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An immune challenge in juvenile or adolescent mice differentially modulates LPS-induced molecular and behavioural sequelae in males and females

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“Dopo la tempesta,
anche il Cielo si specchia
dentro sé stesso”

ABSTRACT

BACKGROUND: Critical time windows are peculiar stages of development where the brain can reorganize in response to experiences. Heightened synaptic plasticity is a double-edged blade: it enables building skills to fit the environment but also increases the vulnerability to stress events, such as neuroinflammatory hits of specific brain areas, like the hippocampus. The term "programming" refers to the imprinting that an adverse event leaves on the individual with consequences that may be also enduring, especially when experienced in a vulnerable window by potentially increasing sensitivity to the development of neuropsychiatric disorders, such as anxiety, bipolar disorder, schizophrenia, and MDD, that emerge during adolescence. Identifying their early markers of onset is crucial given their socio-economic burden. Sex dimorphism is emerging as a key factor for differential stress response and mental disease development.

AIM: This thesis aims to understand how the experience of neuroinflammatory stress during pre-puberty and adolescence may affect the acute central, peripheral and behavioural response later in life.

The systemic injection of LPS is the elective model to study neuroinflammation in rodents, so we employed this model to investigate: 1) the acute transcriptional effects of an immune challenge experienced during juvenile (PND21) and adolescence (PND35), 2) its molecular and behavioural effects on a second inflammatory hit experienced as adults, in male and female mice.

METHODS: Two cohorts were studied: 1) male and female C57BL6J mice were injected i.p. with LPS (100 µg/Kg) or saline at PND21/35 and sacrificed 6 or 24 hours later; 2) mice treated as in point 1 were re-exposed to LPS (830 µg/Kg) or saline at 12wks and sacrificed after 24h. Neuroinflammation-related targets were evaluated in the hippocampus by means of qPCR and metabolites of the kynurenine pathway (KP) were measured in the serum using HPLC-MS/MS. An independent group of adult mice was tested for explorative and anxiety-like behaviour and short-term memory with a battery of tests.

RESULTS: Juvenile and adolescent hippocampal transcriptional response to LPS with respect to inflammatory and microglia-related targets was more intense in females than in males. Consistently, the mRNA expression of the KP limiting enzyme IDO was induced in females only. In adults, this trend was reversed, males showed a stronger transcriptional effect irrespective of experiencing the immune challenge only in early life or also as adults. Serum TRP levels were increased by the immune challenge only in PND21 animals with a different timing between male

and female mice, while serotonin was decreased by LPS at both ages and in both sexes. In adult male animals, the effect of LPS on TRP catabolism was not affected by previous exposure to the immune challenge, while females re-exposed to the inflammatory hit as adults appeared to be more sensible than their saline-receiving counterparts. Behavioural impairments were highlighted: in the OF, LPS reduced the exploratory behaviour of males experiencing the immune challenge only as adults whereas females showed short-term memory impairment after the Y-maze.

CONCLUSIONS: LPS treatment at PND21 or PND35 potentiated the behavioural effects of subsequent exposure to the endotoxin in adulthood especially in female animals exposed to the immune challenge as adolescents. Overall, our data suggest that a neuroinflammatory insult experienced during critical time windows of development has central, peripheral and behavioural long-lasting effects and differs according to biological sex.

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ABBREVIATION TAB.

AA	Anthranilic acid
ACN	Acetonitrile
AD	Alzheimer
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APCI	Atmospheric pressure chemical ionization
AVP	Arginine vasopressin
BBB	Blood-brain barrier
BD	Bipolar disorder
BDNF	Brain-derived neurotrophic factor
BPD	Borderline personality disorder
CD	Cluster of differentiation
CIGS	*Centro interdipartimentale grandi strumenti*
CCL	C-C motif ligand
CNS	Central nervous system
COX2	Cyclooxygenase 2
CRH	Corticotropin-releasing hormone
CRP	C reactive protein
CSSI	*Centro di servizi stabulario interdipartimentale*
Ct	Threshold cycle
Ctrl	Control
CXCL	C-X-C motif ligand
CypA	Cyclophilin A
DAMP	Damage-associated molecular pattern
DC	Dendritic cells
DG	Dentate gyrus
ESI	electrospray ion source
EPM	Elevated plus maze
Fw	Forward
GAD	Generalized anxiety disorder
G.d	Gestational day

GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal axis
HPG	Hypothalamic-pituitary-gonadal axis
HPLC	High-performance liquid chromatography
IDO	Indoleamine 2,3-dioxygenase
iNOS	Inducible Nitric oxide synthase
IL-	Interleukin
ITI	Inter-trial
i.p	Intraperitoneal
KAT	Kynurenine aminotransferase
KMO	Kynurenine 3-monooxygenase
KP	Kynurenine pathway
KYN	Kynurenine
KYNA	Kynurenic acid
KYNU	Kynureninase
LBP	Lipopolysaccharide binding protein
LLOQ	Lower limit of quantification
LPS	Lipopolysaccharide
LTD	Long-term depression
LTP	Long-term potentiation
MDD	Major depressive disorder
MR	Mineralocorticoid receptor
MS	Mass spectrometry
NAD+	Nicotinamide adenine dinucleotide
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLR	NOD-like receptor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
OF	Open field
PAMP	Pathogen-associated molecular pattern
PD	Parkinson disease

PFC	Prefrontal cortex
PGE2	Prostaglandin E2
PND	Post-natal day
PPIA	Cyclophilin A
PRR	Pattern recognition receptors
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
QA	Quinolinic acid
QPRT	Quinolonate phosphoribosyltransferase
RLR	Retinoic acid-inducible gene-I-like receptor
ROS	Reactive oxygen species
RPS29	Ribosomal Protein S29
RT	Room temperature
Rt-	Retro-transcriptase -
RT-PCR	Real-time PCR
Rv	Reverse
SAL	Saline
SCZ	Schizophrenia
SOD	Superoxide dismutase
TDO	Tryptophan dioxygenase
TGF-	Transforming growth factor
TIR	TLR's intracellular domain
TLR	Toll-like receptor
TNF-	Tumour necrosis factor
TRP	Tryptophan
Wks	Weeks
WP	**Work-packages**
XA	Xanthurenic acid
3-HAA	3-hydroxyanthranilic acid
3-HAO	3-hydroxyanthranilate
3-HK	3-hydroxykynurenine

1. BACKGROUND

1.1 JUVENILE AND ADOLESCENCE: CRITICAL TIME WINDOWS TO BE PRESERVED

Critical time windows of development are commonly defined as periods of special sensitivity to environmental stimuli (Burggren et al. 2015; Tierney et al., 2009). These stages include prenatal and perinatal periods, as well as early life/juvenile stages of life such as childhood and adolescence. The brain represents the regulator of processes concerning behavioural adaptation to external/extrinsic happenings and, as a consequence, the responses are reflected in brain structures and function modifications. This is possible because of the great plasticity of the central nervous system (CNS). During critical time windows of development, neural circuits are not fully formed and thus, they are more susceptible to environmental stimuli due to the brain's developmental plasticity: this is why experiences became determining in influencing it (Cisneros-Franco et al., 2020; Tierney et al., 2009). In the last decades, a neurodevelopmental hypothesis for neuropsychiatric disorder was proposed: insults experienced during brain development may activate pathological neural pathways and their consequences can easily emerge during adolescence or in early adulthood (Marco et al., 2009).

Brain structure and its fundamental stages of development are common between vertebrates: complexity and timing are the discriminating factors (Rice et al., 2000). To date, rodents remain one of the most important and widely used translational models to study physiology and physiopathology, especially concerning the central nervous system. Studies have focused on the comparison between human and rodent lifespan and matching stages with comparable moments of criticism were found (Andreollo et al., 2012; Sengupta, 2013; Dutta et al., 2016). The generic correspondence between humans and mice lifespans is that 1 year of human life is almost equivalent to 9 days of life in the mouse (Brust et al., 2015).

1.1.1. An overview of the developing brain in humans and rodents

The first environment that a forming life meets is the maternal one. Risk factors such as stress, smoking, alcohol consumption, infections (Doenni et al., 2016), and malnutrition during pregnancy became crucial for the developing CNS, influencing the underlying mechanisms which are, of course, under genetic control (Tierney et al., 2009). The outcomes are severe congenital abnormalities. The neural tube formation starts 3-4 weeks after conception in humans, on gestational day (g.d.) 10.5-11 in rats, and on g.d 9-9.5 in mice (Semple et al., 2013). The interruption of these processes in this early period can cause abnormal brain development in the spinal cord resulting in *spina bifida* and, in extreme cases, anencephaly (Rice et al., 2000).

Considering anatomical characteristics of the brain, in humans cortex circumvolutions are present at 15 gestational weeks and complete their development after birth (Semple et al., 2013). In other vertebrates, such as rodents, the surface is lissencephalic. In general, vertebrates show a gradient of brain maturation defined as “rostral-to-caudal” with the sole exception of the cerebellum whose development is delayed despite being a caudal structure. As the development is complete, in humans the mass of the neocortex and the visual system, in rodents the mass of the olfactory system, prevails (Rice et al., 2000).

Postmortem studies showed that the human brain reaches 90-95% of adult size during childhood, at 6 years of age, while the most important cortical and subcortical changes happen in early adolescence. White matter increases with age, starting from the second trimester of gestation till the third decade of postnatal life, while grey matter changes are region-specific following a “U-shaped” trajectory but inverted: the maximum volume is reached at 11-12 years of age in the frontal lobe and it slowly declines during adolescence and in early adulthood. For the temporal lobe, the maximum size is reached at 16-17 years followed by a slow decline. The cortical areas are involved in an early thickening of grey matter during childhood and adolescence followed by thinning with age. In general, grey matter continues its decline till a plateau reaches 50 years (Semple et al., 2013) but the most significant structural changes seem to be associated with sex differences (Holder et al., 2014).

In rodents, the total brain percentual gain of adult weight reaches 90% on postnatal day (PND)20, which corresponds to weaning. An increased dendrite density has been demonstrated within the first postnatal week in mice. For rats the main change in grey matter happens in 5 days after birth (Semple et al., 2013).

Focusing on structural brain development, in prenatal life, in both rodents and humans, neurulation represents the first stage for brain formation followed by the proliferation of cells balanced by apoptosis. Cell migration, guided by chemical stimuli and neurotrophic signals, is the process that leads to the cells’ final destination, supervised by glia, involved in supporting by producing myelin and by removing debris. Differentiation, synaptogenesis, and myelination are the final stages (Tierney et al., 2009). During brain maturation in the uterus, the main feature is the massive overproduction of cells and the balance by gradual reduction/pruning became necessary for brain adaptation: it represents the fine-tuning of synapses.

However, the cell's connection refinements, as the stages of brain development previously described, are not only bound to prenatal life: these are processes that continue across years after

birth. Pruning is guided and triggered by experience and it is necessary for individual adaptability. This means that it is characteristic for developing the ability to fit the specific environment in which a subject is involved. In general, the rule is that the repetition of an environmental input reinforces distinctive brain pathways to the detriment of others. Thus, pruning is the stone on which the brain builds the ability to learn. It becomes fundamental especially in early postnatal life for learning basic functions: visual (e.g., face processing), auditory (e.g., language skills), and motor activity. Most of the synapses' remodelling concerning these abilities, occurs in 6-9 months for humans, and the fine-tuning at 4-6 years of age (Tierney et al., 2009). The process of pruning continues also in adolescence for higher cognitive functions such as inhibitory control and emotional regulation (Tierney et al., 2009).

1.1.2 Humans and rodents matching life stages, focus on infancy/juvenile and adolescence vulnerability to develop mental diseases

In humans, postnatal life starts from early childhood which lies between birth and 2 years of age (Gilmore et al., 2018) then childhood till adolescence. In this stage, the main features are visual, motor, and language skills acquisition as mentioned. No autobiographical memory is present before 2 years of age (Occhionero et al., 2023) as the real cognition of the relationship with others which is still forming day by day at this time point. Starting from 3 years, sociability improves (through play behaviour) till adolescence when a switch in its importance happens.

In rodents, infancy starts from PND8 to PND20. It is the stage before weaning (Picut et al., 2015) which belongs to what is called the 'early postnatal phase' (Brust et al., 2015). In general, in this phase, the individual starts to experiment with life in the world after birth and outside the nest (Opendak et al., 2017). During the early postnatal phase, the key moments for pups' vulnerability are birth, weaning, and maternal care. The contact between the dam and its pups is the first connection formed after birth. The mother provides food, protects them, and teaches them how to survive and how to relate to the other littermates. Together with licking, grooming, arched-back nursing, and building the nest, these phenomena belong to what is called maternal care (Caldji et al., 1998). Similarly to humans, the relationship between parents/caregivers as mice mother-pups, and mates, as siblings, are the most important to establish correctly and this is crucial for avoiding susceptibility to stress-related disease (Opendak et al., 2017; Bondar et al., 2018; Gapp et al., 2017; Thomas et al., 2016; Frank et al., 2019; Caldji et al., 1998). Physical abuse, psychological traumas, parental neglect, loss of a dear one or relative, and maternal separation in humans, represent the

most intense and significant events that can perturb the balance of brain development whose effects may be invisible till another critical time window (Opendak et al., 2017; Rivi et al., 2023).

The early postnatal stage in mice ends between PND21-25 with complete independence from the mother but remains still non-reproductive competent (Brust et al., 2015). In mammals, this stage corresponds to “juvenile” (Paquette et al., 2018), followed by adolescence which almost overlaps puberty.

Adolescence represents a peculiar critical time window right through its two-fold factor: the development of the body, guided by hormones that correspond to “puberty” and the social development which is defined as adolescence itself. Puberty is bound to the transition from non-reproductive to reproductive competence. It is characterized by the development of sexual secondary characters from 10-11 years to 15-16 in females and from 11-12 years to 16-17 in males. Adolescence defines the set of cognitive, brain anatomical changes and maturation associated with and resulting from the hormonal changes of puberty (Holder et al., 2014).

In general, brain development and maturation are region-specific and non-linear: hippocampus and amygdala, the elective brain areas for emotional and memory skills acquisition and enhancement, involved even in the traumas and pain elaboration, develop earlier than the cortical ones, implicated, as prefrontal cortex (PFC), in higher cognitive functions: cognitive control, attentional regulation, response inhibition. Their structural maturation ends in late adolescence/early adulthood (Spear et al., 2013; Sisk et al., 2022). This is important because this developmental brain areas-related trajectory determines the pronounced sensitivity to response to risk-taking, novelty, and reward system (Spear et al., 2013) characteristic of teenage vulnerability.

The brain architecture of synaptic structures is influenced by the hypothalamic-pituitary-adrenal (HPA) and the hypothalamic-pituitary-gonadal (HPG) axis regulation which are characterized by a great plasticity during adolescence. Glucocorticoids are the protagonists of stress management both for physical and psychological inputs and outputs, affecting gene expression with consequences on learning, memory, and emotions especially when it is prolonged (Sisk et al., 2022) or repeated later in life. This brings to changes in brain areas directly involved in these processes as the ones just mentioned (Chaby et al., 2015). Thus, the dialog between pubertal hormones, stress hormones, and neurotransmitters, allowed by the blood-brain barrier (BBB), which is permeable to steroid hormones (Kane et al, 2017; Chaby et al., 2015), is at the root of both axis functions. How they “learn” how to answer and fit stressful stimuli is crucial in defining the future responses resulting in resilient or susceptible phenotypes.

Family background and peer relationships represent the key bounds and socially associated increased sensitivity during adolescence is the major shift from childhood. This has direct consequences on well-being: social stressors such as physical and psychological traumas family-related or associated (e.g., bullying, peer rejection, and social isolation) have the greatest impact with respect to other stages of life. On the other hand, positive experiences like social approval and supportive caregiving can determine even a protective effect on the neural level (Sisk et al., 2022).

In rodent females, puberty starts with the onset of ovarian activity. The external sign is vaginal opening, around PND26 (Brust et al., 2015), and the process is considered complete with the first estrus while, in males, the external signs are the preputial separation and the presence of motile sperms in the caput epididymis, after PND21 (Brust et al., 2015). Conventionally, the window of puberty starts on PND28 and ends on PND42 (Holder et al., 2014). Increased exploration and food consumption the high reactivity to stressful events related to HPA and HPG axis maturation and higher sensitivity linked to sexual maturation are, as for humans, the main features of this critical time window (Brust et al., 2015). Brust and colleagues (2015) defined a specific division of mice's adolescence into three stages: “early adolescence”, starting from PND22 to PND34 in which sexual maturation begins and risk-taking behaviour, exploration, and social interest are increased; “mid-adolescence”, from PND35 to PND47, characterised by completed sexual maturity although behavioural maturity is still not reached; “late adolescence”, from PND48 to early adulthood, PND60, in with social interest strongly decreases.

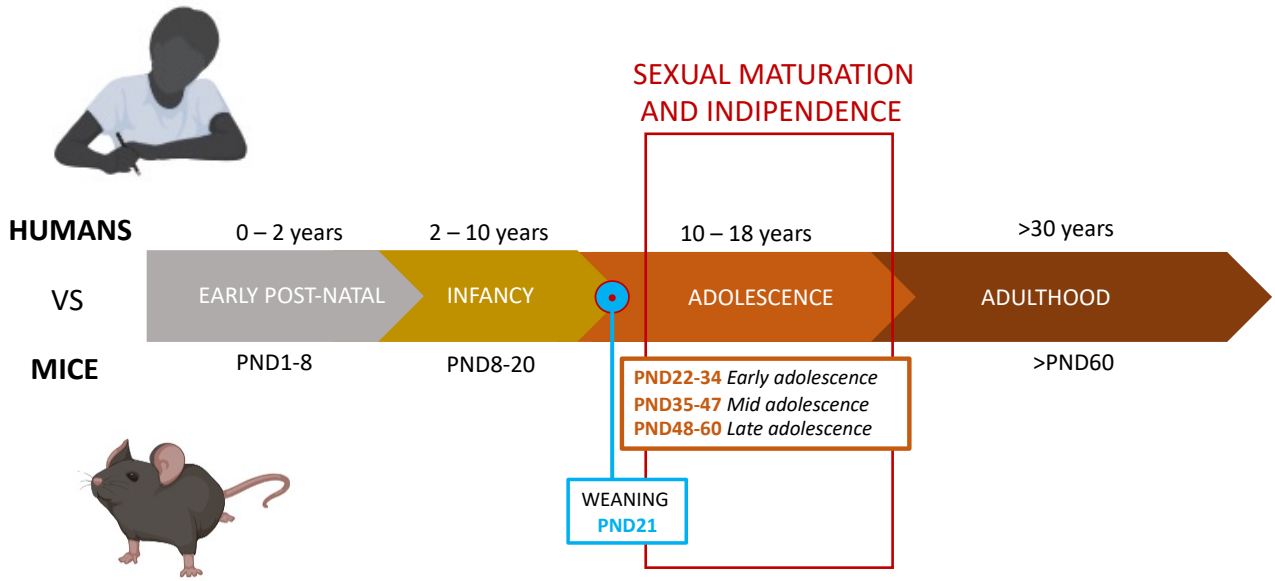

Figure 1. Humans and mice matching stages of life.

The great plasticity of critical time windows is their point of strength but also their weakness: it exposes the individuals to a marked vulnerability to mental disorders, such as anxiety, bipolar disorder (BD), schizophrenia (SCZ), and major depressive disorder (MDD), with the peak of the onset on 14 years, especially associated to the exposure of stressful life experiences (Holder et al., 2014; Fuhrmann et al., 2015). Evidence from clinical studies reports bullying at the age of 7-11 years as impacting in affecting physical and mental health in adulthood: in 50% of adults, a mental disorder is diagnosed before 15 years while 73.9% before 18 years (Fuhrmann et al., 2015).

To date, the socio-economic burden that psychiatric disorders carry is well known, as the distress and suffering experienced by people who live these pathologies directly or indirectly, as relatives.

Considering neurologic disorders' complexity due to their polymorphism and given that most pharmacological treatments ameliorate symptoms but not in all patients, without actually resolving them, the importance of understanding the neurobiological mechanisms underlying mental diseases becomes more and more essential.

