, *Eggerthellaceae*, and *Lachnospiraceae*, with only isolated positives such as *Tyzzerella*.

At the recall phase (T4 SRT vs. T3 microbiome), a subset of these signals was retained (Figure S2). In adults, overlap included *Tuzzerella* in EE/DRSP females, *Bacteroidaceae* and *Erysipelatoclostridium* in EE/LNG females, *Prevotellaceae* and *Paraprevotella* in water males, and *Paludicola* in water females. In pubertal groups, broader cross-stage concordance was observed: EE/DRSP females showed repeated positives associations for *Rikenellaceae*, *Marvinbryantia*, and *Oscillibacter*, alongside negatives associations for *Erysipelatoclostridium* and *Roseburia*, while EE/LNG females retained positives associations for *Acholeplasmataceae*, *Anaerovoracaceae*, *Ruminococcaceae*, *Anaeroplasma*, *Negativibacillus*, and *Tyzzerella*, and negatives associations for *Clostridiaceae*. Together, these results indicate that several taxa associated with learning performance also remained significant during recall, most consistently *Tuzzerella*, *Lachnospiraceae*, and *Clostridiaceae* (negative under COC treatment) and *Bacteroides*, *Roseburia*, *Rikenellaceae*, and *Sutterellaceae* (positive in water groups). These overlapping signals suggest that microbiome–behaviour associations were not limited to initial learning but extended into recall performance, highlighting stable links across both phases of the SRT.

3.6.7. Associations between gut microbiota and cognitive performance (NORT & Barnes Maze)

In the NORT (Figure S3), nominal associations ($p < 0.05$, uncorrected) revealed treatment- and age-specific patterns. In adults, EE/LNG females showed predominantly positive associations, with repeated signals for *Monoglobus*, *Tuzzerella*, and *Turicibacter*, while EE/DRSP females were characterized by largely negative associations, including *Sutterellaceae*

and *Ruminococcaceae*. Adult control males also showed positive profiles, notably involving *Monoglobus*, *Tuzzerella*, and Pseudomonas, whereas adult control females showed fewer and more balanced associations. In pubertal groups, EE/DRSP females exhibited both positive and negative patterns, including Pseudomonas and *Ruminococcaceae*, while EE/LNG females displayed only sparse associations. After FDR correction, significant associations remained restricted to adults: EE/LNG females showed positives for *Turicibacter*, *Monoglobus*, and *Monoglobaceae* and negatives for *Ruminococcaceae* and *Erysipelatoclostridiaceae*, and EE/DRSP females showed a negative association for *Sutterellaceae*.

In the Barnes Maze acquisition phase (Figures S4A–C), associations were more numerous but again differed by treatment and age. Adult EE/LNG females showed consistently negative relationships, with repeated signals for *Lachnospiraceae* groups, *Erysipelatoclostridium*, and *Acholeplasmataceae*, whereas EE/DRSP females yielded dense profiles across Days 1–2 that included both negative *Lachnospiraceae* signals and positive associations with *Blautia*, *Escherichia-Shigella*, and *Ruminococcaceae*. Adult water groups showed more balanced patterns, with positives involving *Rikenellaceae* and *Blautia*. In pubertal groups, EE/DRSP females became strongly positive by Day 3, with recurrent associations involving *Tuzzerella*, *Ruminococcaceae*, and *Escherichia-Shigella*, while EE/LNG females produced few consistent signals. In the probe phase (Day 12; Figure S4D), associations were led by adult EE/DRSP and pubertal EE/LNG females and were dominated by negatives, particularly for duration-related metrics, including repeated effects for *Incertae Sedis* and [*Eubacterium*] *nodatum* group, alongside positives for *Acetitomaculum*. No Barnes Maze associations survived FDR correction.

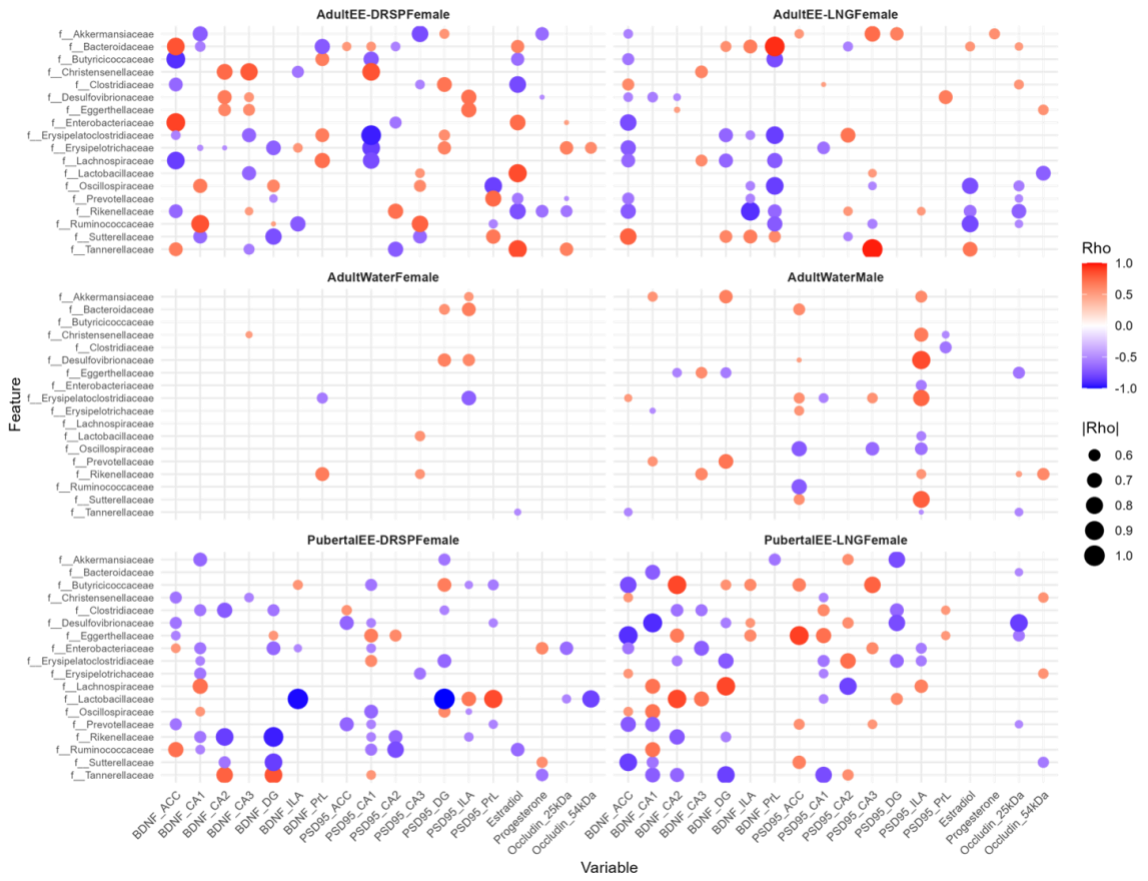

Figure 15. Group specific correlations between gut microbial genera and host IHC/hormonal markers.

Note. Faceted dot plots show Spearman correlations ($|\text{Rho}| \geq 0.5$) between selected genera and host markers across six experimental groups: adult EE/DRSP females, adult EE/LNG females, adult control females, adult control males, pubertal EE/DRSP females, and pubertal EE/LNG females. The x axis lists host variables, ordered by functional category: BDNF regions, PSD-95 regions, hormones (e.g., estradiol, progesterone), and occludin isoforms. Dot color represents the correlation coefficient (red = positive, blue = negative), with intensity indicating magnitude. Dot size corresponds to the absolute correlation coefficient (larger = stronger association). Only taxa–marker pairs meeting the |Rho| threshold within each group are shown.

4. Discussion

COCs have been available to females for more than 60 years and have significantly contributed to their reproductive rights (Bailey & Lindo, 2017; Liao & Dollin, 2012). Despite their safety and widespread use by both adolescent and adult females (Sundstrom & Delay, 2020), our understand of the mechanisms through which COCs may impact brain structure and function remains limited (Cahill, 2018; Pletzer & Kerschbaum, 2014), particularly during critical developmental periods like puberty (Lacasse et al., 2024; Song et al., 2022). The gut-brain axis could have mediating effects that could further explain the mechanisms by which COCs shape cognition. This study examined the impacts of COCs on the brain, cognitive function, and gut microbiome. Our results showed that COC use during puberty has a negative impact on cognition, as well as on BDNF and PSD-95 expressions in the HIPP, but these effects are formulation- and region-dependent. Our results also demonstrate age- and formulation-dependent effects of COCs on the gut microbiota. In addition, we observed significant associations between the microbiome and neuroplasticity measures and cognitive function, suggesting modulating effects of COCs on the gut-brain axis.

Pubertal EE/LNG females displayed increased approach behaviour compared to NC females as well as adults EE/LNG and EE/DRSP when presented with a novel stimulus. This finding indicates increased curiosity, interest in novelty, and recognition of unfamiliar stimulus. Pubertal EE/DRSP showed increased overall chemo-investigation duration in trials three and four, suggesting a sustained interest even towards familiar mice. These findings partially support our hypothesis that COC use during puberty alters cognition relative to COC use during adulthood, particularly in EE/LNG users. Ovariectomized pubertal female rats show impaired social recognition (Yoest et al., 2023), suggesting that ovarian hormones regulate social

behaviour. Given that COCs inhibit the HPG axis, they may interfere with dopaminergic pathways, involved in regulating novelty seeking behaviour and social memory. In mice, dopamine (DA) signaling in the nucleus accumbens (NAc) has been shown to regulate social behaviours (Dai et al., 2021; Dai et al., 2022). DA signaling in the ventral tegmental area (VTA)-NAc circuit is also associated with social interaction and investigation of unfamiliar mice (Bariselli et al., 2018). Nevertheless, the impact of COC use on DA neuron expression in the VTA-NAc pathway and its effects on social behaviour remain unclear. While, a decrease in DA neurons in regions like the striatum has been associated with varying doses of 1st and 2nd COCs generations (Jori & Dolfini, 2008; Pletzer et al., 2023), no research has been conducted on newer generations.

Pubertal EE/LNG mice displayed more primary errors, longer latency to find the escape box, and distance travelled compared to all other groups during spatial acquisition, although performance improved on days 3 and 4. In contrast, pubertal EE/DRSP females displayed a mixed pattern with increased latency and primary errors, but fewer secondary errors and shorter latency compared to NC females and COCs treated adults. These results support our hypothesis that both COC formulation and age of onset would affect cognition, with LNG having slightly more detrimental effects. Levonorgestrel (LNG) is a 2nd generation progestin derived from testosterone, and has high androgenic potency (Chao & Page, 2016; Zaveri & Murphy, 2007). EE/LNG use (4–6 weeks) has been associated with increased expression of γ-aminobutyric acid type A (GABAa) receptor subunits in the rat cerebral cortex and HIPP, as well as elevated hippocampal BDNF protein levels, BDNF mRNA expression, and GABA levels throughout the brain following exposure to 1st and 2nd generation progestins (Boi et al., 2022; Pletzer et al., 2023; Porcu et al., 2012; Porcu et al., 2019; Simone et al., 2015). These findings suggest that

LNG induces specific alterations on neural activity and plasticity which might result in altered cognition. In contrast, DRSP may exert different effects due to low binding affinity to androgen and glucocorticoid receptors and high affinity for progesterone and mineralocorticoid receptors (Dukes, 2009; Elger et al., 2003; Fuhrmann et al., 1996; Oelkers, 2004). Interestingly, EE doses and hormones combination may moderate these effects as well. EE administration display a dose-dependent, nonlinear associations with cognitive deficits (e.g., learning and memory) in intact and ovariectomized female rats (Mennenga et al., 2015; Simone et al., 2015). EE/LNG has been shown to impair learning and memory in intact female rats (Simone et al., 2015), and impairs spatial working memory in middle-aged rodents (Braden et al., 2017; Prakapenka et al., 2018). DRSP has beneficial effects on spatial memory in ovariectomized rodents, but when combined with EE, the effects are reduced (Koebele et al., 2022). These findings suggest that COC-associated cognitive deficits are dependent on formulation and hormone doses.

Pubertal females treated with EE/LNG displayed increased BDNF expression in the CA1 region of the HIPP, potentially indicating disrupted neuroplasticity and supporting our hypothesis. BDNF is a protein involved in neurogenesis, neuroplasticity, learning, and memory (Kowiański et al., 2017; Miranda et al., 2019; Mizuno et al., 2000; Toh et al., 2018). It is highly expressed in regions regulating cognitive function such as the mPFC and HIPP (Chang et al., 2021). The dorsal HIPP in particular, is essential for learning, memory representation, spatial navigation, and exploration (Clark et al., 2000; Fanselow & Dong, 2010; Kim & Frank, 2009; Moser et al., 1993). More specifically, the CA1 region of dorsal HIPP is associated with spatial memory and navigation, learning, episodic memory, and object recognition (Ásgeirsdóttir et al., 2020; Bartsch et al., 2011; Bendel et al., 2005; Danielson et al., 2016). While BDNF is typically associated with improved cognitive function, COC use during puberty may disrupt BDNF

expression (increase or decrease), which could result in increased cognitive deficits such as the ones we observed in the Barnes Maze. This suggests a nonlinear relationship between BDNF expression and cognitive function potentially following an inverted U-shaped curve pattern that could be mediated by neurotransmitters. For example, N-methyl-d-aspartate (NMDA) receptors are essential for synaptic plasticity (Hunt & Castillo, 2012; Malenka & Bear, 2004), and support spatial working memory (Yamada et al., 2017; Yoshihara & Ichitani, 2004). BDNF supports synaptic plasticity by modulating NMDA receptor (NMDARs) function and promoting synaptic accumulation of GluN2β which contain NMDARs (Leal et al., 2017; Lu et al., 2014). BDNF also partially supports synthesis of Pyk2 (PTK2β), which promotes synaptic integration of GluN2B subunits into NMDARs (Afonso et al., 2019). These mediating effects of BDNF on NMDAR may support long-term potentiation and memory function. However, in the context of COC use during puberty, it is plausible that the increased BDNF expression in the CA1 region of EE/LNG pubertal mice may reflect disruptions in NMDAR-mediated pathways in the hippocampus resulting in cognitive impairments, though future research is needed.

In addition, pubertal EE/DRSP females showed increased BDNF expression in the HIPP CA3 region. However, despite this increase, no major cognitive deficits were observed in this group. This could be attributed to the region involved as the CA3 region plays a key role in memory consolidation and retrieval (Dong et al., 2021; Gilbert & Brushfield, 2009; Sammons et al., 2024; Senzai, 2019). Pubertal EE/DRSP females also displayed increased PSD-95 expression in the CA1. PSD-95 is a scaffold protein located in the postsynaptic membrane of excitatory neurons and is part of the membrane associated guanylate kinase group (Xu, 2011). It plays a role in organizing postsynaptic density by aggregating α-amino-3-hydroxy-5-methyl-4-isox-azoleproprionic acid and NMDA receptors, and other scaffolding proteins (Huganir & Nicoll,

2013; Lin et al., 2004). As overexpression of PSD-95 modulates glutamatergic transmission and synaptic plasticity (Stein et al., 2003; Zhang & Lisman, 2012; Zheng et al., 2011), increased PSD-95 expression may have stabilized synaptic connections, mitigating COC-induced disruptions and preserving cognitive functions such as spatial memory.

Our 16S rRNA sequencing analyses revealed that COC use, especially EE/DRSP during puberty, induced significant changes in gut microbiota composition, which might have distinct implications for cognition, neuroplasticity, and gut permeability. While no alterations in alpha and beta diversity were observed, we identified distinct microbial profiles, especially between controls and COC treated pubertal groups which suggests disrupting effects of COCs on microbial composition.

EE/DRSP treated pubertal females displayed an enrichment of *Turicibacter* and *Sutterellaceae* at T3. *Turicibacter* is a genus associated with bile acid changes and lipid metabolism, which might modulate inflammation through bile acid signaling pathways (Lynch et al., 2023; Zhao et al., 2025). In addition, *Sutterellaceae* is a family that displays both pro-inflammatory (e.g., cytokine production) and anti-inflammatory (e.g., IL-10 production) modulating effects (Davoody et al., 2025). It also has modulating effects on tryptophan metabolism, systemic inflammation, and gut barrier integrity, resulting in increased vulnerability for neurological and psychiatric conditions (e.g., anxiety, depression, and autism spectrum disorder) (Davoody et al., 2025). Tryptophan is an amino-acid essential for protein-synthesis and gut microbial composition that impacts tryptophan metabolism (Gao et al., 2020). Tryptophan also plays an important role in serotonin production (Gao et al., 2020; O'Mahony et al., 2015) through tryptophan hydroxylase enzymes (TPH) (Israelyan & Margolis, 2018). Alterations in TPH expression have been associated with anxiety disorder (Donner et al., 2012). Additionally, a

reduction of pro-inflammatory *Escherichia-Shigella* (Ashida et al., 2015; Belotserkovsky & Sansonetti, 2018) is also associated with mental illnesses like anxiety disorders (Baske et al., 2024). These findings highlight complex interactions between bile acid modification, tryptophan metabolism, and inflammation, with *Sutterella's* complex role possibly influencing brain health in a context-dependent manner. Furthermore, pubertal EE/DRSP females displayed an enrichment of *Alistipes*, a genus part of the Bacteroidetes phylum which has been related to depression in humans via downregulation of serotonin availability (Naseribafrouei et al., 2014; Parker et al., 2020). These microbial shifts suggest that EE/DRSP treatment may increase vulnerability to mood disorders by altering serotonin production and inflammatory pathways.

Moreover, EE/LNG-treated pubertal females showed enriched *Akkermansia* and *Lachnospiraceae* at T3 (i.e., 3rd fecal extraction prior to euthanasia) and at which they were 12 weeks of age. Both taxa are associated with greater gut-barrier integrity and short-chain fatty acids (SCFAs) production (Liu et al., 2022; Muriel, 2022). SCFAs are essential gut-produced metabolites (e.g., acetate, propionate, and butyrate) that support gut barrier integrity, as well as immune and neuroendocrine function (Cryan et al., 2019). Interestingly, *Lachnospiraceae* has been shown to produce SCFAs such as butyrate (Halfvarson et al., 2017), which are associated with central nervous system function by influencing neurotransmitter production (e.g., GABA, serotonin, dopamine), as well as supporting blood–brain barrier integrity and synaptic plasticity (Koutromanos et al., 2025). Butyrate promotes serotonin synthesis via enterochromaffin cells, which activates vagal afferents via 5-HT3 receptors and influences gut-brain axis function (Fukumoto et al., 2003). Butyrate also inhibits histone deacetylase, promoting histone acetylation and increasing BDNF gene expression, which may have critical implications for neuroplasticity,

cognitive functioning, and neurological and mental disorders (Calfa et al., 2012; Rajilić-Stojanović et al., 2012; Stilling et al., 2016).

Correlation analyses revealed significant associations between gut composition and both BDNF and PSD-95 expressions among EE/DRSP and EE/LNG treated groups with two- to three-fold stronger correlations compared to controls suggesting that COC use may modulate the gut-brain axis. As such, *Bacteroidaceae* was positively associated with PSD-95 expression in the ACC and negatively associated with BDNF expression in the CA1 in adult groups. These results are consistent with research indicating modulating effects of gut microbiota on synaptic plasticity (Damiani et al., 2023). However, further research is needed to better understand the mechanisms by which *Bacteroidaceae* may shape the brain. *Tannerellaceae* displayed associations that were marker and treatment specific. EE/DRSP adults displayed mixed correlations with BDNF and PSD-95 expression across regions. However, EE/LNG adults had positive associations with PSD-95 expression in the CA3 region which suggests progestin-specific effects on synaptic plasticity. Among *Firmicutes*, *Lachnospiraceae* showed treatment-specific associations: a negative correlation with BDNF expression in ACC of EE/LNG-treated adult mice, but a positive correlation in the HIPP of EE/DRSP-treated adults. These findings suggest that COC may impact the gut-brain axis, with distinct microbial families contributing to region-specific neurobiological outcomes, potentially influencing brain function.

In addition, *Prevotellaceae*, *Coprococcus*, *Butyricicoccaceae*, and UCG-003 were negatively correlated with 25 kDa occludin isoform in EE/DRSP and EE/LNG groups. Higher abundance of these taxa may thus be linked to reduced occludin expression, potentially compromising gut barrier function. *Prevotellaceae's* negative correlation aligns with prior evidence of associations with mucosal inflammation and gut-brain axis dysregulation, which

may contribute to mental health disorders such as anxiety and depression (Simpson et al., 2020). 54 kDa occludin isoform showed positive association with *Oscillibacter* and negative association with *Lactobacillaceae* across COC groups, whereas *Turicibacter* was negatively associated in EE/LNG groups only. These results suggest that specific COC formulations differentially alter gut microbial composition and function.

The SRT showed negative associations between *Tuzzerella*/*Lachnospiraceae* and chemo-investigation in pubertal EE/DRSP and EE/LNG females. *Lachnospiraceae* produce SCFAs which support intestinal epithelial cell function, and their reduction may indicate compromised barrier integrity and immune activation (Furusawa et al., 2013; Keshavarzian et al., 2020). In contrast, adult control males showed positive associations with *Roseburia* known to mitigate inflammation via SCFA production and cytokine regulation (Nie et al., 2021), thus suggesting sex-specific protective effects for some cognitive functions. Moreover, adult EE/LNG females exhibited positive associations with *Turicibacter*, supporting improved object recognition, while adult EE/DRSP females showed negative associations with *Sutterellaceae* which modulates SCFAs metabolism (Peterson et al., 2022). Taken together, these findings suggest COC-induced microbiota modifications that are associated with brain plasticity, cognition and behaviour alterations, with pubertal females showing greater sensitivity.

4.1 Limitations

This study provides insights on the effects of COC use on cognition, neuroplasticity, through gut-microbiota mediating effects. Nevertheless, several limitations remain. First, we examined two COC formulations (EE/LNG and EE/DRSP), despite several other formulations available, which limits the interpretations of our findings to specific progestins and doses. Second, our analyses between gut microbial composition and other measures were correlational,

preventing causal inferences. Future studies should investigate the mechanisms and pathways by which COCs shape the gut-brain axis. Third, the absence of a placebo period, typical in human COC regimens, may not fully reflect clinical use patterns. Fourth, we did not investigate other brain markers (ex., NMDA receptor subunits, GABA receptors) which are known to support cognitive function and modulate cognition via the gut-brain axis (Maqsood & Stone, 2016; Strandwitz et al., 2019). Finally, a critical limitation is the timing discrepancy between behavioural tests and fecal sample collection, which were conducted between T2 and T3 which were collected before and after the completion of behaviour testing, respectively. This limits our ability to draw solid conclusions about the direct relationships between gut microbiota changes and cognitive outcomes.

4.2 Conclusion

This study reveals that COC use significantly influences cognition, neuroplasticity, and gut microbiota in female mice, with distinct effects based on formulation and age. Pubertal EE/LNG females displayed increased social approach behaviour and HIPP CA1 BDNF expression but impaired spatial learning. Females treated with EE/DRSP during puberty showed some spatial learning impairments, sustained chemo-investigation and increased HIPP CA3 BDNF expression and CA1 PSD-95 expression, highlighting formulation-specific brain changes. Adult EE/LNG mice displayed increased mPFC BDNF expression, suggesting region-specific effects of COC use. Microbiome profiles shifted most in pubertal EE/LNG and EE/DRSP groups, supporting treatment and age effects on gut composition. For COC groups, these shifts correlated more strongly with synaptic plasticity markers, suggesting direct interactions between COC use and the gut-brain axis via mechanisms that have yet to be investigated. Finally, our

results highlight puberty as a period of heightened vulnerability to COC use reinforcing the necessity for careful risk–benefit evaluation in adolescents.

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Declaration of Interests: None

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Supplementary material

Software and Packages
All analyses were conducted in R v4.4.1. The following R packages were used (version numbers in parentheses):

- **Core statistical modeling**: MuMIn (1.48.11), lmerTest (3.1.3), lme4 (1.1.37), Matrix (1.7.0), mgcv (1.9.3), nlme (3.1.164).
- **Differential abundance and omics**: DESeq2 (1.44.0), Maaslin2 (1.18.0), limma (3.60.6), genefilter (1.86.0), impute (1.78.0).
- **Data structures**: SummarizedExperiment (1.34.0), Biobase (2.64.0), MatrixGenerics (1.16.0), matrixStats (1.3.0), GenomicRanges (1.56.2), GenomeInfoDb (1.40.1), IRanges (2.38.1), S4Vectors (0.42.1), BiocGenerics (0.50.0), stats4 (4.4.1), BiocParallel (1.38.0).
- **Microbiome-specific**: phyloseq (1.48.0), microbiome (1.26.0), vegan (2.6.8), ape (5.8), permute (0.9.7).
- **Batch correction**: sva (3.52.0).
- **Graph/network analysis**: igraph (2.0.3), ggraph (2.2.1).
- **Visualization**: ggplot2 (3.5.2), patchwork (1.3.0), ggpubr (0.6.0), ggrepel (0.9.6), pheatmap (1.0.12), RColorBrewer (1.1.3), grid (4.4.1), lattice (0.22.6).
- **Data wrangling**: tidyverse (2.0.0), dplyr (1.1.4), tidyr (1.3.1), readr (2.1.5), tibble (3.2.1), stringr (1.5.1), forcats (1.0.0), purrr (1.0.2), reshape2 (1.4.4).
- **Statistical utilities**: rstatix (0.7.2), umap (0.2.10.0).
- **Other data handling**: haven (2.5.4), lubridate (1.9.3).

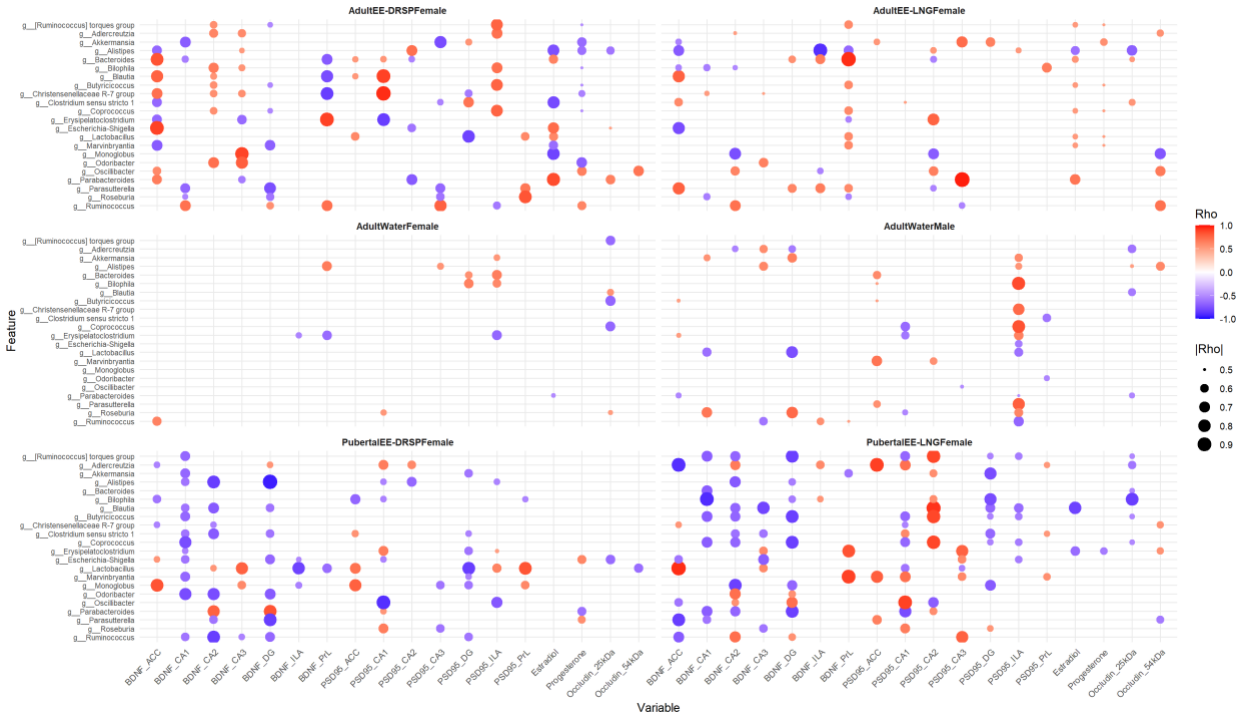

Figure S1. Group specific correlations between gut microbial genus and host IHC/hormonal markers.

Note. Faceted dot plots show Spearman correlations ($|Rho| \geq 0.5$) between selected genus and host markers across six experimental groups: adult EE/DRSP females, adult EE/LNG females, adult control females, adult control males, pubertal EE/DRSP females, and pubertal EE/LNG females. The x axis lists host variables, ordered by functional category: BDNF regions, PSD-95 regions, hormones (e.g. estradiol, progesterone), and occludin isoforms. Dot color represents the correlation coefficient (red = positive, blue = negative), with intensity indicating magnitude. Dot size corresponds to the absolute correlation coefficient (larger = stronger association). Only taxa–marker pairs meeting the |Rho| threshold within each group are shown.

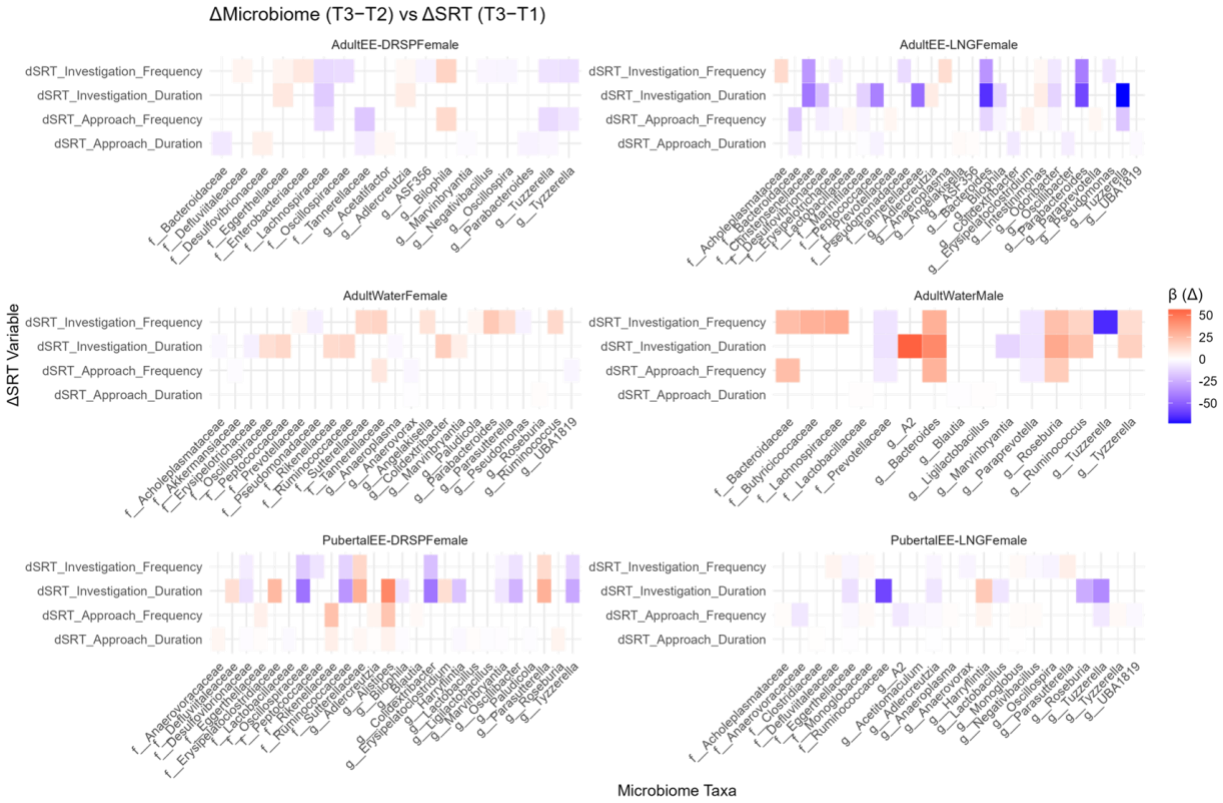

Figure S2. Associations between changes in gut microbial families and social recognition test (SRT) performance. Heatmaps show significant associations (linear mixed models, FDR-adjusted $p < 0.05$) between changes in microbial abundance from timepoint 2 to 3 (ΔMicrobiome, CLR-transformed) and changes in SRT performance from timepoint 1 to 3 (ΔSRT). Behavioral metrics include Investigation Frequency, Investigation Duration, Approach Frequency, and Approach Duration. Tiles are colored by standardized regression coefficients (β), with red indicating positive associations (increases in both microbiome abundance and behavioral performance) and blue indicating negative associations (increases in microbiome abundance associated with decreases in performance). Analyses were performed separately for each experimental group (AdultEE-DRSPFemale, AdultEE-LNGFemale, AdultWaterFemale, AdultWaterMale, PubertalEE-DRSPFemale, PubertalEE-LNGFemale), controlling for housing cage as a random effect.

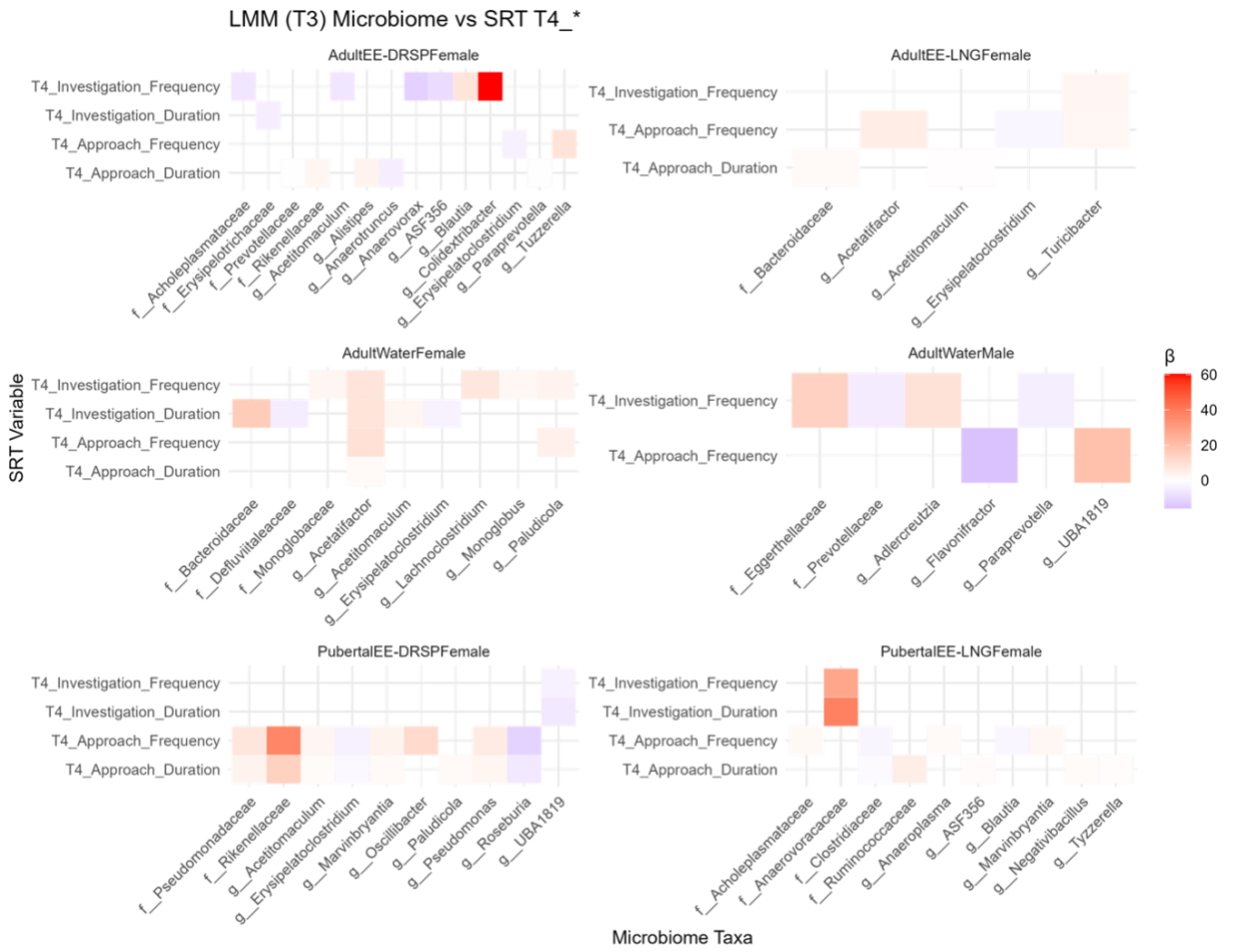

Figure S3. Gut microbiome associations with social recognition test outcomes at T4. Heatmaps show significant associations (linear mixed models, FDR-adjusted $p < 0.05$) between microbial abundance at timepoint 3 (CLR-transformed) and social recognition performance at timepoint 4. Behavioral metrics include Investigation Frequency, Investigation Duration, Approach Frequency, and Approach Duration. Tiles are colored by standardized regression coefficients (β), where red indicates positive associations (greater microbial abundance linked to higher behavioral values) and blue indicates negative associations. Analyses were conducted separately for each experimental group (AdultEE-DRSPFemale, AdultEE-LNGFemale, AdultWaterFemale, AdultWaterMale, PubertalEE-DRSPFemale, PubertalEE-LNGFemale), with housing cage included as a random effect.

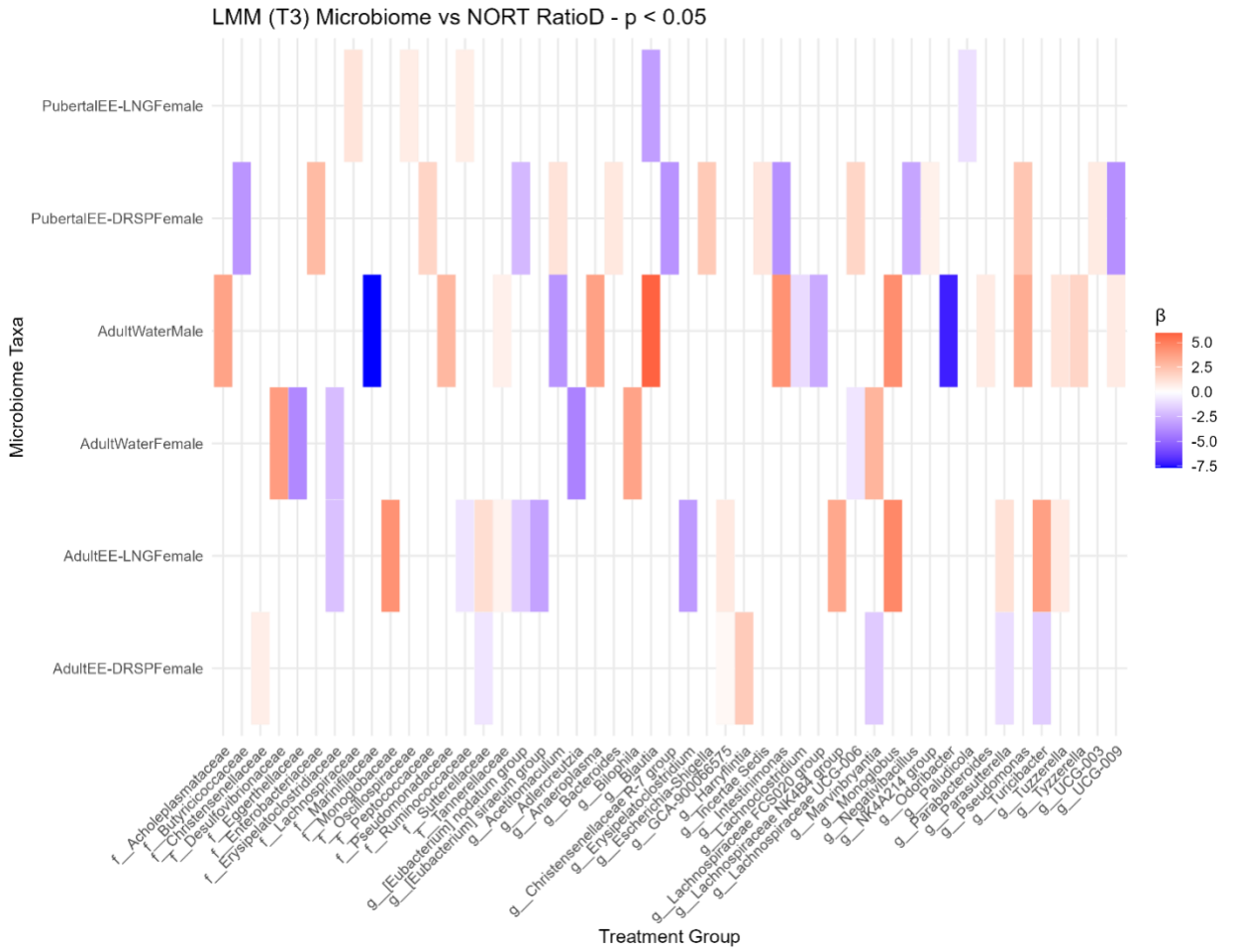

Figure S4. Associations between gut microbiota and novel object recognition (NORT) performance at timepoint 3. Heatmap shows taxa at the family and genus levels significantly associated with the NORT discrimination ratio (RatioD) within each experimental group ($p < 0.05$, linear mixed models with cage as a random effect). Rows indicate groups (PubertalEE-LNGFemale, PubertalEE-DRSPFemale, AdultWaterMale, AdultWaterFemale, AdultEE-LNGFemale, AdultEE-DRSPFemale), and columns represent microbial taxa. Tile color indicates the regression coefficient (β), with red denoting positive associations and blue denoting negative associations between microbial CLR-transformed abundance and NORT performance. Only associations passing the raw p-value threshold are displayed.

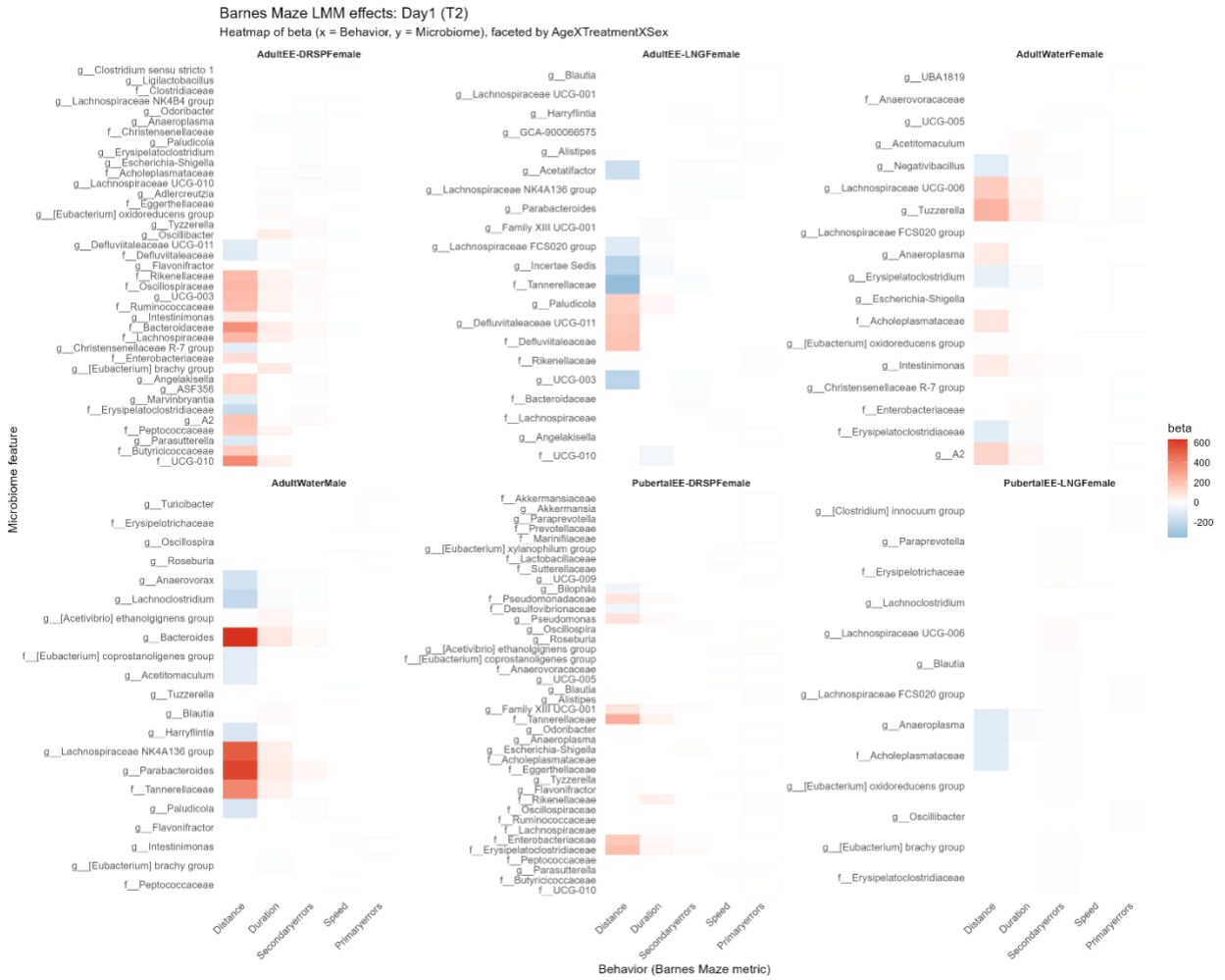

Figure S5A. Heatmap of linear mixed-model associations ($p < 0.05$) between microbiota and Barnes Maze performance on Day 1 (timepoint 2). Rows represent taxa at family and genus levels; columns represent behavioral metrics. Red indicates positive β coefficients, blue negative.

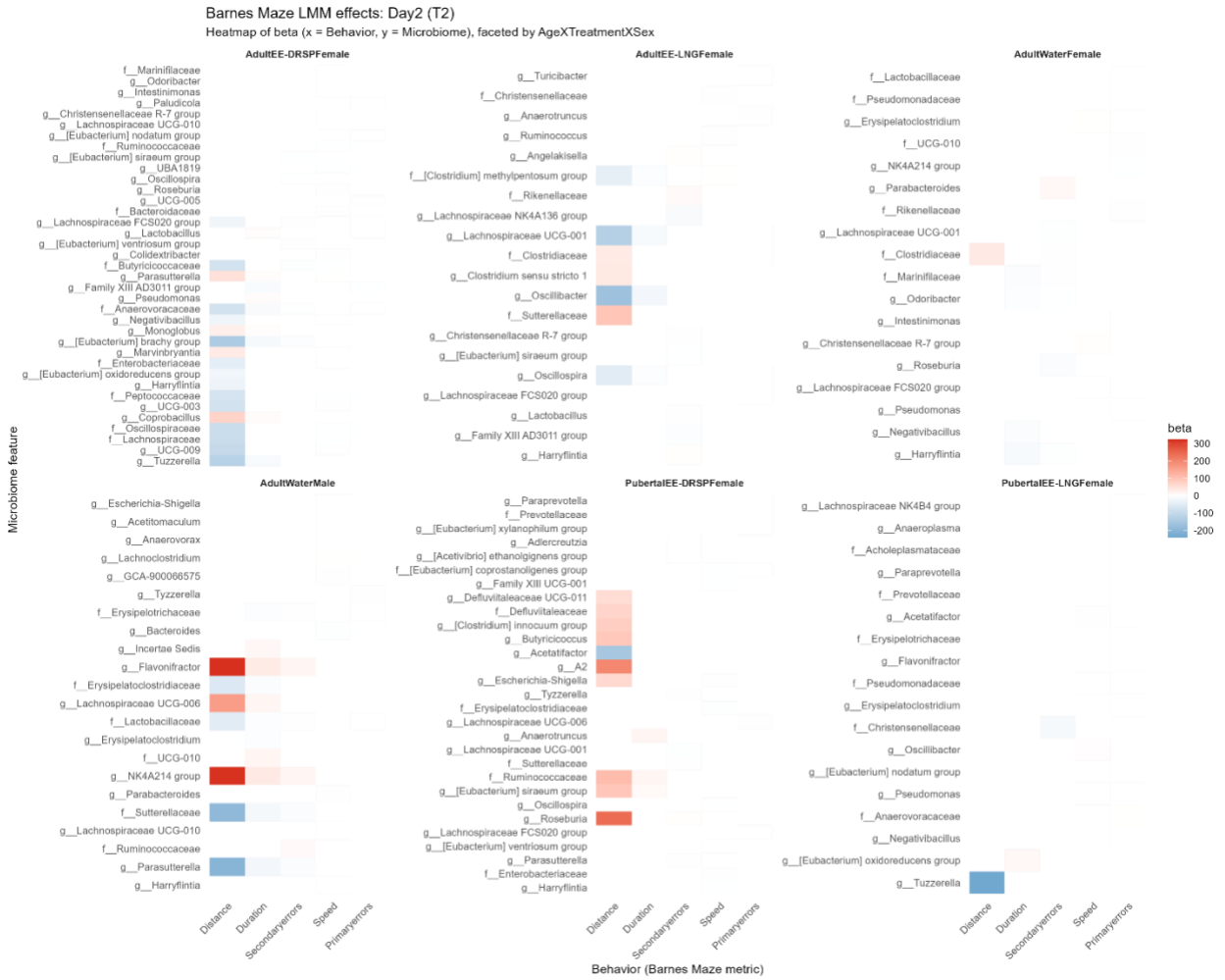

Figure S5B. Heatmap of microbiota–Barnes Maze associations on Day 2 (timepoint 2). Significant taxa–behavior links ($p < 0.05$) are shown, with effect direction and strength coded by tile color.

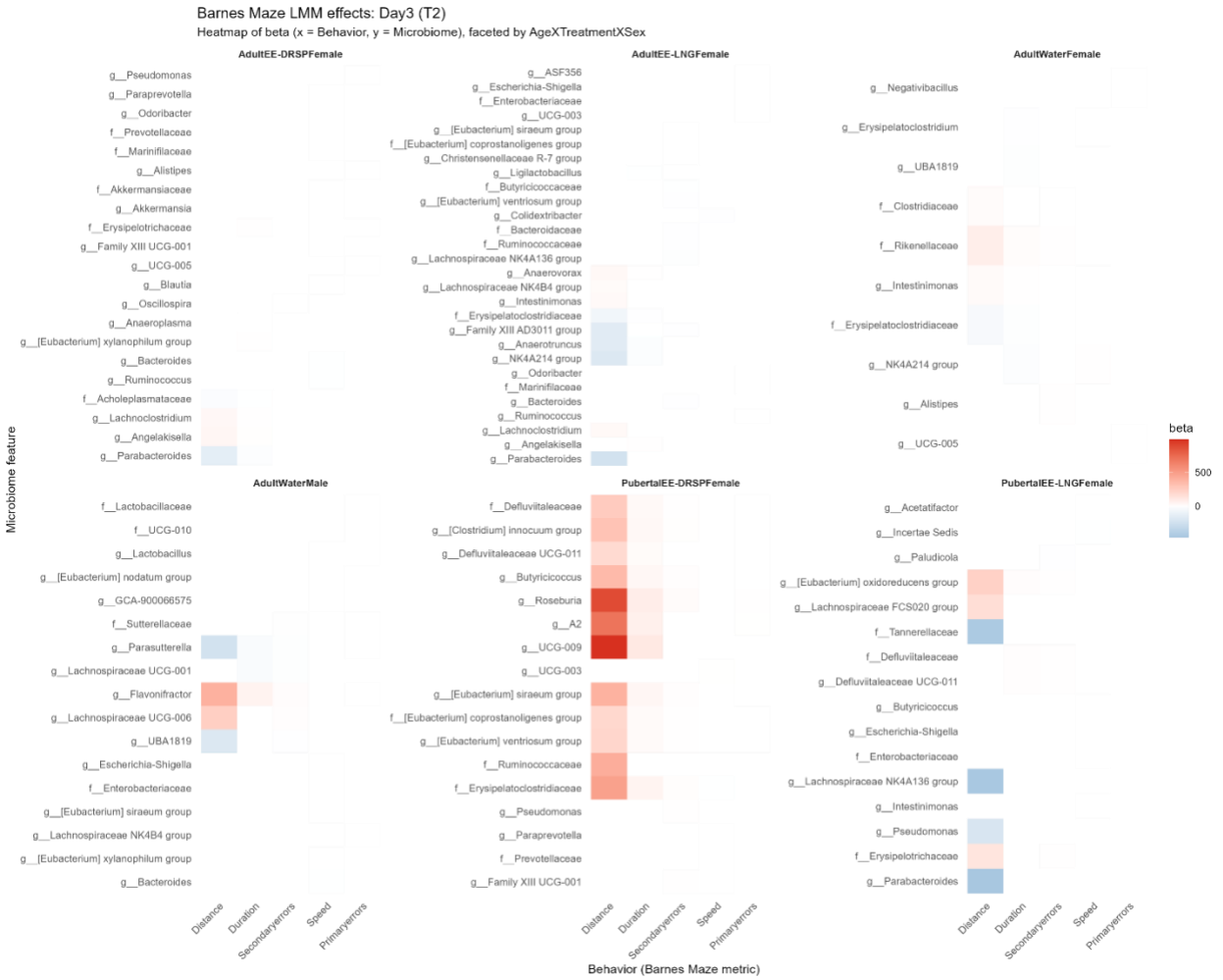

Figure S5C. Heatmap of microbiota–Barnes Maze associations on Day 3 (timepoint 2). Associations are displayed for taxa with nominal significance ($p < 0.05$), highlighting positive (red) and negative (blue) relationships across behavioral readouts.

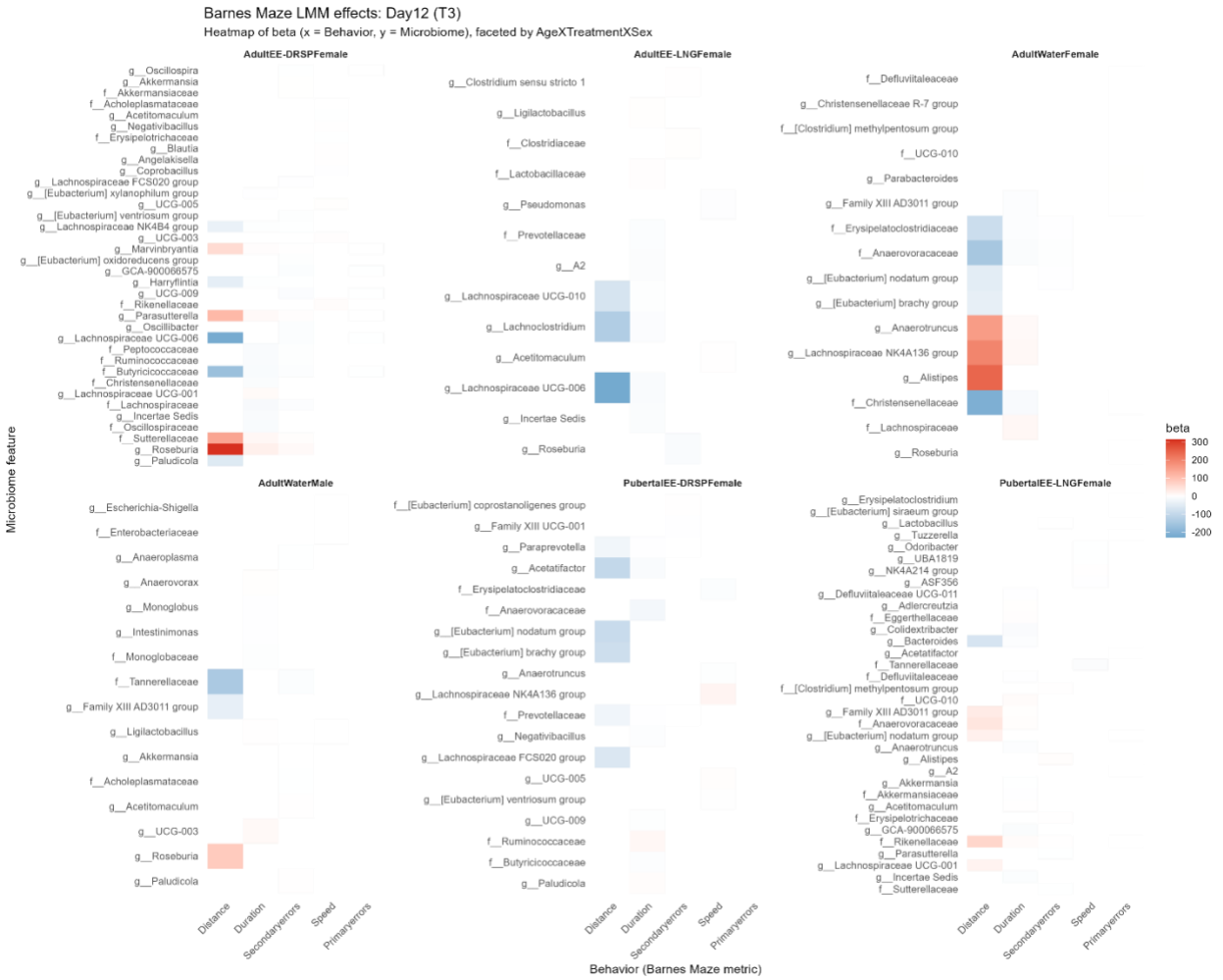

Figure S5D. Heatmap of microbiota–Barnes Maze associations on Day 12 (timepoint 3). Significant associations ($p < 0.05$) are shown at genus and family levels, with color indicating β direction and magnitude.

Supplementary Table 1: Taxa significantly associated with SRT learning (ΔSRT) and recall (probe), identified by linear mixed models (FDR < 0.05).