1.1.3 Exploring the link between stress, immunity, and tryptophan catabolism

"Stress" in 1940 was defined by Hans Selye as a "nonspecific response of the body to any demand made on it" (Cymerblit-Sabba et al., 2014). Nowadays, it is well understood that the body's response to imbalances caused by stressful situations can be highly accurate. This response can be adaptive, enhancing fitness and survival, as it has been crucial for evolution. Positive changes enable adaptation, but if the imbalance persists without resolution, the response can become harmful and detrimental. Family, socio-economic context, life-threatening, accidents, natural disasters, in general, the environment that engulfs the individual can evoke the typical fight or flight response. Anxiety and frustration related to stressful happenings manifest themselves with acute consequences, such as anxious and depressed mood, loss of sleep, eating disorders, drug and alcohol abuse, to chronic outcomes such as post-traumatic stress disorder (PTSD). The brain receives external input and drives the stress response, being healthy or damaging, and directs different systems such as neuroendocrine, autonomic, metabolic, cardiovascular, and of course immune. The dialog with these systems is bidirectional and it has both short- and long-term consequences (McEwen, 2016). The key points are: 1) intensity: exaggerated, persistent stress is maladaptive and, consequently, it is a risk factor for the development of psychopathologies (Bondar et al., 2018; Gapp et al., 2017); 2) time, intended as the moment of the onset of stress: abuses during childhood correlate with adult's medial PFC, hippocampal volume decrease as animal models of early life stress

show compromised neuronal activities and altered neuroplasticity in adulthood (Bondar et al., 2018).

Stress has been proposed as a crucial factor for the onset and progression of psychiatric disorders, such as MDD, and SCZ but also neurodegenerative disorders such as Alzheimer's (AD), Parkinson's (PD), and multiple sclerosis (Esch et al., 2002).

The main feature after a stressful event is the release of corticosteroids by the HPA axis starting from the adrenal cortex. The chain begins through the release of the corticotropin-releasing hormone (CRH) and the arginine vasopressin (AVP) by the paraventricular nucleus (PVN) of the hypothalamus. CRH mainly interacts with CRHR1 or CRHR2 receptors. CRHR1 is located in the PFC, PVN, amygdala, and hippocampus. CRH-CRHR1 interaction is supposed to mediate stress-initiation and it regulates behavioural and neuroendocrine response to the stress input. By binding CRHR2, CRH is supposed to lead to stress resolution. By crossing the BBB, the corticosteroids, released after a stressful stimulus, bind to both glucocorticoid and mineralocorticoid receptors (GRs and MRs), affecting in turn CNS molecular pathways (van Bodegom et al., 2017).

GRs and MRs are differentially distributed in the brain. In particular, MRs are predominant in the limbic brain areas and the higher levels of expression were found in the hippocampus (van Bodegom et al., 2017) to the extent that it has been called “the major feedback site for glucocorticoids” (Sala et al., 2003). This makes this area closely linked to the stress consequences. Besides being involved in memory formation, the hippocampus is fundamental in the inhibition of fear conditioning in the amygdala (Esch et al., 2002) and in emotional control with direct implications in the onset/development of anxiety disorders. Moreover, evidence from neuroimaging studies reports the shrinkage of this limbic area, a reduction in volume, loss of glial cells, and smaller neuronal cell nuclei dimension, together with PFC, in people affected by depressive disorder (Sheline, 2003; McEwen 2016). Hippocampal neuron loss has been found also in PTSD and borderline personality disorder (BPD) (Sala et al., 2003) but it is difficult to understand whether the reduction in the volume of this area is the cause or the effect of stress itself. Hippocampal neurogenesis and neuronal-dependent plasticity are also strictly regulated by important inflammatory mediators, namely cytokines, such as tumor necrosis factor (TNF-) -α, interleukin- (IL-) -1β, and IL-6 with consequences on neuronal proliferation, survival, and differentiation (Ryan et al., 2015; Fourrier et al., 2019)

The immune system is linked to stress. Indeed, it can exacerbate or withdraw its functions. Inflammation is one of the direct outcomes of the immune system activation that occurs in case of infections, traumatic injury, ischemia, degenerative or auto-immune diseases as well as other stressful events already mentioned. Both physical and psychological stress, in both physiological and pathological conditions, increase plasma levels of cytokines which in turn affect the brain from the periphery. However, pivotal sources of these effectors are present in the brain itself as endothelial and epithelial cells of the choroid plexus and ventricles but also glia and neurons located in the dentate gyrus (DG) of the hippocampus (Munhoz et al., 2008). When neuroinflammation occurs, the process develops and ends thanks to the crosstalk between cytokines, chemokines, reactive oxygen species, and second messengers.

Psychiatric disorders are almost influenced by cognitive impairments which leave an imprint on the quality of life and the possibility of recovery. Working memory impairments were associated with the development and the severity of psychotic symptoms in BD and SCZ. Moreover, symptoms such as intrusive thoughts, nightmares, and flashbacks are correlated with deficits in attention, memory, and executive functions in people affected by PTSD. In general, biological processes such as memory, sleep, and eating are influenced and modulated by cytokines. In this regard, the role of peripheral and central inflammatory mediators in affecting learning and memory has been highlighted and falls under the name of the “cytokine model of cognitive function” (Fourrier et al., 2019). Microglia protects the brain by being its alarm/immune system. By secreting inflammatory mediators such as TNF-α, IL-1β, and IL-6, it is also implicated in cognitive function, especially in memory processes and the effect of these cytokines on synaptic plasticity is dose-dependent: both the disruption or the overexpression of the signaling have detrimental effects on memory and learning with direct consequences on impaired neuronal long-term potentiation (LTP) and long-term depression (LTD). Microglia are specialized in driving synaptic pruning during development by prioritizing the most used synapses and eliminating the weak ones. In adulthood, the process of neurogenesis continues, and it is linked to the crosstalk between microglia and neurons. The pathway involves the fractalkine receptor Cx3cr1 and its chemokine ligand 1, Cx3cl1 also named fractalkine (Fourrier et al., 2019). Stress- or infective-induced inflammation determines rapid changes in microglial phenotype. In homeostatic conditions, it is ramified and called “resting” microglia with associated low levels of “activation markers” as CD11b (Bilbo et al., 2012). This is characteristic of a surveillance state. When an injury occurs, microglial cells shift into their pro-

inflammatory M1 phenotype and release various inflammatory mediators, including IL-1β, IL-6, IL-12, TNF-α, and the chemokines CCL2 and CXCL9. This process affects importantly synaptic plasticity, especially due to the changes in calcium release and reuptake, neurotransmitter release, and neuronal excitability (Eyolfson et al., 2020). Moreover, microglia is associated with long-term changes within the brain, because of its characteristic of becoming and remaining chronically sensitized or "primed", crucial for its implication in Alzheimer's, Parkinson's, and Huntington's diseases, and in normal aging (Bilbo et al., 2012). Cytokine activity seems also to modulate the levels and activity of neurotrophic factors such as the brain-derived neurotrophic factor (BDNF) by decreasing its expression and thus altering synaptic plasticity in the hippocampus. Moreover, cytokines influence the HPA-axis hormone production (Fourrier et al., 2019). Pro-inflammatory cytokines also induce the indoleamine 2,3-dioxygenase (IDO) which is the rate-limiting enzyme of the kynurenine pathway (KP). This pathway seizes the circulating tryptophan (TRP) whose decrease is strongly associated with the onset and progression of several psychiatric disorders, especially MDD, BD, and SCZ.

Alterations in the glutamatergic transmission via N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors signaling is also characteristic of these pathologies. KP seems to play a central role in glutamatergic signaling through its neuroactive metabolites. KP's link to the process of inflammation also makes it a pathway of interest for neurodegenerative disorders. Thus, its function is a focus of researchers' attention.

KP starts with the conversion of TRP into kynurenine (KYN) through the catalytic action of IDO in CNS or of tryptophan dioxygenase (TDO) in the periphery, especially in the liver. Although TRP is renowned as a precursor of serotonin and melatonin, KP is responsible for more than 95% of its metabolism. KYN can be degraded into different compounds of KP: the main branch transforms KYN into 3-hydroxykynurenine (3-HK), then 3-hydroxyanthranilic acid (3-HAA) and quinolinic acid (QA) through the catalytic action of kynurenine 3-monooxygenase (KMO), kynureninase (KYNU) and 3-hydroxyanthranilate (3-HAO) respectively. This is also called the QA branch and it is the major endogenous source of nicotinamide and nicotinamide adenine dinucleotide (NAD+) (Bartoli et al., 2021), crucial for energy production both in peripheral tissues and in the brain following quinolonate phosphoribosyltransferase (QPRT) action (Mithaiwala et al., 2021). In the periphery, the transforming step from KYN to 3-HK happens in monocytes and macrophages while in the brain these steps occur in microglia, in fact, astrocytes lack KMO (Mithaiwala et al., 2021). Another branch degrades KYN into anthranilic acid (AA) through KYNU, then is converted in 3-HK thus returning to

the QA main branch. Finally, kynurenine active metabolite can be also transformed by kynurenine aminotransferase (KAT) enzymes into kynurenic acid (KYNA) or in xanthurenic acid (XA) which are competing products of the pathway (Bartoli et al., 2021). KP active metabolites can have neuroprotective or neurotoxic effects on the CNS. KYNA is associated with neuroprotection because of its inhibitory action on NMDA ionotropic glutamate receptors while QA is supposed to be neurotoxic because of its NMDA agonism and its involvement in oxidative stress (Bartoli et al., 2021). TRP, KYN, and 3-HK easily cross the BBB through transporters and AA by passive diffusion while 3-HAA, KYNA, and QA are predominantly synthetized *de novo* following the enzymatic activity of the glial and neuronal cells in CNS. In general, more than 60% of KYN in inflammatory conditions comes from the periphery: IDO, KMO activity, and the BBB passive transport increase in this condition. As the activity of KAT enzymes is not augmented by the immune system activation, the pathway shifts to the production of QA instead of KYNA creating an imbalance (Mithaiwala et al., 2021) thus, moving toward neurotoxic effects. In clinical studies, the increase in KYN/TRP ratio assessed in the periphery is considered an indicator of KP dysregulation and it is associated with CNS diseases (Mithaiwala et al., 2021).

1.1.3.1 An overview of clinical evidence of neuroinflammatory mediators and KP shifts in the main psychiatric diseases

In individuals with MDD, the link between inflammation and depression has been supposed to be related to a feedback mechanism: people with inflammatory disorders manifest depressive symptoms just as patients with depressive disorders show augmented serum levels of TNF-α, IL-1β, IL-6 and IL-8 (Hong et al., 2016) but also augmented C reactive protein (CRP) and IL-1 while decreased IL-6 and CRP were found after antidepressant administration (Tanaka et al., 2021).

Activation of the immune system in the periphery increases the cytokine levels that, after crossing the BBB, may stimulate microglia and astrocytes to produce cytokines within the CNS as well. Stress contributes to microglia activation and thus augment the production of inflammatory mediators. Moreover, evidence of KP imbalance was found in MDD patients (Hong et al., 2016): decreased TRP, KYN, and KYNA in their plasma and increased QA levels were reported in subjects without antidepressant therapy. Increased QA was found also in MDD post-mortem PFC and hippocampus (Tanaka et al., 2021). KYNA/3-HK and KYNA/QA ratios were negatively correlated with hippocampal activity during memory recall in people affected by depression (Fourrier et al., 2019).

The immune system activation was correlated to altered glutamatergic neurotransmission in subjects affected by BD (Bartoli et al., 2021). Increased levels of TNF-α were associated with a

decrease in white matter integrity following alteration in neurogenesis (Fourrier et al., 2019). Serum and plasma samples of BD patients also showed increased CRP, IL-4, IL-6, IL-10, and IL-1β concentrations (Tanaka et al., 2021). Modifications in HPA-axis functioning were related to impaired cognition and higher levels of the 3-HK/KYNA ratio were found in BD patients displaying impairment in declarative memory performances (Fourrier et al., 2019). Besides the 3-HK/KYNA ratio, in other studies, patients affected by this disorder also showed increased 3-HK/KYN and reduced KYNA levels compared to their controls (Tanaka et al., 2021).

Fear- and anxiety-related psychiatric disorders such as PTSD, generalized anxiety disorder (GAD) panic disorder, and several phobias are characterized by strong, exaggerated reactions to stimuli perceived as dangerous even without any apparent reason. This kind of response is sustained by dysfunctional HPA-axis activation and reactivity together with the involvement of immune system activation and pro-inflammatory cytokines release (Michopolous et al., 2017). In the serum of PTSD patients, increased levels of TNF-α, IL-1β, IL-6, and IFN-γ were correlated to its severity while IL-4 was found to be decreased. Transforming growth factor- (TGF) -β is thought to be a predictive indicator for PTSD development while KYN metabolites are measured mainly as evidence of the inflammatory response rather than properly associated with this pathology. TNF-α and IFN-γ were also increased in the serum, plasma, and blood of patients with GAD for increased pro-/anti-inflammatory cytokines ratios (Tanaka et al., 2021).

Besides the dopamine dysfunction and the glutamatergic hypofunction hypothesis (Hong et al., 2016), the importance of peripheral inflammation was widely studied for the etiology of SCZ. Epidemiological studies associate the prenatal or childhood experience of an infection with a higher risk of developing this pathology (Fourrier et al., 2019). SCZ patients reported elevated serum levels of pro-inflammatory cytokines such as IL-1β, IL-6, and TGF-β during psychosis at the first episode, and together with TNF-α, their level decreased after the antipsychotic treatment. In chronic SCZ, IL-1β levels were found to be upregulated while IL-6 increment was associated with the acute effects of the pathology (Tanaka et al., 2021). In SNC, IL-1β, IL-6, and IL-8 were significantly higher in SCZ patients. Considering KP, KYN, and KYN/TRP ratio were higher in the serum of SCZ people. KYN concentration resulted higher also in cerebrospinal fluid in SCZ as KYNA in plasma, brain tissue, and saliva. Despite its neuroprotective effect, KYNA in SCZ seems to boost cognitive impairment (Tanaka et al., 2021). HPLC/MS revealed increased AA concentration while 3-HK was decreased in the serum

of patients with SCZ. Recent evidence suggests AA as a potential target for schizophrenia. In rodents, AA has been associated with autoimmunity (Oxenkrug et al., 2016).

Periphery and CNS influence themselves *in the continuum*. The major neurological disorders seem to have neuroinflammation and KP shifts as a common thread. Limbic areas, such as the hippocampus, are crucial sites of signaling and responses to stress stimuli. The onset and progression of mental diseases appear to be linked to synaptic plasticity vulnerability own of critical time windows of life. Thus, the role of inflammation in the hippocampus needs to be explored to identify early biological hallmarks of these diseases during development. In pre-clinical research, the systemic injection of lipopolysaccharide (LPS) is to date widely used to study neuroinflammation and associated and related pathologies.

1.1.3.2 LPS peripheral administration in rodents as the elective model of neuroinflammation and its application on critical time windows of development

The immune system activation, already described as a common feature of brain diseases, is primarily a line of defence. Infections induced by microorganisms trigger the innate/non-specific immune system activation and a set of metabolic and behavioural responses ensue to fight them and bring the organism back into balance. The main cells involved in this process are monocytes, macrophages, neutrophils, dendritic and natural killer cells (DC and NK) which are equipped with pattern recognition receptors (PRR). PRRs family includes toll-like receptors (TLRs), NOD-like receptors (NLRs), and retinoic acid-inducible gene-I-like receptors (RLRs). They are designed to recognize other patterns expressed by whole pathogens or by their components, called pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Danzer, 2004; Skrzypczak-Wiercioch et al., 2022). In the CNS, the cells called to act as defence line are astrocytes, oligodendrocytes, Schwann cells, neurons, and mainly microglia (Skrzypczak-Wiercioch et al., 2022) that switch from “resting” to its “activate”, M1, ameboid phenotype (Eyolfson et al., 2020; Woodburn et al., 2021). M1 pro-inflammatory phenotype acts through TLRs after an inflammatory insult (Woodburn et al., 2021; Skrzypczak-Wiercioch et al., 2022). These receptors sense PAMPs and DAMPs thanks to their outer leucine-rich repeat domain (LRR) and, after a ligand binding, homo- or heterodimerizes and the activation of TLR’s intracellular domain (TIR) follows. The process induces downstream signalling called the MyD88-dependent signalling pathway that, via nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), unleashes the production of pro-inflammatory mediators (Skrzypczak-Wiercioch et al., 2022). Peripheral and central inflammation are self-sustaining processes: the inflammatory cascade is a signalling of

recruitment, thus other cells are recalled, transcription factors are activated and other cytokines and chemokines are produced. They are also self-regulating processes because of the action of anti-inflammatory molecules. Microglia can turn into an “alternative” state of activation called M2 phenotype, induced by IL-4, IL-10, IL13, and TGF-β. Indeed, the M2 state is associated with anti-inflammatory promotion, tissue repair, and increased phagocytic activity (Eyolfson et al., 2020).

The CNS monitors the peripheral innate immune system activation by different paths working together in parallel. One way involves afferent nerves. They are activated by local cytokine production, depending on the site of the infection. TLRs family on cells, detecting circulating pathogens, represents the humoral pathway dealing with cytokine production. Cytokines then pass BBB according to their volume and thus the possibility of diffusion. A third pathway provides the passage of cytokines by transporter systems. Lastly, IL-1 receptors on perivascular macrophages and endothelial cell activation produce prostaglandin E2 (PGE2), the key immune-to-brain communication that induces microglial cytokines production (Danzer et al., 2008). It is the modulator of fever response (Boissé et al., 2004).

In neuroscience research, LPS is used as an exogenous agent of infection in order to mimic both peripheral and central inflammation. In preclinical studies, the most common way to induce LPS neuroinflammation is the intraperitoneal injection, (i.p.). It is an advantageous procedure because it is easy to perform and considered non-invasive, little stressful and it is effective for its dose-dependent purpose. The (LBP) and lipoproteins carry the LPS to cross BBB (Skrzypczak-Wiercioch et al., 2022) and its systemic administration is associated with altered BBB cytokine transport (Larsson et al., 2016). This promotes the inflammatory process. Lipopolysaccharide is known as the active fragment of Gram-negative bacteria and it works as a PAMP. This molecule consists of hydrophobic lipid A, the most immunogenic component of LPS, and O-antigen.

The common ground between neuroinflammation and LPS-induced inflammation is the TLR4 signaling (Skrzypczak-Wiercioch et al., 2022). Circulating LPS (or the whole Gram-negative microorganism) is detected by LBP thanks to the lipid A presence, then monocytes, macrophages, neutrophils, and DC in the periphery, and microglial cells in CNS, present it to TLR4 through the cluster of differentiation (CD) -14. As a result, IL-1β, IL-6, IL-8, TNF-α, PGE2, nitric oxide (NO) and inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2) and reactive oxygen species (ROS), are released and promote inflammation (Skrzypczak-Wiercioch et al., 2022) together with

chemokines as CXCL1, CXCL2, CXCL10, CCL2 and CCL7, as well as an increase in circulating corticosterone (Bilbo et al., 2012).

The systemic administration of lipopolysaccharide, to both human and animal models as rodents, unleashes central and peripheral molecular responses which are linked to physical symptoms, including fever, which fall under the name of sickness behaviour. The peak occurs in 2-6 hours after LPS administration (Danzer et al., 2008). In humans, inability to concentrate, general discomfort, and lethargy as little interest in the environment and refusal to eat and drink arise (Danzer et al., 2004). In rodents, huddling, ptosis, and piloerection are other characteristics, together with lethargy and reduced feeding, drinking (Kane et al., 2017), and hippocampal impairment (Danzer et al., 2008). The main cytokines associated with the development of these symptoms are TNF-α and IL-1β (Danzer et al., 2008). Sickness behaviour and fever are proposed as precise responses to fight against the infection (Danzer et al., 2004). After 24 hours, LPS induces depressive-like behaviour. This mechanism is related to IDO-increased activity. Unlike sickness, depressive-like behaviour is not considered an adaptive response to the immune challenge. Rather, it appears to be the result of a prolonged response associated with chronic conditions after an initial acute-phase response (Frenois et al., 2007; O'Connor et al., 2008).