Group	Shared between Learning & Recall	Unique to Learning	Unique to Recall
Adult EE/DRSP Female	*Tuzzerella*	*Tyzzerella*, Lachnospiraceae	ASF356
Adult EE/LNG Female	Bacteroidaceae	Desulfovibrionaceae, Marinifilaceae, *Bacteroides*	*Erysipelatoclostridium*
Adult Water Male	Prevotellaceae, *Paraprevotella*	*Bacteroides*, *Roseburia*, *Ruminococcus*	–
Adult Water Female	*Paludicola*	Rikenellaceae, Sutterellaceae, *Ruminococcus*	–
Pubertal EE/DRSP Female	Rikenellaceae, *Marvinbryantia*, *Oscillibacter*	Desulfovibrionaceae, Eggerthellaceae, Marinifilaceae	*Erysipelatoclostridium*, *Roseburia*
Pubertal EE/LNG Female	*Tuzzerella*, Acholeplasmataceae, Anaerovoracaceae, Ruminococcaceae, *Anaeroplasma*, *Negativibacillus*	Eggerthellaceae, Lachnospiraceae	Clostridiaceae

Supplementary Table 2: Pairwise PERMANOVA comparisons of 16S microbiome data collected from treated mice across three different timepoints

Comparison Type	Timepoint1	Timepoint2	Group1	Group2	PERMANOVA_R2	PERMANOVA_p	Dispersion_p	PERMANOVA_p_adj	Dispersion_p_adj
WithinTimepoint	1	1	PubertalEE-LNGFemale	PubertalEE-DRSPFemale	0,045	0,855	0,095	0,873	0,299
WithinTimepoint	1	1	PubertalEE-LNGFemale	AdultEE-DRSPFemale	0,075	0,014	0,059	0,029	0,225
WithinTimepoint	1	1	PubertalEE-LNGFemale	AdultEE-LNGFemale	0,077	0,014	0,037	0,029	0,174
WithinTimepoint	1	1	PubertalEE-LNGFemale	AdultWaterFemale	0,054	0,005	0,014	0,014	0,111
WithinTimepoint	1	1	PubertalEE-LNGFemale	AdultWaterMale	0,094	0,001	0,053	0,004	0,221
WithinTimepoint	1	1	PubertalEE-DRSPFemale	AdultEE-DRSPFemale	0,091	0,001	0,790	0,004	0,915
WithinTimepoint	1	1	PubertalEE-DRSPFemale	AdultEE-LNGFemale	0,090	0,006	0,838	0,016	0,937
WithinTimepoint	1	1	PubertalEE-DRSPFemale	AdultWaterFemale	0,055	0,006	0,618	0,016	0,816
WithinTimepoint	1	1	PubertalEE-DRSPFemale	AdultWaterMale	0,081	0,007	0,913	0,017	0,967
WithinTimepoint	1	1	AdultEE-DRSPFemale	AdultEE-LNGFemale	0,058	0,261	0,545	0,311	0,798
WithinTimepoint	1	1	AdultEE-DRSPFemale	AdultWaterFemale	0,045	0,076	0,392	0,118	0,768
WithinTimepoint	1	1	AdultEE-DRSPFemale	AdultWaterMale	0,100	0,001	0,657	0,004	0,850
WithinTimepoint	1	1	AdultEE-LNGFemale	AdultWaterFemale	0,034	0,453	0,734	0,498	0,892

WithinTime point	1	1	AdultEE-LNGFemale	AdultWaterMale	0,089	0,004	0,931	0,012	0,972
WithinTime point	1	1	AdultWaterFemale	AdultWaterMale	0,043	0,098	0,687	0,147	0,860
WithinTime point	2	2	PubertalEE-LNGFemale	PubertalEE-DRSPFemale	0,056	0,340	0,835	0,380	0,937
WithinTime point	2	2	PubertalEE-LNGFemale	AdultEE-DRSPFemale	0,057	0,257	0,874	0,308	0,941
WithinTime point	2	2	PubertalEE-LNGFemale	AdultEE-LNGFemale	0,067	0,190	0,607	0,244	0,812
WithinTime point	2	2	PubertalEE-LNGFemale	AdultWaterFemale	0,048	0,141	0,414	0,193	0,768
WithinTime point	2	2	PubertalEE-LNGFemale	AdultWaterMale	0,079	0,002	0,563	0,007	0,800
WithinTime point	2	2	PubertalEE-DRSPFemale	AdultEE-DRSPFemale	0,070	0,038	0,590	0,068	0,812
WithinTime point	2	2	PubertalEE-DRSPFemale	AdultEE-LNGFemale	0,068	0,154	0,292	0,207	0,615
WithinTime point	2	2	PubertalEE-DRSPFemale	AdultWaterFemale	0,040	0,470	0,574	0,508	0,800
WithinTime point	2	2	PubertalEE-DRSPFemale	AdultWaterMale	0,080	0,007	0,257	0,017	0,572
WithinTime point	2	2	AdultEE-DRSPFemale	AdultEE-LNGFemale	0,059	0,456	0,525	0,498	0,794
WithinTime point	2	2	AdultEE-DRSPFemale	AdultWaterFemale	0,047	0,166	0,176	0,218	0,447
WithinTime point	2	2	AdultEE-DRSPFemale	AdultWaterMale	0,086	0,002	0,488	0,007	0,770
WithinTime point	2	2	AdultEE-LNGFemale	AdultWaterFemale	0,049	0,227	0,036	0,283	0,174

WithinTime point	2	2	AdultEE-LNGFemale	AdultWaterMale	0,084	0,014	0,982	0,029	0,987
WithinTime point	2	2	AdultWaterFemale	AdultWaterMale	0,051	0,057	0,017	0,094	0,120
WithinTime point	3	3	PubertalEE-LNGFemale	PubertalEE-DRSPFemale	0,066	0,227	0,677	0,283	0,860
WithinTime point	3	3	PubertalEE-LNGFemale	AdultEE-DRSPFemale	0,070	0,121	0,090	0,175	0,297
WithinTime point	3	3	PubertalEE-LNGFemale	AdultEE-LNGFemale	0,063	0,218	0,397	0,275	0,768
WithinTime point	3	3	PubertalEE-LNGFemale	AdultWaterFemale	0,046	0,118	0,696	0,172	0,861
WithinTime point	3	3	PubertalEE-LNGFemale	AdultWaterMale	0,145	0,001	0,001	0,004	0,025
WithinTime point	3	3	PubertalEE-DRSPFemale	AdultEE-DRSPFemale	0,061	0,266	0,440	0,315	0,768
WithinTime point	3	3	PubertalEE-DRSPFemale	AdultEE-LNGFemale	0,054	0,504	0,849	0,531	0,939
WithinTime point	3	3	PubertalEE-DRSPFemale	AdultWaterFemale	0,040	0,308	0,396	0,355	0,768
WithinTime point	3	3	PubertalEE-DRSPFemale	AdultWaterMale	0,089	0,002	0,054	0,007	0,221
WithinTime point	3	3	AdultEE-DRSPFemale	AdultEE-LNGFemale	0,062	0,170	0,437	0,221	0,768
WithinTime point	3	3	AdultEE-DRSPFemale	AdultWaterFemale	0,046	0,087	0,008	0,134	0,072
WithinTime point	3	3	AdultEE-DRSPFemale	AdultWaterMale	0,119	0,001	0,037	0,004	0,174
WithinTime point	3	3	AdultEE-LNGFemale	AdultWaterFemale	0,032	0,662	0,122	0,683	0,345

WithinTimepoint	3	3	AdultEE-LNGFemale	AdultWaterMale	0,098	0,001	0,008	0,004	0,072
WithinTimepoint	3	3	AdultWaterFemale	AdultWaterMale	0,063	0,001	0,001	0,004	0,025
AcrossTimepoints_AllGroups	1	1	PubertalEE-LNGFemale	PubertalEE-DRSPFemale	0,045	0,850	0,086	0,872	0,295
AcrossTimepoints_AllGroups	1	1	PubertalEE-LNGFemale	AdultEE-DRSPFemale	0,075	0,008	0,063	0,019	0,235
AcrossTimepoints_AllGroups	1	1	PubertalEE-LNGFemale	AdultEE-LNGFemale	0,077	0,018	0,038	0,036	0,175
AcrossTimepoints_AllGroups	1	1	PubertalEE-LNGFemale	AdultWaterFemale	0,054	0,004	0,013	0,012	0,111
AcrossTimepoints_AllGroups	1	1	PubertalEE-LNGFemale	AdultWaterMale	0,094	0,003	0,043	0,010	0,189
AcrossTimepoints_AllGroups	1	2	PubertalEE-LNGFemale	PubertalEE-LNGFemale	0,063	0,115	0,310	0,169	0,637
AcrossTimepoints_AllGroups	1	2	PubertalEE-LNGFemale	PubertalEE-DRSPFemale	0,095	0,001	0,138	0,004	0,380
AcrossTimepoints_AllGroups	1	2	PubertalEE-LNGFemale	AdultEE-DRSPFemale	0,099	0,001	0,240	0,004	0,553
AcrossTimepoints_AllGroups	1	2	PubertalEE-LNGFemale	AdultEE-LNGFemale	0,117	0,001	0,473	0,004	0,768
AcrossTimepoints_AllGroups	1	2	PubertalEE-LNGFemale	AdultWaterFemale	0,084	0,001	0,007	0,004	0,072
AcrossTimepoints_AllGroups	1	2	PubertalEE-LNGFemale	AdultWaterMale	0,114	0,001	0,439	0,004	0,768
AcrossTimepoints_AllGroups	1	3	PubertalEE-LNGFemale	PubertalEE-LNGFemale	0,123	0,001	0,004	0,004	0,061

AcrossTimepoints_AllGroups	1	3	PubertalEE-LNGFemale	PubertalEE-DRSPFemale	0,109	0,001	0,056	0,004	0,221
AcrossTimepoints_AllGroups	1	3	PubertalEE-LNGFemale	AdultEE-DRSPFemale	0,120	0,001	0,074	0,004	0,266
AcrossTimepoints_AllGroups	1	3	PubertalEE-LNGFemale	AdultEE-LNGFemale	0,116	0,001	0,017	0,004	0,120
AcrossTimepoints_AllGroups	1	3	PubertalEE-LNGFemale	AdultWaterFemale	0,091	0,001	0,001	0,004	0,025
AcrossTimepoints_AllGroups	1	3	PubertalEE-LNGFemale	AdultWaterMale	0,142	0,001	0,864	0,004	0,940
AcrossTimepoints_AllGroups	1	1	PubertalEE-DRSPFemale	AdultEE-DRSPFemale	0,091	0,001	0,820	0,004	0,937
AcrossTimepoints_AllGroups	1	1	PubertalEE-DRSPFemale	AdultEE-LNGFemale	0,090	0,002	0,857	0,007	0,939
AcrossTimepoints_AllGroups	1	1	PubertalEE-DRSPFemale	AdultWaterFemale	0,055	0,003	0,607	0,010	0,812
AcrossTimepoints_AllGroups	1	1	PubertalEE-DRSPFemale	AdultWaterMale	0,081	0,005	0,912	0,014	0,967
AcrossTimepoints_AllGroups	1	2	PubertalEE-DRSPFemale	PubertalEE-LNGFemale	0,066	0,047	0,858	0,082	0,939
AcrossTimepoints_AllGroups	1	2	PubertalEE-DRSPFemale	PubertalEE-DRSPFemale	0,073	0,036	0,933	0,065	0,972
AcrossTimepoints_AllGroups	1	2	PubertalEE-DRSPFemale	AdultEE-DRSPFemale	0,105	0,001	0,639	0,004	0,836
AcrossTimepoints_AllGroups	1	2	PubertalEE-DRSPFemale	AdultEE-LNGFemale	0,115	0,001	0,265	0,004	0,583
AcrossTimepoints_AllGroups	1	2	PubertalEE-	AdultWaterFemale	0,074	0,001	0,424	0,004	0,768

			DRSPFemale						
AcrossTimepoints_AllGroups	1	2	PubertalEE-DRSPFemale	AdultWaterMale	0,104	0,001	0,207	0,004	0,493
AcrossTimepoints_AllGroups	1	3	PubertalEE-DRSPFemale	PubertalEE-LNGFemale	0,120	0,001	0,120	0,004	0,345
AcrossTimepoints_AllGroups	1	3	PubertalEE-DRSPFemale	PubertalEE-DRSPFemale	0,090	0,006	0,455	0,016	0,768
AcrossTimepoints_AllGroups	1	3	PubertalEE-DRSPFemale	AdultEE-DRSPFemale	0,121	0,001	0,967	0,004	0,982
AcrossTimepoints_AllGroups	1	3	PubertalEE-DRSPFemale	AdultEE-LNGFemale	0,110	0,001	0,481	0,004	0,768
AcrossTimepoints_AllGroups	1	3	PubertalEE-DRSPFemale	AdultWaterFemale	0,081	0,001	0,015	0,004	0,114
AcrossTimepoints_AllGroups	1	3	PubertalEE-DRSPFemale	AdultWaterMale	0,114	0,001	0,072	0,004	0,264
AcrossTimepoints_AllGroups	1	1	AdultEE-DRSPFemale	AdultEE-LNGFemale	0,058	0,307	0,574	0,355	0,800
AcrossTimepoints_AllGroups	1	1	AdultEE-DRSPFemale	AdultWaterFemale	0,045	0,059	0,376	0,097	0,752
AcrossTimepoints_AllGroups	1	1	AdultEE-DRSPFemale	AdultWaterMale	0,100	0,002	0,682	0,007	0,860
AcrossTimepoints_AllGroups	1	2	AdultEE-DRSPFemale	PubertalEE-LNGFemale	0,072	0,044	0,951	0,078	0,977
AcrossTimepoints_AllGroups	1	2	AdultEE-DRSPFemale	PubertalEE-DRSPFemale	0,085	0,001	0,759	0,004	0,895
AcrossTimepoints_AllGroups	1	2	AdultEE-DRSPFemale	AdultEE-DRSPFemale	0,049	0,633	0,744	0,660	0,895
AcrossTimepoints_AllGroups	1	2	AdultEE-DRSPFemale	AdultEE-LNGFemale	0,091	0,007	0,162	0,017	0,428

AcrossTimepoints_AllGroups	1	2	AdultEE-DRSPFemale	AdultWaterFemale	0,060	0,010	0,248	0,023	0,558
AcrossTimepoints_AllGroups	1	2	AdultEE-DRSPFemale	AdultWaterMale	0,105	0,001	0,155	0,004	0,420
AcrossTimepoints_AllGroups	1	3	AdultEE-DRSPFemale	PubertalEE-LNGFemale	0,091	0,012	0,024	0,026	0,153
AcrossTimepoints_AllGroups	1	3	AdultEE-DRSPFemale	PubertalEE-DRSPFemale	0,074	0,048	0,312	0,083	0,637
AcrossTimepoints_AllGroups	1	3	AdultEE-DRSPFemale	AdultEE-DRSPFemale	0,059	0,250	0,756	0,302	0,895
AcrossTimepoints_AllGroups	1	3	AdultEE-DRSPFemale	AdultEE-LNGFemale	0,084	0,002	0,277	0,007	0,596
AcrossTimepoints_AllGroups	1	3	AdultEE-DRSPFemale	AdultWaterFemale	0,072	0,001	0,001	0,004	0,025
AcrossTimepoints_AllGroups	1	3	AdultEE-DRSPFemale	AdultWaterMale	0,133	0,001	0,021	0,004	0,143
AcrossTimepoints_AllGroups	1	1	AdultEE-LNGFemale	AdultWaterFemale	0,034	0,456	0,783	0,498	0,912
AcrossTimepoints_AllGroups	1	1	AdultEE-LNGFemale	AdultWaterMale	0,089	0,007	0,920	0,017	0,969
AcrossTimepoints_AllGroups	1	2	AdultEE-LNGFemale	PubertalEE-LNGFemale	0,065	0,127	0,747	0,178	0,895
AcrossTimepoints_AllGroups	1	2	AdultEE-LNGFemale	PubertalEE-DRSPFemale	0,073	0,049	0,952	0,084	0,977
AcrossTimepoints_AllGroups	1	2	AdultEE-LNGFemale	AdultEE-DRSPFemale	0,061	0,159	0,478	0,213	0,768
AcrossTimepoints_AllGroups	1	2	AdultEE-LNGFemale	AdultEE-LNGFemale	0,055	0,560	0,110	0,587	0,325
AcrossTimepoints_AllGroups	1	2	AdultEE-LNGFemale	AdultWaterFemale	0,061	0,007	0,554	0,017	0,798
AcrossTimepoints_AllGroups	1	2	AdultEE-LNGFemale	AdultWaterMale	0,107	0,001	0,083	0,004	0,293
AcrossTimepoints_AllGroups	1	3	AdultEE-LNGFemale	PubertalEE-	0,070	0,126	0,088	0,178	0,295

				LNGFemale					
AcrossTimepoints_AllGroups	1	3	AdultEE-LNGFemale	PubertalEE-DRSPFemale	0,072	0,090	0,471	0,137	0,768
AcrossTimepoints_AllGroups	1	3	AdultEE-LNGFemale	AdultEE-DRSPFemale	0,074	0,033	0,873	0,061	0,941
AcrossTimepoints_AllGroups	1	3	AdultEE-LNGFemale	AdultEE-LNGFemale	0,056	0,339	0,500	0,380	0,780
AcrossTimepoints_AllGroups	1	3	AdultEE-LNGFemale	AdultWaterFemale	0,055	0,016	0,004	0,033	0,061
AcrossTimepoints_AllGroups	1	3	AdultEE-LNGFemale	AdultWaterMale	0,128	0,001	0,014	0,004	0,111
AcrossTimepoints_AllGroups	1	1	AdultWaterFemale	AdultWaterMale	0,043	0,071	0,715	0,114	0,879
AcrossTimepoints_AllGroups	1	2	AdultWaterFemale	PubertalEE-LNGFemale	0,036	0,323	0,555	0,368	0,798
AcrossTimepoints_AllGroups	1	2	AdultWaterFemale	PubertalEE-DRSPFemale	0,045	0,074	0,753	0,118	0,895
AcrossTimepoints_AllGroups	1	2	AdultWaterFemale	AdultEE-DRSPFemale	0,047	0,029	0,275	0,055	0,596
AcrossTimepoints_AllGroups	1	2	AdultWaterFemale	AdultEE-LNGFemale	0,045	0,104	0,057	0,155	0,221
AcrossTimepoints_AllGroups	1	2	AdultWaterFemale	AdultWaterFemale	0,028	0,494	0,673	0,523	0,860
AcrossTimepoints_AllGroups	1	2	AdultWaterFemale	AdultWaterMale	0,060	0,002	0,033	0,007	0,172
AcrossTimepoints_AllGroups	1	3	AdultWaterFemale	PubertalEE-LNGFemale	0,065	0,007	0,095	0,017	0,299
AcrossTimepoints_AllGroups	1	3	AdultWaterFemale	PubertalEE-DRSPFemale	0,050	0,029	0,529	0,055	0,794
AcrossTimepoints_AllGroups	1	3	AdultWaterFemale	AdultEE-DRSPFemale	0,057	0,005	0,642	0,014	0,836
AcrossTimepoints_AllGroups	1	3	AdultWaterFemale	AdultEE-LNGFemale	0,049	0,033	0,595	0,061	0,812

AcrossTimepoints_AllGroups	1	3	AdultWaterFemale	AdultWaterFemale	0,039	0,012	0,005	0,026	0,066
AcrossTimepoints_AllGroups	1	3	AdultWaterFemale	AdultWaterMale	0,073	0,001	0,006	0,004	0,072
AcrossTimepoints_AllGroups	1	2	AdultWaterMale	PubertalEE-LNGFemale	0,073	0,012	0,782	0,026	0,912
AcrossTimepoints_AllGroups	1	2	AdultWaterMale	PubertalEE-DRSPFemale	0,078	0,014	0,994	0,029	0,994
AcrossTimepoints_AllGroups	1	2	AdultWaterMale	AdultEE-DRSPFemale	0,098	0,001	0,512	0,004	0,786
AcrossTimepoints_AllGroups	1	2	AdultWaterMale	AdultEE-LNGFemale	0,082	0,018	0,159	0,036	0,425
AcrossTimepoints_AllGroups	1	2	AdultWaterMale	AdultWaterFemale	0,058	0,011	0,470	0,025	0,768
AcrossTimepoints_AllGroups	1	2	AdultWaterMale	AdultWaterMale	0,052	0,472	0,121	0,508	0,345
AcrossTimepoints_AllGroups	1	3	AdultWaterMale	PubertalEE-LNGFemale	0,128	0,001	0,106	0,004	0,318
AcrossTimepoints_AllGroups	1	3	AdultWaterMale	PubertalEE-DRSPFemale	0,085	0,006	0,478	0,016	0,768
AcrossTimepoints_AllGroups	1	3	AdultWaterMale	AdultEE-DRSPFemale	0,107	0,001	0,949	0,004	0,977
AcrossTimepoints_AllGroups	1	3	AdultWaterMale	AdultEE-LNGFemale	0,095	0,002	0,504	0,007	0,780
AcrossTimepoints_AllGroups	1	3	AdultWaterMale	AdultWaterFemale	0,065	0,004	0,008	0,012	0,072
AcrossTimepoints_AllGroups	1	3	AdultWaterMale	AdultWaterMale	0,058	0,267	0,032	0,315	0,172
AcrossTimepoints_AllGroups	2	2	PubertalEE-LNGFemale	PubertalEE-DRSPFemale	0,056	0,361	0,821	0,402	0,937
AcrossTimepoints_AllGroups	2	2	PubertalEE-LNGFemale	AdultEE-DRSPFemale	0,057	0,269	0,853	0,315	0,939

AcrossTimepoints_AllGroups	2	2	PubertalEE-LNGFemale	AdultEE-LNGFemale	0,067	0,206	0,594	0,261	0,812
AcrossTimepoints_AllGroups	2	2	PubertalEE-LNGFemale	AdultWaterFemale	0,048	0,139	0,440	0,192	0,768
AcrossTimepoints_AllGroups	2	2	PubertalEE-LNGFemale	AdultWaterMale	0,079	0,004	0,516	0,012	0,786
AcrossTimepoints_AllGroups	2	3	PubertalEE-LNGFemale	PubertalEE-LNGFemale	0,076	0,094	0,194	0,142	0,474
AcrossTimepoints_AllGroups	2	3	PubertalEE-LNGFemale	PubertalEE-DRSPFemale	0,067	0,126	0,470	0,178	0,768
AcrossTimepoints_AllGroups	2	3	PubertalEE-LNGFemale	AdultEE-DRSPFemale	0,079	0,014	0,826	0,029	0,937
AcrossTimepoints_AllGroups	2	3	PubertalEE-LNGFemale	AdultEE-LNGFemale	0,073	0,033	0,490	0,061	0,770
AcrossTimepoints_AllGroups	2	3	PubertalEE-LNGFemale	AdultWaterFemale	0,052	0,029	0,032	0,055	0,172
AcrossTimepoints_AllGroups	2	3	PubertalEE-LNGFemale	AdultWaterMale	0,095	0,001	0,349	0,004	0,705
AcrossTimepoints_AllGroups	2	2	PubertalEE-DRSPFemale	AdultEE-DRSPFemale	0,070	0,028	0,614	0,055	0,816
AcrossTimepoints_AllGroups	2	2	PubertalEE-DRSPFemale	AdultEE-LNGFemale	0,068	0,154	0,297	0,207	0,619
AcrossTimepoints_AllGroups	2	2	PubertalEE-DRSPFemale	AdultWaterFemale	0,040	0,458	0,605	0,498	0,812
AcrossTimepoints_AllGroups	2	2	PubertalEE-DRSPFemale	AdultWaterMale	0,080	0,009	0,246	0,021	0,558
AcrossTimepoints_AllGroups	2	3	PubertalEE-	PubertalEE-	0,090	0,022	0,186	0,044	0,460

			DRSPFemale	LNGFemale					
AcrossTimepoints_AllGroups	2	3	PubertalEE-DRSPFemale	PubertalEE-DRSPFemale	0,040	0,920	0,551	0,929	0,798
AcrossTimepoints_AllGroups	2	3	PubertalEE-DRSPFemale	AdultEE-DRSPFemale	0,080	0,006	0,962	0,016	0,982
AcrossTimepoints_AllGroups	2	3	PubertalEE-DRSPFemale	AdultEE-LNGFemale	0,070	0,050	0,572	0,085	0,800
AcrossTimepoints_AllGroups	2	3	PubertalEE-DRSPFemale	AdultWaterFemale	0,051	0,033	0,027	0,061	0,162
AcrossTimepoints_AllGroups	2	3	PubertalEE-DRSPFemale	AdultWaterMale	0,079	0,004	0,093	0,012	0,299
AcrossTimepoints_AllGroups	2	2	AdultEE-DRSPFemale	AdultEE-LNGFemale	0,059	0,484	0,535	0,518	0,796
AcrossTimepoints_AllGroups	2	2	AdultEE-DRSPFemale	AdultWaterFemale	0,047	0,165	0,171	0,218	0,440
AcrossTimepoints_AllGroups	2	2	AdultEE-DRSPFemale	AdultWaterMale	0,086	0,003	0,477	0,010	0,768
AcrossTimepoints_AllGroups	2	3	AdultEE-DRSPFemale	PubertalEE-LNGFemale	0,077	0,053	0,032	0,090	0,172
AcrossTimepoints_AllGroups	2	3	AdultEE-DRSPFemale	PubertalEE-DRSPFemale	0,071	0,075	0,289	0,118	0,615
AcrossTimepoints_AllGroups	2	3	AdultEE-DRSPFemale	AdultEE-DRSPFemale	0,034	0,964	0,556	0,969	0,798
AcrossTimepoints_AllGroups	2	3	AdultEE-DRSPFemale	AdultEE-LNGFemale	0,068	0,065	0,209	0,105	0,493
AcrossTimepoints_AllGroups	2	3	AdultEE-DRSPFemale	AdultWaterFemale	0,048	0,057	0,002	0,094	0,040
AcrossTimepoints_AllGroups	2	3	AdultEE-DRSPFemale	AdultWaterMale	0,104	0,001	0,184	0,004	0,460
AcrossTimepoints_AllGroups	2	2	AdultEE-LNGFemale	AdultWaterFemale	0,049	0,231	0,037	0,286	0,174

AcrossTimepoints_AllGroups	2	2	AdultEE-LNGFemale	AdultWaterMale	0,084	0,012	0,975	0,026	0,985
AcrossTimepoints_AllGroups	2	3	AdultEE-LNGFemale	PubertalEE-LNGFemale	0,092	0,035	0,003	0,064	0,054
AcrossTimepoints_AllGroups	2	3	AdultEE-LNGFemale	PubertalEE-DRSPFemale	0,070	0,246	0,138	0,299	0,380
AcrossTimepoints_AllGroups	2	3	AdultEE-LNGFemale	AdultEE-DRSPFemale	0,075	0,076	0,222	0,118	0,517
AcrossTimepoints_AllGroups	2	3	AdultEE-LNGFemale	AdultEE-LNGFemale	0,043	0,889	0,055	0,903	0,221
AcrossTimepoints_AllGroups	2	3	AdultEE-LNGFemale	AdultWaterFemale	0,045	0,177	0,001	0,229	0,025
AcrossTimepoints_AllGroups	2	3	AdultEE-LNGFemale	AdultWaterMale	0,092	0,001	0,448	0,004	0,768
AcrossTimepoints_AllGroups	2	2	AdultWaterFemale	AdultWaterMale	0,051	0,056	0,026	0,094	0,161
AcrossTimepoints_AllGroups	2	3	AdultWaterFemale	PubertalEE-LNGFemale	0,078	0,002	0,207	0,007	0,493
AcrossTimepoints_AllGroups	2	3	AdultWaterFemale	PubertalEE-DRSPFemale	0,047	0,206	0,722	0,261	0,882
AcrossTimepoints_AllGroups	2	3	AdultWaterFemale	AdultEE-DRSPFemale	0,049	0,112	0,468	0,165	0,768
AcrossTimepoints_AllGroups	2	3	AdultWaterFemale	AdultEE-LNGFemale	0,048	0,139	0,886	0,192	0,948
AcrossTimepoints_AllGroups	2	3	AdultWaterFemale	AdultWaterFemale	0,031	0,328	0,024	0,371	0,153
AcrossTimepoints_AllGroups	2	3	AdultWaterFemale	AdultWaterMale	0,058	0,008	0,005	0,019	0,066
AcrossTimepoints_AllGroups	2	3	AdultWaterMale	PubertalEE-LNGFemale	0,133	0,001	0,001	0,004	0,025
AcrossTimepoints_AllGroups	2	3	AdultWaterMale	PubertalEE-DRSPFemale	0,083	0,014	0,099	0,029	0,306

AcrossTimepoints_AllGroups	2	3	AdultWaterMale	AdultEE-DRSPFemale	0,107	0,001	0,170	0,004	0,440
AcrossTimepoints_AllGroups	2	3	AdultWaterMale	AdultEE-LNGFemale	0,088	0,002	0,039	0,007	0,176
AcrossTimepoints_AllGroups	2	3	AdultWaterMale	AdultWaterFemale	0,063	0,002	0,001	0,007	0,025
AcrossTimepoints_AllGroups	2	3	AdultWaterMale	AdultWaterMale	0,029	0,992	0,463	0,992	0,768
AcrossTimepoints_AllGroups	3	3	PubertalEE-LNGFemale	PubertalEE-DRSPFemale	0,066	0,237	0,690	0,290	0,860
AcrossTimepoints_AllGroups	3	3	PubertalEE-LNGFemale	AdultEE-DRSPFemale	0,070	0,141	0,088	0,193	0,295
AcrossTimepoints_AllGroups	3	3	PubertalEE-LNGFemale	AdultEE-LNGFemale	0,063	0,237	0,409	0,290	0,768
AcrossTimepoints_AllGroups	3	3	PubertalEE-LNGFemale	AdultWaterFemale	0,046	0,126	0,691	0,178	0,860
AcrossTimepoints_AllGroups	3	3	PubertalEE-LNGFemale	AdultWaterMale	0,145	0,001	0,002	0,004	0,040
AcrossTimepoints_AllGroups	3	3	PubertalEE-LNGFemale	AdultEE-DRSPFemale	0,061	0,281	0,468	0,327	0,768
AcrossTimepoints_AllGroups	3	3	PubertalEE-DRSPFemale	AdultEE-LNGFemale	0,054	0,491	0,832	0,523	0,937
AcrossTimepoints_AllGroups	3	3	PubertalEE-DRSPFemale	AdultWaterFemale	0,040	0,320	0,411	0,366	0,768
AcrossTimepoints_AllGroups	3	3	PubertalEE-DRSPFemale	AdultWaterMale	0,089	0,003	0,057	0,010	0,221
AcrossTimepoints_AllGroups	3	3	AdultEE-DRSPFemale	AdultEE-LNGFemale	0,062	0,165	0,426	0,218	0,768
AcrossTimepoints_AllGroups	3	3	AdultEE-DRSPFemale	AdultWaterFemale	0,046	0,085	0,008	0,131	0,072

AcrossTimepoints_AllGroups	3	3	AdultEE-DRSPFemale	AdultWaterMale	0,119	0,001	0,033	0,004	0,172
AcrossTimepoints_AllGroups	3	3	AdultEE-LNGFemale	AdultWaterFemale	0,032	0,653	0,105	0,677	0,318
AcrossTimepoints_AllGroups	3	3	AdultEE-LNGFemale	AdultWaterMale	0,098	0,002	0,007	0,007	0,072
AcrossTimepoints_AllGroups	3	3	AdultWaterFemale	AdultWaterMale	0,063	0,001	0,001	0,004	0,025

CHAPTER 3 : EFFECTS OF COMBINED ORAL CONTRACEPTIVES ON DEPRESSION- AND ANXIETY-LIKE BEHAVIOURS AND ON 5HT-1A AND GABA CELL EXPRESSION AND IN ADULT AND PUBERTAL CD-1 MICE

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Abstract

Puberty is a critical period of development during which exposure to combined oral contraceptives (COCs) could impact brain development and increase the vulnerability to mental illness. However, research on the effects of COCs on mental health is sparse and the mechanisms underlying these effects are unknown. This study investigated the effects of COC use on depression- and anxiety-like behaviours in pubertal and adult CD-1 mice. As of 5 or 10 weeks of age, mice received 250 μL of either one of two COCs formulations (ethinyl estradiol and levonorgestrel (EE/LNG) or ethinyl estradiol and drospirenone (EE/DRSP) or a vehicle via oral gavage daily. Five weeks later, when pubertal mice reached adulthood, all mice were exposed to the Tail suspension, Open field, Elevated Plus Maze, and Forced swim tests to assess depression-like and anxiety-like behaviours. Mice were then euthanized and brains were collected. Results revealed that pubertal COCs groups and males had increased active coping during the Forced swim test, whereas adults treated with EE/LNG displayed increased immobility. Adults treated with EE/LNG adult also displayed less anxiety as they spent more time in the center of the Open Field. EE/DRSP-treated adult females displayed increased GABA immunoreactive cell expression in the CA2 and CA1, while EE/LNG-treated adult females had lower GABA expression in the CA3. Males had higher 5HT-1A immunoreactive cell expression in the CA3. Our results indicate that the effects of COCs on the brain and behaviour are formulation- and age-dependent, and they also vary across tests. This study provides insights on the role of COCs use during puberty in shaping vulnerability to mental illness.