Neuroinflammation LPS-mediated was investigated also in critical time windows of development. Prenatal exposure to 0.01 mg/kg of LPS is correlated to the development of SCZ. Pups showed decreased myelination in cortical and limbic brain regions and a reduction of Parv-expressing cells in PFC, hippocampus, and locomotor hyperactivity and prepulse inhibition impairment as adolescents (Wischhoff et al., 2015). The systemic injection of 0.01 mg/kg of LPS also increased peripheral cytokine production and their mRNA expression in the hippocampus, hypothalamus, amygdala, and PFC at PND14 in CD1 mice, 3 hours after the i.p. injection. Alteration in anxiety-like behaviour, assessed with the novelty-suppressed feeding test, was reported in adolescence (Dinel et al., 2014). Thus, this dose is suitable to trigger the inflammatory response in early postnatal life. The same dose of LPS was administered to C57BL6 mice at PND14 to study variation in the synaptic hippocampal transmission which failed to be demonstrated in adolescence but was present in adult animals (Gomez et al., 2021).

1.2 THE SO CALLED "PROGRAMMING EFFECT" AND ITS IMPORTANCE IN ADULTHOOD

The term "*allostasis*," introduced in 1988, describes the body's continuous and dynamic process of maintaining stability and balance, also known as homeostasis. Its literal meaning is: "achieving stability through change". Excessive or inadequate management of allostasis is defined as "allostatic overload" intended as the failure to switch off the response when it is unnecessary or the inability to switch on the adequate one (McEwen et al., 2016). Responses are both biological, including increased blood pressure, and chemical mediators, such as catecholamine, release but also behavioural, manifesting as an increase in anxiety, freezing, and aggressivity. Indeed, distinct strategies are pursued by animals, both humans and rodents, to answer to environmental stimuli based mainly on previous experiences. This effect can be almost unconscious and falls under the name of "programming": which is a fundamental system of adaptation that confers to recognize threats and drives the use of energy resources. When a subject is exposed to the same repeated stimulus, that one is defined as homotypic. In the case of stress, in general, animals display a kind of "habituated" response compared to the ones who experience it for the first time. When the stimuli are different, they're defined as heterotypic, and the responses can be sensitizing. Prolonged or exaggerated exposure to adverse stimuli is related to dysfunctional programming and allostatic response leading to a higher risk of developing psychopathologies, especially when stressful events occur during critical time windows of development. The programming effect has widely been studied in the field of prenatal and perinatal critical periods, but little is known about adolescence (Sharma et al., 2019; McEwen et al., 2016).

1.2.1 Programming and inflammation: molecular implications

The exposure to an immune challenge, as LPS systemic administration, may be involved in "programming" the response to a subsequent one later in life (Sharma et al., 2019). Ellis and collaborators demonstrated attenuated febrile response and reduced peripheral TNFα, IL-6, and NFkB activation, in adult rats exposed neonatally to 0.01 mg/kg of LPS compared to saline-receiving counterparts, while CRH was increased, indicating the involvement of HPA-axis action (Ellis et al., 2005). The group of Boissé demonstrated variation of the COX2 later in life after an immune challenge experienced in the perinatal period: western blot indicated a hypothalamic reduction of COX2 in adult rats pre-treated neonatally with LPS and responding to a second immune challenge (Boissé et al., 2004). Sharma and collaborators focused on puberty as a sensitive time window of development to stress to immune challenges and their eventual enduring effects. They demonstrated that homotypic stress induced through the i.p. administration of LPS (1.5 mg/kg) in

CD-1 adolescent mice (PND42) determined an attenuated/programmed response in adult animals (10 wks) when re-exposed to the same insult: TNF-α, IL-1β and IL-6 expression in PFC and peripheral IFN-γ and IL-6 were reduced after the second hit and the hypothermic response was attenuated compared to saline controls (Sharma et al., 2019). A programmed immune-mediated effect was also reported for microglia: higher microglial cells number was found in cortex-associated areas, ventral striatum and DG in adolescent (PND30) BALB/c mice as in frontal association cortex of adult animals (PND100) following a viral (Poly(I: C), 20 mg/kg) immune challenge experienced in prenatal life, at embryonic day 9 (Manitz et al., 2013; Juckel et al., 2011). Prenatal exposure to infection also induced changes in KP but the long-lasting effects are still under investigation (Notarangelo et al., 2017). Notarangelo et al. found increased levels of 3-HK in basal brain tissue of CD-1 mice at PND60 after an i.p LPS injection (1 mg/kg and not 0.1 mg/kg) when previously exposed (gestational day 15) to both LPS (0.1 mg/kg) or saline, indicating that this response seems not to be associated with a programming effect (Notarangelo et al., 2021).

1.2.2 Programming and inflammation: behavioural implications

Behaviour is the visible and final output of the CNS (Crawley, 2008), strictly related to the biological changes induced by the environment via stimuli experienced by individuals, positive or negative when perceived as dangerous and stressful. Literature reports a wide plethora of well-validated behavioural tests used to assess sensory abilities, motor functions, learning and memory, feeding and drinking, as well as social interactions, drug and alcohol self-administration, anxiety traits, depressive-like behaviour, and schizophrenia (Crawley 2008). Importantly, the use of multiple tests is needed to obtain a broad understanding of the different behavioural domains in animal models (Ramos 2008). The use of a behavioural test battery allows this task. Moreover, evaluating multiple behavioural responses in the same mouse reduces the total number of animals needed according to the reduction principle belonging to the "three R rule". The order of tests within the battery is generally designed starting from the least invasive tests before being tested onto the more invasive assays (McIlwain et al., 2001).

In human and animal models, the cognitive ability of the individuals is part of the behavioural output: it is essential in determining reproductive success, learning ability, and memory skills. The ability to form an association between a predictive stimulus and a reinforcer, namely associative learning, is well-conserved in animals and can facilitate to fit of the environment by optimising resource research or prediction and avoidance of a threat. Reversal learning allows animals to cope

with environmental changes, abandoning previously established associations for alternative cues. Associative and reversal learning can be impaired shortly after stress exposure in rodent models. Working memory, involved in maintaining information in memory for immediate use or its manipulation, allows reasoning, reading comprehension, and other individual skills which can be impaired after exposure to stress with consequences on the quality of life (Chaby et al., 2015). "*Fear* is the emotional response to a real or perceived imminent threat, whereas *anxiety* is the anticipation of a future threat". Anxiety is not necessarily only a pathological state, as it can prevent exposure to dangers. However, when anxiety is sustained and/or raised by non-threatening stimuli, it becomes maladaptive (Daviu et al., 2019). Considering the relationship between stress and the onset of anxiety, since stressful experiences such as immune challenges (background, section 1.1.3) can impair cognitive ability, experiencing them during critical time windows can have long-lasting effects also on adult behaviour, thus, long-term changes have been studied following stress in early stages of life. Importantly, evidence reports that systemic inflammation, associated with brain inflammation and affecting mood, learning, and memory through processes related to neurodegeneration and structural remodeling occurs mainly in the hippocampus (Dinel et al., 2011). Impairments in anxiety-like behaviour in the elevated plus maze (EPM) and the open field (OF) or a novel environment were observed later in life following juvenile stress in rats (Cymerblit-Sabba et al., 2015): N: NIH Norway rats injected with 50 µg/kg of LPS at PND3 and PND5 displayed a significant increase in emergence latency in an open field arena compared to the saline-treated controls once they reached adulthood (P70-100) (Claypoole et al., 2017) while Wistar rats treated with the same dose of LPS at PND3 showed a higher number of entries, spent more time and traveled a longer distances in the open arms than controls as adolescents (PND40-46) in the EPM (Rodríguez Rico et al., 2010). LPS-treated (0.1 mg/kg) Sprague–Dawley rats injected at PND14 showed significantly reduced exploration of a novel objects introduced in an open field arena as adults (8 wks), indicating an effect of an early-life immune challenge on anxiety-traits in adulthood (Spencer et al., 2005).
When considering short-term memory, CD-1 mice injected with LPS (0.1 mg/kg) at PND14 displayed no significant effect in adolescence (PND30) or adulthood (PND90) in the Y-maze test (inter-trial of 30 minutes). After a second administration of LPS (0.1 mg/kg) in adulthood, impaired spatial memory was revealed after 24h: animals spent the same time exploring the novel and the familiar arms (Dinel et al., 2014).

These studies revealed that a single (Claypoole et al., 2017; Rodríguez Rico et al., 2010; Gomez et al., 2021) or double (Dinel et al., 2014) inflammatory event as the LPS injection experienced in a

critical window of development may produce long-lasting alterations in CNS, endocrine and also behavioural.

1.3 THE ROLE OF SEX ON BRAIN DISEASE: WHAT DO WE KNOW?

The presence of sex-specific prevalence of mental and physical disorders is to date a topic of world relevance. The total burden calculated in 2014 for psychiatric disorders was 30.1% in women and 24.1% in men (Kokras et al., 2014). Sex and gender have an active role in maintaining/affecting individual homeostasis (Wang et al., 2007). The term "sex differences" stands for biological characteristics which are specific to male or female subjects while "gender differences" refers to the differential effect of the environment on them. The term "sex" is almost used for animal translational model studies while both "sex" and "gender" for humans depend on the topic considered (Kokkosis et al., 2020). Nowadays, the use of both male and female animals in experimental research cannot be neglected given the importance of their different responses concerning both models of psychiatric disorders and the efficacy of drugs (Kokras et al., 2014). Introducing sex/gender in preclinical and clinical research has been crucial for trying to understand normal brain development, reproduction, and aging, as well as social behaviour, sleep and substance abuse, and cognitive and emotional responses concerning learning and memory, language, fear, anxiety, and nociception, as well as the risk and consequences of traumatic brain injury and stroke. In the human population, higher rates of autoimmune diseases, chronic pain among women, and the generally higher susceptibility of men to infectious disease, hypertension, cardiovascular diseases, aggressive behaviour, and abuse of alcohol or drugs were highlighted (Wang et al., 2007). Dyslexia and stuttering are three/four times more frequent in boys than girls as for attention deficit hyperactivity disorder (10:1 for boys). The relative risk of developing autism or autism spectrum disorder is up to 4:1 for males, as early-onset schizophrenia. MDD/anxiety/panic disorders and anorexia/bulimia are more frequently diagnosed in women, 2:1 and 13:1 respectively when compared to men (McCarthy et al., 2012). Sex-based differences in the frequency of these diseases may lie in structural brain area development and molecular variation responses which are distinct among males and females.

1.3.1 An overview of sex differences in the developing male or female brain

Neurodevelopment displays differences among the sexes. The total brain size, as the regional grey matter volumes (background, section 1.1.1), follows an inverted U-shaped trajectory during development, with a peak at nearly 10.5 and 14.5 years in females and males respectively. While grey matter reaches its peak earlier in females, white matter grows faster in males with age. Importantly, total and regional-size differences in the brain are reported. In children, in vivo imaging studies report a 9–12% greater brain size in males. Studying three independent paediatric cohorts,

the caudate nucleus, temporal lobes, thalamus, and basomedial diencephalons were found to be larger in females. During puberty, the hippocampus and the right striatum were found to be larger in girls while in boys this occurred in a region of the amygdala (Lenroot et al., 2010). Consistently with these results, in a previous cross-sectional study of 121 healthy children and adolescents aged between 4 and 18 years, amygdala and hippocampal volume were found to be increased in both sexes but the amygdala increased significantly more in males while hippocampal volume grew in females during their development (Giedd et al., 1997).

MRI studies show that the brain size and the white-to-grey matter proportions are maintained even in adult individuals and women display a greater cortical complexity in the frontal and parietal regions. Differences in cortical complexity may enhance or not sex-specific skills and different behavioural attitudes (Darnall et al., 2009). Frontal and medial paralimbic brain regions were found to be larger in women, while the frontal medial cortex, hypothalamus, amygdala, and angular gyrus were bigger in men (Lenroot et al., 2010). In addition to these structural differences, at the cellular level, women show a higher neuron density in parts of the temporal lobe cortex linked to language both processing and comprehension and they have access to brain areas linked to pain and imagination more frequently than men. Moreover, men and women process emotional memories by using different parts of the amygdala (Darnall et al., 2009). The whole set of hormonal, psychological, social/environmental, and cultural factors together with morphological sex-based differences may help to understand both behavioural and subsequent immune responses (Darnall et al., 2009).

1.3.2 Clinical and preclinical evidence of sex-dependent neuroinflammatory response in the main psychiatric diseases

As mentioned, HPA-axis functioning, immune dysregulation and neuroplasticity are the main systems of interest implicated in the pathogenesis of mental diseases and sex differences emerge as contributing factors in their progression.

In both anxiety and depressive disorders, males and females have nearly the same rates of depression (5%) before the onset of puberty, then the ratio shifts to 2:1 for females. One potential mechanism is attributed to the sex-differential development of the HPA axis and to its susceptibility to stress which is higher in females with advancing puberty. This phenomenon is associated with higher activation of the HPA axis. In males, this response is decreased due to testosterone levels (Lenroot et al., 2010). Evidence of sex-dependent response to inflammation was also highlighted: in a meta-analysis of 26 studies, women were found to be more sensitive to developing MDD following

IFN-α treatment, and an association between systemic inflammation and depression was demonstrated in this gender by displaying higher depression scores and levels of CRP, IL-6, and fibrinogen (Kokkosis et al., 2020). Importantly, suicidal peripartum depression has been linked to KP pathway shifts followed by increased plasma IL-6, IL-8, IL-2, and QA, and serotonergic signaling dysregulations (Kokkosis et al., 2020). Animal model studies highlighted the presence of sex dimorphism in the microglial-mediated immune responses during mental disorders. In particular, an alteration of cx3cr1 signalling was reported in the PFC of female rats (Bollinger et al., 2019) and of microglial morphologic features in the amygdala, orbitofrontal cortex, and hippocampus, possibly affecting differential neuronal plasticity (Bollinger et al., 2017). On the other hand, Millet and colleagues hypothesized a protective role of the female sex from molecular and behavioural shifts induced by an immune challenge LPS-mediated. In their study, only male mice (C57BL6), and not females, showed depressive-like behaviour and increased levels of superoxide dismutase (SOD) I in the hippocampus 24h after an LPS (0.83 mg/kg) i.p. injection when compared to saline-receiving males (Millet et al., 2019).

As mentioned, HPA-axis functioning changes in PTSD are strictly associated with immune system activation (Michopolous et al., 2017; Tanaka et al., 2017). Evidence of sex dimorphism was found in the immune responses associated with this disease. In the study of Neylan and colleagues, gene microarrays of circulating CD14+ monocytes showed general suppressed gene expression of genes involved in chemotaxis, cytokine-cytokine interaction, and immune activation in PTSD male samples while females displayed their increase (Neylan et al., 2010). In a rodent model of trauma, the sex-specific effect was detected in transcriptional upregulation of NFkB via TNF-α induction and hippocampal-altered synaptic plasticity in female animals (Kokkosis et al.,2020).

Schizophrenia incidence rises during adolescence as a critical time window of development and sex-dimorphism associated are reported: male results as the more sensitive to develop SCZ considering severity and frequency. The mean disease onset calculated is 5 years earlier for men compared to women. They also have a distinct peak age of onset during late adolescence and young adulthood, while in females the progression is more gradual, and a second rise in incidence is reported near menopause (Lenroot et al., 2010; Kokkosis et al., 2020). Females show a higher vulnerability to SCZ and an increase of psychotic episodes also during postpartum. This phenomenon is linked to sudden estrogen levels dropping. The “estrogen hypothesis” postulates that this hormone can exert a protective effect on SCZ (Kokkosis et al., 2020). Interestingly, the link between the immune response

and the development of this disease seems to be associated not only with sex dimorphism but also with the effect of programming (Hui et al., 2018).

1.3.3 Immune-mediated programming effect according to sex differences in rodents

The bidirectional relationship between the “programming” induced by neuroinflammation and sex is still a topic of research that remains to be explored. Anyway, given its importance, some aspects were recently investigated and some initial findings were highlighted. Little is known concerning the behavioural features yet.

In a mouse model of SCZ, prenatal infection was found to differentially program microglial shifts according to sex later in life. Indeed, increased inflammatory response, “dark'' microglial density, synapse interaction, and oxidative stress were demonstrated in the hippocampus of adult (PND60-90) C57BL/6 males after the exposure to prenatal (embryonic day 9.5) polyinosinic: polycytidylic acid (5 mg/kg). In addition to microglial alterations, behavioural impairment was also elicited by altered sensorimotor gating after an acoustic PPI test in male adults (Hui et al., 2018). Age and sex differences in acute stress and immune responses in mice were found following restraint stress: prepubertal outbred females displayed reduced corticosterone levels compared with their adult counterparts. Similarly, following LPS treatment, pubertal females displayed lower levels of corticosterone than both adult females and male counterparts (Sharma et al., 2019).

A recent publication from Sharma and colleagues has shown age- and sex-related peripheral immune response after LPS treatment. The pretreatment with LPS attenuated the immune response to a second homotypic challenge: males pretreated with LPS during puberty displayed an attenuated hypothermic response following the second LPS treatment compared with saline-controls and attenuated peripheral IL-6 and IFN-γ concentrations. Females pretreated with LPS during puberty displayed lower IL-1β, TNF-α, and IL-6 mRNA expression in the PFC following the second inflammatory hit compared with saline controls. Thus, experiencing LPS during puberty programs the peripheral and central immune responses which result in attenuation following a subsequent homotypic stressor (Sharma et al., 2018).

To date, the field of sex-dimorphism modulating the programming of immune-mediated and behavioural responses remains unexplored, especially focusing on adolescence as a sensitive time window of development in which various hits can contribute to mental illness onset or exacerbation.

2.0 AIMs

Human beings and animals are strongly influenced in their development by genetic factors and the environment. Stressors, like early life immune challenges, act as disturbing environmental factors and can affect the balance of an individual's brain development, especially during specific stages of life called "critical time windows" of development such as prenatal and perinatal periods, like infancy and adolescence. During these stages of life, individuals are immensely receptive to physical and psychological stimuli. After a stressful event, an amplified immune system, HPA-axis activation, and 5HT-system dysregulation represent the main outcomes. These processes are involved in the alteration of brain maturity and neuroplasticity and may ultimately lead to the emergence of mental disease. Adverse events can shape physical and psychological development, resulting in biological, psychological balance, and adaptive behavioural phenotype in resilient individuals, while exerting detrimental effects in susceptible ones. The experiences in early life can have "programming" effects within the brain that are fundamental in determining the predisposition to develop neuropsychiatric disorders in adult individuals.

In rodents, the peripheral systemic injection of LPS is the elective model used to mimic neuroinflammation by activating cytokine cascade and microglia. Importantly, it is also responsible for triggering the kynurenine pathway (KP), crucial for tryptophan catabolism, whose dysregulation has been linked to the development of several psychiatric disorders including MDD.

Moreover, it has been observed that early-life exposure to LPS increases the risk of developing emotional behaviours as well as cognitive alterations in adulthood.

Given that biological sex is being recognized as a key discriminant in defining vulnerability to mental illness, the **general aim** of this thesis is to explore in male and female animals the different effects evoked by an immune activation during critical windows of development, like infancy or adolescence, and the short-term and long-term molecular and behavioural consequences.

The aim was addressed with the following work packages (WP):

Work-package 1:

Evaluation of the acute effects of an immune challenge experienced during juvenile, PND21, or adolescence, PND35, on the expression levels of pro-inflammatory, anti-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-4) microglial markers (CD11b, CD14, CD206, the fractalkine receptor cx3cr1) and the KP limiting enzyme IDO in the hippocampus and on the serum levels of TRP, 5HT and KP metabolites (KYN, KYNA, and AA) of male and female C57BL6J mice.