Keywords : Puberty; Combined oral contraceptives; Brain; Mental health; Depression; Anxiety; Serotonin

1.0 Introduction

Combined oral contraceptives (COCs) are the most commonly used and effective methods of reversible hormonal contraception worldwide (UN, 2019). COCs combine synthetic analogs of ovarian hormones, such as ethinyl estradiol (EE) and progestins. However, COC formulations differ based on the type of progestins and their binding affinity to androgen receptors (Edwards & Can, 2023). Progestins possess either high (e.g., levonorgestrel), low (e.g., norgestimate), or anti-androgenic activity (e.g., drospirenone). Nevertheless, all COCs prevent pregnancy by inhibiting the hypothalamic-pituitary-gonadal axis (HPG) and ovulation (Edwards & Can, 2023), and by thickening the cervical mucus to impede sperm mobility (Bronson, 1981; Dickey, 1987; Lacasse, Gomez-Perales, et al., 2022). However, the effects of COCs are not limited to the reproductive system. Recent findings show that COCs can impact brain structure and function (Hampson, 2023; Heller et al., 2022; Sharma, Fang, et al., 2020; Sharma, Smith, et al., 2020). COCs may also increase the vulnerability to depression and anxiety (Anderl et al., 2020; Bengtsdotter et al., 2018; Johansson et al., 2023; Lundin et al., 2017; Skovlund et al., 2016), but evidence remains mixed (de Wit et al., 2021; Kraft et al., 2024).

Depression and anxiety are the most commonly experienced mood disorders (Mnookin, 2016; Stein et al., 2017). Depression is characterized by symptoms ranging from social and cognitive impairments to low self-esteem and suicidal ideation (Meeus, 2016; Rock et al., 2014; Twenge et al., 2018), while anxiety disorders are marked by prolonged fear or avoidance of perceived threats (Craske et al., 2017). Both illnesses can be long-lasting and are associated with lower subjective quality of life and impaired social functioning (Brown & Roose, 2011; Hansson, 2002). Dysregulation of some neurotransmitter systems (e.g., GABA, serotonin) and their specific subtypes along with imbalance in dopaminergic pathways have been associated

with increased HPA axis reactivity, neuroinflammation increasing vulnerability for both depression and anxiety (Jauhar et al., 2023; K. S. V. et al., 2026; Liu et al., 2018; Żmudzka et al., 2018).

Depression and anxiety disorders are two times more prevalent in females compared to males (Albert, 2015; Pavlidi et al., 2023; Whiteford et al., 2013). This sex difference can partially be explained by fluctuating levels of estradiol and progesterone and their effect on neurotransmitters like serotonin, dopamine and $GABA_a$ (Borrow & Cameron, 2014; Bristot et al., 2014; Hwang et al., 2020; Krolick et al., 2018; Östlund et al., 2003; Steiner et al., 2003). COCs can also increase symptoms of depression and anxiety in some women, particularly adolescent female users (Anderl et al., 2021; Kraft et al., 2024; Lundin et al., 2017; Skovlund et al., 2016). However, the mechanism underlying the effects of COCs on brain function and mental health remains unclear. It is also unclear why some COCs users are more vulnerable to anxiety and depression than others as there is multiple risk factors involved in these two conditions (Davies et al., 2019). Differences in the age of onset of COC use could explain these discrepancies.

COCs are commonly prescribed to adolescent females to manage medical conditions like hormonal acne, dysmenorrhea, and premenstrual symptoms (Bahamondes et al., 2015). However, puberty is a critical period of development characterized by the activation of the HPG axis, resulting in physiological (e.g., sexual maturation), behavioural, and cognitive maturation (Fuhrmann et al., 2015; Herting & Sowell, 2017; Larsen & Luna, 2018; Plant, 2015). During this period, several changes in brain structure, function and connectivity (Goddings et al., 2019) occur, rendering the brain more vulnerable to internal and external factors (Eiland & Romeo, 2013; Laube et al., 2020; Naulé et al., 2020). COC use during puberty inhibits the HPG axis

which may result in altered brain structure and function (Hilz, 2022). For example, females who start using COCs during puberty have increased white matter volume in the right fusiform gyrus compared to non-users (Sharma, Smith, et al., 2020). They also show higher BOLD responses in the left fusiform gyrus, right cuneus, bilateral and left mid-temporal lingual gyri, right insula, and pre-central gyrus during a working memory task (Sharma, Smith, et al., 2020) and also exhibit greater functional connectivity in the salience network during the resting state (Sharma, Fang, et al., 2020). COC users also display reduced responsiveness to exposure therapy when treating anxiety (Anderl et al., 2021; Anderl et al., 2020; Bengtsdotter et al., 2018; Buggio et al., 2022; Bui et al., 2023; Kraft et al., 2024; Lundin et al., 2017; Raeder et al., 2019; Skovlund et al., 2016) and have higher rates of antidepressant use (Lindberg et al., 2012; Skovlund et al., 2016; Wiréhn et al., 2010). While many studies show associations between COC use and mental illness, some studies found no association (de Wit et al., 2021; Worly et al., 2018) while others even note a reduced vulnerability to mood disorders following COC use (Cheslack-Postava et al., 2015). The lack of systematic research and methodological discrepancies between studies, like COC formulae, androgenicity, age of onset, and history of mood disorders might explain the inconsistent findings (Kraft et al., 2024; Schaffir et al., 2016). Moreover, the mechanisms through which COCs could impact brain development and increase the vulnerability to mood disorders are unclear. Animal models would provide insights and a better understanding of these possible mechanisms (Tronson & Schuh, 2022).

Therefore, this study aimed to determine whether COC use since puberty or only in adulthood increases depression- and anxiety-like behaviours in CD-1 mice. We also investigated the effects of COCs on neurotransmitters, like serotonin and GABA. We hypothesize that mice that were exposed to COCs since puberty would exhibit significantly higher anxiety- and

depression-like behaviours and reduced expression of serotonin and GABA in brain regions associated with depression and anxiety compared to mice treated with COC in adulthood, naturally cycling females, and males.

2.0 Methods

2.1 Animals

Sixty-five male and female CD-1 mice were shipped from Charles River Laboratories Inc. (Kingston, New-York, United States) at 3 weeks of age. Upon arrival in our facility, mice were housed in groups of three or four in either all-male or all-female rooms in polycarbonate Lexan housing cages (17 × 28 × 12 cm [width x length x height]) lined with Teklad Corn Cob bedding (Harlan Laboratories, Inc., Madison, WI, USA). Mice were provided with two square pieces of nestlet (Ancare Corp., Bellmore, NY, USA) and a cardboard refuge hut (Ketchum Manufacturing, Inc., Brockville, ON, Canada) for enrichment, and had *ad libitum* access to food (Harlan Laboratories, Inc., Madison, WI, US, T2018 – Global 18% rodent) and water. Rooms were kept on a reversed light cycle (14h:10h light/dark, lights off at 10 A.M.). Room temperature (24 ± 2°C) and humidity (40 ± 5 %) were kept constant. All manipulations were approved by the Animal Care Committee of the University of Ottawa.

2.2 Combined oral contraceptives administration

Mice in the pubertal groups (n=10/group) began hormone treatment at 5 weeks of age, while mice in the adult groups (n=10/group) began hormone treatment at 10 weeks of age. In both age groups, mice were administered either a vehicle or one of two COC formulations, either 0.22 µg of ethinyl estradiol and 1.0762 µg of levonorgestrel, (i.e., EE/LNG groups) or 0.22 µg of ethinyl estradiol and 21.525 µg of drospirenone (i.e., EE/DRSP groups) dissolved in 248.4 µl of water and 1.6 µl of dimethyl sulfoxide (DMSO) for solubility through oral gavage for 6 weeks.

Finally, adult males (n=10) and adult naturally cycling females (n=15) served as controls and were administered sterile water and 1.6 µl of DMSO. The dose of synthetic hormones was selected based on the typical doses found in combined oral contraceptives and converted to a mouse equivalent dose using an Animal Equivalent Dose conversion factor (Nair & Jacob, 2016), and then multiplied by the average weight of CD-1 mice (35 g).

2.3 Estrus cycle

Estrus cycle was assessed daily via vaginal lavage for one week prior to behaviour testing to ensure acyclicity in COC-treated mice (Lacasse, Gomez-Perales, et al., 2022) and also on each day of behaviour testing. Vaginal cells were collected by inserting a latex transfer pipette in the vaginal canal (2-3mm), and by gently flushing 2-3 times approximately 25-50 µl of 0.9% saline (McLean et al., 2012). Cells were then placed on Fisherbrand Superfrost Plus microscope slides and coverslipped with Fisherbrand coverslips. Cycle staging was completed using a Leica DAS microscope (10X magnification) attached to a SONY DXC-390P digital camera and Northern Eclipse 8.0 software (Empix Imaging, Mississauga, Ontario, Canada) to identify the types of cells present in each sample. Acyclicity in COCs-treated mice was confirmed using vaginal cytology staging criteria as described in Ajayi and Akhigbe (2020). Mice treated with COCs remained predominantly in the metestrus/diestrus phase, characterized by the presence of leukocytes, nucleated epithelial cells, and some cornified squamous epithelial cells (Kheloui et al., In preparation).

2.4 Behaviour testing

Prior to each behaviour testing session, mice were acclimated for 30 min before behaviour testing in sex-segregated rooms. All testing sessions were conducted under dim red light during the dark phase of the light-dark cycle as of noon. The arena was cleaned with 70% ethanol between each trial to eliminate olfactory cues.

2.4.1 Forced swim test

Forced swim test was conducted to assess depression-like behaviour (Kraeuter et al., 2018b). Animals were individually placed in a glass cylinder (25 cm x 10 cm) filled with water (23-25°C) for 5 minutes. Durations and frequency of escape, swimming, and immobility (e.g., minimal movement to maintain the head above the water) behaviours were recorded using a camera recording apparatus and scored manually by two raters that were blind to treatment conditions. A threshold of 20% coefficient of variation (CV) was applied to ensure consistency in the scores between the two raters. Greater duration of immobility indicated increased behavioural despair and depression-like behaviour (Can, Dao, Arad, et al., 2012).

2.4.2 Elevated plus maze

Anxiety-like behaviour was also assessed using the elevated plus maze (Kraeuter et al., 2018a). Mice were placed at the center of a plus-shaped apparatus with two open and two closed arms (6 x 75 cm) raised 75 cm off the floor for 5 minutes (Komada et al., 2008). Time spent in the center, open and closed arms, as well as the number of entries in both arms, were recorded and analyzed using a video tracking system (Ethovision XT). Reduced time and number of entries in the open arms as well as increased time and number of entries spent in the closed arms are representative of greater anxiety-like behaviour (Kraeuter et al., 2018a).

2.4.3 Tail suspension test

Tail suspension test was carried out to assess depression-like behaviour (Can, Dao, Terrillion, et al., 2012). Each mouse was suspended approximately 1 meter above the base of the testing enclosure with a 2-cm piece of adhesive lab tape placed on the last third of the mouse's tail, while ensuring the mouse's ventral side was oriented towards the rear of the enclosure. Each session lasted 5 minutes. Duration and frequency of immobility behaviour was recorded using a camera and analyzed using Ethovision XT software. Greater duration of immobility indicated increased behavioural despair and depression-like behaviour (Can, Dao, Terrillion, et al., 2012).

2.4.4 Open Field

Open Field Test was used to assess general locomotor activity and anxiety-like behaviours (Seibenhener & Wooten, 2015). Mice were individually placed in a square arena (100 x 100 cm), and exploratory behaviour was recorded over 5 minutes. The peripheral area (30 cm from each wall) surrounded the central zone (40 x 40 cm). An overhead video tracking system (Ethovision XT) was used to record, and all variables were analyzed using EthoVision software including time spent as well as number of entries in the center (Bailey & Crawley, 2009). Increased time spent in the center zone indicated reduced anxiety-like behaviour.

2.5 Brain tissue collection

Twenty-four hours following behaviour testing, mice were euthanized using a Euthanyl injection (Sodium pentobarbital; 500 mg/kg, i.p.). Mice were assessed for motor reflexes by gently pinching their feet and toes. Once no motor reflexes were detected, intracardial perfusion was performed with 20 ml of 0.9% saline, followed by 20 ml of 4% paraformaldehyde. After perfusion, brains were post-fixed with 10 ml of 4% PFA for 2 hours at 4 °C. The PFA solution was replaced with 10 ml of 30% sucrose for 24 hrs. Following that, brains were transferred to

fresh 30% sucrose and stored at 4 °C until processing. Brain slicing was conducted using a Leica VT1200 S automated vibrating blade microtome and sectioned at a thickness of 30 µm. Sections were then stored in tubes containing cryoprotectant at −20 °C until immunostaining.

2.6 Immunohistochemistry

Brain sections were rinsed three times with tris-buffered saline (TBS) (pH 7.6) for five minutes. Following this, sections were incubated for 30 minutes in an antigen retrieval solution containing 0.05M sodium citrate tribasic dihydrate, followed by rinsing 3 x 5 minutes in TBS and a 30-minute incubation in a 0.1M glycine solution. Subsequently, the sections were rinsed 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% Triton X-100, and 1% hydrogen peroxide. Then, sections were transferred to a solution (TBS, 2% NGS, 0.1% Triton X-100) containing a diluted primary antibody for serotonin (5HT-1A) (1/300; Abcam; new cat: ab85615) or gamma-aminobutyric acid (GABA) (1/800; Abcam; cat: ab17413) and incubated overnight at room temperature. After incubation, sections were washed in a diluted blocking solution (TBS, 1% NGS, 0.1% Triton X-100) 3 x 5 minutes. Subsequently, sections were incubated for 60 minutes at room temperature in a secondary antibody solution (TBS, 2% NGS, 0.1% Triton X-100) contained either biotinylated goat anti-rabbit polyclonal IgG (1/500; Vector Laboratories; cat: BA-1000) for 5HT-1A, or biotinylated goat anti-guinea pig polyclonal IgG (1/500; Vector Laboratories; cat: BA-7000) for GABA. Following secondary antibody incubation, sections underwent 3 x 10-minute washes with 0.1% Triton X-100 and PBS, followed by a 1-hour incubation in the VECTASTAIN® Elite ABC-HRP Kit (Vector Laboratories; cat: PK-6100). Then, the sections underwent 3 x 10-minute washes in TBS and were then incubated in freshly prepared 3,3'-diaminobenzidine (DAB) solution (Vector Laboratories; cat: SK-4100), followed

by 3 x 5-minute rinses in cold TBS. Finally, sections were mounted on Fisherbrand Superfrost Plus microscope slides and coverslipped with Fisherbrand coverslips after applying Permount solution.

2.7 Image analysis and cell counting

Brain regions including the ventral tegmental area (VTA; Bregma -2.97 mm), nucleus accumbens (NAc; Bregma +1.13 mm), paraventricular nucleus of the hypothalamus (PVN; Bregma -0.67 mm), and hippocampus (e.g., CA1, CA2, CA3, DG) (HIPP; Bregma - 1.94 mm) were identified using The Mouse Brain Atlas in Stereotaxic Coordinates (Paxinos & Franklin, 2019). Microscopy was performed using a Zeiss AxioImager M2 microscope with MBF Stereo Investigator software and a Zeiss EC Plan-Neofluar 20×/0.50 NA objective. Cell quantification (e.g., total number of immunoreactive cells) for both 5HT-1A and GABA was performed using Fiji Image J software (Schindelin et al., 2012) to ensure accurate analysis of immunoreactive cell populations.

2.8 Statistical analyses

Boxplots were used to identify outliers and all cases that exceeded the 1.5 interquartile range were considered statistical outliers and winsorized to the next extreme data point in the group (Anscombe, 1973). Data from the behavioural testing and immunohistochemistry were analyzed using one-way ANOVAs on the dependent variables. In the case of main effects, pairwise comparisons were used to determine intergroup differences with Fisher's Least Significant Difference (LSD) test. 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 Forced swim test

A significant difference in duration of escape behaviour was found ($F_{(5,63)} = 4.952$, $p < .001$, $\eta^2 = .30$). Post-hoc tests showed that EE/LNG pubertal females had significantly longer escape duration than NC females (*MD* = 25.48, *SE* = 8.89, *p* = .006), EE/LNG adults (*MD* = 32.28, *SE* = 9.74, *p* = .002), and EE/DRSP adults (*MD* = 26.95, *SE* = 9.74, *p* = .008). Similarly, EE/DRSP pubertal females displayed significantly longer escape duration than NC females (*MD* = 28.17, *SE* = 9.19, *p* = .003), EE/LNG adults (*MD* = 34.97, *SE* = 10, *p* < .001), and EE/DRSP adults (*MD* = 29.64, *SE* = 10, *p* = .004). Males also displayed significantly longer escape duration than NC females (*MD* = 18.65, *SE* = 8.89, *p* = .04), EE/LNG adults (*MD* = 25.45, *SE* = 9.74, *p* = .01), as well as EE/DRSP adults (*MD* = 20.12, *SE* = 9.74, *p* = .04).

The ANOVA showed that there was a significant difference in swimming duration ($F_{(5,63)} = 2.656$, $p = .03$, $\eta^2 = .19$). Post-hoc tests showed that EE/DRSP adult females displayed shorter swimming duration than males (*MD* = -44.15, *SE* = 17.88, *p* = .02), EE/LNG adults (*MD* = -45.67, *SE* = 17.89, *p* = .01), EE/LNG pubertal (*MD* = -42.13, *SE* = 17.89, *p* = .02), and EE/DRSP pubertal (*MD* = -40.04, *SE* = 18.38, *p* = .03) females. In addition, NC females displayed reduced swimming duration compared to males (*MD* = -33.32, *SE* = 16.33, *p* = .046) and EE/LNG adults (*MD* = -34.84, *SE* = 16.33, *p* = .04).

A significant difference in swimming frequency was also found ($F_{(5,63)} = 3.215$, $p = .01$, $\eta^2 = .22$). Post-hoc tests revealed that EE/DRSP pubertal mice had increased frequency compared to NC females (*MD* = 11.49, *SE* = 3.86, *p* = .004), EE/DRSP adults (*MD* = 14.81, *SE* = 4.21, *p* < .001), and EE/LNG pubertal females (*MD* = 12.01, *SE* = 4.21, *p* = .006).

The ANOVA revealed that there was a significant difference in immobility duration ($F_{(5,63)} = 4.335$, $p = .002$, $\eta^2 = .27$). Post-hoc tests showed that EE/LNG pubertal females had significantly lower immobility than NC females (*MD* = -49.10, *SE* = 16.86, $p = .005$), EE/LNG adults (*MD* = -48.04, *SE* = 18.47, $p = .01$), and EE/DRSP adults (*MD* = -51.47, *SE* = 18.47, $p = .007$). Similarly, EE/DRSP pubertal females displayed lower immobility duration than NC females (*MD* = -45.16, *SE* = 17.42, $p = .01$), EE/LNG adults (*MD* = -44.09, *SE* = 18.98, $p = .02$), and EE/DRSP adults (*MD* = -47.53, *SE* = 18.98, $p = .02$). Males also displayed significantly lower immobility duration than NC females (*MD* = -48.93, *SE* = 16.86, $p = .005$), EE/LNG adults (*MD* = -47.87, *SE* = 18.47, $p = .01$), and EE/DRSP adults (*MD* = -51.31, *SE* = 18.47, $p = .007$).

The ANOVA also found a significant difference in immobility frequency ($F_{(5,63)} = 5.024$, $p < .001$, $\eta^2 = .30$). Post-hoc tests revealed that EE/LNG adults displayed higher immobility frequency compared to males (*MD* = 21.10, *SE* = 6.14, $p = .001$), EE/DRSP adults (*MD* = 12.35, *SE* = 6.14, $p = .049$), EE/LNG pubertal (*MD* = 27.25, *SE* = 6.14, $p < .001$) as well as EE/DRSP pubertal females (*MD* = 18.58, *SE* = 6.30, $p = .005$). In contrast, EE/LNG pubertal females showed significantly lower immobility frequency compared to NC females (*MD* = -17.72, *SE* = 5.60, $p = .002$) and EE/DRSP adults (*MD* = -14.90, *SE* = 6.14, $p = .02$). Finally, males had lower immobility frequency than NC females (*MD* = -11.57, *SE* = 5.60, $p = .04$).

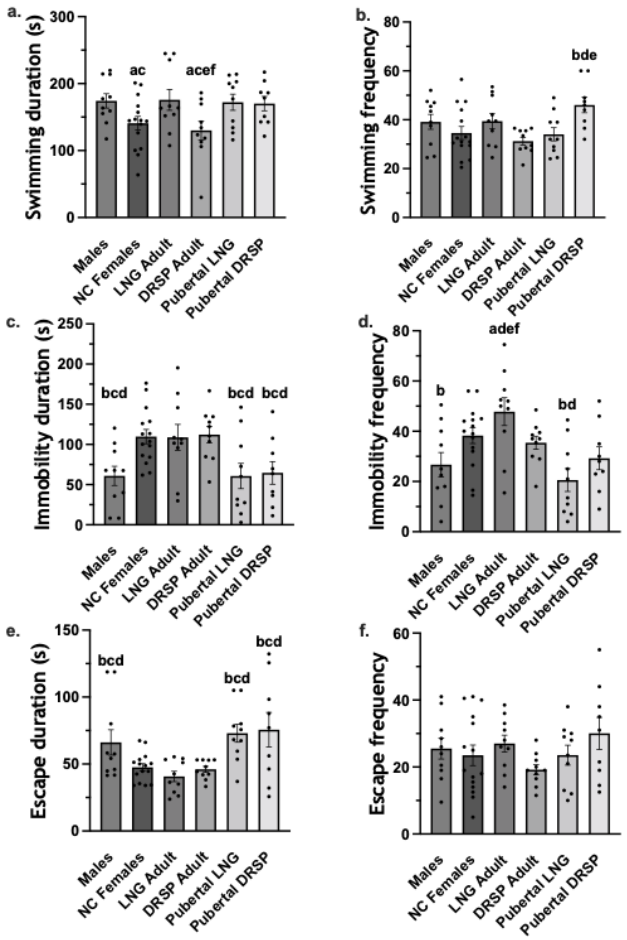

Figure 1. Forced swim test

Note. Group means (±*SEM*) for swimming, escape and immobility. Data presented as (a.) swimming duration, (b.) swimming frequency, (c.) immobility duration, (d.) immobility frequency, (e.) escape duration, and (f.) escape frequency.

The following labels represent pairwise comparisons between groups in the study:

a. Males vs. NC females, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **b.** NC females vs. males, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **c.** LNG Adult vs. males, NC females, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **d.** DRSP Adult vs. males, NC females, LNG Adult, LNG Pubertal, and DRSP Pubertal; **e.** LNG Pubertal vs. males,

NC females, LNG Adult, DRSP Adult, and DRSP Pubertal; **f.** DRSP Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and LNG Pubertal.

3.2 Elevated plus maze

The ANOVA found no significant difference in open arm duration ($F_{(5,63)} = .363$, $p = .87$, $\eta^2 = .03$) and number of open arm entries ($F_{(5,63)} = .336$, $p = .89$, $\eta^2 = .03$). Moreover, no differences were observed for both closed arm duration ($F_{(5,63)} = 1.850$, $p = .12$, $\eta^2 = .14$) and number of closed arm entries ($F_{(5,63)} = .422$, $p = .83$, $\eta^2 = .04$). Finally, no differences were found for frequency ($F_{(5,63)} = .266$, $p = .93$, $\eta^2 = .02$) and time spent ($F_{(5,63)} = 1.603$, $p = .17$, $\eta^2 = .12$) in the center zone.

3.3 Tail suspension test

The ANOVA found no significant difference in mobility (i.e., active movement) duration ($F_{(5,63)} = .917$, $p = .48$, $\eta^2 = .15$) and frequency ($F_{(5,63)} = .666$, $p = .65$, $\eta^2 = .12$). The ANOVA revealed that there was a significant difference in latency to mobility (i.e., first active movement) ($F_{(5,63)} = 5.270$, $p < .001$, $\eta^2 = .31$). Post-hoc tests showed that NC females had a higher latency to first active movement compared to males ($MD = 2.36$, $SE = .74$, $p = .002$), EE/LNG adults ($MD = 2.96$, $SE = .74$, $p < .001$), EE/DRSP adults ($MD = 2.69$, $SE = .74$, $p < .001$), EE/LNG pubertal females ($MD = 2.55$, $SE = .74$, $p = .001$), and EE/DRSP pubertal females ($MD = 3.03$, $SE = .77$, $p < .001$).

No differences were found for immobility duration ($F_{(5,63)} = .220$, $p = .95$, $\eta^2 = .02$) and frequency ($F_{(5,63)} = 1.223$, $p = .31$, $\eta^2 = .10$), as well as latency to first immobility ($F_{(5,63)} = 1.593$, $p = .18$, $\eta^2 = .12$).