The critical time windows chosen were PND21 which corresponds to the infancy-juvenile stage of life, an “early” stage characterized by weaning and maternal separation, and PND35, corresponding to adolescence, is the stage of animals' total independence and is characterized by sex maturation. Animals were injected with 100 µg/Kg of LPS based on previous studies in pups and adolescent mice and rats that confirmed as the minimum range to trigger the cytokine cascade. Mice response to the stressor LPS was analyzed after 6h and 24h. Transcriptional effects were evaluated in the maturing hippocampus area of the limbic system, sensitive to neuroinflammation, involved in memory formation and in regulating stress response, affected by important abnormalities and atrophy in the presence of mental disorders (Sala et al., 2004; Millet et al., 2019).

Work-package 2:

Evaluation of the programming effects of a neurodevelopmental immune hit on a subsequent challenge later in life. C57BL6J mice were first injected with 100 µg/Kg of LPS during juvenile, PND21, or adolescence, PND35, then as adults (at 12 weeks of age) were exposed again to LPS at the concentration of 830 µg/Kg. Again, we were particularly interested in the eventual differences between males and females and in the additive-synergic effect of LPS experienced both in early life and as adults. The hippocampal expression and serum levels of TRP and KP metabolites in serum were evaluated for WP1.

Previous studies in mice have demonstrated that LPS at a concentration of 830 µg/Kg triggers the inflammatory cytokine cascade and induces a sickness behaviour syndrome that includes a reduction of food intake and explorative activity (peaking at 2-6 hours) which is followed by depressive-like behaviours (24-48 hours after LPS injection) including reduced motivation, anhedonia, and cognitive impairments (Danzer et al., 2008).

Work-package 3:

Evaluation of the long-term behavioural effects of an immune challenge experienced during juvenile, PND21, or adolescence, PND35 combined with a subsequent challenge later in life. Animals were treated as in WP2.

In mice, characteristic behaviours of the species are compromised in stressful situations: exploration is reduced, anxiety is marked and animals manifest memory impairments. Considering the complexity of the behavioural response, we used a battery of tests to assess more than one behavioural domain through a specific test in each animal. We evaluated the explorative behaviour, the anxiety-like behaviour, and spatial memory through the open field maze, the elevated plus maze, and the Y maze respectively.

3.0 METHODS

3.1 ANIMALS

Adult male and female C57BL/6J mice (Charles River Laboratories, Lecco, Italy), bred in *Centro di Servizi Stabulario Interdipartimentale* (CSSI), University of Modena and Reggio Emilia, were mated in 1:3 ratio and separated after 3 days. Pups were weaned at PND21 and arranged by sex (n = 5 per cage before treatment). Animals were housed in polycarbonate cages (30 × 30 × 15 cm) with *ad libitum* access to food and water and maintained under a 12/12 h light-dark cycle (lights on 8:00 am to 8:00 pm), at room temperature, RT (21 ± 3 °C), with relative controlled humidity. Mice were handled once a week to habituate them to the experimenters and checked daily for signs of discomfort as indicated by the animal care and use guidelines "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research" (National Research Council 2003). All procedures were carried out by the EC guidelines (EEC Council Directive 86/609 1987) and the Italian legislation on animal experimentation (*Decreto Legislativo* 26/2014) with the approval of the local Ethical Committee.

3.2 TREATMENT AND EXPERIMENTAL DESIGN

Male and female animals were injected i.p. with saline (SAL, ctrls) or treated with LPS from *Escherichia coli* (serotype 0127:B8; Merck Life Science) dissolved in saline (SAL) at the concentration of 100 µg/Kg or 830 µg/Kg according to the following experimental design:

- *Cohort 1:* juvenile (PND21) and adolescent (PND35) male and female animals were injected i.p. with saline or 100 µg/Kg; LPS concentration had been chosen considering literature ranges used for mouse or rat pups which variates from 0.1 mg/kg to 1.5 mg/kg (Wischhoff et al., 2015; Dinel et al., 2014; Gomez et al., 2021; Ellis et al., 2005; Boissé et al., 2004; Sharma et al., 2019; Notarangelo et al., 2021; Spencer et al., 2005). Animals were sacrificed 6 or 24 hours after treatment.
- *Cohort 2:* juvenile (PND21) and adolescent (PND35) male and female animals injected saline or 100 µg/Kg of LPS were left undisturbed up to 12 weeks (wks) of age when they were injected with 830 µg/Kg of LPS or saline. Animals were then sacrificed 24 hours after treatments.
- *Cohort 3:* juvenile (PND21) and adolescent (PND35) male and female animals injected with saline or 100 µg/Kg LPS exposed at 12wks of age to either 830 µg/Kg of LPS or saline underwent a battery of behavioural tests 24 hours after treatment: first the open field (OF)

test, to assess locomotor activity, followed by the elevated plus maze (EPM), to study anxiety-like behaviour and the Y-maze, a spatial memory recognition test. Mice were sacrificed after the last behavioural test.

On the day of the treatment, animals were weighed before and after LPS or saline administration for the evaluation of mean body weight. Food and water were also weighed for the evaluation of treatment-induced effects on feeding and drinking.

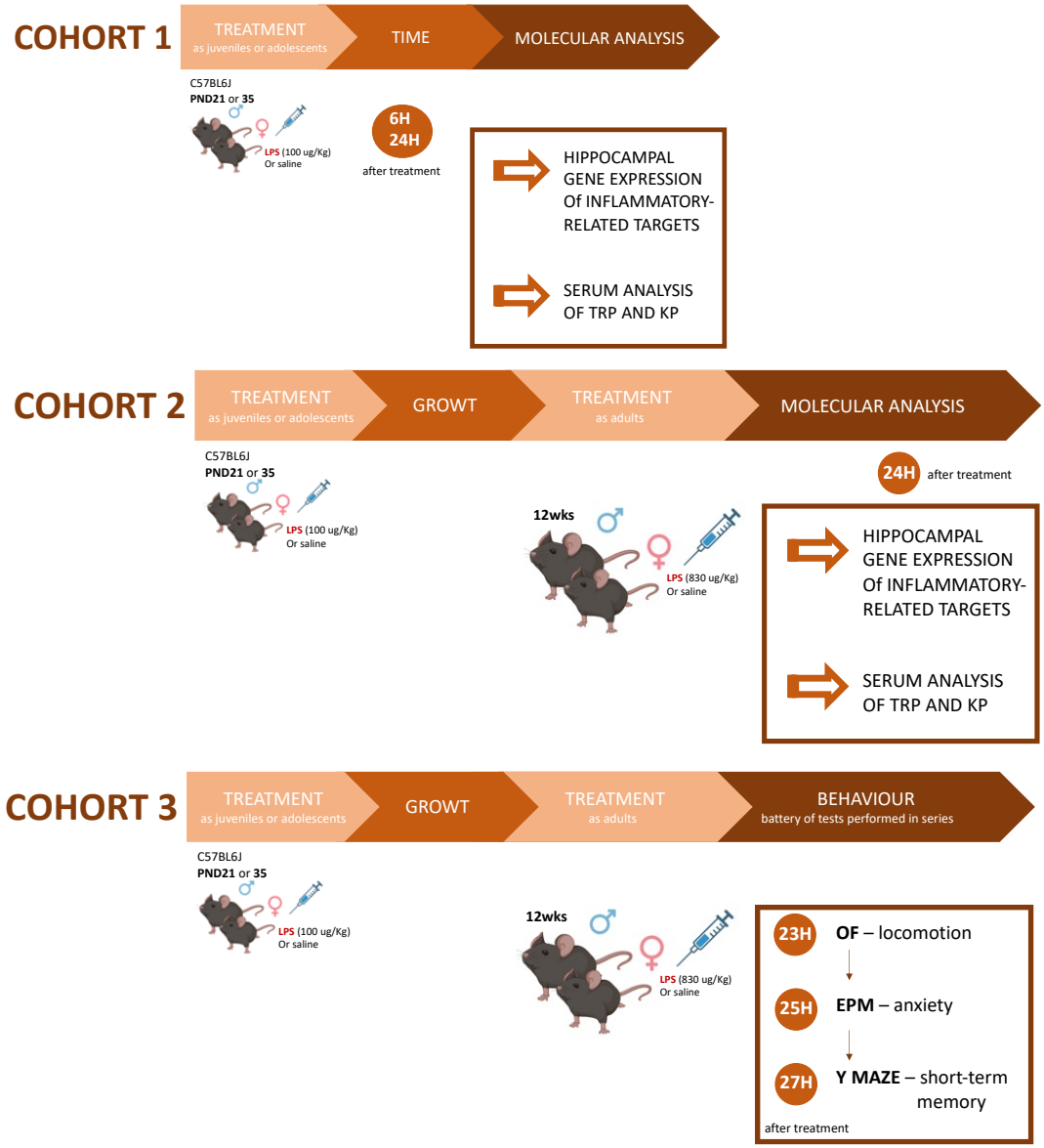

Figure 2. Treatment and experimental design. The present figure illustrates the three cohorts of animals postulated in this study: in the *Cohort 1,* male and female animals were injected i.p. with saline or treated with LPS (100 µg/Kg) at PND21 or 35 then sacrificed after 6 or 24h. Gene expression and serum analysis followed; in the *Cohort 2*, PND21 or PND35 male and female animals injected saline or LPS (100 µg/Kg) grew up and they were injected with 830 µg/Kg of LPS or saline at 12 wks of age. Animals were then sacrificed 24h after treatments. Gene expression and serum analysis followed; in the *Cohort 3,* PND21 or PND35 male and female animals injected saline or LPS (100 µg/Kg) grew up and injected with 830 µg/Kg of LPS or saline at 12 wks of age then underwent a battery of behavioural tests: OF (locomotion), EPM (anxiety-like behaviour), Y-MAZE (short-term memory).

3.3 MOLECULAR ANALYSIS

Trunk blood and brain areas were collected from animals belonging to all the 3 cohorts.

The frontal cortex, cortex, cerebellum, hypothalamus, and spleen were dissected and maintained in a 96 multi-well in dry ice immediately after sacrifice and stored at -80ºC for subsequent analysis.

3.3.1 Total RNA Extraction, Reverse transcription, Real Time PCR

Total mRNA extraction was performed on frozen hippocampi as previously described (Alboni, Benatti, et al. 2013) with Polytron (ULTRATURRAX®, Janke& Kunkel) tissue homogenizer and high RNA purification was achieved using the RNeasy® Mini kit (QIAGEN), combined with DNase digestion (RNase-Free DNase Set, QIAGEN). Following quantification with Nanodrop, 1200 ng of the total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription (Thermo Scientific) kit in a thermal cycler (SimplyAMP thermal cycler, Thermo Scientific) following these steps: 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C.

Hippocampal gene expression of inflammation and microglial markers was evaluated on 7.5 ng/µL of cDNA which was amplified using the CFX Connect™ Real-Time PCR (Bio-rad) thermocycler. The reaction mix was prepared using RNAse-free water, specifically designed primers forward (Fw) and reverse (Rv), at the final concentration of 300 nM, and the SsoAdvanced Universal SYBR-Green Supermix (Bio-Rad). Specific primers used are reported in the following table:

PRIMER	*forward*	*reverse*
CypA	AGCATACAGGTCCTGGCATC	TTCACCTTCCCAAAGACCAC
Rps29	TGAAGGCAAGATGGGTCAC	GCACATGTTCAGCCCGTATT
TNF-α	GGCCTCCCTCTCATCAGTTC	CACTTGGTGGTTTGCTACGA
IL-1β	TGAAAGCTCTCCACCTCAATG	CCAAGGCCACAGGTATTTTG
IL-6	CTTCACAAGTCGGAGGCTTA	CAAGTGCATCATCGTTGTTC
IL-4	CCAAGGTGCTTCGCATATTT	ATCGAAAAGCCCGAAAGAGT
Cx3cr1	TCAGCAGAATCGTCATACTCAAA	CGTGAGACTGGGTGAGTGACT
IDO	TGCAGACTGTGTCCTGGCAAACT	GCCCTTGTCGCAGTCCCCAC
CD14	AACTTTCAGAATCTACCGACCATG	AAGTTGCAGGAACAACTTTCCTC
CD206	CAAGGAAGGTTGGCATTTGT	CCTTTCAGTCCTTTGCAAGT
CD11b	ACGCCATCTACATGATTGTCAC	AAGACTACACTGACAGGGAGGC

Table 1. Specific primer forward and reverse and related sequences. Primers were designed through the Primer-Blast designing tool. CypA and Rps29 housekeeping were used as reference. All primers were used at the final concentration of 300 ng.

Each High-Profile 96-Well PCR Plates (Bio-Rad) well was filled with 18 µL of reaction mix and 2 µL of sample (15 ng). The plates were sealed with an adhesive protective foil centrifuged to avoid the presence of bubbles and placed in the CFX Connect™ Real-Time PCR thermocycler. The cycling parameters were: 95°C for 30 seconds, repeated 10 seconds more, and 60°C for 30 seconds. The process was repeated x 39 cycles. The dissociation curve analysis followed the amplification: from 60°C to 95°C for 5 seconds and from 65°C to 95°C for 5 seconds. The process consisted of one cycle.

Cycle threshold (Ct) values were determined by CFX MaestroTM Software (Bio-Rad).

Target's gene amount was expressed through the relative quantification which was calculated using the 2-ΔΔCt method. PND21 and 35 were analyzed separately. The calibrator chosen was the male control group: the one treated with saline and sacrificed at 6h for cohort 1 and the one subjected to the double injection with saline for cohort 2. Gene expression was normalized using two housekeeping genes as a reference, cyclophilin A (CypA), and ribosomal protein S29 (RPS29), unaltered by experimental conditions, assuming a 100% amplification efficiency. CypA, or PPIA, is known to be the most stable in rat cerebrum (Kim et al., 2014) particularly in the frontal cortex and hippocampus even after a stressor interference as a restrictive diet (Shwarz et al., 2023) or brain injury in mice (Timaru-Kast et al., 2014) as RPS29 which is widely used as a reference gene in conditions of neuroinflammation in mice and rats brain, in particular in their hippocampus (Yanguas-Yanguas-Casas et al., 2016; Yanguas-Casas et al., 2017; Csanova et al., 2017). The stability of mRNA expression of the housekeeping was assessed using Normfinder® considering intra and intergroup variations. The mean between the two genes was found to be the most stable value across groups and was the one used for the normalization.

3.3.2 Serum collection

Trunk blood was collected, and serum was separated from whole blood: firstly, samples were left to clot for 15 minutes at RT and then left for 1h in ice before centrifugation (1,000 × g for 15 min). Serum was transferred into Eppendorf and stored at -80ºC and then prepared for high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) analysis.

3.3.2.1 Chemicals, sample preparation for metabolomic analysis, HPLC-MS/MS Analysis

Tryptophan (TRP), kynurenine (KYN), serotonin (5-HT), L-Tryptophan-(indole-d5), anthranilic acid (AA), kynurenic acid (KYNA), HPLC-grade acetonitrile, and methanol were obtained from Merck Life Sciences. L-kynurenine (d4) was obtained by Buchen BV (Netherlands). Analytical grade formic acid, acetonitrile, and perchloric acid were obtained from Carlo Erba (Milan, Italy). Water was purified using a Milli-Q water purification system (Millipore, Milan, Italy).

To each 100 µL of serum was added 100 µL of perchloric acid fortified with deuterated internal standard (final concentration 1 µM). After thorough mixing, the precipitated proteins were removed by centrifugation (16,000 x g for 15 min at 4 °C), and the supernatant was collected for direct injection into the LC-MS/MS.

The analyses were performed using an Agilent HP 1200 liquid chromatograph (Agilent, Milan, Italy) consisting of a binary pump, an autosampler, and a thermostated column compartment. Chromatographic separations were carried out using a Discovery HS-F5 column (3µm particle size, 150 x 2.1 mm, Supelco, Milan, Italy) using 0.1% formic acid in water and acetonitrile (ACN) as mobile phase. The HPLC analyses were carried out using a linear elution profile of 15 min from 5% to 90% of ACN.

The column was washed with 90% ACN for 3.5 minutes, then equilibrated for 5 minutes with 5% ACN. The flow rate was 0.5 mL/min. The injection volume was 20 µL.

The HPLC-MS/MS analysis was performed by *Centro Interdipartimentale Grandi Strumenti*, CIGS. An Agilent 6410 triple quadrupole-mass spectrometer with an electrospray ion source (ESI) operating in positive mode was used for detection. The calibration curves were constructed using calibration standards and were linear over the concentration range of 0.0391–10.000 µM with a correlation coefficient (r2) of 0.999.

3.4 BEHAVIOURAL ASSESSMENT

Experiments were performed from 9:00 am to 4:00 pm during the light period. Animals belonging to Cohort 3, treated (LPS/SAL) at PND21-35 and during adulthood at 12 weeks of age, were habituated to manipulation before the beginning of the behavioural tests. All the trials were performed blindfolded in a soundproofed experimental room inside CSSI. Animal tests were registered through a video camera linked to the video tracking system ANYMaze (San Diego Instruments, http://www.anymaze.com) and analyzed offline. Each test apparatus's walls and floor were wiped with distilled water and ethanol 70% to avoid odor cues which could affect the behaviour of subsequent animals. Each animal underwent behavioural tests performed in series with 2h of spacing between each: 1) OF, 2) EPM, and 3) Y-maze. Tests began 24 hours after the adult treatment with LPS or saline. Animals were habituated to the experimental room before the beginning of each behavioural test's battery session for 1h. Male and female mice were tested on different days to avoid odour cues influencing the behaviour of the animals.

3.4.1 Open field

The open field is commonly used to evaluate motor function (McIlwain et al., 2001; Camara et al., 2015; Carton et al., 2021), spontaneous exploration and anxiety (McIlwain et al., 2001; Dinel et al., 2011). These behavioural characteristics were measured in an apparatus (40 cm × 40 cm × 30 cm) characterised by white walls and neutral grey floor as previously described (Benatti et al., 2011) with

few modifications. It was virtually divided into periphery surrounding the most illuminated central zone.

The test duration was 5 minutes (Benatti et al., 2011). Each mouse was placed individually in the centre of the apparatus. During the test, the total distance travelled and the total number of line crossings were recorded and evaluated as parameters of general locomotor activity while the total time immobile was recorded and evaluated as an index of anxiety/exploration. Moreover, some parameters were considered singularly in the central area: the per cent distance travelled in the centre ((centre distance travelled/ total distance travelled) x 100) was registered and evaluated for anxiety/exploration while the number of line crossings was considered as a locomotor parameter.

3.4.2 Elevated Plus Maze

The Elevated plus maze is widely used to test anxiety-like behaviour. No test can directly measure anxiety but it is possible to quantify alterations in animals' behaviour as an index of becoming anxious (Wahlsten et al., 2011; Ramos, 2008).

Our EPM apparatus consisted of two open and two closed arms (75 cm long, 5 cm wide, enclosed by a wall of 25 cm high) assembled to create a plus shape with a little central square as the intersection between them as previously described with few modifications (Benatti et al., 2011). Closed arms walls and the apparatus floor were grey neutral coloured.

Each trial duration was 5-min (Benatti et al., 2011). At the beginning of the test, each mouse was placed individually in the centre facing an open arm, the same for all the animals. The general parameters measured to study anxiety-like behaviour or exploration were the per cent time spent in the open or closed arms ((open or closed arms time/ total time) x 100) and the per cent distance travelled in the open or closed arms ((open or closed arms distance/ total distance) x 100) and per cent mean visits in both areas were recorded to evaluate locomotor activity. Moreover, in the open arms the per cent number of entries ((number of entries in the open arms/total number of entries)x100), was recorded as an additional parameter to assess anxiety and exploration.

3.4.3 Y Maze

The Y maze is a test characterised by an apparatus composed of three arms shaped to form a “Y” with a triangle as a centre. It is a greatly used test to study memory, in particular, spatial memory recognition (Dellu et al., 1992; Camara et al., 2015), learning (Wahlsten, 2011) and it makes it possible to evaluate the explorative behaviour considering the innate preference of mice for novelty

in an unfamiliar environment (Camara et al., 2015). The memory task is assessed by closing one of the arms during a first trial of variable duration. After an inter-trial interval (ITI), in the second trial, the arm, called also “novel” arm (NA) is opened and the animal is free to explore all the arms. The new arm will be the one in which the animal will spend most of the time and, by comparing the exploration of the three arms, it is possible to understand if the mouse can distinguish between novelty and familiarity under particular experimental conditions.

The apparatus used for our experiments, characterised by black walls and a neutral grey floor, was made of three arms named A, B, and C (22x7x20 cm each), arranged to create a “Y”. The central zone was a triangle: the intersection between the three arms. The apparatus had been equipped with a system for arms closure with a sliding door. The arm chosen to be closed was changed for each test session, after apparatus wiping, to avoid errors due to odour cues presence. Cues of different forms and colours were placed on the walls of the room to allow the animal's orientation during trials (Labrousse et al., 2009).

Each test session was made of two trials: the first was called “acclimatisation” and the second was called “test”, separated by an ITI of 30 minutes (Dellu et al., 2000; Dinel et al., 2011; Camara et al., 2015). The acclimatation duration was 5 minutes. During this phase, one arm was closed. At the beginning of the trial, the animal was placed individually in the centre facing the arm on the left with respect to the one closed. Thus, the mouse was free to explore just two arms and the centre. After the ITI passed in its cage with mates, during the “test” the animal was free to explore all the arms for 5 minutes. Again, each mouse started from the centre in front of the same arm faced during acclimatation. The parameters analysed for the stage “test” were the per cent distance travelled in the novel arm and the familiar arms ((distance travelled in the novel or familiar arms/ total distance travelled)x100) and the per cent number of entries in the novel arm and the familiar arms ((n° of entries in the novel or familiar arms/ total n° of entries)x100) and per cent number of alternations ((n°of entries in the arm A or B or C/total number of entries)x100), as parameters to assess the memory performance and the distance travelled to the first entry in the NA and latency, considered as indices of anxiety.

3.5 STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS software version 28 (IBM Corp., Armonk, NY, United States) and GraphPad Prism version 9.0 (GraphPad Software Inc., La Jolla, CA, USA). Extreme outliers were excluded before statistical analysis using the boxplot tool in SPSS (more than 3x the

interquartile range outside of the end of the interquartile box). Juveniles (PND21) / juvenile-adults (PND21-12wks) and adolescents (PND35) / adolescent-adults (PND35-12wks) were analysed separately. In cohort 1, weights and molecular data were analysed with three-way ANOVA for main effects of treatment (LPS/SAL), time (6h/24h), sex or interaction between the factors (treatment*sex*time) followed by Bonferroni's correction. In Cohort 2, weights were analysed with repeated measure ANOVA and molecular data with three-way ANOVA for main effects of adolescent/adult treatment (SAL/SAL or LPS, LPS/SAL or LPS), sex or interaction between the factors (adolescent treatment*adult treatment *sex) all followed by Bonferroni's correction. In Cohort 3, behavioural data were analysed with a two-tailed paired t-test or three-way ANOVA for main effects of adolescent/adult treatment, sex or interaction between these factors followed by Bonferroni's correction. Statistical significance was considered for p values <0.05.

4.0 RESULTS

4.1 COHORT 1

In Cohort 1 we evaluated whether the inflammatory response induced by the stressor LPS (100 µg/Kg) was different in male or female mice at two different critical time windows of development: juvenile (PND21) and adolescent (PND35) stages of life.

Six and 24 hours after receiving either saline or LPS animals were sacrificed and we evaluated:

1) hippocampal gene expression of pro-inflammatory and anti-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-4), indoleamine 2,3-dioxygenase (IDO) enzyme, microglial markers (CD11b, CD14, CD206) and fractalkine receptor (Cx3cr1);
2) TRP, 5-HT and KP metabolite KYN concentrations and KYNA/KYN, AA/KYN ratios in serum.

4.1.1.1 Effect of an immune challenge on the body weight of male and female PND21 mice

To assess the effect of the immune challenge on body weight, animals were weighed before, 6h or 24h after treatment. The animal's weight was significantly decreased 24h after the immune challenge at PND21.

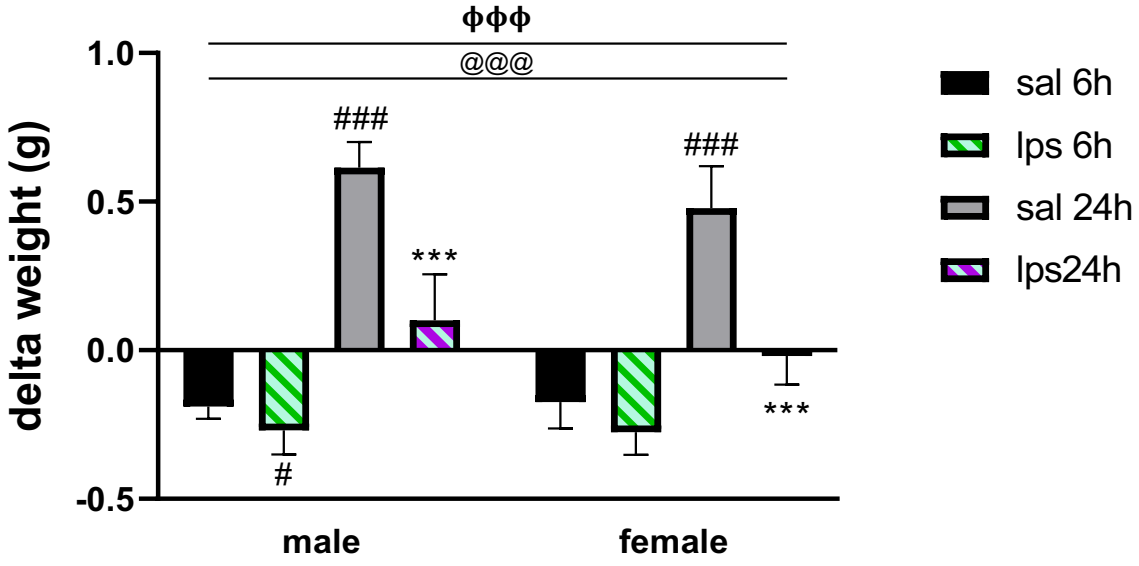

Figure 3. Effect of exposure to LPS on body weight of PND21 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-10 per group) and then sacrificed after 6 or 24h. Data were expressed as mean variation in body weight (g) and ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **@@@** = main effect of treatment p<0.001, **ϕϕϕ** = main effect of time after treatment p<0.001. *Post hoc*: ******* = vs respective saline p<0.001, **###** = vs time p<0.001, **#** = vs time p=0.011.

The three-way ANOVA revealed the main effects of treatment ($F(1,69)=16.488$, $p<0.001$), time after treatment ($F(1,69)=50.152$, $p<0.001$) and of the interaction of treatment and time after treatment ($F(1,69)=7.997$, $p=0.006$) on PND21 weight.

Following Bonferroni pairwise comparisons, we observed that male and female PND21 mice receiving either saline or LPS had a similar weight 6 hours after treatment. At the 24-hour time point animals injected with saline displayed a significant weight increase which was not present in LPS-treated male ($p=0.001$) and female ($p<0.001$) mice.

4.1.1.2.1 Effect of an immune challenge on the expression levels of inflammation-related targets in the hippocampus of male and female PND21 mice

When considering the main pro- and anti-inflammatory cytokines evaluated, TNF-α and IL-1β mRNA levels were significantly upregulated following the immune challenge in the hippocampus of mice at PND21, while no effects were present for both IL-6 and IL-4.

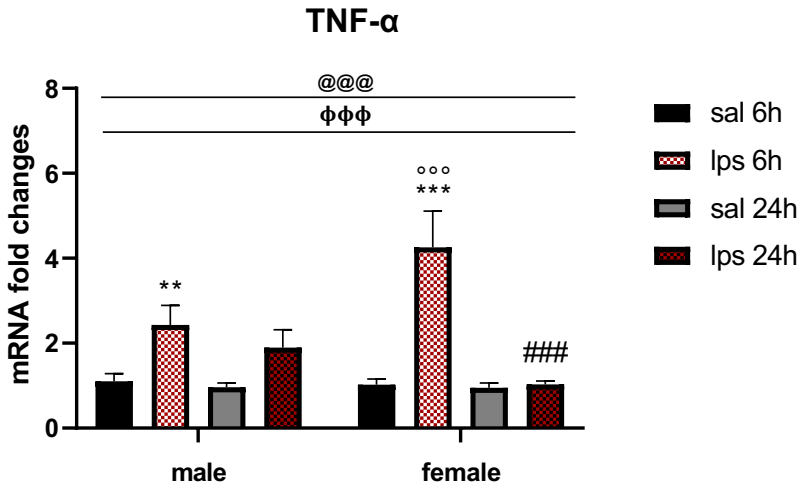

Figure 4. Effect of exposure to LPS on the hippocampal mRNA levels of TNF-α of PND21 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate TNF-α expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **@@@** = main effect of treatment $p<0.001$, **ϕϕϕ** = main effect of time after treatment $p<0.001$. *Post hoc*: ******* = vs respective saline $p<0.001$, ****** = vs respective saline $p=0.01$, **°°°** = vs sex $p<0.001$, **###** = vs time $p<0.001$).

Three-way ANOVA revealed for TNF-α hippocampal expression a main effect of treatment ($F(1;58)=25.976$; $p<0.001$), time after treatment ($F(1;58)=17.670$, $p<0.001$) and significant interactions between treatment and time after treatment ($F(1;58)=14.230$, $p<0.001$), sex and time after treatment ($F(1;58)=4.973$, $p=0.030$), and sex*treatment*time after treatment ($F(1;58)=5.556$; $p=0.022$).

Bonferroni *post hoc* analysis demonstrated a significant upregulation of TNF-α mRNA in the hippocampus 6h after LPS treatment in both male (p=0.01) and female animals (p<0.001). The target returned to control levels in animals sacrificed 24h after the immune challenge but its decrease was statistically significant from LPS-receiving counterparts and sacrificed after 6 hours, only in females (p<0.001). We found a significant difference in the expression levels of this target between sexes: females treated with LPS seemed to be more sensitive to the immune challenge than males at the earlier time point, in fact, 6 hours after treatment the hippocampal levels of TNF-α mRNA were nearly twice as high with respect to their matching male counterparts (p<0.001).

However, in males, the decrease of TNF-α expression 24h after LPS was less marked than what was observed in female animals. Hippocampal levels of this cytokine in males sacrificed 24h after the immune challenge remained higher than their matching saline counterparts, without reaching statistical significance.

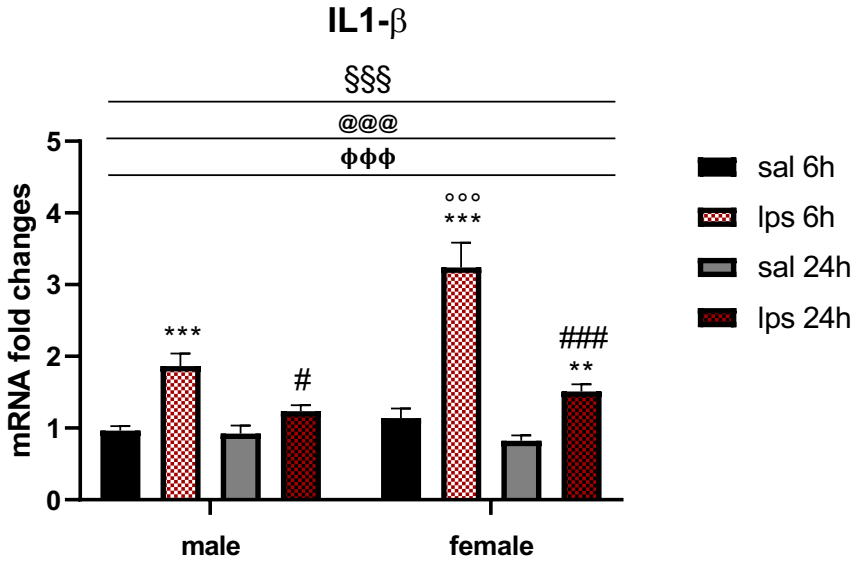

Figure 5. Effect of exposure to LPS on the hippocampal mRNA levels of IL-1β of PND21 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate IL-1β expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§§** = main effect of sex p<0.001, **@@@** = main effect of treatment p<0.001, **ϕϕϕ** = main effect of time after treatment p<0.001. *Post hoc*: ******* = vs respective saline p<0.001, ****** = vs respective saline p=0.004, **°°°** = vs sex p<0.001, **###** = vs time p<0.001, **#** = vs time p=0.011.

When considering IL-1β gene expression in the hippocampus, three-way ANOVA revealed main effects of time after treatment ($F(1,59)=32.24$, $p<0.001$), treatment ($F(1,59)=69.848$, $p<0.001$), sex ($F(1,59)=12.942$, $p<0.001$) and the interactions between treatment and time after treatment ($F(1,59)=17.417$, $p<0.001$), sex and time after treatment ($F(1,59)=8.203$, $p=0.006$), and sex and treatment ($F(1,59)=10.810$, $p=0.002$).

Bonferroni's *post hoc* analysis showed a significant upregulation of IL-1β mRNA 6h after LPS treatment in both male and female animals ($p<0.001$). As for TNF-α, a significant difference in the hippocampal expression of this target was present between sexes: in females, 6h after the exposure to LPS, the expression levels of IL-1β were significantly higher with respect to the matching male animals ($p<0.001$). In both males and females, 24h after the immune challenge IL-1β expression was significantly decreased with respect to their matching counterparts and sacrificed 6 hours later ($p=0.011$ and $p<0.001$ respectively). However, LPS-mediated upregulation of IL-1β was still present in female animals sacrificed at the 24h time point with respect to their saline-receiving counterparts ($p=0.004$).

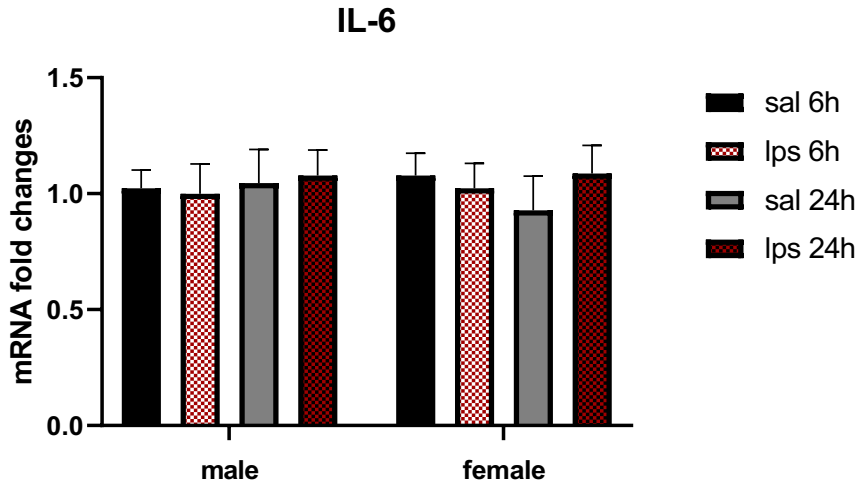

Figure 6. Effect of exposure to LPS on the hippocampal mRNA levels of IL-6 of PND21 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate IL-6 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni.

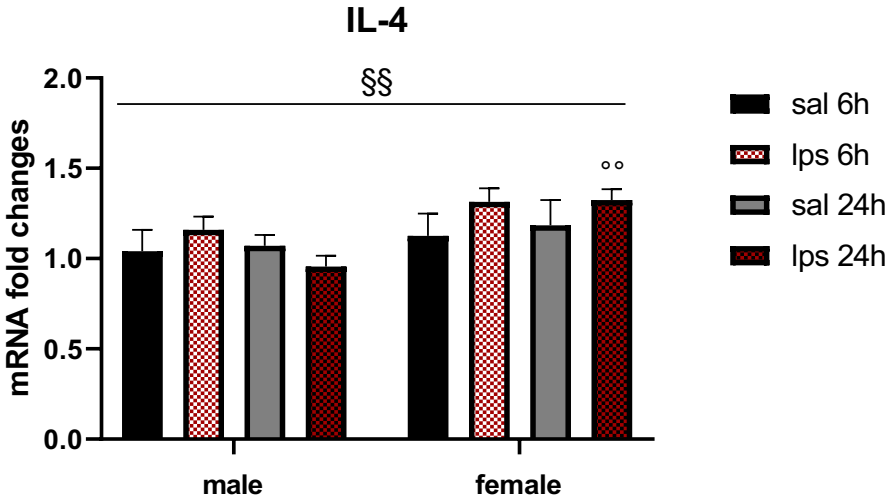

Figure 7. Effect of exposure to LPS on the hippocampal mRNA levels of IL-4 of PND21 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate IL-4 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping

genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§** = main effect of sex p=0.003. *Post hoc*: **°°** = vs sex p=0.007.

IL-6 mRNA levels were not affected in our experimental conditions while three-way ANOVA showed a main effect of sex (F(1,58)=9.435, p=0.003) for IL-4 hippocampal expression.

In PND21 animals treated with LPS and sacrificed at 6h or 24h we did not observe any significant difference with respect to their saline-controls. Following Bonferroni's *post hoc* analysis, females treated with LPS and sacrificed after 24h showed a significant increase in hippocampal IL-4 expression levels with respect to their male counterparts (p=0.007).

4.1.1.2.2 Effect of an immune challenge on the expression levels of microglial activation markers in the hippocampus of male and female PND21 mice

The expression levels of the main microglial markers evaluated, CD11b, CD14 and CD206, were significantly upregulated following the immune challenge in the hippocampus of mice at PND21, while the mRNA levels of the fractalkine receptor Cx3cr1 were not affected in PND21 mice.

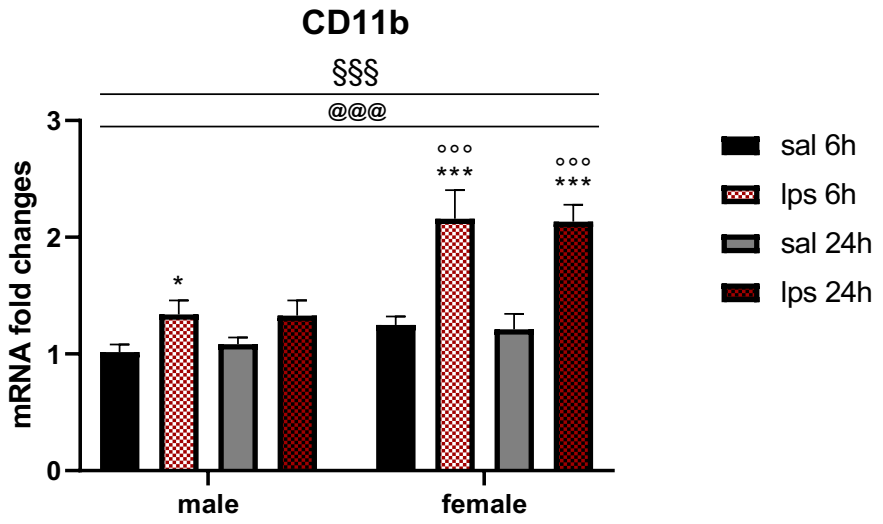

Figure 8. Effect of exposure to LPS on the hippocampal mRNA levels of CD11b of PND21 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate CD11b expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **@@@** = main effect of treatment p<0.001, **§§§** = main effect of sex p<0.001. *Post hoc*: ******* = vs respective saline p<0.001, ***** = vs respective saline p=0.046, **°°°** = vs sex p<0.001.

Three-way ANOVA revealed for CD11b mRNA levels main effects of treatment (F(1,59)=45.411, p<0.001), sex (F(1,59)=24.612, p<0.001) and the interaction between treatment and sex (F(1,59)=11.206, p=0.002).

Bonferroni *post hoc* analysis showed a significant upregulation of CD11b mRNA in the hippocampus 6h after LPS treatment in both males (p=0.046) and females (p<0.001) with respect to their saline-receiving controls. As observed previously, the immune challenge affected the expression of this target differently between sexes: exposure to LPS resulted in a higher increase of the expression levels of CD11b in females compared to male counterparts both 6 and 24 hours after treatment (p<0.001). Moreover, this upregulation was still present at the 24h time point for female animals (p<0.001), while in male mice it returned to control levels.