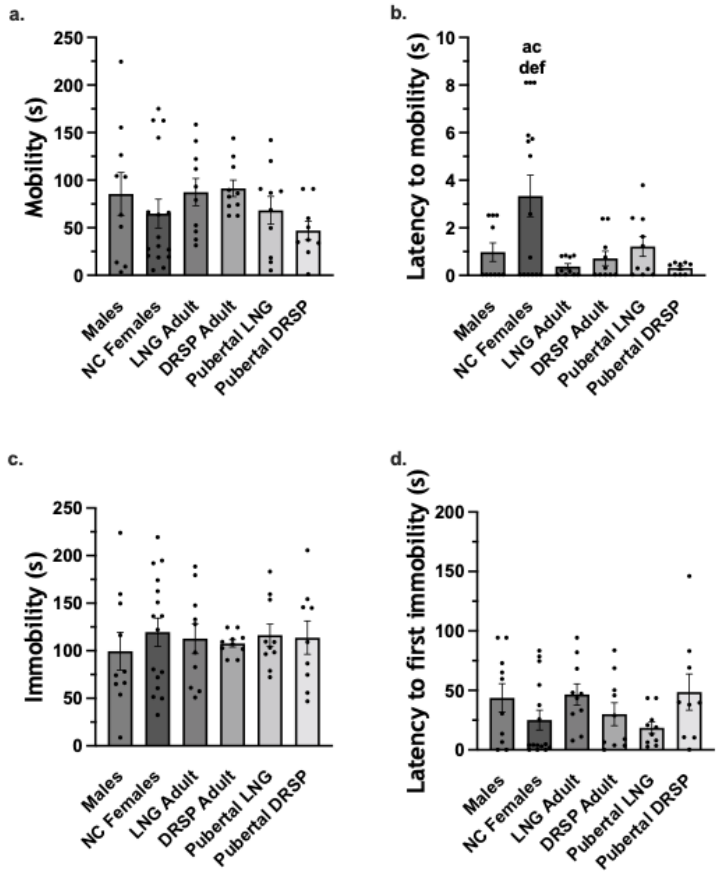

Figure 2. Tail suspension test

Note. Group means (±*SEM*) for mobility and immobility. Data presented as (a.) mobility duration, (b.) latency to first mobility, (c.) immobility duration, and (d.) latency to first immobility frequency.

The following labels represent pairwise comparisons between groups in the study:

a. Males vs. NC females, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **b.** NC females vs. males, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **c.** LNG Adult vs. males, NC females, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **d.** DRSP Adult vs. males, NC females, LNG Adult, LNG Pubertal, and DRSP Pubertal; **e.** LNG Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and DRSP Pubertal; **f.** DRSP Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and LNG Pubertal.

3.4 Open field

A significant difference was found for the frequency of entries in the center ($F_{(5,64)}$ = 3.224, p = .01, η^2 = .22). EE/DRSP pubertal mice entered the center significantly less than NC females (*MD* = -6.40, *SE* = 1.67, p < .001), EE/LNG adults (*MD* = -5.33, *SE* = 1.84, p = .005), EE/DRSP adults (*MD* = -4.83, *SE* = 1.84, p = .01) and EE/LNG pubertal females (*MD* = -4.43, *SE* = 1.84, p = .02).

Moreover, the ANOVA revealed significant differences for time spent in the center ($F_{(5,64)}$ = 6.471, p < .001, η^2 = .35). EE/LNG adults spent significantly more time in the center compared to males (*MD* = 6.79, *SE* = 2.93, p = .02), EE/DRSP adults (*MD* = 9.25, *SE* = 2.94, p = .003), EE/LNG pubertal mice (*MD* = 10.09, *SE* = 2.94, p = .001) as well as EE/DRSP (*MD* = 14.86, *SE* = 3.01, p < .001) pubertal females. In addition, EE/DRSP pubertal females spent significantly less time in the center compared to males (*MD* = -8.07, *SE* = 3.02, p = .01) and NC females (*MD* = -11.43, *SE* = 2.74, p < .001).

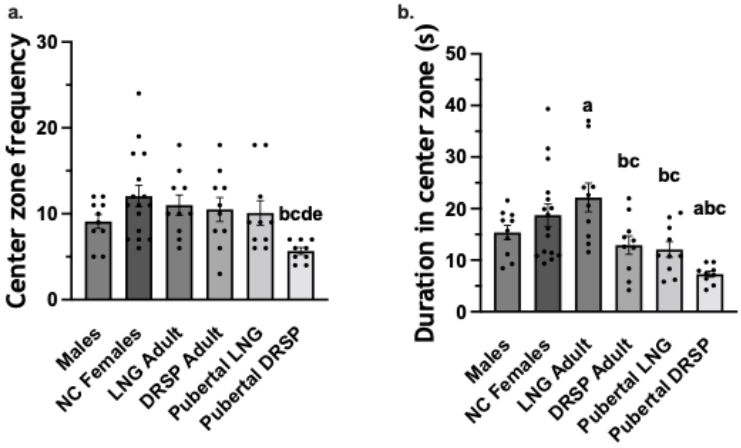

Figure 3. Open field test

Note. Group means (±*SEM*) for center frequency and duration. Data presented as (a.) center zone frequency, and (b.) center zone duration.

The following labels represent pairwise comparisons between groups in the study:

a. Males vs. NC females, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **b.** NC females vs. males, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **c.** LNG Adult vs. males, NC females, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **d.** DRSP Adult vs. males, NC females, LNG Adult, LNG Pubertal, and DRSP Pubertal; **e.** LNG Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and DRSP Pubertal; **f.** DRSP Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and LNG Pubertal.

3.5 Immunohistochemistry

3.5.1 5HT-1A cell expression in the HIPP

The ANOVA revealed no significant difference in 5HT-1A expression in the CA1 ($F_{(5,58)} = 1.121$, $p = .36$, $\eta^2 = .10$), CA2 ($F_{(5,58)} = 1.014$, $p = .42$, $\eta^2 = .09$) and DG ($F_{(5,58)} = 1.933$, $p = .10$, $\eta^2 = .15$) regions of the hippocampus. However, a significant group difference was found in the CA3 region of the hippocampus ($F_{(5,58)} = 3.124$, $p = .02$, $\eta^2 = .23$). Post-hoc analyses revealed that males had significantly higher 5HT-1A expression compared to NC females ($MD = 205.68$, $SE = 65.51$, $p = .003$), EE/LNG adults ($MD = 257.38$, $SE = 70.98$, $p < .001$), and EE/LNG pubertal females ($MD = 159.38$, $SE = 70.98$, $p = .03$).

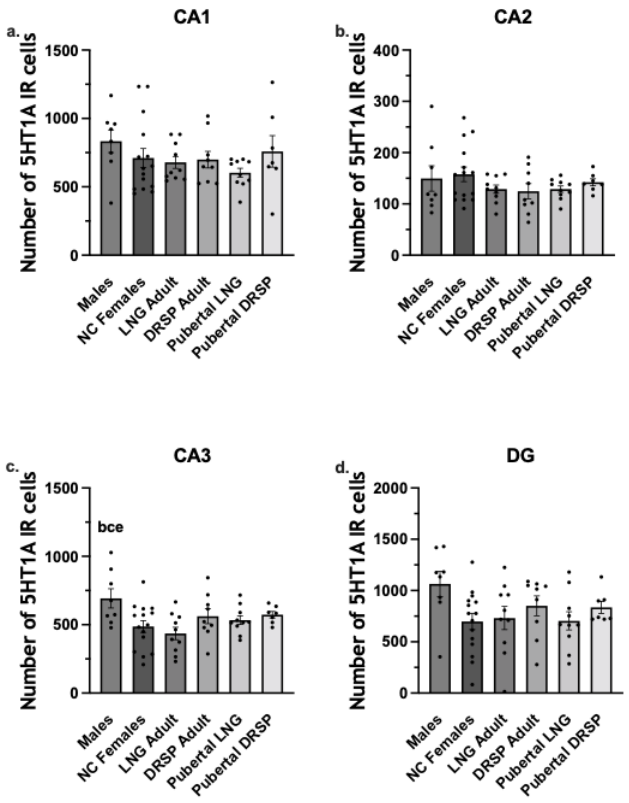

Figure 4. Hippocampal 5HT-1A immunoreactive (IR) cell expression

Note. Group means (±*SEM*) in one-way ANOVA for IHC. Data presented as (a.) CA1, (b.) CA2, (c.) CA3, and (d.) Dentate gyrus.

The following labels represent pairwise comparisons between groups in the study:

a. Males vs. NC females, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **b.** NC females vs. males, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **c.** LNG Adult vs. males, NC females, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **d.** DRSP Adult vs. males, NC females, LNG Adult, LNG Pubertal, and DRSP Pubertal; **e.** LNG Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and DRSP Pubertal; **f.** DRSP Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and LNG Pubertal.

3.5.2 5HT-1A cell expression in the VTA and NAc

The ANOVA found no significant group differences in 5HT-1A expression in the VTA region ($F_{(5,54)} = .690$, $p = .63$, $\eta^2 = .07$) as well as the NAc region ($F_{(5,44)} = 1.639$, $p = .17$, $\eta^2 = .17$).

3.5.3 GABA cell expression in the HIPP

The ANOVA found a significant group difference in GABA expression in the CA1 region of the HIPP ($F_{(5,61)} = 2.422$, $p = .04$, $\eta^2 = .18$). EE/LNG adult females displayed significantly lower GABA expression compared to EE/DRSP adults (*MD* = -495.60, *SE* = 182.56, $p = .009$) and EE/LNG pubertal females (*MD* = -568.17, *SE* = 187.57, $p = .004$).

In addition, a significant group difference was also observed in the CA2 and CA3 regions of the HIPP ($F_{(5,61)} = 5.901$, $p < .001$, $\eta^2 = .35$; $F_{(5,61)} = 2.837$, $p = .02$, $\eta^2 = .20$, respectively). Post hoc tests showed that EE/DRSP adults had significantly higher GABA expression than males (*MD* = 145.90, *SE* = 58.95, $p = .02$; *MD* = 344.70, *SE* = 143.88, $p = .02$), NC females (*MD* = 208.59, *SE* = 53.14, $p < .001$; *MD* = 343.78, *SE* = 129.70, $p = .01$), EE/LNG adults (*MD* = 254.70, *SE* = 58.95, $p < .001$; *MD* = 441.50, *SE* = 143.88, $p = .003$), and EE/DRSP pubertal females (*MD* = 216.47, *SE* = 64.96, $p = .002$; *MD* = 398.26, *SE* = 158.56, $p = .02$). No significant difference was found in the DG ($F_{(5,61)} = 1.364$, $p = .25$, $\eta^2 = .11$).

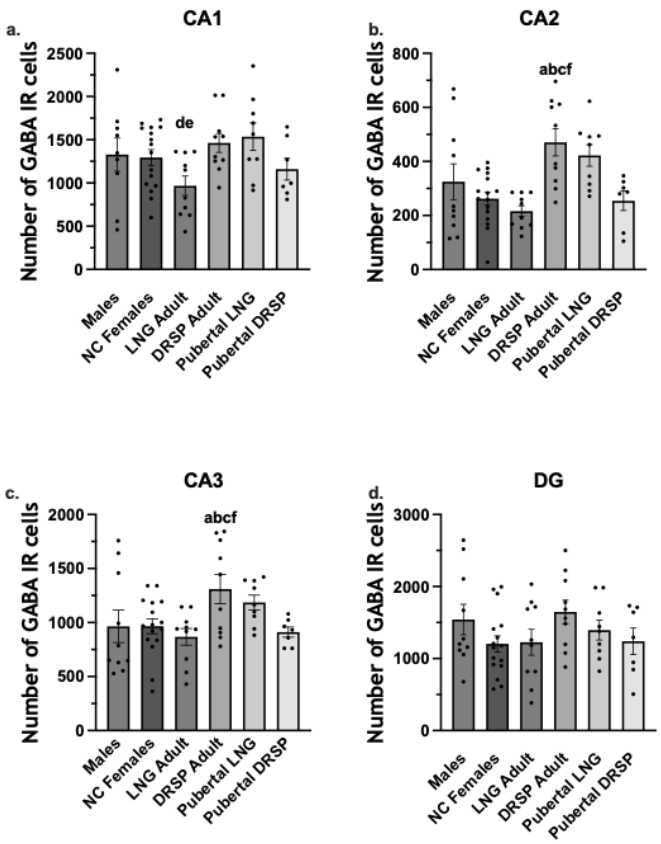

Figure 5. Hippocampal GABA IR cell expression

Note. Group means (±*SEM*) in one-way ANOVA for IHC. Data presented as (a.) CA1, (b.) CA2 , (c.) CA3, and (d.) Dentate gyrus.

The following labels represent pairwise comparisons between groups in the study:

a. Males vs. NC females, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **b.** NC females vs. males, LNG Adult, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **c.** LNG Adult vs. males, NC females, DRSP Adult, LNG Pubertal, and DRSP Pubertal; **d.** DRSP Adult vs. males, NC females, LNG Adult, LNG Pubertal, and DRSP Pubertal; **e.** LNG Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and DRSP Pubertal; **f.** DRSP Pubertal vs. males, NC females, LNG Adult, DRSP Adult, and LNG Pubertal.

3.5.4 GABA cell expression in the VTA and NAc

The ANOVA found no significant group difference in GABA expression in the VTA and NAc regions ($F_{(5,50)} = .687$, $p = .64$, $\eta^2 = .07$; $F_{(5,44)} = 1.553$, $p = .20$, $\eta^2 = .17$, respectively).

4.0 Discussion

The current study investigated the effects of COC exposure since puberty on depression- and anxiety-like behaviours, as well as 5HT-1A and GABAergic expressions in the brain. Our results demonstrated that there were age- and formulation-dependent effects of COC on forced swim stress-coping and open field exploration behaviours, but effects were not observed across all anxiety- and depression-related test measures. There was also formulation-dependent effects in GABA expression (e.g., HIPP), but none for 5HT-1A expression.

Our findings revealed that mice treated with EE/LNG or EE/DRSP during puberty displayed increased escape and lower immobility durations compared to NC females and their adult counterparts, suggesting increased stress coping strategies following COC exposure during puberty. These results are not consistent with our hypothesis that mice treated with COCs during puberty would display more depression-like behaviours. Our results differ from findings indicating that late pubertal (P40-47) and 8 weeks old young adult mice display more immobility in the FST compared to mice experiencing early puberty (i.e., P24 and P28, respectively) (Boivin et al., 2017; Hefner & Holmes, 2007), likely because of age-related differences across development, rather than enduring effects of COC exposure during puberty. Thus, our results suggest that COC treatment during puberty may alter the developmental trajectory of stress-coping behaviour, promoting greater active coping in adulthood. Interestingly, recent work showed that pubertal chronic stress exposure was associated with increased coping behaviours during the FST in female mice, an effect which is mediated by the action of allopregnanolone on

GABA receptors (GABAr) (Kenney et al., 2025). COC exposure, similar to stress exposure, may therefore induce changes in neurosteroid function and GABAr expression, and HPA-axis reactivity during puberty, fostering resilience in specific stress-coping strategies.

Like females treated with EE/LNG and EE/DRSP during puberty, males also displayed increased escape behaviour. These results are not consistent with previous reports showing that females consistently display reduced immobility and more active coping behaviours (e.g., swimming) compared to males (Brotto et al., 2000; Colom-Lapetina et al., 2017; Colom-Lapetina et al., 2019). Nevertheless, these differences in coping strategies in the FST may not solely be due to sex differences. Rather, they could also change as a function of developmental timing and stress exposure. Indeed, pubertal stress has been shown to increase immobility in both sexes, whereas escape-related behaviours seem to be altered in a sex-specific manner with males displaying reduced climbing and females reduced swimming (Harris et al., 2022). These behavioural alterations may be modulated by sex-dependent changes in neurotransmission (Harris et al., 2022), reduced HPA axis activation (Wulsin et al., 2016), decreased hippocampal neurogenesis (Nickle et al., 2020), and reduced synaptic plasticity (Leussis & Andersen, 2008). Therefore, it is possible that the similar pattern in escape behaviour observed in males and COC-treated pubertal females may rise from puberty-related remodeling of the stress response.

In addition, females treated with EE/LNG in adulthood displayed increased time spent in the center of the open field test which indicates less anxiety-like behaviour. This result partially supports our hypothesis and suggests that EE and LNG during adulthood might provide some protective effects on mood, but the specific role of each hormone remains unclear. For instance, estradiol treatment has been linked to increased time in the center and reduced time in the periphery of the OFT in adult female rats, suggesting that estradiol has anxiolytic effects

(Renczés et al., 2020). Estradiol has been shown to reduce anxiety-like behaviours via its actions on regions like the hippocampus and amygdala, although the mechanisms underlying these effects remain unclear (Walf et al., 2006). This aligns with reports of dose-dependent effects of EE, LNG and a combination of EE/LNG on anxiety-like behaviours in adult female rats – effect which was reversed with high EE doses (Simone et al., 2015). Similarly, EE/LNG treatment in female adult rats increased time spent and number of entries in the center in the OFT, indicative of reduced anxiety-like behaviours (Koebele et al., 2021). Taken together, these findings suggest that EE/LNG exposure during adulthood may have modest protective effects on anxiety-like behaviours. However, these effects may depend on doses and experimental conditions, and the action mechanisms of each hormone remain difficult to disentangle.

In contrast, progesterone (P4) and its metabolite allopregnanolone (3α,5α-THP) have been associated with increased number of entries in the center in the OFT, which may be mediated by neurosteroid metabolism and BDNF signaling in the hippocampus and amygdala (Frye et al., 2025). Moreover, blocking 5α-reductase in the amygdala suppresses P4 anxiolytic action, whereas direct allopregnanolone administration restores these effects (Koonce & Frye, 2013; Walf et al., 2006). This highlights the key role allopregnanolone might play in regulating anxiety behaviours. As such, LNG administration has been shown to alter allopregnanolone levels in female rats, resulting in altered social and sexual behaviours (Santoru et al., 2014). Subcutaneous EE/LNG administration in female rats has also been associated with reduced allopregnanolone levels, but only LNG seems to increase anxiety-like behaviours (Porcu et al., 2012). However, it remains uncertain whether estradiol administration alone increases allopregnanolone levels (Pluchino et al., 2009). In the current study, EE/LNG treatment reduced anxiety-like behaviour, suggesting that EE may have counteracted potential anxiogenic effects of

LNG—possibly through its interactions with allopregnanolone. However, as we did not measure allopregnanolone levels, future studies should directly investigate these possible underlying interactions.

In addition, females treated with EE/DRSP during puberty spent less time in the center (i.e., increased thigmotaxis) during the OFT, indicating increased anxiety. This finding partially aligns with our hypothesis regarding the age-dependent effects of hormonal treatments on anxiety behaviours. However, the specific role of EE/DRSP and whether it causes anxiolytic effects requires further investigation. As such, evidence shows that DRSP alleviates mood and premenstrual symptoms in adult women as well as anxiety symptoms during postpartum (Caruso et al., 2023; Pearlstein et al., 2005; Yonkers et al., 2005). Moreover, EE/DRSP use in adult female mice did not alter HPA axis reactivity and anxiety and depressive-like behaviours (Schuh et al., 2024). These findings suggest that pubertal mice may be particularly vulnerable to EE/DRSP's action, potentially inducing adverse effects for specific anxiety-related behaviours that were not observed in adults.

These effects of COCs on behaviours also seem to be task-specific as treatment differences were observed in the FST and the OFT, but not in the TST or EPM. These findings suggest a selective change in stress-coping strategies rather than a general shift in affect, locomotion, or depression- and anxiety-like behaviours across tests. Others have also reported inconsistencies in findings between the TST and FST. Despite conceptual overlap (e.g., both assessing depression), the behaviours during these tests can rely on distinct mechanisms and cognitive processes, resulting in different outcomes/symptoms assessed (Chatterjee et al., 2012; Li et al., 2025). Similarly, anxiety behaviour can be divided in state anxiety which reflects threat perception at a precise time, and trait anxiety which indicates anxiety levels over time

(Figueiredo Cerqueira et al., 2023). As such, the EPM is a measure of state anxiety (i.e., a perceived threat via open arm exposure), whereas the OFT assesses trait anxiety (i.e., free exploration paradigm) (Goes et al., 2009, 2015). Therefore, anxiety and depression are multifaceted conditions and using a battery of tests provides a comprehensive understanding of behavioural despair/anxiety and coping strategies.

Males displayed higher 5HT-1A expression in the CA3 region of the hippocampus than females. The CA3 is involved in supporting cognitive functions such as short-term working memory, memory encoding and consolidation, as well as pattern recognition (Cherubini & Miles, 2015; Lin et al., 2021; Sammons et al., 2024). It has also been associated with anxiety through serotonin's action on CA3 neurons (Karayol et al., 2021). Modulation of 5-HT4 receptors in the CA3 reduces anxiety-like behaviour in rats, increasing the anxiolytic effects of NMDA receptors antagonist D-AP5, suggesting interactions between serotonin and glutamate signaling in anxiety related behaviours (Charousaei et al., 2021). Therefore, the sex difference observed in 5HT-1A expression might have further implications for anxiety- and depression-related behaviours. Nevertheless, how this result translates in altered behaviour in males specifically and for 5HT-1A receptors, remains unclear, as serotonin can have differential effects on anxiety and depression depending on receptor subtype (Albert et al., 2014; Żmudzka et al., 2018). In addition, serotonergic cell bodies are highly concentrated in the brainstem raphe nuclei, whereas HIPP, VTA, and NAc are projections targets (Hornung, 2003). As a result, our 5HT-1A measures yield sparse somatic labeling, potentially limiting sensitivity to more subtle COC- or age-related effects. However, these findings do not rule out COCs effects on serotonin as they may be more important on serotonergic signaling (e.g., receptor availability) rather than 5HT-1A immunoreactivity in specific regions. Consistent with this, one study found that COC users

display lower brain 5-HT4 receptor binding, especially in the hippocampus (Larsen et al., 2020), but further research is needed to investigate 5HT-1A receptors.

GABA showed robust effects with EE/DRSP adults displaying significantly higher expression in the HIPP (CA2 and CA3) compared to all the groups, except EE/LNG pubertal mice. This pattern suggests altered GABA signaling in HIPP subregions that contain GABA interneurons and receptors (Nagarajan et al., 2026; Pelkey et al., 2017). This may promote resilience to depressive-like states by modulating stress induced hippocampal activity (Lacasse et al., 2024). Since CA2/CA3 host GABAergic circuitry and receptor expression (Chen & Sharma, 2025), and GABA deficits and receptor dysfunction are associated with anxiety/depression (Möhler, 2009), this pattern may indicate a formulation-specific shift in hippocampal GABA inhibitory activity. Indeed, DRSP has a neutral or non-suppressive effect on allopregnanolone which is a potent positive secondary binding site modulator of GABAa receptors (Pletzer et al., 2023). This might contribute to preserve GABA signaling in EE/DRSP adults. In contrast, EE/LNG adults displayed lower GABA expression in the CA1 region. This is consistent with evidence showing that androgenic progestins have more disruptive effects on hippocampal GABAa receptor expression than anti-androgenic progestins (Follesa et al., 2002; Lacasse et al., 2024; Porcu et al., 2012; Porcu et al., 2019). This may explain the increased OFT exploratory and the FST coping behaviours observed among EE/LNG adults.

4.1 Limitations and future directions

Given that depression and anxiety are complex, highly comorbid mental illnesses (i.e., rates between 60–90%), share overlapping symptoms, genetic risks, and neurobiological pathways while exhibiting distinct etiology, neurotransmitter pathways, and treatment responses (Cui et al., 2024; Liu et al., 2018; Pizzagalli, 2016), the current finding should be interpreted

while considering several limitations. First, it provides a broad, preliminary investigation of COC effects on mood disorders and neurochemistry, limiting our ability to determine the specific mechanisms underlying the relationship between COC use and vulnerability to depression and anxiety. Future studies should investigate in more depth the disorder-specific processes involved in relation to COC use which could provide better insights into the etiology of these conditions. Moreover, no analyses were performed in key brain regions due to limitations in samples which significantly reduced statistical power. These regions, such as the amygdala, the paraventricular nucleus of the hypothalamus and the dorsal raphe are involved in mood disorders and fear regulation (AbuHasan et al., 2025; Bao & Swaab, 2019; Fox & Shackman, 2024; Herman & Tasker, 2016; Walker & Tadi, 2025). Similarly, we did not assess other relevant neurotransmitters (e.g., dopamine and norepinephrine), which have been associated with mood disorders (Sekhon & Gupta, 2025).

4.2 Conclusion

To our knowledge, this study is the first to examine how developmental timing (puberty vs adulthood) and COC formulation (EE/LNG vs EE/DRSP) interact to shape vulnerability to mood disorders using an animal model. We show that COC use is associated with age- and formulation-dependent changes in specific anxiety and depressive behaviours as well as neurobiological outcomes (i.e., 5HT-1A and GABA expression). Pubertal COC exposure is associated with increased active coping in the FST, suggesting a bias towards stress-coping strategies, although these effects were test-specific and not observed across all anxiety- and depressive-related tests. Formulation and age-specific changes in hippocampal GABA expression were observed in COCs treated adult females, whereas 5HT-1A immunoreactivity was largely unchanged. Together, these findings suggest COCs impacts on mood disorders are

dependent on developmental timing, and progestin type. This highlights the importance of considering the age of onset and formulation choice when evaluating depression and anxiety in COC users.

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Declaration of Interests: None

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CHAPTER 4 : EFFECTS OF COMBINED ORAL CONTRACEPTIVES AND LIPOPOLYSACCHARIDE ADMINISTRATION ON ACUTE IMMUNE AND STRESS RESPONSES DURING PUBERTY AND ADULTHOOD

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Abstract

Combined oral contraceptives (COCs) are the most widely used method of contraception worldwide. However, their effects on the hypothalamic-pituitary-adrenal (HPA) axis and on the immune system, particularly during puberty, remain poorly understood. Given that puberty is a period of maturation for the HPA axis and immune system, and exposure to stress during this period can have enduring effects on the brain and behaviour, this study investigated whether COC use during puberty and adulthood exacerbates lipopolysaccharide (LPS)-induced inflammation and stress responses. Female CD-1 mice received either one of two COC formulae (ethinyl estradiol/levonorgestrel (EE/LNG) or ethinyl estradiol/drospirenone (EE/DRSP)) or a vehicle either from 4 to 6 weeks (pubertal) or from 10 to 12 weeks (adult). At 6 (pubertal) or 12 (adult) weeks of age, mice were injected with either saline or LPS and sickness behaviours were monitored. Plasma and brain tissue samples were collected 8 hours following saline or LPS treatment. Results showed that COCs- treated pubertal mice displayed increased sickness behaviour despite higher anti-inflammatory cytokine (e.g., IL-10) concentrations than adults. LPS-treated EE/LNG adults had greater corticosterone and pro-inflammatory cytokine plasma concentrations and cytokine expressions in the brain than LPS-treated EE/LNG pubertal females in a region-specific manner. Regardless of age of onset of COC use, EE/LNG potentiated, while EE/DRSP reduced, LPS-induced inflammatory responses. These findings indicate that COC formulation and age of onset of COC exposure modulate LPS-induced immune and HPA-axis responses.

Keywords: Puberty; Combined oral contraceptives; Lipopolysaccharide; Stress; Hypothalamic-pituitary-adrenal axis; Drospirenone; Levonorgestrel

1.0 Introduction

Oral contraceptives are one of the most effective forms of reversible birth control and are used daily by millions of females worldwide (Brown et al., 2017). Combined oral contraceptives (COCs) which contain both synthetic forms of estradiol (i.e., ethinyl estradiol) and progesterone (e.g., levonorgestrel, drospirenone, etc.) have been shown to alter brain structure and function, as well as mood and cognition (Armbruster et al., 2017; Hampson, 2023; Jensen et al., 2022; Laird et al., 2019; Montoya & Bos, 2017; Sharma, Fang, et al., 2020; Sharma, Smith, et al., 2020).

COCs are often used by females during puberty and adolescence to prevent pregnancy, but also to manage conditions such as premenstrual dysphoric disorder, acne, menstrual irregularities, and endocrine issues (Bahamondes et al., 2015; Carey & Allen, 2012; Schrager et al., 2020). However, puberty is a critical developmental period characterized by the activation and maturation of the HPG axis, resulting in fluctuating sex hormone levels that drive sex-dependent brain maturation, as well as physiological and behavioural changes (Becker, 2013; Breehl & Caban, 2024). COC use during puberty has been associated with region-specific structural and functional brain alterations, such as increased white matter volume in the right fusiform gyrus compared to naturally cycling human females (Sharma, Smith, et al., 2020). Moreover, compared to adult users, human females who began taking COCs during puberty also display higher BOLD responses to negative affective stimuli in the left fusiform gyrus, right cuneus, bilateral and left mid-temporal lingual gyri, right insula, and precentral gyrus during an emotional working memory task (Sharma, Smith, et al., 2020). In addition, females exposed to COCs during puberty also demonstrate enhanced resting state functional connectivity within the salience network compared to adult COC users (Sharma, Fang, et al., 2020).

Puberty is also a period of heightened neuroplasticity, and exposure to stress during this period can have enduring effects on the brain and behaviour (Marshall, 2016; McCormick et al., 2017). During this period, due to rising levels of sex hormones, the hypothalamic-pituitary-adrenal (HPA) axis undergoes sex-dependent maturation (Duan & Sun, 2017; Goel et al., 2014; McCormick & Mathews, 2007; Smith et al., 2023). Sex hormones have both organizational and activational effects on the HPA axis, contributing to sex differences in stress responses across the lifespan (Heck & Handa, 2019a). Sex hormones such as androgens typically mitigate the stress response, while estradiol exacerbates it (Handa et al., 2022; Zuloaga et al., 2024), which could explain sex differences in stress reactivity. Specifically, higher estradiol levels are associated with increased corticotropin-releasing (CRH) and adrenocorticotropic (ACTH) hormones which leads to increased glucocorticoid production in both humans and rodents in response to stress (Handa & Weiser, 2014). As such, ovariectomized female rats show decreased corticosterone (CORT) and ACTH release following a stressor and this effect is suppressed with estradiol administration (Serova et al., 2010; Weiser & Handa, 2009). Estrogen receptors (ERα and ERβ) also modulate HPA axis activity through their expression in neurons within HPA-relevant regions (e.g., paraventricular nucleus of the hypothalamus (PVN)) and in regions that project to the PVN (e.g., hippocampus, bed nucleus of the stria terminalis, medial preoptic area) (Handa & Weiser, 2014; Suzuki & Handa, 2005). Moreover, glucocorticoids have also been shown to influence ERα and ERβ expression as they have been shown to inhibit ERα transcriptional activity (Karmakar et al., 2013). In addition, adrenalectomy has been shown to downregulate ERβ mRNA expression in the PVN of female rats: an effect that is reversed with CORT treatment (Handa & Weiser, 2014; Isgor et al., 2003). In contrast, progesterone and allopregnanolone in females, along with androgens and their metabolites, such as

dihydrotestosterone in males, are associated with reduced concentrations of CORT and ACTH (Heck & Handa, 2019a; Ring, 2025; Seale, Wood, Atkinson, Bate, et al., 2004; Stephens et al., 2016; Zuloaga et al., 2024). These differences in endogenous sex hormone concentrations contribute to sex differences in stress response and could explain the increased vulnerability to stress-related disorders in females (Heck & Handa, 2019a). For instance, adult female rats exposed to recurrent restraint stress during puberty have higher basal CORT levels in adulthood when compared to males and non-stressed female controls (Barha et al., 2011). Females generally display a faster and more pronounced stress response compared to males (Goel et al., 2014; Heck & Handa, 2019a). Interestingly, when statistically controlling for sex hormone levels, both cortisol reactivity and stress response patterns were reduced in human males, COC users, and naturally cycling females in the luteal phase (Barel et al., 2018), highlighting the pivotal role of sex hormones in shaping the stress response.

Similarly, sex hormones also modulate the immune response (Hoffmann et al., 2023) through their action on differentiation processes from precursor cells, lifespan and survival of immune cells, as well as their ability to fight pathogens and malignancies (e.g., phagocytic activity, cytokine, and antibody production) (Mendes et al., 2018; Sciarra et al., 2023). In addition, sex hormone receptors are expressed in specific immune cells, thus modulating their function (Bouman et al., 2005; Brundin et al., 2021). Estrogens usually enhance both innate and adaptive immune responses through the stimulation of pro- and anti-inflammatory cytokines, B cell proliferation, and humoral immunity (Chakraborty et al., 2022; Harding & Heaton, 2022; Kovats, 2015). ERα have also been shown to modulate gene expression in immune cells via their interactions with nuclear transcription factor kappa B (NF-κB), signaling pathways in macrophages and dendritic cells (Kalaitzidis & Gilmore, 2005; Kovats, 2015). In contrast,

progesterone and androgens seem to have immunosuppressive effects through their action on antibody production, cytokine production, and immune cell function (Trigunaite et al., 2015; Zwahlen & Stute, 2024). These hormonal influences impact health and development particularly during puberty (Quatrini et al., 2021; Ucciferri & Dunn, 2022), resulting in sex differences in inflammation and immune response (Klein & Flanagan, 2016).

Given that both the HPA axis and the immune system undergo maturation during puberty, there are age and sex differences in acute responses to stress and immune challenges. For example, mice treated with lipopolysaccharide (LPS), a bacterial endotoxin (Galanos & Freudenberg, 1993), during puberty, display less sickness behaviour (Sharma et al., 2018), less hypothermia (Cai et al., 2016), and reduced concentrations of pro-inflammatory cytokines (i.e., tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6, IL-12p70, and interferon γ (IFNγ)), and CORT (Cai et al., 2016) compared to mice exposed to LPS in adulthood. Findings from our laboratory also show that pubertal LPS treatment induces greater sickness behaviour, hypothermia, and serum pro- and anti-inflammatory cytokines concentrations in males than in females (Cai et al., 2016; Sharma et al., 2018). However, pubertal LPS treatment induces greater serum corticosterone concentration in females than in males (Girard-Joyal & Ismail, 2017). Moreover, pubertal activation of both stress and immune systems can have enduring effects on brain and behaviour (Holder & Blaustein, 2014; Kane & Ismail, 2017; Lafuse et al., 2017; Mantovani & Fucic, 2014; McCormick et al., 2017). For example, exposure to LPS during puberty has been associated with an enduring decrease in sexual receptivity in females (Ismail et al., 2011), and impairments in spatial learning (Ismail & Blaustein, 2013; Kolmogorova et al., 2019) and dopamine-sensitive behaviours in males (Girard-Joyal & Ismail, 2017), as well as an

increase in anxiety- and depression-like behaviours in both males and females (Murray et al., 2019).

Given that the synthetic hormones found in COCs bind to the same receptors as endogenous gonadal hormones, COCs could disrupt these sex hormone-mediated processes on the HPA axis and immune function, especially during puberty, a period of increased vulnerability to stress, and interfere with the maturation and development of these systems. Therefore, this study aimed to determine whether COC use during puberty alters immune system and HPA axis reactivity following LPS treatment. We hypothesized that COC use would increase corticosterone and pro-inflammatory cytokines concentrations and reduce anti-inflammatory cytokine concentration compared to controls. In addition, we expected that mice treated with COCs during puberty would display more pro-inflammatory cytokines and corticosterone concentration compared to adulthood.

2.0 Methods

2.1 Animals

One hundred twenty female CD-1 mice were shipped at 3 weeks of age from Charles River Laboratories Inc. (Kingston, New-York, United States). Upon arrival, mice were housed in groups of three or four in polycarbonate Lexan cages (17 × 28 × 12 cm [width x length x height]) filled with Teklad Corn Cob bedding (Harlan Laboratories, Inc., Madison, WI, USA, 0.25 in. diameter). Mice were kept in an all-female room on a reversed light/dark cycle (14 hours light, 10 hours dark) with lights off at 10 A.M. and under controlled temperature (24 ± 2°C) and humidity (40 ± 5 %) conditions. For environmental enrichment, mice were provided two square pieces of nestlet (Ancare Corp., Bellmore, NY, USA) and a cardboard refuge hut (Ketchum Manufacturing, Inc., Brockville, ON, Canada). Mice had *ad libitum* access to food (Harlan

Laboratories, Inc., Madison, WI, US, T2018 – Global 18% rodent) and water. All procedures were approved by the Animal Care Committee of the University of Ottawa.

2.2 Oral contraceptive treatment

Female mice began synthetic hormone treatment at either 4 (pubertal groups) or 10 (adult groups) weeks of age, respectively. Mice were administered either a vehicle containing 248.4 µl of water and 1.6 µl of dimethyl sulfoxide (DMSO) of (n = 20 pubertal mice and 20 adult mice) or one of two COC formulae through oral gavage daily for 2 consecutive weeks. A subset of pubertal (n=20) and adult (n = 20) female mice received a mixture of 0.22 µg of ethinyl estradiol and 1.0762 µg of levonorgestrel (i.e., EE/LNG) dissolved in 248.4 µl of water and 1.6 µl of DMSO for solubility. Another subset of pubertal (n = 20) and adult (n = 20) female mice received a combination of 0.22 µg of ethinyl estradiol and 21.525 µg of drospirenone (i.e., EE/DRSP) dissolved in 248.4 µl of water and 1.6 µl of DMSO for solubility. The dosage of synthetic hormones was selected based on the doses found in most commonly used COCs and converted to a mouse equivalent dose using an Animal Equivalent Dose conversion factor (Nair & Jacob, 2016), and then multiplied by the average weight of CD-1 mice (35 g).

2.3 Lipopolysaccharide administration

At 6 (pubertal groups) or 12 (adult groups) weeks old, mice received an intraperitoneal (i.p.) injection of either 1.5 mg/kg of LPS (Escherichia coli serotype O26:B6; L3755; 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 has been shown to induce sickness behaviours for approximately 24-48 hours (Cai et al., 2016).

2.4 Sickness monitoring

Sickness behaviour monitoring occurred at 30 min, 2, 4, 6, and 8 hours post LPS or saline injection. The progression of sickness behaviours was evaluated using a non-intrusive and impartial method, employing two raters who were blinded to the experimental conditions, as outlined in Kolmogorova et al. (2017). Raters visually inspected the mice for signs of lethargy (i.e., reduced movement), huddling (i.e., curled body posture), ptosis (i.e., drooping eyelids), and piloerection (i.e., raised fur). Each mouse was scored based on the number of symptoms observed (0-4) at each time point. The sickness scores assigned by both raters at each time point were averaged for further statistical analyses.

2.5 Euthanasia and plasma extraction

Eight hours after the saline or LPS treatment, mice were anesthetized with Euthanyl (Sodium pentobarbital; 120 mg/kg, i.p.). Motor reflexes of the mice were assessed by gently pinching their feet. Once no motor reflexes were observed, blood was obtained via cardiac puncture and placed into Microvette CB 300 K2E blood extraction tubes (Sarstedt AG & Co, Nümbrecht, Germany) containing an EDTA anti-coagulant. The tubes were stored at 4°C until plasma extraction. Within four hours of blood collection, samples were centrifuged at 1,100 x g at 20 °C for 15 minutes to separate the plasma, which was then aliquoted and stored at -80 °C.

2.6 Brain tissue extraction

After blood collection, mice were decapitated and brains were collected, flash-frozen in liquid nitrogen, and stored at -80 °C until further processing. Using a LEICA CM1950 cryostat, the brain tissue was sliced into coronal sections of 300 μm thickness. Tissue samples from the amygdala, hypothalamus, and hippocampus were punched-dissected and kept into RNA-free tubes. Tubes were stored at -80 °C until RNA extraction.

2.7 Enzyme-linked immunosorbent assay (ELISA)

Plasma concentrations of corticosterone were measured in duplicate with an ELISA kit (No. ADI-901-097; Enzo Life Sciences Inc.) according to the manufacturer's instructions. Each plate contained one pooled sample to monitor inter-assay variation. All plates were read with a Biotek Powerwave XS2 and analyzed with the Gen 5 V2.0 software. Samples with %CV greater than 15% were excluded from the analyses.

2.8 Multiplex immunoassay

Plasma levels of interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 12 (p70) (IL12p70), interferon γ (IFNγ), and tumor necrosis factor (TNF-α) were assessed using a multiplex bead-based Luminex immunoassay. Utilizing multiplex kits (No. MTH17MAG-47K; Millipore-Sigma) in accordance with the manufacturer's instructions, plasma samples were analyzed in duplicate. Each assay plate included a pooled sample to monitor inter-assay variability. Cytokine concentrations were measured using the MAGPIX system.

2.9 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 hypothalamus (HYP), prefrontal cortex (PFC), and hippocampus (HIPP) tissue. cDNA was synthesized using the QuantiTect Reverse Transcription kit (No. 205311; QIAGEN) according to the manufacturer's instructions. cDNA was stored at -80 °C until subsequent real-time quantitative PCR.

2.10 Real-time quantitative polymerase chain reaction (RT-qPCR)

Gene expressions were evaluated utilizing the SsoAdvanced Universal SYBR Green Supermix (No. 1725274; Bio-Rad) in triplicate of 10µL reactions, performed on the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Primer efficiency was assessed by

analyzing the slope between RNA quantity and cycle thresholds using CFX Maestro software (Bio-Rad). All primer pairs exhibited reaction efficiency within the range of 90-110%. The housekeeping genes β-actin and GAPDH remained stable across experimental conditions. The quantitative threshold amplification cycle number (Cq) was determined for each reaction and log-transformed as described previously (Taylor et al., 2019). Primers were obtained from Integrated DNA Technologies, with sequences provided in Table 2.

Table. 2 Summary of primer sequences

Target Gene	Forward	Reverse
β-actin	GAACCCTAAGGCCAACCGTG	GGTACGACCAGAGGCATACAGG
GAPDH	TCAACAGCAACTCCCACTCT	CCACCACCCTGTTGCTGTA
IL-6	GCCTTCTTGGGACTGATGCT	GCCATTGCACAACTCTTTTCTC
IL-1β	TGTCTGAAGCAGCTATGGCAAC	CTGCCTGAAGCTCTTGTTGATG
TNF-α	GCCTATGTCTCAGCCTCTTCTC	GCCATTTGGGAACTTCTCATCC

2.11 Statistical analyses

All statistical analyses were performed using IBM SPSS v20 software. Cases that exceeded the 1.5 interquartile range in boxplot analyses (RT-qPCR, ELISA, multiplex, and sickness behaviour data) were considered statistical outliers and were winsorized 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 (30 minutes, 2 hours, 4 hours, 6 hours, and 8 hours) and the between-subject effects of age (adult or pubertal), COC formulation (EE/LNG or EE/DRSP), and LPS treatment (LPS or saline). Greenhouse-Geisser corrections were applied to F-values that violated Mauchly's test of sphericity. For other measures (i.e., RT-qPCR, ELISA, multiplex), a 2

x 2 x 2 ANOVA was performed for age (adult or pubertal), COC formulation (EE/LNG or EE/DRSP), and LPS treatment (LPS or saline). When appropriate, statistically significant effects were followed by pairwise comparisons with Fisher's Least Significant Difference test. 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 ($p < 0.05$). Therefore, all within-subjects effects were assessed with Greenhouse-Geisser corrections. The ANOVA revealed a within-subjects main effect of time ($F_{(2.695, 291.047)} = 243.227$, $p < 0.001$, $\eta_p^2 = 0.69$), as well as within-subjects interactions of time x age ($F_{(2.695, 291.047)} = 6.151$, $p < 0.001$, $\eta_p^2 = 0.05$), time x LPS ($F_{(2.695, 291.047)} = 96.809$, $p < 0.001$, $\eta_p^2 = 0.47$), and time x age x LPS ($F_{(2.695, 291.047)} = 4.083$, $p = 0.01$, $\eta_p^2 = 0.04$). Pairwise comparisons revealed that LPS-treated pubertal mice exhibited greater sickness behaviour than adults at 2 ($MD = 0.68$, $SE = 0.15$, $p < .001$), 4 ($MD = 0.93$, $SE = 0.15$, $p < .001$), 6 ($MD = 0.72$, $SE = 0.17$, $p < .001$), and 8 ($MD = 0.77$, $SE = 0.22$, $p < .001$) hours post-injection. In addition, adult LPS-treated mice showed increased sickness behaviour compared to their saline-treated counterparts at 30 minutes ($MD = 0.30$, $SE = 0.07$, $p < .001$), 2 ($MD = 0.95$, $SE = 0.15$, $p < .001$), 4 ($MD = 1.35$, $SE = 0.15$, $p < .001$), 6 ($MD = 2.03$, $SE = 0.17$, $p < .001$), and 8 ($MD = 2.13$, $SE = 0.22$, $p < .001$) hours following treatment. Similarly, LPS-treated pubertal mice displayed increased sickness behaviour at 30 minutes ($MD = 0.15$, $SE = 0.07$, $p = .04$), 2 ($MD = 1.45$, $SE = 0.15$, $p < .001$), 4 ($MD = 2.17$, $SE = 0.15$, $p < .001$), 6 ($MD = 2.52$, $SE = 0.17$, $p < .001$), and 8 ($MD = 2.83$, $SE = 0.22$, $p < .001$) hours following treatment compared to saline-treated pubertal mice. (Figure 1A).

Moreover, a time x treatment x LPS interaction was also found ($F_{(5.390, 291.047)} = 2.636$, $p = 0.02$, $\eta_p^2 = 0.05$). Pairwise comparisons showed that LPS-treated EE/DRSP mice had greater sickness behaviour than LPS-treated controls and LPS-treated EE/LNG mice (*MD* = 0.28, *SE* = 0.09, *p* = .002; *MD* = 0.20, *SE* = 0.09, *p* = .02; respectively) 30 minutes post LPS injection. In addition, 4 hours post-injection, LPS-treated EE/DRSP mice displayed higher sickness scores than EE/LNG mice (*MD* = 0.40, *SE* = 0.18, *p* = .03). LPS-treated EE/DRSP mice also had higher sickness scores than controls (*MD* = 0.48, *SE* = 0.20, *p* = .02) at the 6 hours mark. See Figure 1(B).

The mixed ANOVA also found a significant between-subjects main effects of age ($F_{(1,108)} = 22.278$, $p < 0.001$, $\eta_p^2 = 0.17$), treatment ($F_{(2,108)} = 5.838$, $p = 0.004$, $\eta_p^2 = 0.10$), and LPS ($F_{(1,108)} = 429.684$, $p < 0.001$, $\eta_p^2 = 0.80$). A significant between-subjects age x LPS ($F_{(1,108)} = 9.406$, $p = 0.003$, $\eta_p^2 = 0.08$) interaction was also found. Pairwise comparisons revealed that LPS-treated pubertal mice had significantly higher sickness behaviour scores than adult LPS-treated mice (*MD* = 0.60, *SE* = 0.11, *p* < .001). In addition, both adults (*MD* = 1.35, *SE* = 0.11, *p* < .001), and pubertal (*MD* = 1.82, *SE* = 0.11, *p* < .001) LPS-treated mice exhibited higher sickness behaviour than their saline-treated counterparts.

3.2 ELISA

The ANOVA found significant main effects of age ($F_{(1,107)} = 24.921$, $p < 0.001$, $\eta_p^2 = 0.19$) and LPS ($F_{(1,107)} = 141.475$, $p < 0.001$, $\eta_p^2 = 0.57$). Significant age x LPS ($F_{(1,107)} = 4.151$, $p = 0.04$, $\eta_p^2 = 0.04$), treatment x LPS ($F_{(2,107)} = 5.016$, $p = 0.008$, $\eta_p^2 = 0.09$), and age x treatment x LPS interactions were also observed ($F_{(2,107)} = 4.993$, $p = 0.008$, $\eta_p^2 = 0.09$). Pairwise comparisons revealed that LPS-treated adult control females displayed significantly higher corticosterone concentration than LPS-treated pubertal controls (*MD* = 157661.20, *SE* = 36122.71, *p* < .001). Similarly, LPS-treated EE/LNG adults (*MD* = 140748.40, *SE* = 36122.71, *p*

< .001) displayed significantly higher corticosterone concentration than their pubertal counterparts. In addition, saline-treated EE/DRSP adults (*MD* = 85592.76, *SE* = 36122.71, *p* = .02) displayed increased corticosterone concentrations compared to saline-treated EE/DRSP pubertal mice. However, LPS-treated EE/DRSP adults displayed decreased corticosterone levels compared to LPS-treated adult controls (*MD* = -115393.08, *SE* = 36122.71, *p* = .002) and LPS-treated EE/LNG adults (*MD* = -117391.16, *SE* = 36122.71, *p* = .002). In addition, saline-treated EE/DRSP adults had significantly higher corticosterone levels (*MD* = 93836.48, *SE* = 36122.71, *p* = .01) than their EE/LNG counterparts. (Figure 2)

3.3 Multiplex

3.3.1 Peripheral plasma IFNγ concentrations

The ANOVA found significant main effects of age ($F_{(1,107)} = 12.968$, $p < 0.001$, $\eta_p^2 = 0.11$), treatment ($F_{(2,107)} = 4.423$, $p = 0.01$, $\eta_p^2 = 0.08$), and LPS ($F_{(1,107)} = 63.122$, $p < 0.001$, $\eta_p^2 = 0.37$). There were also significant age x treatment ($F_{(2,107)} = 5.238$, $p = 0.007$, $\eta_p^2 = 0.09$), age x LPS ($F_{(1,107)} = 8.804$, $p = 0.004$, $\eta_p^2 = 0.08$), treatment x LPS ($F_{(2,107)} = 6.126$, $p = 0.003$, $\eta_p^2 = 0.10$), and age x treatment x LPS ($F_{(2,107)} = 8.306$, $p < 0.001$, $\eta_p^2 = 0.13$) interactions. Pairwise comparisons revealed that LPS-treated control adults had higher IFNγ levels compared to their pubertal (*MD* = 3897.68, *SE* = 1112.03, *p* < .001) counterparts and LPS-treated EE/DRSP adults (*MD* = 3104.17, *SE* = 1112.03, *p* = .006). Similarly, LPS-treated EE/LNG adults displayed significantly more IFNγ concentration than their pubertal counterparts (*MD* = 6552.88, *SE* = 1112.03, *p* < .001). Moreover, LPS-treated EE/LNG adults had significantly higher IFNγ concentrations compared to LPS-treated control adults (*MD* = 4220.78, *SE* = 1112.03, *p* < .001) and LPS-treated EE/DRSP adults (*MD* = 7324.95, *SE* = 1112.03, *p* < .001). Interestingly, LPS-

treated EE/DRSP pubertal mice had significantly higher IFNγ levels than their LPS-treated control counterparts (*MD* = 2256.99, *SE* = 1112.03, $p = .045$). (Figure 3A).

3.3.2 Peripheral plasma IL-1β concentrations

The ANOVA detected significant main effects of age ($F_{(1,107)} = 9.930$, $p = 0.002$, $\eta_p^2 = 0.09$) and LPS ($F_{(1,107)} = 46.387$, $p < 0.001$, $\eta_p^2 = 0.30$). There were also age x LPS ($F_{(1,107)} = 7.656$, $p = 0.007$, $\eta_p^2 = 0.07$), treatment x LPS ($F_{(2,107)} = 3.803$, $p = 0.03$, $\eta_p^2 = 0.07$), and age x treatment x LPS ($F_{(2,107)} = 3.488$, $p = 0.03$, $\eta_p^2 = 0.06$) interactions. Pairwise comparisons revealed that LPS-treated control adults had higher IL-1β levels compared to pubertal counterparts (*MD* = 22.28, *SE* = 6.64, $p = .001$). Similarly, LPS-treated EE/LNG adults had higher IL-1β levels (*MD* = 27.89, *SE* = 6.64, $p < .001$) compared to pubertal counterparts. However, LPS-treated EE/DRSP adults displayed reduced IL-1β concentrations compared to controls (*MD* = -24.14, *SE* = 6.64, $p < .001$) and EE/LNG (*MD* = -30.79, *SE* = 6.64, $p < .001$) counterparts. (Figure 3B).

3.3.3 Peripheral plasma IL-6 concentrations

The ANOVA found significant main effects of age ($F_{(1,107)} = 12.831$, $p < 0.001$, $\eta_p^2 = 0.11$), treatment ($F_{(2,107)} = 4.439$, $p = 0.01$, $\eta_p^2 = 0.08$), and LPS ($F_{(1,107)} = 110.347$, $p < 0.001$, $\eta_p^2 = 0.51$). There were also significant age x treatment ($F_{(2,107)} = 4.090$, $p = 0.02$, $\eta_p^2 = 0.07$), age x LPS ($F_{(1,107)} = 8.612$, $p = 0.004$, $\eta_p^2 = 0.07$), treatment x LPS ($F_{(2,107)} = 7.568$, $p < 0.001$, $\eta_p^2 = 0.12$), and age x treatment x LPS ($F_{(2,107)} = 7.070$, $p = 0.001$, $\eta_p^2 = 0.12$) interactions. Pairwise comparisons revealed that LPS-treated control adults had higher IL-6 levels compared to pubertal counterparts (*MD* = 2128.59, *SE* = 446.47, $p < .001$). In addition, LPS-treated EE/LNG adults had higher IL-6 levels (*MD* = 1965.06, *SE* = 446.47, $p < .001$) compared to pubertal counterparts. However, LPS-treated EE/DRSP adults displayed lower IL-6 levels compared to

controls (*MD* = -2609.06, *SE* = 446.47, $p < .001$), and EE/LNG counterparts (*MD* = -2611.15, *SE* = 446.47, $p < .001$). (Figure 3C).

3.3.4 Peripheral plasma IL-10 concentrations

The ANOVA found significant main effects of age ($F_{(1,107)} = 11.857$, $p < 0.001$, $\eta_p^2 = 0.10$) and LPS ($F_{(1,107)} = 141.621$, $p < 0.001$, $\eta_p^2 = 0.57$). There was a significant age x LPS ($F_{(1,107)} = 14.860$, $p < 0.001$, $\eta_p^2 = 0.12$) interaction. Pairwise comparisons showed that, regardless of COC treatment, LPS-treated pubertal mice had higher !L-10 levels (*MD* = 122.18, *SE* = 23.57, $p < .001$) compared to LPS-treated adults. Moreover, both LPS-treated adult (*MD* = 134.69, *SE* = 23.57, $p < .001$) and pubertal (*MD* = 263.75, *SE* = 23.78, $p < .001$) mice displayed significantly more !L-10 concentrations than their saline-treated counterparts. (Figure 3D).

3.3.5 Peripheral plasma IL-12p70 concentrations

Significant main effects of age ($F_{(1,107)} = 30.190$, $p < 0.001$, $\eta_p^2 = 0.22$), treatment ($F_{(2,107)} = 3.813$, $p = 0.03$, $\eta_p^2 = 0.07$), and LPS ($F_{(1,107)} = 71.943$, $p < 0.001$, $\eta_p^2 = 0.40$) were observed. There were also significant age x LPS ($F_{(1,107)} = 19.292$, $p < 0.001$, $\eta_p^2 = 0.15$), treatment x LPS ($F_{(2,107)} = 10.304$, $p < 0.001$, $\eta_p^2 = 0.16$), and age x treatment x LPS ($F_{(2,107)} = 6.323$, $p = 0.003$, $\eta_p^2 = 0.11$) interactions. Pairwise comparisons revealed that LPS-treated control adults had higher IL-12p70 levels (*MD* = 21.48, *SE* = 4.56, $p < .001$) compared to LPS-treated control pubertal mice. In addition, LPS-treated EE/LNG adults had higher IL-12p70 levels (*MD* = 29.37, *SE* = 4.56, $p < .001$) compared to LPS-treated EE/LNG pubertal mice. However, LPS-treated EE/DRSP adults displayed lower IL-12p70 concentration than LPS-treated control adults (*MD* = -21.74, *SE* = 4.56, $p < .001$) and LPS-treated EE/LNG adults (*MD* = -27.74, *SE* = 4.56, $p < .001$) (Figure 3E).

3.3.6 Peripheral plasma TNF-α concentrations

The ANOVA detected significant main effects of treatment ($F_{(2,107)} = 6.028$, $p = 0.003$, $\eta_p^2 = 0.10$) and LPS ($F_{(1,107)} = 170.670$, $p < 0.001$, $\eta_p^2 = 0.62$). A significant two-way interaction between treatment x LPS ($F_{(2,107)} = 9.891$, $p < 0.001$, $\eta_p^2 = 0.16$) was also found. Pairwise comparisons revealed that LPS-treated EE/LNG mice displayed higher TNF-α concentrations compared to LPS-treated controls (*MD* = 39.06, *SE* = 8.61, $p < .001$) and LPS-treated EE/DRSP mice (*MD* = 43.58, *SE* = 8.61, $p < .001$). (Figure 3F).

3.4 RT-qPCR

3.4.1 IL-1β mRNA expressions in the HYP, PFC, and HIPP

Hypothalamus (HYP): The ANOVA found significant main effects of age ($F_{(1,107)} = 6.069$, $p = 0.02$, $\eta_p^2 = 0.05$) and LPS ($F_{(1,107)} = 64.976$, $p < 0.001$, $\eta_p^2 = 0.38$) in the HYP. A significant two-way interaction between age X LPS ($F_{(1,107)} = 5.039$, $p = 0.03$, $\eta_p^2 = 0.05$) was also found. Pairwise comparisons revealed that LPS-treated adult mice displayed significantly greater IL-1β mRNA expression in the HYP (*MD* = 14.98, *SE* = 4.48, $p < .001$) than their pubertal counterparts. In addition, both LPS-treated adult and pubertal mice displayed more IL-1β mRNA expression in the HYP than their saline-treated adult and pubertal counterparts (*MD* = 32.79, *SE* = 4.48, $p < 0.001$; *MD* = 18.50, *SE* = 4.52, $p < 0.001$; respectively) (Figure 4A).

Prefrontal cortex (PFC) : The ANOVA detected significant main effects of age ($F_{(1,107)} = 20.642$, $p < 0.001$, $\eta_p^2 = 0.16$) and LPS ($F_{(1,107)} = 120.455$, $p < 0.001$, $\eta_p^2 = 0.53$) IL-1β mRNA expression in the PFC. A significant two-way interaction between age X LPS ($F_{(1,107)} = 17.303$ $p < 0.001$, $\eta_p^2 = 0.14$) was also found. Pairwise comparisons showed that LPS-treated adult mice displayed significantly greater IL-1β mRNA expression in the PFC (*MD* = 5.85, *SE* = 0.95, $p < .001$) than their pubertal counterparts. In addition, both LPS-treated adult and pubertal mice

displayed more IL-1β mRNA expression in the PFC than their saline-treated adult and pubertal counterparts (*MD* = 10.18, *SE* = 0.95, $p < 0.001$; *MD* = 4.58, *SE* = 0.95, $p < 0.001$; respectively) (Figure 4B).