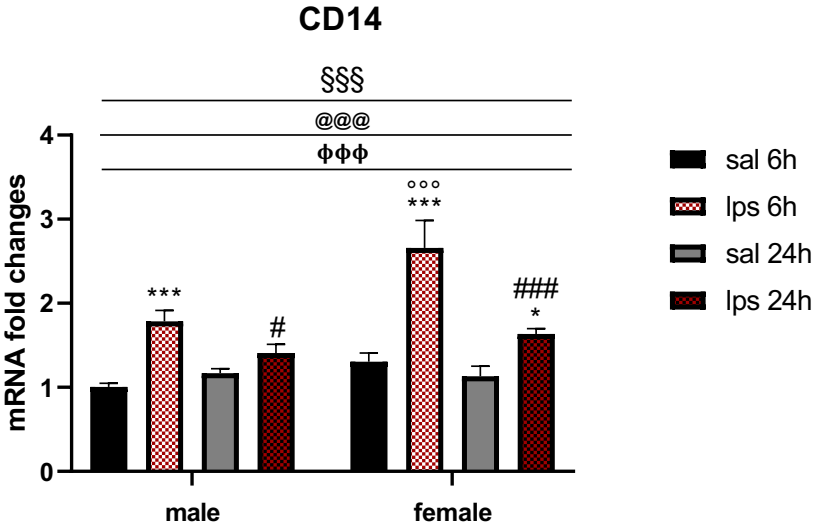

Figure 9. Effect of exposure to LPS on the hippocampal mRNA levels of CD14 of PND21 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate CD14 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **@@@** = main effect of treatment p<0.001, **§§§** = main effect of sex p<0.001, **ϕϕϕ** = main effect of time after treatment p<0.001. *Post hoc*: ******* = vs respective saline p<0.001, ***** = vs respective saline p=0.016, **°°°** = vs sex p<0.001, ### = vs time p<0.001, # = vs time p=0.03.

When analysing CD14 mRNA hippocampal expression, three-way ANOVA revealed a main effect of sex (F(1,60)=13.523, p<0.001), time after treatment (F(1,60)=14.428, p<0.001), treatment (F(1,60)=43.299, p<0.001) and the following interactions: treatment*time after treatment (F(1,60)=11.707, p=0.001), sex*time after treatment (F(1,60)=5.440, p=0.024) and sex*treatment (F(1,60)=4.369, p=0.041).

Bonferroni's pairwise comparisons demonstrated an upregulation of CD14 mRNA in the hippocampus 6h after LPS treatment in male and female animals compared to their saline-receiving controls (p<0.001). The immune challenge differentially affected the hippocampal expression of this target in the two sexes: 6h after the receiving LPS, both males (p=0.03) and females (p<0.001) displayed significantly increased mRNA levels of CD14 compared to the groups sacrificed at the 24h

time point, but female animals showed higher expression levels of this target when compared to their male counterparts (p<0.001). Twenty-four hours after LPS, CD14 expression levels were not significantly different from controls in males, while in females the upregulation was still present with respect to their saline-receiving counterparts (p=0.016).

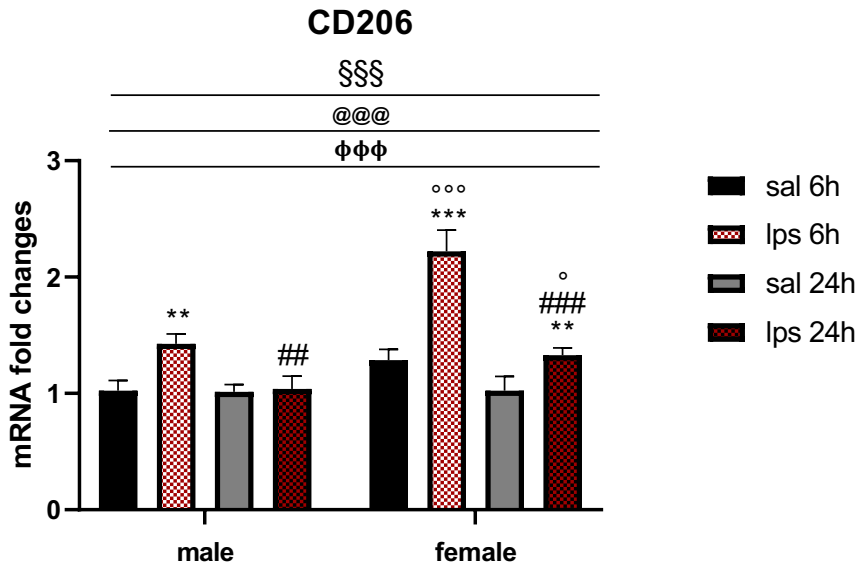

Figure 10. Effect of exposure to LPS on the hippocampal mRNA levels of CD206 of PND21 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate CD206 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **@@@** = main effect of treatment p<0.001, **§§§** = main effect of sex p<0.001, **ϕϕϕ** = main effect of time after treatment p<0.001. *Post hoc*: ******* = vs respective saline p<0.001, ****** = vs respective saline p=0.007, ****** = vs respective saline p=0.006, **°°°** = vs sex p<0.001, **°** = vs sex p=0.047, ### = vs time p<0.001, ## = vs time p=0.009.

Considering CD206 mRNA expression, three-way ANOVA showed a main effect of sex (F(1,61)=18.454, p<0.001), time after treatment (F(1,61)=33.225, p<0.001), treatment (F(1,61)=37.857, p<0.001) and by their interaction: treatment*time after treatment (F(1,61)=9.542, p=0.003), sex*time after treatment (F(1,61)=9.068, p=0.004) and sex*treatment (F(1,61)=10.193, p=0.002).

CD206 mRNA pattern in our experimental conditions was similar to what was observed for CD11b expression. Bonferroni *post hoc* analysis demonstrated a significant upregulation of CD206 mRNA in the hippocampus 6h after LPS treatment in male (p=0.007) and female (p<0.001) animals with respect to their saline-receiving controls. Females showed significantly higher induction of the target 6h after the exposure to LPS with respect to the group with matching treatment sacrificed after 24h (p<0.001) and to their male counterparts (p<0.001). At the 24h time point, the expression

of this target in LPS-treated males was lower compared to animals sacrificed 6h after the immune challenge (p=0.009). In female animals, the LPS-induced upregulation was still present compared to saline-receiving controls (p=0.006) after 24h, and to their corresponding male counterparts (p=0.047).

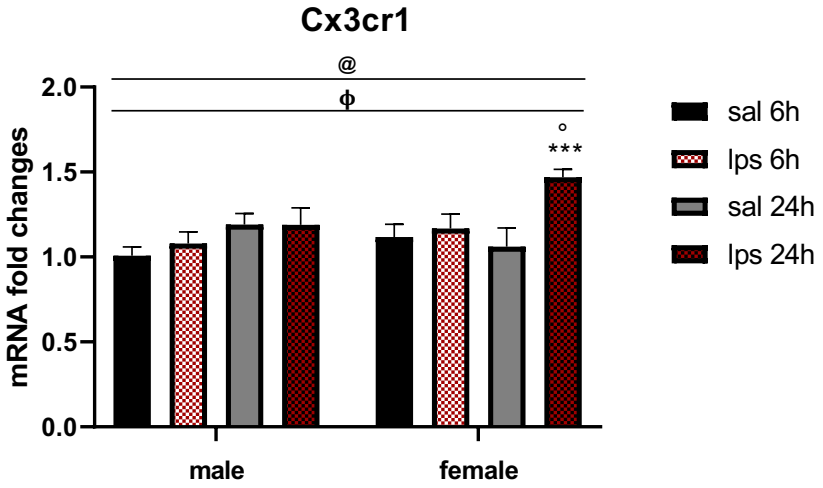

Figure 11. Effect of exposure to LPS on the hippocampal mRNA levels of Cx3cr1 of PND21 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate Cx3cr1 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **@** = main effect of treatment p=0.022, **ϕ** = main effect of time after treatment p=0.019. *Post hoc*: ******* = vs respective saline p<0.001, **°** = vs sex p=0.013.

Three-way ANOVA revealed main effects of time after treatment (F(1,61)=5.879, p=0.019) and treatment (F(1,61)=5.587, p=0.022) for Cx3cr1 mRNA hippocampal levels.

Following Bonferroni's pairwise comparison Cx3cr1 mRNA levels were not affected by LPS, 6h after treatment in both males and females when compared to their respective saline-receiving counterparts. At the 24h time point, only in females, the mRNA levels of this target were significantly increased following LPS exposure with respect to their control group (p<0.001). Moreover, the mRNA expression in this group was significantly higher with respect to their male counterparts (p=0.013).

4.1.1.2.3 Effect of an immune challenge on the expression levels of kynurenine pathway limiting enzyme, indoleamine 2,3-dioxygenase, in the hippocampus of male and female PND21 mice

Hippocampal IDO mRNA expression was significantly induced after LPS injection only in female animals.

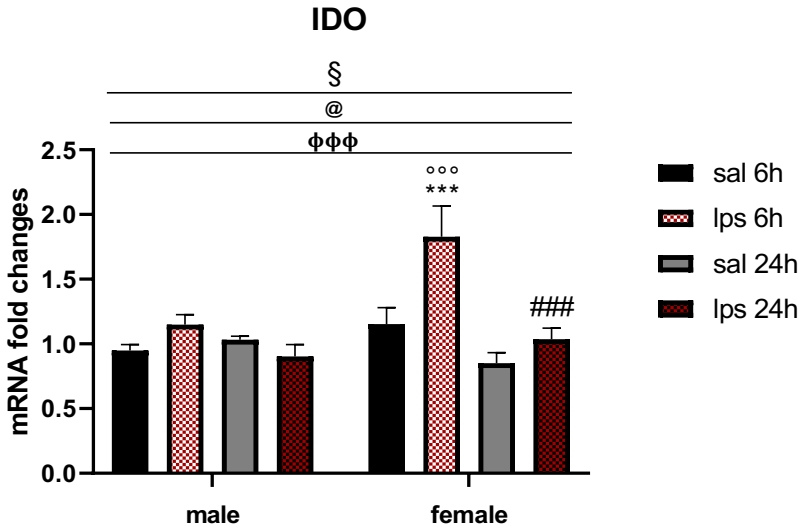

Figure 12. Effect of exposure to LPS on the hippocampal mRNA levels of IDO of PND21 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate IDO expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **@** = main effect of treatment p=0.013, **§** = main effect of sex p=0.026, **ϕϕϕ** = main effect of time after treatment p<0.001. *Post hoc*: ******* = vs respective saline p<0.001, **°°°** = vs sex p<0.001, ### = vs time p<0.001.

When analyzing IDO expression, three-way ANOVA revealed main effects of time after treatment ($F(1,62)=15.717$, $p<0.001$), treatment ($F(1,62)=6.658$, $p=0.13$), sex ($F(1,62)=5.271$, $p=0.26$) and the interactions of treatment*time after treatment ($F(1,62)=5.043$, $p=0.029$), sex*time after treatment ($F(1,62)=6.708$, $p=0.12$), and sex*treatment ($F(1,62)=6.592$, $p=0.13$).

Following Bonferroni's pairwise comparisons no difference in IDO expression was found between male animals injected with LPS and sacrificed after 6 or 24h. A significant induction of IDO mRNA was observed in females 6h after the immune challenge when compared to their corresponding control group ($p<0.001$), to their matching male counterparts ($p<0.001$), and female animals sacrificed 24h after LPS ($p<0.001$).

4.1.1.3 Effect of an immune challenge on the serum levels of KP metabolites in male and female PND21 mice

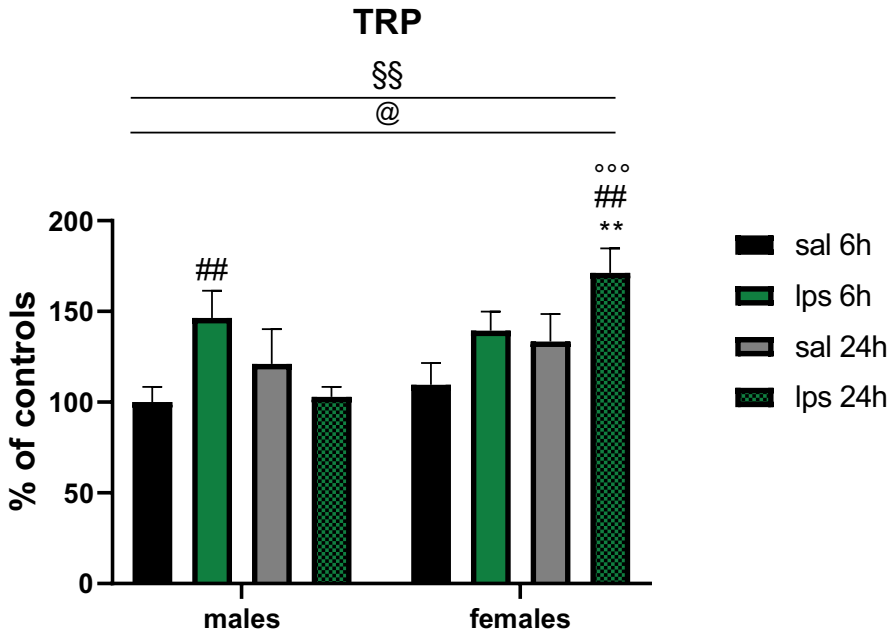

Figure 13. Effect of the treatment with LPS at PND21 on serum TRP concentration in mice: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21 and then sacrificed after 6 or 24h (n=7-8 per group). HPLC-MS/MS was performed to evaluate TRP metabolite levels. Concentrations (µM) were expressed as a percent of controls (male sal sal) and as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§** = main effect of sex p=0.006, **@** = main effect of treatment p=0.042. *Post hoc*: ****** = vs respective saline p=0.008, **°°°** = vs sex p<0.001, **##** = vs time p=0.003, **##** = vs time p=0.008.

Three-way ANOVA revealed the main effects of sex ($F(1,60)=8.300$, $p=0.006$), treatment ($F(1,60)=4.326$, $p=0.042$) and the interactions of sex and treatment ($F(1,60)=5.484$, $p=0.023$), sex and time after treatment ($F(1,60)=13.832$, $p<0.001$) and sex*treatment*time after treatment ($F(1,60)=4.412$, $p=0.04$) for TRP levels in mice serum.

Bonferroni *post hoc* analysis demonstrated that TRP levels were significantly increased 24h after the immune challenge in female serum with respect to the corresponding saline-control group ($p=0.008$), matching male counterparts ($p<0.001$) and also the 6h time point ($p=0.003$). TRP concentration was higher in males 6h after the injection with respect to animals LPS-treated and sacrificed after 24h ($p=0.008$).

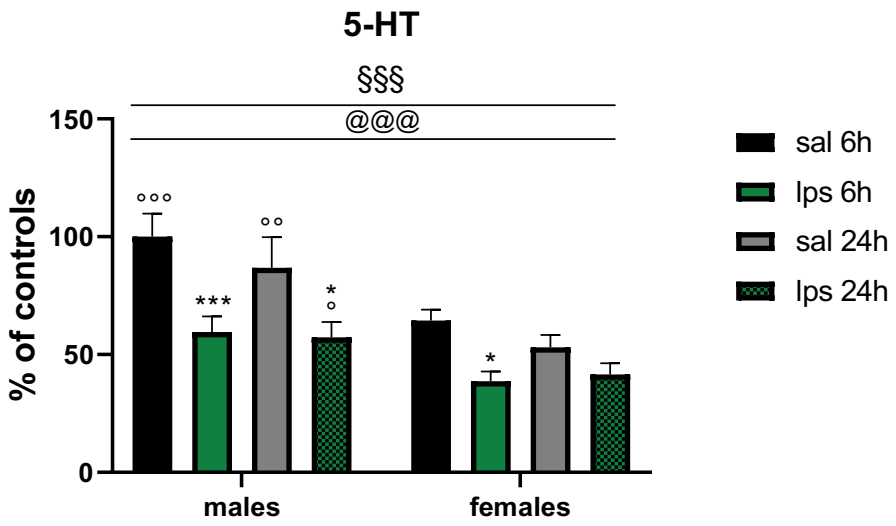

Figure 14. Effect of the treatment with LPS at PND21 on 5-HT concentration in mice serum: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21 and then sacrificed after 6 or 24h (n=7-8 per group). HPLC-MS/MS was performed to evaluate 5-HT metabolite levels. Concentrations (µM) were expressed as a percent of controls (male sal sal) and as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§§** = main effect of sex p<0.001, **@@@** = main effect of treatment p<0.001. *Post hoc*: ******* = vs respective saline p<0.001, ***** = vs respective saline p=0.03, ***** = vs respective saline p=0.015, **°°°** = vs sex p<0.001, **°°** = vs sex p=0.002, **°** = vs sex p=0.041.

Three-way ANOVA revealed the main effects of sex ($F(1,61)=28.847$, $p<0.001$) and treatment ($F(1,61)=23.476$, $p<0.001$) for 5-HT presence in mice serum. Bonferroni's pairwise comparisons showed downregulation of 5-HT levels in males ($p<0.001$) and females ($p=0.015$), with respect to their control groups, 6h after the immune challenge. The effect was present also at 24h time point but only in males ($p=0.03$). Males showed higher serum concentrations of serotonin with respect to females when they were sacrificed both 6 ($p<0.001$) and 24h ($p=0.02$) after saline injection and 24h after they received LPS ($p=0.041$).

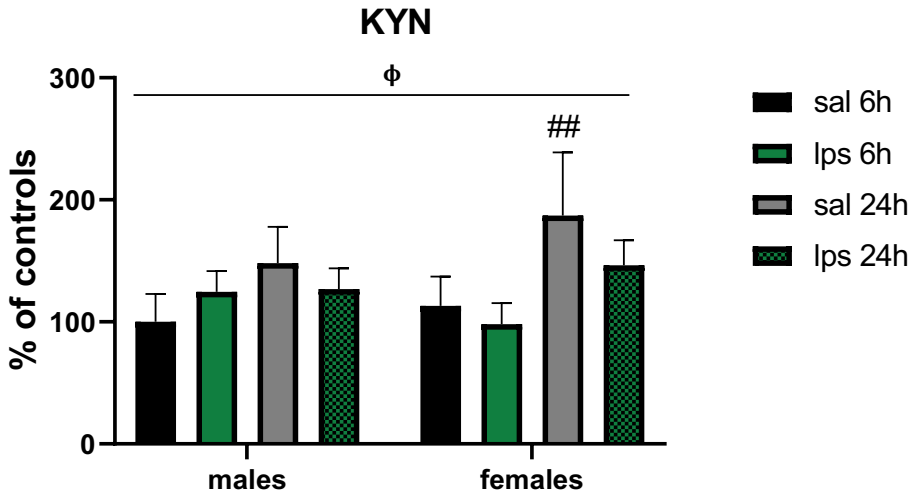

Figure 15. Effect of the treatment with LPS at PND21 on KYN concentration in mice serum: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21 and then sacrificed after 6 or 24h (n=7-8 per group). HPLC-MS/MS was performed to evaluate KYN metabolite levels. Concentrations (µM) were expressed as a percent of controls (male sal sal) and as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **ϕ** = main effect of time after treatment p=0.024. *Post hoc*: **#** = vs time p=0.035.

When analyzing the first active metabolite of KP, KYN, three-way ANOVA revealed the main effect of time after treatment ($F(1,60)=5.377$, $p=0.024$). Bonferroni *post hoc* showed a higher concentration of KYN in females 24h after receiving saline with respect to the counterpart sacrificed after 6h ($p=0.035$) while no differences were observed in males. LPS failed to affect the KYN serum levels of the animals in our experimental conditions.