Hippocampus (HIPP): The ANOVA revealed significant main effects of age ($F_{(1,107)} = 4.138$, $p = 0.04$, $\eta_p^2 = 0.04$), treatment ($F_{(2,107)} = 3.235$, $p = 0.04$, $\eta_p^2 = 0.06$), and LPS ($F_{(1,107)} = 154.641$, $p < 0.001$, $\eta_p^2 = 0.59$) in the HIPP. There were also age x treatment ($F_{(2,107)} = 6.363$, $p = 0.002$, $\eta_p^2 = 0.11$), treatment x LPS ($F_{(2,107)} = 4.066$, $p = 0.02$, $\eta_p^2 = 0.07$), and age x treatment x LPS ($F_{(2,107)} = 7.698$, $p < 0.001$, $\eta_p^2 = 0.13$) interactions. Pairwise comparisons revealed that both LPS-treated adult controls and adult EE/LNG mice had significantly higher IL-1β mRNA expression in the HIPP compared to LPS-treated pubertal controls and pubertal EE/LNG mice (*MD* = 8.19, *SE* = 2.55, $p = 0.002$; *MD* = 10.30, *SE* = 2.55, $p < 0.001$; respectively). However, LPS-treated EE/DRSP pubertal mice had significantly higher IL-1β mRNA expression in the HIPP compared to LPS-treated EE/DRSP adults (*MD* = 7.22, *SE* = 2.55, $p = .005$). Moreover, LPS-treated EE/DRSP adults displayed significantly lower IL-1β mRNA expression compared to both LPS-treated adult controls and EE/LNG adults (*MD* = -11.26, *SE* = 2.55, $p = 0.002$; *MD* = -15.66, *SE* = 2.55, $p < 0.001$; respectively) (Figure 4C).

3.4.2 IL-6 mRNA expression in the HYP, PFC, and HIPP

HYP: Significant main effects of LPS were detected ($F_{(1,107)} = 55.756$, $p < 0.001$, $\eta_p^2 = 0.34$) in the HYP. A significant two-way interaction between treatment X LPS ($F_{(2,107)} = 3.928$, $p = 0.02$, $\eta_p^2 = 0.07$) was also found. Pairwise comparisons revealed that LPS-treated EE/LNG mice had greater IL-6 mRNA expression in the HYP compared to both LPS-treated controls and EE/DRSP mice (*MD* = 2.62, *SE* = 0.97, $p = 0.008$; *MD* = 2.98, *SE* = 0.97, $p = 0.003$; respectively). See Figure 4(D).

PFC: The ANOVA also revealed significant main effects of age ($F_{(1,107)} = 13.224$, $p < 0.001$, $\eta_p^2 = 0.11$), treatment ($F_{(2,107)} = 5.011$, $p = 0.008$, $\eta_p^2 = 0.09$), and LPS ($F_{(1,107)} = 51.197$, $p < 0.001$, $\eta_p^2 = 0.32$) on IL-6 mRNA expression in the PFC. There were also age x treatment ($F_{(2,107)} = 4.592$, $p = 0.01$, $\eta_p^2 = 0.08$), age x LPS ($F_{(1,107)} = 12.519$, $p < 0.001$, $\eta_p^2 = 0.11$), treatment x LPS ($F_{(2,107)} = 6.036$, $p = 0.003$, $\eta_p^2 = 0.10$), and age x treatment x LPS ($F_{(2,107)} = 4.936$, $p = 0.009$, $\eta_p^2 = 0.08$) interactions. Pairwise comparisons revealed that LPS-treated adult controls had greater IL-6 mRNA expression in PFC than pubertal counterparts (*MD* = 3.49, *SE* = 1.51, $p = .02$). Similarly, LPS-treated EE/LNG adults had higher IL-6 mRNA expression than their pubertal counterparts (*MD* = 9.58, *SE* = 1.51, $p < .001$). Moreover, LPS-treated EE/LNG adults displayed significantly greater IL-6 mRNA expression compared to both LPS-treated control and EE/DRSP adults (*MD* = 6.13, *SE* = 1.51, $p < 0.001$; *MD* = 9.66, *SE* = 1.51, $p < 0.001$; respectively). In addition, LPS-treated control adults displayed higher expression (MD = 3.53, SE = 1.51, p = .02) compared to LPS-treated EE/DRSP adults. (Figure 4E).

HIPP: The ANOVA found significant main effects of age ($F_{(1,107)} = 5.384$, $p = 0.02$, $\eta_p^2 = 0.05$), treatment ($F_{(2,107)} = 7.181$, $p = 0.001$, $\eta_p^2 = 0.12$), and LPS ($F_{(1,107)} = 73.999$, $p < 0.001$, $\eta_p^2 = 0.41$) on IL-6 mRNA expression in the HIPP. There was also a significant treatment x LPS ($F_{(2,107)} = 9.013$, $p < 0.001$, $\eta_p^2 = 0.14$) interaction. Pairwise comparisons revealed that LPS-treated EE/LNG mice displayed more IL-6 mRNA expression in the HIPP compared to both LPS-treated EE/DRSP and control mice (*MD* = 6.26, *SE* = 1.27, $p < 0.001$; *MD* = 6.26, *SE* = 1.27, $p < 0.001$; respectively) (Figure 4F).

3.4.3 TNF-α mRNA expression in the HYP, PFC, and HIPP

HYP: There were significant main effects of age ($F_{(1,107)} = 6.422$, $p = 0.01$, $\eta_p^2 = 0.06$) and LPS ($F_{(1,107)} = 102.228$, $p < 0.001$, $\eta_p^2 = 0.49$) on TNF-α mRNA expression in the HYP.

There was also a significant age X LPS ($F_{(1,107)} = 7.454$, $p = 0.007$, $\eta_p^2 = 0.07$) interaction. Pairwise comparisons showed that LPS-treated pubertal mice displayed significantly greater TNF-α mRNA expression (*MD* = 7.70, *SE* = 2.06, $p < .001$) than their LPS-treated adult counterparts. Moreover, both LPS-treated adult and pubertal mice displayed more TNF-α mRNA expression than their saline-treated adult and pubertal counterparts (*MD* = 10.79, *SE* = 2.058, $p < 0.001$; *MD* = 18.77, *SE* = 2.08, $p < 0.001$; respectively). (Figure 4G).

PFC: The ANOVA found significant main effects of age ($F_{(1,107)} = 29.797$, $p < 0.001$, $\eta_p^2 = 0.22$), treatment ($F_{(2,107)} = 3.924$, $p = 0.02$, $\eta_p^2 = 0.07$), and LPS ($F_{(1,107)} = 137.934$, $p < 0.001$, $\eta_p^2 = 0.56$) on TNF-α mRNA expression in the PFC. There were also age x treatment ($F_{(2,107)} = 7.299$, $p = 0.001$, $\eta_p^2 = 0.12$), age x LPS ($F_{(1,107)} = 23.385$, $p < 0.001$, $\eta_p^2 = 0.18$), treatment x LPS ($F_{(2,107)} = 4.485$, $p = 0.01$, $\eta_p^2 = 0.08$), and age x treatment x LPS ($F_{(2,107)} = 7.908$, $p < 0.001$, $\eta_p^2 = 0.13$) interactions. Pairwise comparisons showed that both LPS-treated control and EE/LNG adults had higher TNF-α mRNA expression compared to their LPS-treated control and EE/LNG pubertal mice counterparts (*MD* = 2.93, *SE* = 1.21, $p = .02$; *MD* = 10.58, *SE* = 1.21, $p < 0.001$; respectively). Moreover, LPS-treated EE/LNG adults displayed significantly greater TNF-α mRNA expression compared to both LPS-treated control and EE/DRSP adults (*MD* = 6.73, *SE* = 1.21, $p < 0.001$; *MD* = 7.58, *SE* = 1.21, $p < 0.001$; respectively) (Figure 4H).

HIPP: The ANOVA found significant main effects of age ($F_{(1,107)} = 5.093$, $p = 0.03$, $\eta_p^2 = 0.05$), treatment ($F_{(2,107)} = 4.216$, $p = 0.02$, $\eta_p^2 = 0.07$), and LPS ($F_{(1,107)} = 194.676$, $p < 0.001$, $\eta_p^2 = 0.65$) in the HIPP. There were also significant age x treatment ($F_{(2,107)} = 5.218$, $p = 0.007$, $\eta_p^2 = 0.09$), treatment x LPS ($F_{(2,107)} = 6.486$, $p = 0.002$, $\eta_p^2 = 0.11$), and age x treatment x LPS ($F_{(2,107)} = 8.013$, $p < 0.001$, $\eta_p^2 = 0.13$) interactions. Pairwise comparisons showed that both LPS-treated control and EE/LNG adults had higher TNF-α mRNA expression compared to their

LPS-treated control and EE/LNG pubertal counterparts (*MD* = 4.78, *SE* = 2.10, *p* = .03; *MD* = 10.32, *SE* = 2.10, *p* < 0.001; respectively). Moreover, LPS-treated EE/LNG adults displayed significantly greater TNF-α mRNA expression compared to both LPS-treated control and EE/DRSP adults (*MD* = 8.65, *SE* = 2.10, *p* < 0.001; *MD* = 13.48, *SE* = 2.10, *p* < 0.001; respectively). In addition, LPS-treated control adults displayed higher expression (*MD* = 4.83, *SE* = 2.10, *p* = .02) compared to LPS-treated EE/DRSP adults. Finally, LPS-treated EE/DRSP pubertal mice had increased TNF-α mRNA expression compared to LPS-treated pubertal controls (*MD* = 4.72, *SE* = 2.10, *p* = .03). (Figure 4I).

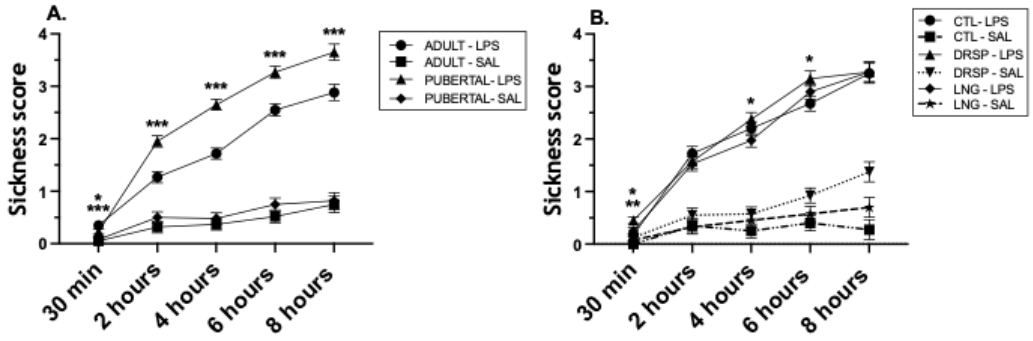

Figure 1. Sickness behaviour

(A) Mean (± SEM) sickness scores following LPS or saline (SAL) in pubertal and adult females. (B) Mean (± SEM) sickness scores over time across treatment groups (CTL, DRSP, LNG) following LPS or SAL. Asterisks denote significant differences at the same timepoint (*p < 0.05, **p < 0.01, ***p < 0.001; comparisons as indicated in the legend), n = 10/group. Abbreviations: LPS, lipopolysaccharide; SAL, saline; CTL, control; DRSP, drospirenone; LNG, levonorgestrel.

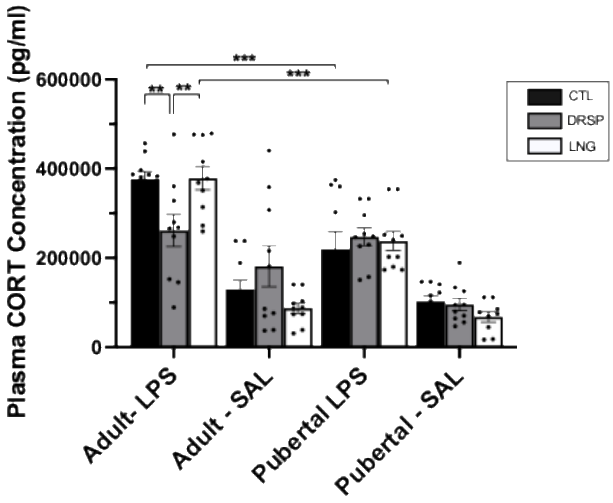

Figure 2. Corticosterone (CORT) concentration

Mean (±SEM) plasma CORT concentrations following SAL or LPS treatment in pubertal or adult mice treated with either CTL, DRSP, or LNG. Asterisks denote significant differences at the same timepoint (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; comparisons as indicated in the legend), n = 10/group. Abbreviations: LPS, lipopolysaccharide; SAL, saline; CTL, control; DRSP, drospirenone; LNG, levonorgestrel.

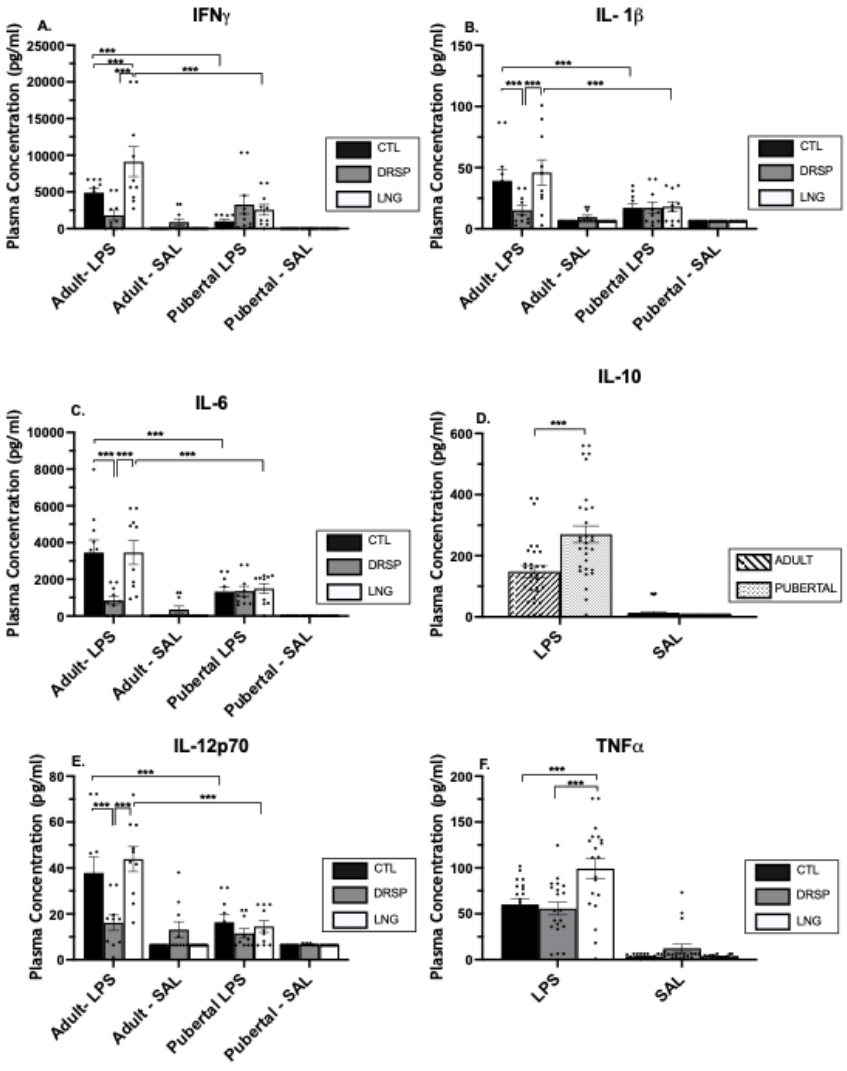

Figure 3. Cytokines concentrations

Mean (±SEM) acute plasma (A) IFNγ concentration, (B) IL-1β concentration, (C) IL-6 concentration, (D) IL-10 concentration, (E) IL-12p70 concentration, and (F) TNF-α concentration following SAL or LPS treatment in pubertal or adult mice treated with either CTL, DRSP, or LNG. Asterisks denote significant differences at the same time point (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; comparisons as indicated in the legend), n = 10/group. Abbreviations: LPS, lipopolysaccharide; SAL, saline; CTL, control; DRSP, drospirenone; LNG, levonorgestrel.

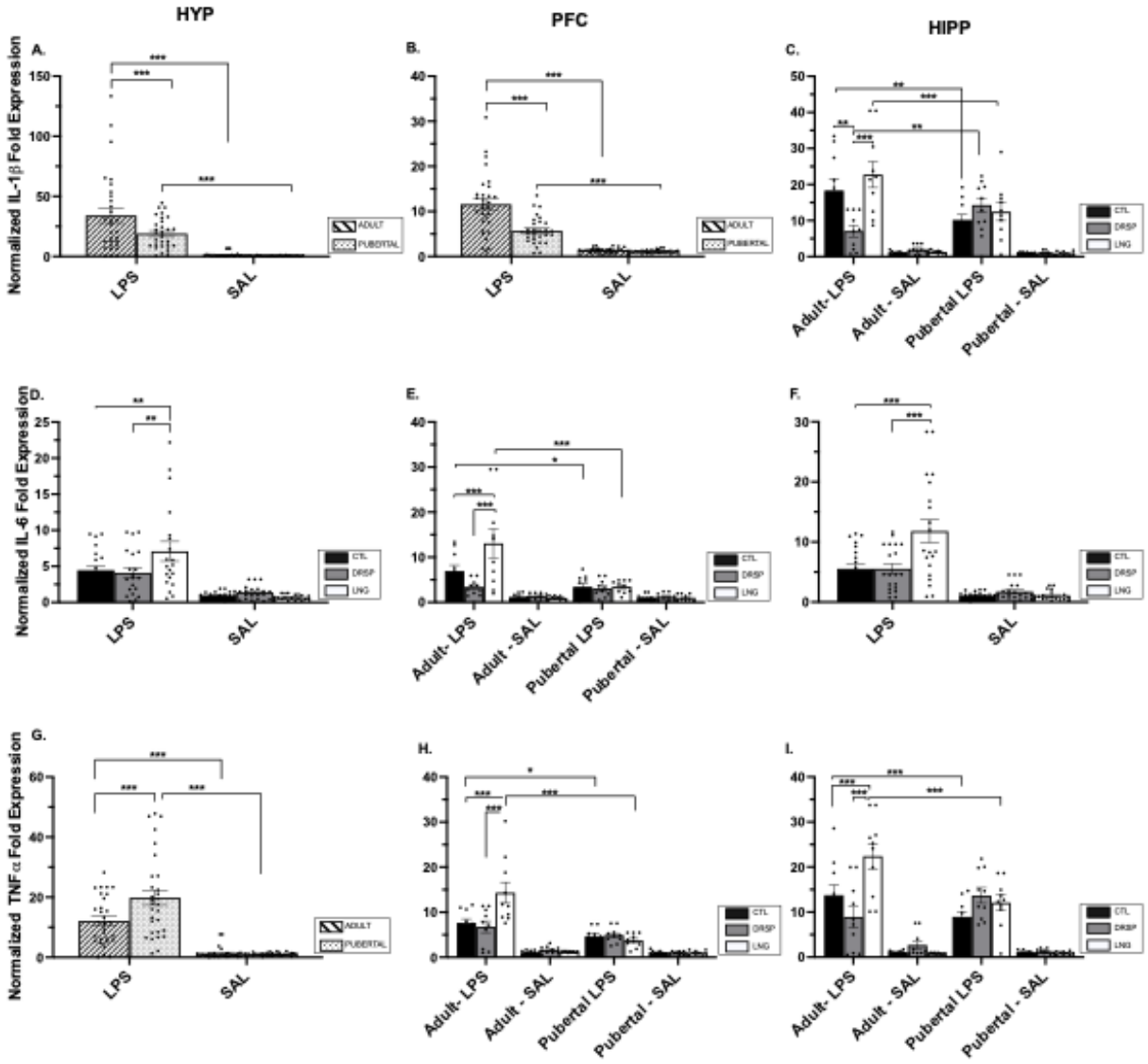

Figure 4. IL-1β, IL-6 and TNF-α mRNA expression

Geometric mean (±SEM) of acute mRNA (A) IL-1β expression in the HYP, (B) IL-1β expression in the PFC, (C) IL-1β expression in the HIPP, (D) IL-6 expression in the HYP, (E) IL-6 expression in the PFC, (F) IL-6 expression in the HIPP, (G) TNF-α expression in the HYP, (H) TNF-α expression in the PFC, and (I) TNF-α expression in the HIPP following SAL or LPS treatment in pubertal or adult mice treated with either CTL, DRSP, or LNG. Asterisks denote significant differences at the same timepoint (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$;

comparisons as indicated in the legend), n = 10/group. Abbreviations: LPS, lipopolysaccharide; SAL, saline; CTL, control; DRSP, drospirenone; LNG, levonorgestrel.

4.0 Discussion

To our knowledge, this study is the first to investigate the effects of COC use on immune and stress markers during puberty – a critical period of development during which the brain is more sensitive to stress and immune challenges (Holder & Blaustein, 2014; Kane & Ismail, 2017; Kolmogorova et al., 2021). As neuroinflammation can lead to conditions such as depression, schizophrenia, and neurodegenerative disorders (Adamu et al., 2024; Lurie, 2018; Najjar et al., 2013), altered immune signaling during puberty could potentially increase vulnerability to such illnesses. In addition, the underlying mechanisms and whether effects vary by contraceptive formulation remain understudied, despite evidence indicating formulation-specific differences (Kangasniemi et al., 2020). As such, this study examined whether puberty and COC formulations modulate immune and HPA-axis responses following an acute immune challenge.

As expected and similar to previous reports from our laboratory (Esposito et al., 2024; Esposito, Gandelman, et al., 2022; Esposito, Kearns, et al., 2022; Murray et al., 2019), LPS treatment induced sickness behaviours in both adult and pubertal mice. However, we found that pubertal mice displayed greater sickness behaviour than adults at nearly all post-injection time points, whereas evidence from our laboratory typically showed greater LPS-induced sickness behaviour in adults compared to pubertal mice (Cai et al., 2016). Notably, part of the pubertal female mice in our experiment were also treated with COCs whereas in Cai et al. (2016), pubertal groups included non COCs-treated males and females. Age is a critical factor modulating LPS-induced immune activation, sickness behaviour, and microglial priming (Lima

et al., 2022). LPS-induced immune response is characterized by increased sensitivity of microglia to an immune challenge resulting in increased pro-inflammatory and anti-inflammatory cytokine release and hyperactivation of microglia following LPS treatment (Henry et al., 2009; Hoogland et al., 2015; Lima et al., 2022; Norden & Godbout, 2013). Microglial priming has also been associated with increased vulnerability to neurodegenerative diseases and altered neuroplasticity (Lima et al., 2022). Puberty is a critical period of development that is characterized by ongoing maturation of immune–brain signaling (Bilbo & Schwarz, 2012; Ucciferri & Dunn, 2022). COCs have been shown to alter microglial structure and activity in regions like the mPFC and the HIPP (Gilfarb et al., 2025). Considering this evidence, the unexpected increase in sickness behaviour in pubertal mice suggests that COC treatment may have altered microglial activity which could potentially contribute to altered immune signaling and increased sickness behaviour, but further investigation is needed.

In addition, we observed a formulation-specific effect with mice treated with EE/DRSP mice displaying greater sickness behaviour than controls and those treated with EE/LNG at specific timepoints (30 min, 4 hours and 6 hours). DRSP is a progestin that has high affinity to progesterone receptors (PRs) and acts as a receptor agonist (Giatti et al., 2016). Progesterone has neuroprotective and anti-inflammatory effects on the central nervous system and modulates neurons and glial cells which express PRs (Bassani et al., 2023). Progesterone treatment has also been shown to dampen TNF-α, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 expressions in a dose-dependent manner, and suppress nuclear factor kappa B (NF-κB) activation (Lei et al., 2014). Similarly, synthetic progestins, such as nestorone, a 4th generation progestin with high PR affinity and no androgenic activity (Kumar et al., 2000), also exert neuroprotective and anti-inflammatory effects. Specifically, it has been shown to reduce

astrogliosis and microglial activation, suppress TNF-α, iNOS, and NF-κB signaling, and increase HIPP cell proliferation (Garay et al., 2014; Giatti et al., 2016; Meyer et al., 2015). In PRs knockout mice, the effects of nestorone on glial cell activity were suppressed, indicating that nestorone's neuroprotective effects are mediated by intracellular PRs (Hussain et al., 2011). Taken together, these findings suggest that some progestins may influence sickness behaviour by modulating glial cell activity and PRs signaling. Despite evidence indicating that progesterone and progestins have anti-inflammatory effects, our results do not provide direct evidence of dampened inflammation following COC use. Rather, it may reflect altered sensitivity to immune challenges or changes in behavioural expression of sickness associated with specific progestin formulations.

LPS-treated adult mice displayed higher corticosterone levels compared to LPS-treated pubertal mice. These results are also in-line with previos results indicating that there are age differences in LPS-induced activation of the HPA axis (Girard-Joyal et al., 2015), though the underlying mechanisms remain to be examined. Furthermore, we observed that mice treated with EE/DRSP in adulthood, displayed lower corticosterone plasma levels compared to controls and EE/LNG-treated counterparts, suggesting an effect of COC formula on HPA axis reactivity (e.g., CORT levels). Ethinyl estradiol combined with norgestrel, a LNG-derived progestin with half its potency (Furman, 2018), has been linked to increased corticosterone in female rats (Olatunji et al., 2017), highlighting the heterogeneity of COC formulations effects on the HPA axis.

LPS significantly increased peripheral cytokines levels. However, adults consistently showed higher pro-inflammatory cytokine (e.g., IFNγ, IL-1β, IL-6, IL-12p70) concentrations in the plasma compared to pubertal mice, whereas pubertal mice exhibited higher levels of the anti-inflammatory cytokine IL-10. These findings are consistent with prior research from our

laboratory showing greater LPS-induced increases in pro-inflammatory cytokines in adults and larger anti-inflammatory increases in pubertal mice (Cai et al., 2016; Murray et al., 2023; Sharma et al., 2018). Prepubertal rats also display faster recovery of corticosterone and IL-6 levels as well as faster return to baseline in the paraventricular nucleus of the HYP compared to adults following LPS administration (Goble et al., 2011). Importantly, increased IL-10 levels in pubertal mice may reflect age-dependent changes in microglial activity that are known to release anti-inflammatory cytokines (e.g., IL-10) to dampen the inflammatory response (da Silva et al., 2024; Munshi et al., 2019).

Nevertheless, our peripheral inflammation patterns did not parallel the sickness behaviour findings, as pubertal mice displayed greater sickness behaviour despite showing lower pro-inflammatory cytokine levels and higher IL-10. These findings suggest that peripheral inflammatory response magnitude alone does not fully account for sickness behaviour. As such, evidence has shown a non-linear relationship between peripheral inflammation and sickness behaviour that is mediated by CNS immune activation (Dantzer, 2004; Tyagi & Bartley, 2025).

Results also demonstrated that LPS-treated EE/LNG mice had increased peripheral pro-inflammatory cytokines (IFN-γ, IL-1β, IL-6, IL-12p70, TNF-α) compared to controls and EE/DRSP mice. This finding indicates that EE/LNG can exacerbate peripheral inflammatory responses, whereas EE/DRSP may attenuate them. Interestingly, testosterone has been shown to exert immunosuppressive effects through the downregulation of both innate and adaptive immune responses, reduction of antibody production, and suppression of Th-1 cell type responses, which heightens the risk for infections (Borráz-León et al., 2022; Foo et al., 2017; Trigunaite et al., 2015). LNG is a progestin with high androgenic properties and is a 19-nortestosterone derivative synthesized via the removal of the 19-methyl group in the testosterone

molecule (Patel & Bajaj, 2025). Such high-androgenic progestins have been shown to upregulate the expression of receptor activator of NF-κB ligand (RANKL), which may promote NF-κB activation in hormone-responsive epithelial cells (Shamseddin et al., 2021), skewing inflammatory signaling pathways toward a pro-inflammatory state. This mechanism may explain our observation that EE/LNG amplified peripheral pro-inflammatory markers, whereas the anti-androgenic effects of DRSP formulation attenuated this response pattern.

Central IL-1β, IL-6, and TNF-α mRNA expressions largely paralleled peripheral cytokine profiles in these mice. More specifically, mice treated with LPS in adulthood displayed greater IL-1β mRNA expression in the HYP and PFC, IL-6 mRNA expression in the PFC, and TNF-α mRNA expression in the PFC and HIPP compared to pubertal mice. These patterns are consistent with the higher plasma concentration of pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α). Interestingly, adults appear more susceptible to LPS-induced neuroinflammation, may be due to age-related increased blood-brain barrier (BBB) permeability (Banks et al., 2015; Galea, 2021; Peng et al., 2021). Age-related changes in permeability may be driven by shifts in transcellular transport mechanisms (Yang et al., 2020), potentially allowing greater influx of peripheral cytokines into the brain, and amplifying microglial activity and cytokine mRNA expression in regions such as the PFC and the HIPP (da Silva et al., 2024; Kalyan et al., 2022; Smith et al., 2012). Consistent with this possibility, cecal ligation and perforation have been associated with increased BBB permeability and oxidative stress in aging male Wistar rats versus younger rats (Margotti et al., 2020).