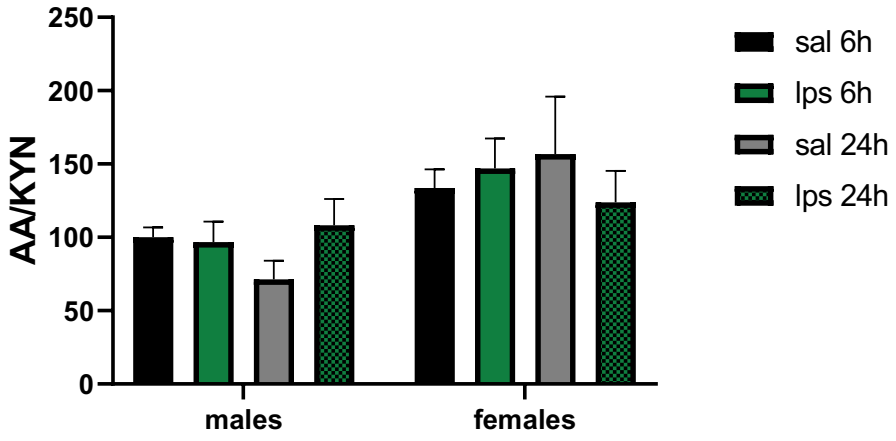

Figure 16. Effect of the treatment with LPS at PND21 on AA/KYN ratio in mice serum: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21 and then sacrificed after 6 or 24h (n=7-8 per group). HPLC-MS/MS was performed to evaluate the AA/KYN ratio. The ratio was expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni.

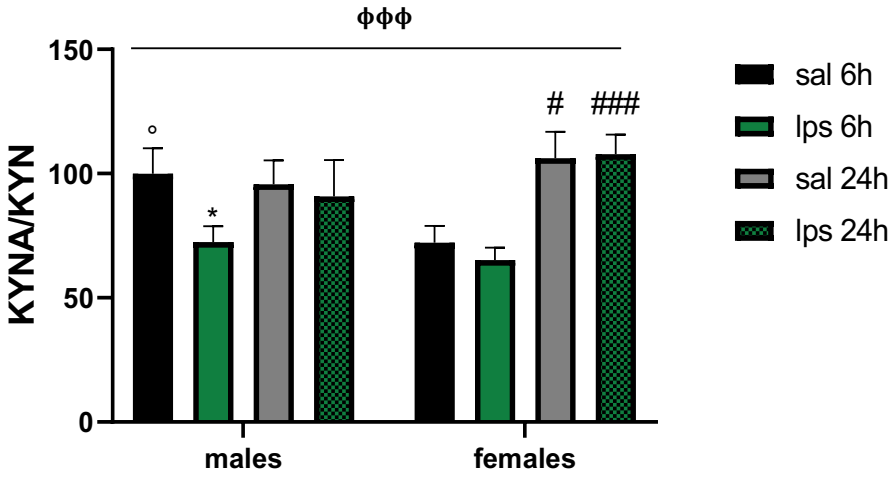

Figure 17. Effect of the treatment with LPS at PND21 on KYNA/KYN ratio in mice serum: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21 and then sacrificed after 6 or 24h (n=7-8 per group). HPLC-MS/MS was performed to evaluate the KYNA/KYN ratio. The ratio was expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **ϕϕϕ** = main effect of time after treatment p=0.001. *Post hoc*: ***** = vs respective saline p=0.048, **°** = vs sex p=0.046, **###** = vs time p=0.001, **#** = vs time p=0.014.

The AA/KYN ratio was not affected in our experimental conditions, whereas, for KYNA/KYN ratio, three-way ANOVA demonstrated a main effect of time after treatment ($F(1,59)=11.713$, $p=0.001$) and the interaction of sex and time after treatment ($F(1,59)=5.644$, $p=0.021$).

While Bonferroni's pairwise comparisons showed a lower KYNA/KYN ratio in males 6h after the immune challenge with respect to their controls ($p=0.048$), 24 hours after the immune challenge, KYNA/KYN ratio was higher in females irrespective of the treatment received if compared to their respective female saline ($p=0.014$) or LPS ($p=0.001$) -receiving counterpart then sacrificed after 6h. Moreover, saline-exposed males sacrificed after 6h showed higher levels of this ratio with respect to matching female counterparts ($p=0.046$).

4.1.2.1 Effect of an immune challenge on the body weight of male and female PND35 mice.

The animal's weight profile significantly decreased between 6h and 24h after the immune challenge induced by LPS at PND35.

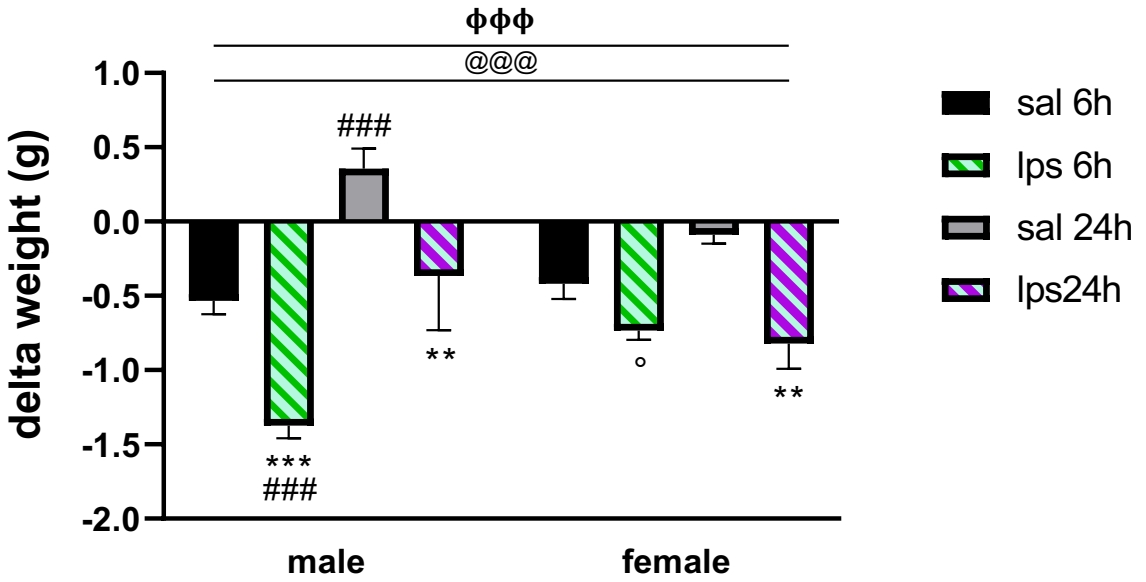

Figure 18. Effect of exposure to LPS on body weight of PND35 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-10 per group) and then sacrificed after 6 or 24h. Data were expressed as mean variation in body weight (g) and ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **@@@** = main effect of treatment p<0.001, **ϕϕϕ** = main effect of time after treatment p<0.001. *Post hoc*: ******* = vs respective saline p<0.001, ****** = vs respective saline p=0.003, **°** = vs sex p=0.011, **###** = vs time p<0.001.

The three-way ANOVA highlighted the main effects of treatment ($F(1,69)=30.229$, $p<0.001$), time after treatment ($F(1,69)=19.209$, $p<0.001$) and sex*time after treatment ($F(1,69)=12.322$, $p<0.001$) on PND35 weight profile.

Bonferroni *post hoc* pairwise comparison showed a significant decrease in weight of LPS-treated males ($p<0.01$) compared to both their saline counterparts, and to their LPS-receiving counterparts sacrificed after 24 hours ($p<0.001$). The decrease in weight observed in males was significantly higher than what was observed in the matching females ($p=0.011$), in this group exposure to the immune challenge caused a slight decrease in the weight that failed to reach statistical significance. In females, 24h following LPS exposure the animals still weighed significantly less than their matching saline control group ($p=0.003$).

4.1.2.2.1 Effect of an immune challenge on the expression levels of inflammation-related targets in the hippocampus of male and female PND35 mice

Similarly to what was observed for PND21 mice, in PND35 animals TNF-α and IL-1β mRNA levels were significantly upregulated following the immune challenge in the hippocampus of mice, while no effects were revealed for both IL-6 and IL-4.

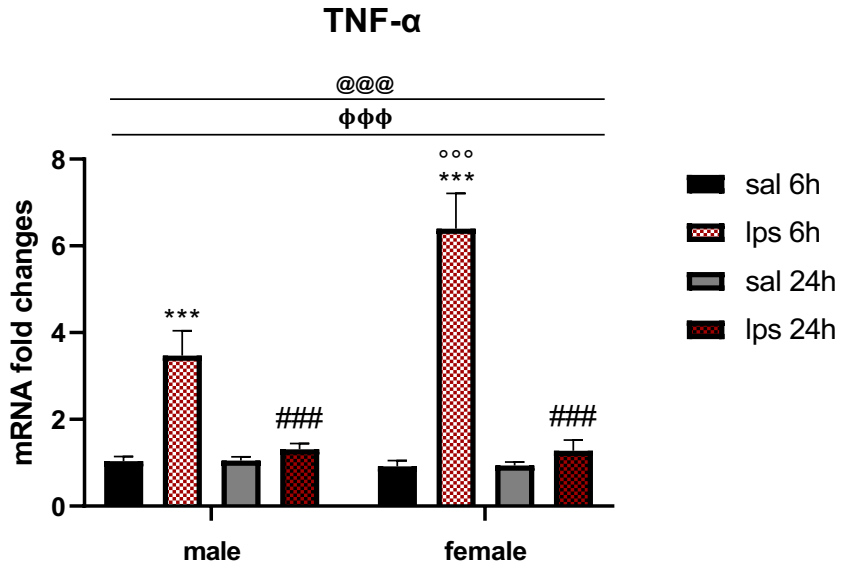

Figure 19. Effect of exposure to LPS on the hippocampal mRNA levels of TNF-α of PND35 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate TNF-α expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **@@@** = main effect of treatment p<0.001, **ϕϕϕ** = main effect of time after treatment p<0.001. *Post hoc*: ******* = vs respective saline p<0.001, **°°°** = vs sex p<0.001, **###** = vs time p≤0.001.

Three-way ANOVA revealed for TNF-α mRNA levels main effects of time after treatment ($F(1,62)=31.336$, $p<0.001$), treatment ($F(1,62)=44.496$, $p<0.001$), and the interactions between treatment and time after treatment ($F(1,62)= 31.993$, $p<0.001$) and sex*treatment ($F(1,62)=4.495$, $p=0.039$). Bonferroni *post hoc* analysis showed a significant upregulation of TNF-α mRNA levels 6h after the immune challenge in males and females ($p<0.001$). The induction following LPS was significantly higher in females 6h after the treatment compared to their male counterparts ($p<0.001$). TNF-α expression returned to control levels at the 24-hour time point both in females ($p<0.001$) and also in males ($p=0.001$).

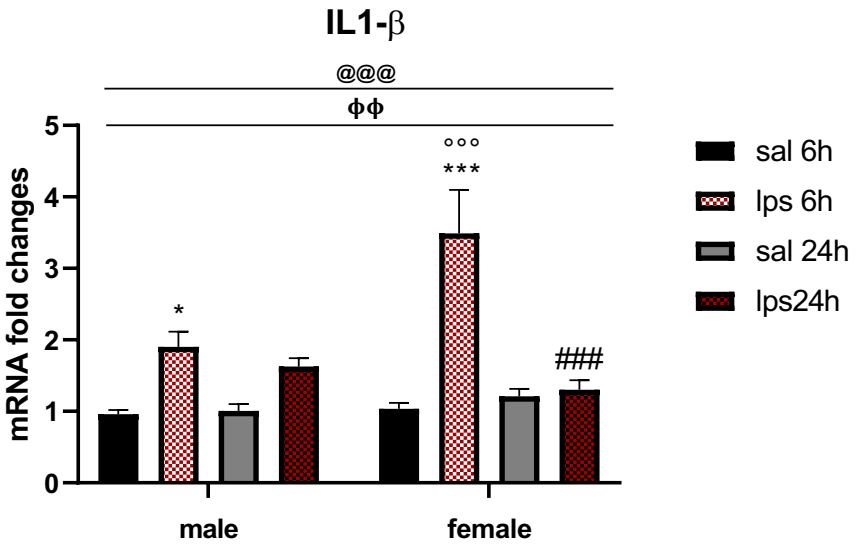

Figure 20. Effect of exposure to LPS on the hippocampal mRNA levels of IL-1β of PND35 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate IL-1β expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **@@@** = main effect of treatment p<0.001, **ϕϕ** = main effect of time after treatment p=0.008. *Post hoc*: ******* = vs respective saline p<0.001, ***** = vs respective saline p=0.011, **°°°** = vs sex p<0.001, **###** = vs time p<0.001.

When analyzing IL-1β gene expression levels in the hippocampus three-way ANOVA revealed a main effect of time after treatment ($F(1,62)=7.718$, $p=0.008$), treatment ($F(1,62)=29.578$, $p<0.001$), and the interactions between treatment and time after treatment ($F(1,62)= 11.647$, $p=0.001$), sex and time after treatment ($F(1,62)= 4.560$, $p=0.037$), and sex*treatment*time after treatment ($F(1,62)=6.209$, $p=0.016$).

Bonferroni pairwise comparisons revealed that IL-1β mRNA expression levels were significantly upregulated 6h after LPS treatment in males ($p=0.011$) and in females ($p<0.001$). LPS-induced upregulation of IL-1β hippocampal expression was significantly higher in females 6h after the immune challenge compared to their matching male counterparts ($p<0.001$). This effect returned to control-saline levels in animals sacrificed 24h after the immune challenge, this decrease was statistically significant only in females with respect to the matching female group sacrificed at a 6h time point ($p<0.001$).

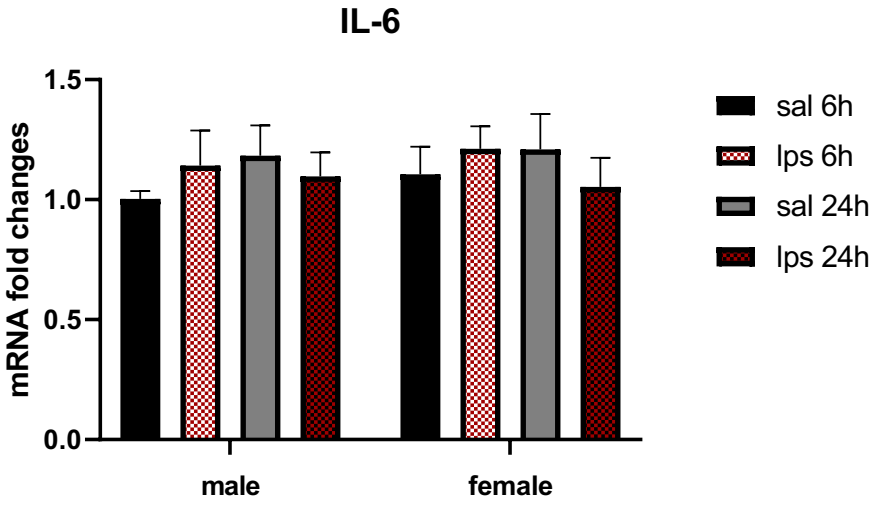

Figure 21. Effect of exposure to LPS on the hippocampal mRNA levels of IL-6 of PND35 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate IL-6 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni.

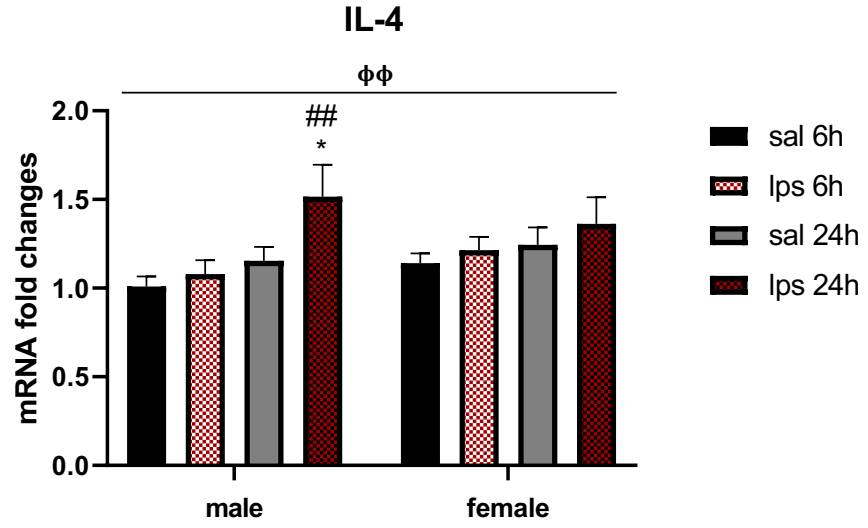

Figure 22. Effect of exposure to LPS on the hippocampal mRNA levels of IL-4 of PND35 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate IL-4 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **ϕϕ** = main effect of time after treatment p=0.007. *Post hoc*: ***** = vs respective saline p=0.012, **##** = vs time p=0.003.

As observed in PND21 animals, the three-way ANOVA showed that the IL-6 expression levels were not affected in our experimental conditions while for IL4 mRNA expression we found a significant main effect of time after treatment ($F(1,61)=7.728$, $p=0.007$).

Bonferroni *post hoc* pairwise comparisons demonstrated a significant increase in the levels of IL-4 mRNA in males treated with LPS and sacrificed after 24h ($p=0.012$) compared to their saline-receiving controls. This increase was significantly higher even when compared to the animals treated with LPS and sacrificed after 6h ($p=0.003$). No significant effects were found in female animals.

4.1.2.2.2 Effect of an immune challenge on the expression levels of microglial activation markers in the hippocampus of male and female PND35 mice

When considering the main microglial markers, in PND35 animals CD11b, CD14, and CD206 mRNA levels were significantly upregulated following the immune challenge in the hippocampus of mice, while no effects were revealed for Cx3cr1, similarly to what was observed in PND21 animals.

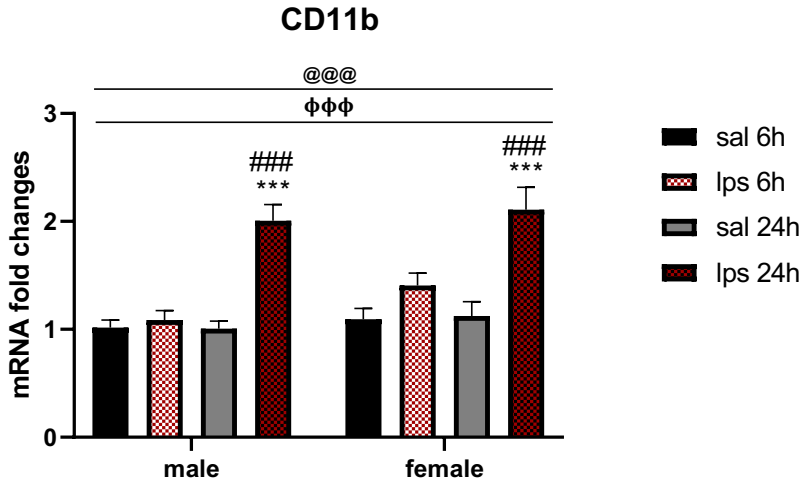

Figure 23. Effect of exposure to LPS on the hippocampal mRNA levels of CD11b of PND35 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate CD11b expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **@@@** = main effect of treatment $p<0.001$, **ϕϕϕ** = main effect of time after treatment $p<0.001$. *Post hoc*: ******* = vs respective saline $p<0.001$, **###** = vs time $p<0.001$.

Three-way ANOVA revealed significant main effects of treatment ($F(1,60)=59.370$, $p<0.001$), time after treatment ($F(1,60)=20.419$, $p<0.001$), and the interaction between treatment and time after treatment ($F(1,60)=25.999$, $p<0.0001$) for CD11b mRNA hippocampal expression levels.

Bonferroni's pairwise analysis showed no difference between LPS-treated animals sacrificed after 6h and their controls. Twenty-four hours after treatment, CD11b mRNA expression was significantly

upregulated in the hippocampus of both male and female animals injected with LPS with respect to their saline-receiving control groups (p<0.001) and to their counterparts sacrificed after 6h (p<0.001).