Interestingly, regardless of COC treatment, pubertal mice displayed higher TNF-α mRNA expression in the HYP compared to adult counterparts, indicating region-specific neuroinflammatory responses. The hypothalamus plays a major role in the onset of puberty via

the release of gonadotropin-releasing hormone (GnRH), which stimulates the pituitary to produce luteinizing hormone and follicle-stimulating hormone, driving sex hormone production and sexual maturation (Krishna & Witchel, 2000; Naulé et al., 2021). This process is supported by neural networks (e.g., kisspeptin, neurokinin B, and GABAergic neurons) and glial cells, which undergo major transformations during puberty (Naulé et al., 2021). TNF-α, an essential mediator of hypothalamic inflammation, activates microglia and may disrupt neuroendocrine pathways critical for pubertal development (Brás et al., 2020; Raffaele et al., 2020). Chronic and elevated TNF-α levels have been shown to activate microglia and impair the growth hormone/insulin-like growth factor 1 axis (Amaro & Chiarelli, 2020; Witkowska-Sędek & Pyrżak, 2020), potentially impairing growth and delaying pubertal development. Thus, the mechanisms underlying this increase in hypothalamus-specific TNF-α mRNA expression remain unclear. In addition, the fact that this effect was specific to puberty suggests possible lasting effects on development, warranting further investigation.

Our results also demonstrated a combined effect of COC formulation and age on neuroinflammation. LPS-treated EE/LNG adults had increased IL-6 and TNF-α mRNA brain expression, consistent with the plasma results for pro-inflammatory cytokines. However, LPS-treated EE/DRSP adults displayed reduced hippocampal IL-1β and TNF-α expression. DRSP is a spironolactone-derived progestin with anti-mineralocorticoid and anti-androgenic properties (Rapkin & Winer, 2007), and diminishes fluid-retention and hypertension that is induced by EE (Oelkers, 2004). Mineralocorticoid receptors (MRs) are highly expressed in microglia cells (Carrillo-de Sauvage et al., 2013; Sierra et al., 2008; Zhang et al., 2025), and when activated by aldosterone or low-to-moderate glucocorticoid levels, they stimulate NF-κB pathway activity and pro-inflammatory cytokines (e.g., IL-6 and TNF-α) release (Chansawhang et al., 2022; Chantong

et al., 2012). Similarly, anti-mineralocorticoid drugs such as drospirenone have been shown to reduce pro-inflammatory cytokine production and microglial activity (Qualls et al., 2024; Zhang et al., 2025). Therefore, it is possible that the anti-mineralocorticoid activity of DRSP could impact microglial activity and NF-κB signaling resulting in reduced neuroinflammation, but further research is needed. Interestingly, LPS-treated EE/DRSP pubertal mice showed, elevated hippocampal IL-1β and TNF-α expressions, which suggests a developmental sensitivity that may counteract the anti-inflammatory effect seen in adults. LPS exposure during critical developmental periods has been shown to disrupt microglial phagocytosis, synaptic pruning, and spine density resulting in altered hippocampal-dependent contextual learning (Osborne et al., 2021). Therefore, the increased IL-1β and TNF-α expressions in the HIPP of LPS-treated EE/DRSP pubertal mice suggests that puberty may uniquely modulate DRSP's anti-mineralocorticoid effects on microglia – reversing the anti-inflammatory response seen in adults and promoting inflammation through mechanisms that have yet to be investigated.

4.1 Limitations and future directions

The current study provides valuable insights into the effects of different COC formulations during puberty on immune and stress responses. Nevertheless, several limitations must be acknowledged. First, this study focused on only two specific COC formulations (e.g., EE/LNG and EE/DRSP), which does not characterize the diversity of COCs and, more specifically, progestins available on the market (De Leo et al., 2016; Lacasse et al., 2024) – particularly given that our results suggest formulation-specific effects. Second, our study administered COCs via oral gavage for two weeks. This regimen may not fully mimic the typical human COC use pattern, often spanning months or years, and may have also limited our ability to detect long-term effects on immune and HPA axis reactivity. Third, our study did not assess other relevant

immune and stress markers, such as glucocorticoid receptor expression, which has been shown to modulate LPS effects on neuroinflammation (Molina et al., 2017; Munhoz et al., 2010). Similarly, our focus on specific pro- and anti-inflammatory cytokines (e.g., IL-1β, IL-6, IL-10, TNF-α, IFNγ, IL-12p70) may have overlooked other immune mediators (e.g., C-reactive protein, CCL2, CXCL10) that could be disrupted by COC use. Fourth, we did not measure microglial activity which may be a key mediator in shaping the effects of age and COC use on inflammation. Future studies should therefore investigate the role of microglia, considering they have been associated with LPS-induced sickness behaviour (Henry et al., 2009). Fifth, we did not examine the gut microbiome, as it may play a critical role in modulating the association between LPS and neuroinflammation through the gut-brain axis (An et al., 2022; Ling et al., 2020; Wei et al., 2025). Moreover, COCs are suspected to alter gut microbiota composition (Mihajlovic et al., 2021; Zim & Bommareddy, 2025), raising the possibility of microbiome-mediated effects on neuroinflammation. As such, further research should investigate whether COC-related immune and stress responses are mediated by gut microbial composition.

4.2 Conclusion

Our study aimed to investigate the impact of COC use during puberty on the immune system and HPA axis following LPS injection. Our results revealed complex, age- and formulation-dependent effects. EE/LNG adults displayed more pro-inflammatory cytokines compared to EE/DRSP adults, suggesting that COC formulation may play a critical role in shaping neuroinflammation. However, this pattern seemed to be reversed in EE/DRSP pubertal mice in the hippocampus, which suggests distinct age effects. Taken altogether, our findings indicate that COC use, particularly during puberty, modulates immune and stress responses, with formulation-specific effects. These results highlight the need to further investigate the underlying

mechanisms and their potential long-term consequences on neurodevelopment and health in females.

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GENERAL DISCUSSION

COCs were introduced in 1960 and have since significantly changed in composition and dosing (Dhont, 2010; Kheloui & Ismail, 2024). Until recently, very few researchers examined the effects of COCs on the brain of adolescents and adults. Moreover, the limited published findings on the effects of COCs on the brain remain contradictory and inconsistent (Hampson, 2023; Lacasse et al., 2024). As such, knowledge on the mechanisms by which COCs impact the brain and whether these effects are reversible or enduring remains limited because of several recurring limitations. These limitations include (1) scarcity of animal models (2) heterogeneity in the study designs (e.g., doses, routes of administration, age of onset, use duration, prior exposure to COCs and/or other forms of hormonal contraception), and (3) a wide range of COC brands and formulations (e.g., progestins pharmacological profile) available on the market (Hampson, 2020, 2023; Kheloui et al., 2023; Lacasse, Gomez-Perales, et al., 2022; Pletzer & Kerschbaum, 2014; Tronson & Schuh, 2022).

This doctoral thesis builds on previous research from our laboratory demonstrating structural and functional brain changes that are associated with COCs use initiated either in adolescence or in adulthood only (Sharma, Fang, et al., 2020; Sharma, Smith, et al., 2020), and explores possible mechanisms underlying these effects. Therefore, the objectives of this thesis were to investigate whether exposure to different COC formulations during puberty and adulthood might impact cognitive function (e.g., spatial learning and memory, object and social recognition) and the gut microbial composition (**Study 1**), mental health (e.g., depression and anxiety) (**Study** 2), as well as immune function (e.g., cytokine concentration and expression) and stress reactivity (e.g., corticosterone levels) (**Study 3**) in a mouse model as well as the possible mechanisms underlying these effects. A summary of the main findings is provided in Table 1.

Puberty represents a critical developmental period marked by several changes in the gut microbiome (Sisk-Hackworth et al., 2023), neuroplasticity and cognition (Ciccia et al., 2009; Naulé et al., 2021), and the maturation of immune function and the HPA axis (Holder & Blaustein, 2014; Kane & Ismail, 2017). These changes in neurobiological processes are partially driven by the surge and variations in sex hormones that occur during puberty (Sisk & Zehr, 2005; Vigil et al., 2016). As such, we hypothesized that COC exposure during puberty might interfere with these developing systems and impact brain structure and function.

Table 1
Summary of Main Findings

Chapter	Outcome variable	Measures	Summary of findings
II	Spatial learning and memory	Barnes Maze	• Pubertal EE/LNG: Impairments in spatial learning (e.g. more errors, longer latency, and greater distance travelled) and memory (e.g. more errors during long-term memory). • Pubertal EE/DRSP: Partial impairments in spatial learning.
II	Social and object recognition	Social Recognition Test; NORT	• Pubertal EE/LNG and EE/DRSP: ⇑ social approach; ⇑ chemo-investigation was also observed. • NORT: no significant group differences.
II	Neuroplasticity	BDNF IR cell expression (IHC); PSD-95 IR cell expression (IHC)	• Pubertal EE/LNG: ⇑ BDNF in HIPP CA1 and ⇓ PSD-95 in mPFC ILA. • Pubertal EE/DRSP: ⇑ BDNF in CA3 and ⇑ PSD-95 in CA1. • Adult EE/LNG: ⇑ BDNF in mPFC ACAd and PrL.
II	Endogenous hormones and gut permeability	Estradiol and progesterone (ELISA); Occludin (Western Blot)	• Adult EE/LNG: ⇑ Estradiol plasma concentration • ⇑ Progesterone concentrations in NC and pubertal EE/LNG females • Occludin: Males had ⇑ occludin expression at 54 kDa. No group differences detected at 25 kDa.

Chapter	Outcome variable	Measures	Summary of findings
II	Gut microbiome	Alpha diversity; beta diversity / PERMANOVA; relative abundance	• No significant group differences in alpha and beta diversity. • Community composition differed as a function of age and treatment, with age- and treatment-related effects of comparable magnitude. • Pubertal groups displayed ⇑ compositional shifts over time.
II	Gut microbiome	Enrichment	• Pubertal EE/DRSP: T3 enrichment of *Turicibacter,* and *Sutterellaceae* • Pubertal EE/LNG: T3 enrichment of *Akkermansia, Lachnospiraceae,* and *UCG-010.*
II	Gut-brain associations	Correlations between microbiota genera and BDNF/PSD-95 /Behaviour	• COC-treated groups showed 2-3 times more strong microbiota-neuroplasticity correlations than controls. • The direction and taxa involved varied according to formulation, age, and brain region examined.
III	Depression-related behaviours	Forced Swim Test; Tail Suspension Test	• NC females showed latency to first mobility in the TST.
III	Anxiety-related behaviours	Open Field; Elevated Plus Maze	• Adult EE/LNG: ⇑ time in the center of the OFT. • Pubertal EE/DRSP: ⇓ center entries frequency in the OFT. • EPM: no significant group differences.
III	Serotonin receptor	5HT-1A IR cell expression (IHC)	• Control males: ⇑ 5HT-1A expression in HIPP CA3.
III	GABA	GABA IR cell expression (IHC)	• Adult EE/DRSP: ⇑ HIPP GABA cell expression in CA2 and CA3. • Adult EE/LNG: ⇓ GABA expression in CA1.
IV	Sickness behaviour	Sickness scores	• LPS increased sickness behaviour. • Pubertal LPS mice: greater sickness behaviour than adults from 2-8 h post-LPS injection. • EE/DRSP: greater sickness scores than selected groups at specific timepoints.
IV	HPA axis response	Plasma CORT concentrations	• LPS adults (e.g. control adults and EE/LNG adults): greater CORT concentrations than pubertal mice. • Adult EE/DRSP: lower CORT than LPS adult controls and LPS adult EE/LNG mice.

Chapter	Outcome variable	Measures	Summary of findings
IV	Peripheral immune response	IFN-γ, IL-1β, IL-6, IL-10, IL-12p70, TNF-α plasma concentrations	• LPS ⇑ cytokine concentrations. • LPS adults: ⇑ pro-inflammatory responses than LPS pubertal mice (e.g. IFN-γ, IL-1β, IL-6, and IL-12p70). • LPS pubertal mice : ⇑ anti-inflammatory cytokine concentration (e.g. IL-10). • LPS EE/LNG adults displayed greater pro-inflammatory cytokine levels than LPS EE/DRSP adults (e.g. IFN-γ, IL-1β, IL-6, TNF-α and IL-12p70).
IV	Central inflammatory response	IL-1β, IL-6, TNF-α mRNA in HYP, PFC, HIPP	• Effects varied by brain region, age, and formulation. • LPS adults : ⇑ IL-1β expression in HYP, PFC; ⇑ IL-6 in PFC; ⇑ TNF-α in HIPP and PFC. • LPS pubertal mice : ⇑ TNF-α in the HYP. • LPS EE/LNG adults: ⇑ expression for IL-6 in HYP/PFC/HIPP; ⇑ TNF-α in HIPP and PFC. ⇑ IL-1β expression in HIPP. • LPS EE/DRSP adults : ⇓ inflammatory response than LPS EE/LNG adults • LPS pubertal EE/DRSP ⇑ HPP IL-1β than their adult counterparts.

Note. Arrows indicate the direction of effects with ⇑ indicating an increase and ⇓ indicating a reduction/lower. HIPP = hippocampus; mPFC = medial prefrontal cortex; HYP = hypothalamus; PFC = prefrontal cortex; OFT = open field test; EPM = elevated plus maze; NORT = novel object recognition test; CORT = corticosterone.

Puberty as a window of vulnerability and resilience

This thesis confirms that age of onset of COC use impacts certain aspects of health examined in this thesis. More specifically, **Study 1** demonstrated that COC exposure since puberty is associated with alterations in spatial learning and hippocampal neuroplasticity markers, suggesting that COCs can interfere with normal neurodevelopment during puberty and result in cognitive alterations (Vijayakumar et al., 2018). **Study 2** showed that mice treated with COCs since puberty displayed more active coping in the forced swim test. Finally, **Study 3** showed that mice treated with COCs since puberty displayed greater sickness behaviour following immune challenge.

Puberty is characterized by brain reorganizing and remodelling (e.g., synaptic pruning, myelination), which can increase experience-associated neuroplasticity in networks that support

executive functions and learning (Baker et al., 2025; Sisk & Gee, 2022). Consequently, COC exposure during puberty may shift developmental trajectories during a period when neural systems are still developing. Interestingly, research shows that exposure to a mild predictable stress during puberty can improve cognitive functioning and provide resilience to future stress, whereas chronic unpredictable stress exposure has negative effects on the brain and cognitive function (Cotella et al., 2023). Similarly, a systematic review showed that the effects of pubertal stress on the adult brain were dependent on the outcome examined (Borsini et al., 2023). These findings suggest that stress during puberty can yield outcomes that are not strictly negative or positive, but conditional to the specific characteristics of the challenge and the outcome measured. In the current thesis, all studies also included groups that were only exposed to COCs as of adulthood. The inclusion of these groups allowed us to compare the effects of COC use across developmental stages and to confirm whether some of the effects of COCs are limited to the onset of COC use during puberty.

Study 2 showed that pubertal COC exposure was associated with a shift toward more active coping in the forced swim test**.** This finding indicates that COC use during puberty alters stress-coping strategy rather than simply increasing depression-like behaviour, which is consistent with previous research (Romeo, 2017). One possible underlying mechanism which was investigated in **Study 2** was GABA expression. Evidence shows that puberty-related remodeling of stress reactivity and increased sensitivity to neurosteroids such as allopregnanolone – a modulator of GABAa receptor function can increase the likelihood of anxiety- and depression-like behaviours during adolescence (Kenney et al., 2025; Möhler, 2009; Möhler, 2012). Pubertal female mice exposed repeatedly to a stressor displayed behavioural resilience and reduced depression-like behavior, which was associated with allopregnanolone

action on GABAa receptors (Kenney et al., 2025). These findings support a mixed pattern of both vulnerability and resilience in affective disorders, via modulation of GABAa signaling, which may contribute to shifts in stress coping behaviours.

In **Study 3**, mice treated with COCs since puberty displayed greater sickness behaviour following immune challenge, yet showed reduced peripheral inflammation (e.g., higher IL-10 and lower pro-inflammatory cytokines than adults), highlighting that behavioral reactivity during puberty does not necessarily imply greater inflammation. This pattern is consistent with evidence showing greater pro-inflammatory cytokine concentration in adults than in pubertal mice, but greater anti-inflammatory cytokines in pubertal mice compared to adults (Cai et al., 2016). The age difference in sickness behaviour could be partly driven by shifts in immune sensitivity through specific mechanisms (e.g., vagal signaling, microglial activation, NF-κB signaling) resulting in stronger sickness behaviour response in pubertal mice despite lower peripheral cytokine levels (Dantzer & Kelley, 2007; Bilbo & Schwarz, 2012; Hoogland et al., 2015). These results support a framework in which puberty can confer both vulnerability (Holder & Blaustein, 2014; Kane & Ismail, 2017) and resilience depending on the outcome measured. Interestingly, gut-targeted interventions such as probiotic consumption during puberty can reduce LPS-induced cytokine levels, which could prevent anxiety- and depression-like behaviours (Esposito, Kearns, et al., 2022; Murray et al., 2019; Yahfoufi et al., 2020), and provide insights that build upon our microbiome findings in **Study 1**.

Formulation effects: Composition and progestins matter

Progestins vary in several characteristics (e.g., pharmacokinetics, composition, receptor activity, side effects induced) and therefore play a central role in how COCs impact brain structure and function (Pletzer et al., 2023; Stanczyk, 2007). This thesis confirms that COCs

effects are formula-dependent. Across studies, EE/LNG consistently biased outcomes toward greater neural, behavioural, and immune disruptions, including spatial learning impairments in pubertal mice (**Study 1**), reduced GABA expression in the CA1 of the HIPP in adult mice (**Study 2**), and greater pro-inflammatory cytokine levels and neuroinflammation (e.g., IL-6 and TNF-α) following LPS injection (**Study 3**). These findings are consistent with evidence showing that androgenic progestins can modulate GABA receptor expression and immune signaling pathways (Follesa et al., 2002; Porcu et al., 2019). However, since LNG was administered only in combination with EE in this thesis, these effects likely reflect LNG and EE interactions, rather than solely LNG-related effects. Indeed, while EE and LNG have been shown to improve spatial working memory in adult ovariectomized rats when administered separately, their effects differ when they are administered concurrently (Prakapenka et al., 2018), indicating that the EE-LNG interaction should be considered when interpreting the results.

In contrast, EE/DRSP revealed more context- and age-dependent effects, including a region-specific increase in hippocampal expression of neuroplasticity markers without robust effects on cognition (**Study 1**), heightened hippocampal GABA expression (**Study 2**), and reduced neuroinflammation and corticosterone levels (**Study 3**) in adult mice. These patterns might be explained by DRSP's anti-androgenic and anti-mineralocorticoid properties and the role of mineralocorticoid receptor signaling in microglial NF-κB–linked cytokine release (Chantong et al., 2012; Oelkers, 2004). However, the benefits observed in adults did not uniformly extend to pubertal mice. Interestingly, when administered during puberty, EE/DRSP was associated with increased thigmotaxis in the OFT (**Study 2**) and increased hippocampal neuroinflammation (**Study 3**). These findings suggest that a specific formulation can yield different outcomes when administered during different developmental stages. Similarly,

clinical evidence indicates that age is an important factor in shaping risk for mood disorders following hormonal contraceptive use (Mu & Kulkarni, 2022). Furthermore, evidence indicates that anti-androgenic formulations (e.g., nomegestrol acetate with 17β-estradiol) are better tolerated in adult women with mood disorders (Robertson et al., 2021), suggesting that progestin types influence tolerability and health-related outcomes. Taken together, these findings suggest that the effects of COCs on the brain and behaviour are dependent on formula and on the age of onset of COC use.

Additionally, formulation-specific microbial profiles associated with bile acid, SCFA, and tryptophan metabolism (**Study 1**) provide an interesting avenue to explain how progestins action could differently modulate gut-derived metabolites relevant to neuroplasticity, stress reactivity, and immune function (Cryan et al., 2019; Gao et al., 2020; O'Mahony et al., 2015). These mechanisms offer a plausible link between formulation-specific microbial shifts and neural and immune changes observed in **Studies 2 and 3**. Together, these findings indicate that COCs should not be considered as a single homogenous factor but rather a multi-dimensional factor that differently shapes neural, immune, and behavioral outcomes based on composition and progestin's action.

Task- and region-specific effects

This thesis also shows that age- and formulation-specific effects vary based on the outcome measured and brain region examined, indicating that COCs effects are selective and emerge in task-, context-, and brain region-specific manners. Formulation-dependent alterations in pubertal mice co-occurred with subregion-specific changes in neuroplasticity markers (e.g., increased BDNF expression in the CA1 of EE/LNG pubertal mice as well as increased BDNF expression in the CA3 and PSD-95 expression in the CA1 of EE/DRSP pubertal mice) (**Study 1**).

It is plausible that COC-use contributes to alterations in glutamatergic/NMDA-dependent pathways, which are modulated by gut microbiota and are essential to cognitive functioning (Lu et al., 2014; Maqsood & Stone, 2016). Similarly, previous human research shows that EE/DRSP adult users display selective alterations in specific cognitive abilities (e.g., verbal memory, visuospatial ability) and improved performance during inhibition tasks (Lagunas et al., 2026), supporting the idea that brain region and cognitive functions are not equally and unilaterally impacted by COC use.

Interestingly, our results showed more alterations in neuroplasticity in the hippocampus rather than the prefrontal cortex of pubertal mice. This effect may relate to developmental timing and regional differences in maturation. Puberty is associated with remodeling across many brain regions (Herting & Sowell, 2017; Kretzer et al., 2024). However, the prefrontal cortex, a critical region for executive function and decision-making, undergoes maturation later than the hippocampus, and continues to develop into the late 20s. This extended window for maturation may allow greater potential for reorganization and adaptation relative to the hippocampus (Drzewiecki & Juraska, 2020), which could explain discrepancies in how COC impacts specific brain regions and behaviour.

Study 2 also demonstrated significant age- and formulation-dependent effects in certain behaviours tests (e.g., FST and OFT), but not in others (e.g., TST and EPM). These findings highlight the task-specific nature of COC effects which is consistent with research showing that depression- or anxiety-like behaviours are sensitive to the specific testing paradigms used (Chatterjee et al., 2012; Goes et al., 2009). Similarly, **Study 3** demonstrated brain region-specific effects as cytokine expression varied based on COC formulation and developmental stage. This pattern is partially consistent with human research suggesting that COC effects on inflammation

are context-dependent. For instance, human females that are exposed to oral contraceptives display more pro-inflammatory markers (e.g., C-reactive protein, TNF-α, IL-6) and oxidative stress, and less acute plasma cortisol levels compared to non-users (De Groote et al., 2009; Gervasio et al., 2022; Masama et al., 2022; Mengelkoch et al., 2024). In contrast, some studies report no difference in basal cytokine concentrations between oral contraceptive users and non-users (Eagan et al., 2021; Mengelkoch et al., 2024). These results suggest that the effects of COCs on inflammation may be context-dependent as they vary according to the marker measured, the presence of a stressor and/or immune challenge. Overall, across all three studies, COC effects are age- and formulation-dependent, resulting in selective changes in specific neurobehavioral and neuroimmune processes rather than broad, unidirectional shifts.

COCs modulating effects on the gut-brain axis

This thesis also demonstrated that COC exposure induced significant changes in gut microbiota composition and abundance (e.g., *Turicibacter*, *Sutterellaceae*, Escherichia–Shigella), particularly in pubertal EE/DRSP-treated mice (**Study 1**). These changes could potentially be attributed to gut–brain–immune pathways involving bile acid signaling, SCFA production, and tryptophan metabolism (Cryan et al., 2019; Lynch et al., 2023; Zhao et al., 2025). Adult COC-treated groups also displayed strong associations between microbial families and hippocampal and anterior cingulate cortex plasticity markers (BDNF, PSD-95). More specifically, *Bacteroidaceae* showed associations in adults (positive with PSD-95 in the anterior cingulate cortex and negative with BDNF in the CA1), while *Lactobacillaceae* displayed positive correlations with PSD-95 in the CA3. In comparison, pubertal COC-treated groups displayed strong associations between microbiota family and performance on the social recognition task, particularly for *Roseburia*, *Rikenellaceae*, *Desulfovibrionaceae*, and

Eggerthellaceae. These associations were modulated by COC formulation, with EE/DRSP and EE/LNG often producing distinct or even opposing associations across developmental stages. Nevertheless, our findings are consistent with evidence showing that neuroplasticity and cognition are modulated by the gut-brain axis (Damiani et al., 2023; Hameed et al., 2024). Our results also suggest that COC use can alter this association in an age- and formulation-dependent manner, although the mechanisms underlying these effects remain to be investigated. Taken together, our findings identify puberty as a period of increased susceptibility to COC-associated gut microbiota shifts which are associated with alterations in brain structure and function, emphasizing the necessity to further assess the mechanisms and potential long-term consequences of COC use for women's health.

Limitations and future directions

This thesis is not without limitations and several should be considered when interpreting these findings. First, this thesis focused on a limited set of COCs formulations (e.g., EE/LNG and EE/DRSP) and doses whereas a wide range of contraceptives with distinct pharmacological profiles are available (Hampson, 2023). Future work should expand on investigating different formulations. This limitation is particularly relevant given that newer versions of estradiol have been developed recently like estetrol, a natural estradiol component metabolized by the human liver and is reported to be better tolerated by users (Fruzzetti et al., 2021). Similarly, progestins only contraceptive pills are often used by women and future studies should examine whether progestin-only exposure produces similar or distinct neurobehavioral, neuroimmune, and gut microbiome effects.

Second, our model did not include an equivalent to the pill-free week used in many human COC regimens. Because the rodent estrous cycle is typically 4-5 days long, incorporating

a human-like placebo week remains difficult, reducing the ecological validity of our model. Third, although the synthetic hormone regimen was administered orally, many hormonal contraceptives rely on alternative routes (e.g., implant, hormonal IUD, vaginal ring). Future studies should therefore compare routes of administration and examine whether they have similar or distinct effects. Fourth, the exposure duration in our model was relatively short compared to typical use, which often spans years in adolescents and adult women. Longitudinal studies would provide interesting insights as to whether prolonged exposure induces different effects and whether outcomes persist after discontinuation. Fifth, although it remains difficult to determine the magnitude of the stress induced by weaning and shipping at three weeks of age, these factors may have influenced some of the outcome measures and represent a potential limitation of the study. However, previous work indicates that the long-term consequences of shipping stress is age-dependent, and that mice shipped at three weeks of age display fewer or no persistent behavioral alterations compared to mice shipped at 6 weeks of age, during the pubertal stress sensitive period (Ismail et al., 2011; Laroche et al., 2009), suggesting that mice are resistant to shipping stress at 3 weeks of age. Moreover, to minimize these effects, all mice were shipped under the same conditions and at the same age and were given an acclimation period before the start of the experiment.

Finally, this thesis identified composition- and age-dependent shifts in gut microbial community patterns. However, given that these analyses were primarily correlational, causality cannot be inferred. Future studies should aim to determine causality by using experimental paradigms such as fecal microbiota transplantation, colonization of germ-free animal models, and direct supplementation of gut-derived metabolites, particularly SCFA and tryptophan. This information would provide insights and clarity on the mechanisms and pathways by which gut

microbial composition may modulate the brain, cognition, and immune signaling. In addition, an interesting avenue would be to examine whether gut-targeted interventions (e.g., probiotics) can mitigate COC-associated changes and may have protective effects on women's health.

Concluding remarks

This thesis provides evidence that COCs use has effects on brain, behaviour, and neuroimmune outcomes, that depend on both developmental timing and COC formulation. Across studies, we demonstrated that COC exposure can alter cognitive performance and neuroplasticity marker expression (e.g., BDNF and PSD-95), and that these changes can differ by brain region and COCs composition. In addition, we demonstrated formulation-dependent modifications in gut microbial profiles and detected associations between microbial families and both cognitive and neuroplasticity measures, suggesting that the gut–brain axis could modulate the effects of COCs on the brain and behavior. In addition, we showed that COC use alters depression- and anxiety-like behaviours as well as hippocampal GABA cell expression in an age- and formulation-dependent manner. Finally, we also provided evidence that acute COC use during puberty and adulthood impacts stress reactivity and both peripheral and central inflammation distinctly, following an immune challenge. Taken altogether, these results confirm that age of onset and COC formulations are critical factors for understanding how hormonal contraception shapes women's health. Importantly, our findings reveal that puberty represents a period of vulnerability, but also resilience to COC exposure potentially mitigating negative health effects and suggesting a more complex depiction of these effects. Considering that millions of women rely on combined oral contraceptives, our findings highlight the critical need for increased neuroendocrine research to investigate their effects on women's health further. This research will contribute to a paradigm shift in the field of neuroendocrinology, which has

historically marginalized women and will allow for informed decisions and appropriate representation in biomedical research.

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