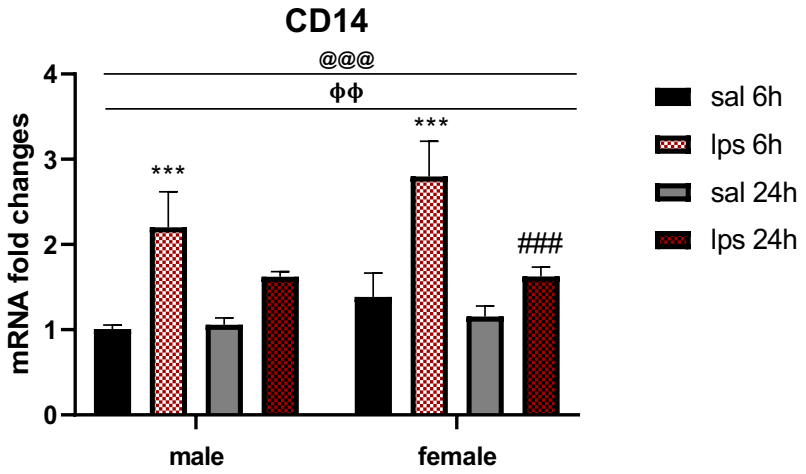

Figure 24. Effect of exposure to LPS on the hippocampal mRNA levels of CD14 of PND35 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate CD14 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **@@@** = main effect of treatment p<0.001, **ϕϕ** = main effect of time after treatment p=0.006. *Post hoc*: ******* = vs respective saline p<0.001, **###** = vs time p=0.001.

Considering CD14 mRNA hippocampal levels, three-way ANOVA revealed the main effects of time after treatment (F(1,63)=8.062, p=0.006), treatment (F(1,63)=28.604, p<0.001) and their interaction (F(1,63)=5.338, p=0.025).As for proinflammatory cytokines, Bonferroni pairwise comparison demonstrated that CD14 mRNA was significantly upregulated 6h after LPS treatment in both male and female animals with respect to their respective saline controls (p<0.001). The levels of the target returned to control levels at the 24h time point in both sexes. In female animals, CD14 expression was significantly higher in animals sacrificed 6h after LPS with respect to those sacrificed after 24h (p=0.001). This difference was present also in males, but it failed to reach statistical significance.

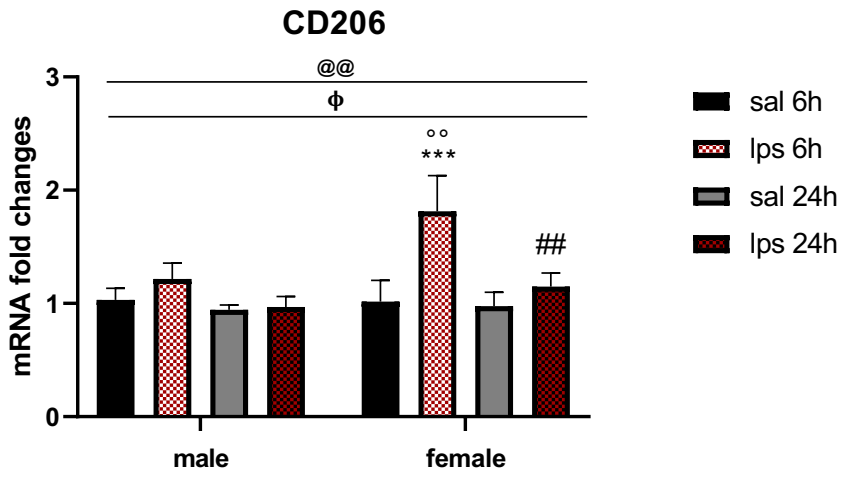

Figure 25. Effect of exposure to LPS on the hippocampal mRNA levels of CD206 of PND35 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate CD206 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **@@** = main effect of treatment p=0.007, **ϕ** = main effect of time after treatment p=0.042. *Post hoc*: ******* = vs respective saline p<0.001, **°°** = vs sex p=0.01, **##** = vs time p=0.004.

For CD206 mRNA expression, main effects of time after treatment (F(1,62)=4.338, p=0.042) and treatment (F(1,62)=7.887, p=0.007) were found by three-way ANOVA.

No effect on CD206 mRNA was reported in males in our experimental conditions, while Bonferroni's pairwise comparisons showed that LPS induced a significant increase in CD206 expression in female animals 6h after treatment (p<0.001) with respect to their saline-receiving control group and their matching-male counterparts (p=0.01). Females showed also significantly higher levels of the target 6h after the LPS with respect to the 24h time point (p=0.004).

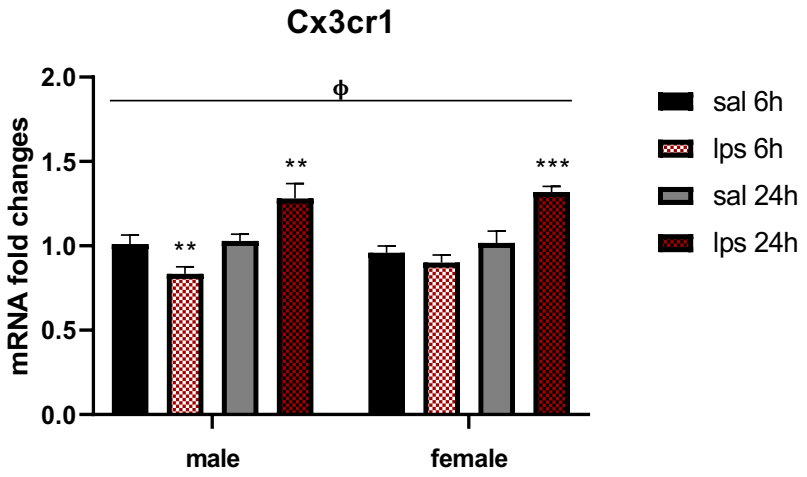

Figure 26. Effect of exposure to LPS on the hippocampal mRNA levels of Cx3cr1 of PND35 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate Cx3cr1 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **ϕ** = main effect of time after treatment p=0.042. *Post hoc*: ****** = vs respective saline p=0.01, ****** = vs respective saline p=0.002, ******* = vs respective saline p<0.001.

Three-way ANOVA analysis revealed the main effect of time after treatment ($F(1,57)=38.562$, $p<0.001$) and the interaction between treatment and time after treatment ($F(1,57)=27.669$, $p<0.001$) for Cx3cr1 mRNA hippocampal expression in adolescent mice.

Bonferroni *post hoc* analysis demonstrated that 6h after the treatment with LPS Cx3cr1 expression was downregulated in males compared to their saline-receiving controls (p=0.01), while no effect was present in female animals. The mRNA levels of this target increased in 24h groups injected with LPS in both males (p=0.002) and females (p<0.001) with respect to their saline-receiving controls.

4.1.2.2.3 Effect of an immune challenge on the expression levels of the kynurenine pathway limiting enzyme, indoleamine 2,3-dioxygenase, in the hippocampus of male and female PND35 mice

In PND35 animals IDO mRNA levels were significantly upregulated following the immune challenge in the hippocampus of both male and female animals.

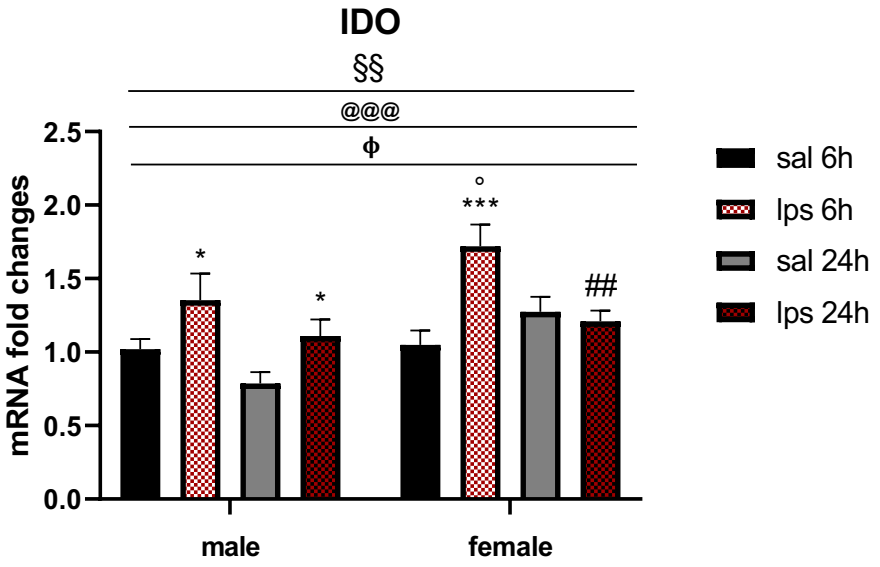

Figure 27. Effect of exposure to LPS on the hippocampal mRNA levels of IDO of PND35 mice sacrificed after 6 or 24h: Male and female mice were injected with LPS (100 µg/kg) or saline (n=7-8 per group) and then sacrificed after 6 or 24h. Real-time PCR was performed to evaluate IDO expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: §§ = main effect of sex p=0.003, **@@@** = main effect of treatment p<0.001, **ϕ** = main effect of time after treatment p=0.019. *Post hoc*: ******* = vs respective saline p<0.001, ***** = vs respective saline p=0.039, ***** = vs respective saline p=0.046, **°** = vs sex p=0.028, **###** = vs time p=0.002.

For IDO hippocampal mRNA expression, the three-way ANOVA showed significant main effects of time after treatment ($F(1,59)=5.817$, $p=0.019$), treatment ($F(1,59)=16.054$, $p<0.001$) and sex ($F(1,59)=9.705$, $p=0.003$) and the significant interactions of treatment*time after treatment ($F(1,59)=5.622$, $p=0.021$) and sex*treatment*time after treatment ($F(1,59)=5.289$, $p=0.026$). Bonferroni *post hoc* showed a significant upregulation of IDO mRNA in males ($p=0.039$) and females ($p<0.001$) 6h after the LPS with respect to their saline-control group. While the expression of this target returned to control levels in females, in males IDO mRNA expression remained significantly higher also 24h after LPS with respect to their respective control group ($p=0.046$). Moreover, IDO mRNA levels were differentially regulated between sexes: at the 6-hour time point, the effect of LPS was significantly higher in females with respect to the matching male counterparts ($p=0.028$) and with respect to females LPS-treated and sacrificed after 24h ($p=0.002$).

4.1.2.3 Effect of an immune challenge on serum levels of KP metabolites in male and female PND35 mice

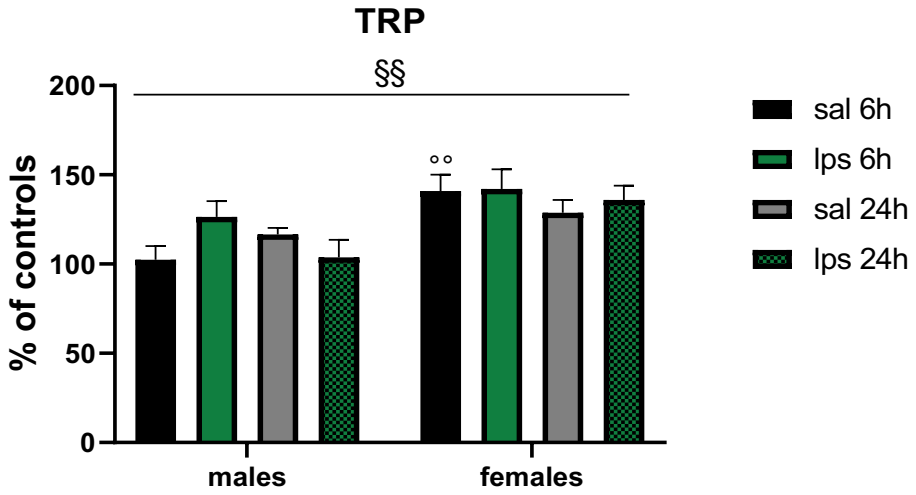

Figure 28. Effect of the treatment with LPS at PND35 on TRP concentration in mice serum: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35 and then sacrificed after 6 or 24h (n=7-8 per group). HPLC-MS/MS was performed to evaluate TRP metabolites levels. Concentrations (µM) were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§** = main effect of sex p=0.008. *Post hoc*: **°°** = vs sex p=0.003.

Three-way ANOVA showed a main effect of sex (F(1,61)=7.555, p=0.008). Following Bonferroni *post hoc* analysis, no differences induced by the treatment were demonstrated for TRP concentration. In fact, following the immune challenge, we did not observe any significant difference in animals sacrificed after 6h or 24h compared to their saline-matching controls. Females injected with saline showed significantly higher serum levels of tryptophan with respect to males matching counterparts at the 6h time-point.

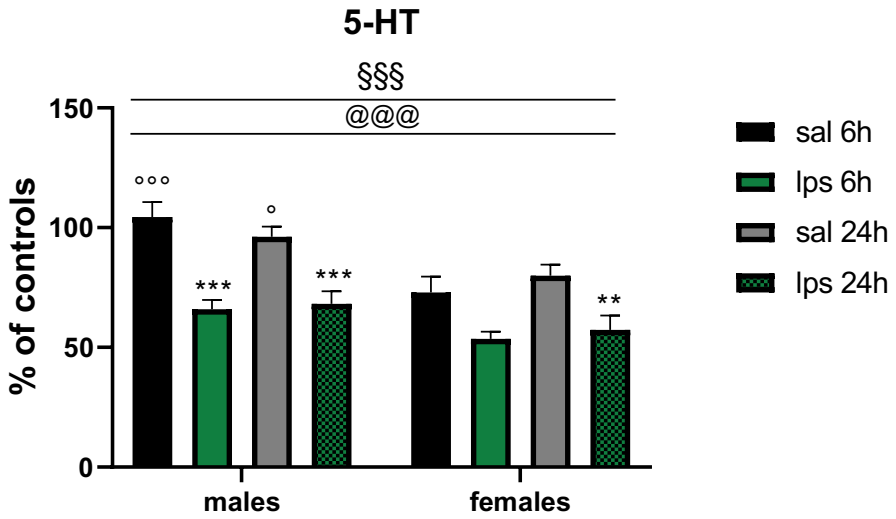

Figure 29. Effect of the treatment with LPS at PND35 on 5-HT concentration in mice serum: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35 and then sacrificed after 6 or 24h (n=7-8 per group). HPLC-MS/MS was performed to evaluate 5-HT metabolites levels. Concentrations (µM) were expressed as mean ± SEM (standard error of the mean). Statistical analysis was

performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§§** = main effect of sex p<0.001, **@@@** = main effect of treatment p<0.001. *Post hoc*: **°°°** = vs sex p<0.001, **°** = vs sex p=0.013, ******* = vs respective control p<0.001, ****** = vs respective control p =0.007.

When analyzing serum concentration of 5-HT, three-way ANOVA showed the main effects of sex (1,63)=23.303, p<0.001) and treatment (F(1,63)=42.431, p<0.001). Bonferroni's pairwise comparisons revealed higher levels of 5-HT in saline-receiving males with respect to females both at 6h (p<0.001) and 24h (p=0.013) as observed in PND21 animals. A significant decrease in the concentration of serotonin was demonstrated 6 and 24h after the immune challenge induced by LPS in males (p<0.001) while in females this effect was similar, but it reached statistical significance only after 24h (p=0.007).

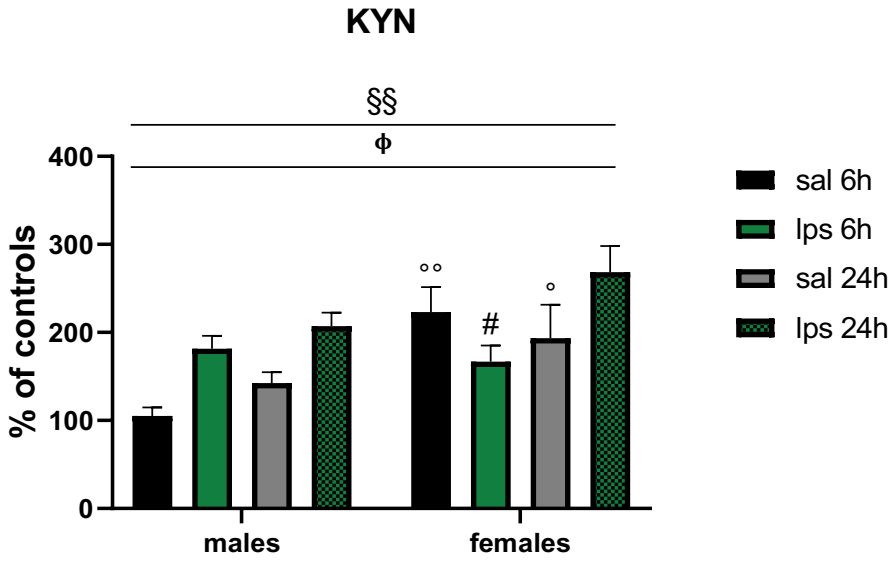

Figure 30. Effect of the treatment with LPS at PND35 on KYN concentration in mice serum: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35 and then sacrificed after 6 or 24h (n=7-8 per group). HPLC-MS/MS was performed to evaluate KYN metabolite levels. Concentrations (µM) were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§** = main effect of sex p=0.003, **ϕ** = main effect of time after treatment p=0.046. *Post hoc*: **°°** = vs sex p=0.01, **°** = vs sex p=0.021, **#** = vs time p =0.012.

The main effects of sex (F(1,61)=9.536, p=0.003) and time (F(1,61)=4.158, p=0.046) were observed for KYN concentration in the serum of mice following three-way ANOVA. Bonferroni *post hoc* analysis showed increased levels of KYN metabolite in the serum of females 6h (p=0.01) and 24h (p=0.021) after the injection with saline if compared to males in the same conditions. Following LPS, kynurenine serum levels were significantly lower in females sacrificed 6 hours after treatment with respect to their matching 24h counterparts (p=0.012).

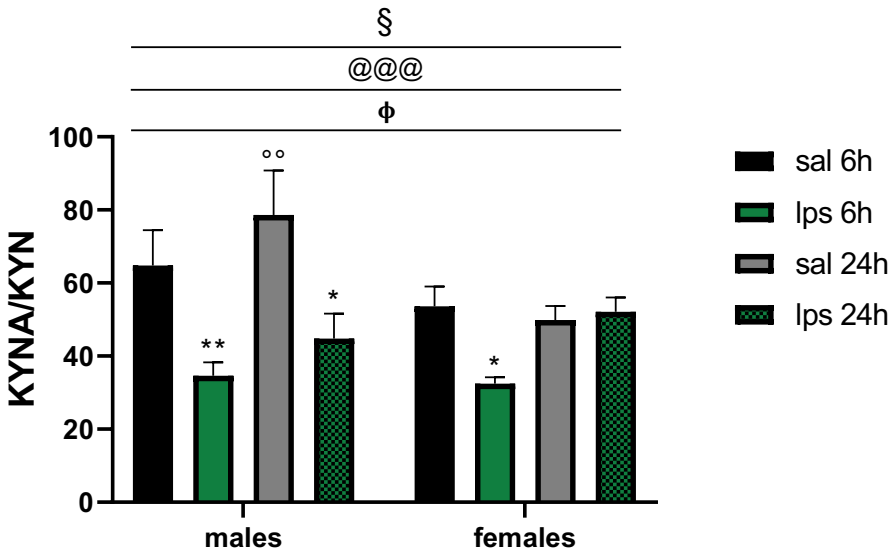

Figure 31. Effect of the treatment with LPS at PND35 on KYNA/KYN ratio in mice serum: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35 and then sacrificed after 6 or 24h (n=7-8 per group). HPLC-MS/MS was performed to evaluate the KYNA/KYN ratio. The ratio was expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§** = main effect of sex p=0.045, **@@@** = main effect of treatment p<0.001, **ϕ** = main effect of time after treatment p=0.023. *Post hoc*: **°°** = vs sex p=0.006, ****** = vs respective control p=0.005, ***** = vs respective control p=0.012, ***** = vs respective control p=0.043.

Three-way ANOVA revealed the main effects of sex ($F(1,63)=4.222$, $p=0.045$), treatment ($F(1,63)=13.681$, $p<0.001$) and time after treatment ($F(1,63)=5.468$, $p=0.023$). Bonferroni *post hoc* analysis demonstrated that the immune challenge decreased the KYNA/KYN ratio in males after both 6h ($p=0.005$) and 24h ($p=0.012$). The effect was present also in females but only at a 6h time point ($p=0.043$). Higher values of this ratio were observed in the male group injected with saline with respect to female counterparts at 24h time point ($p=0.006$).

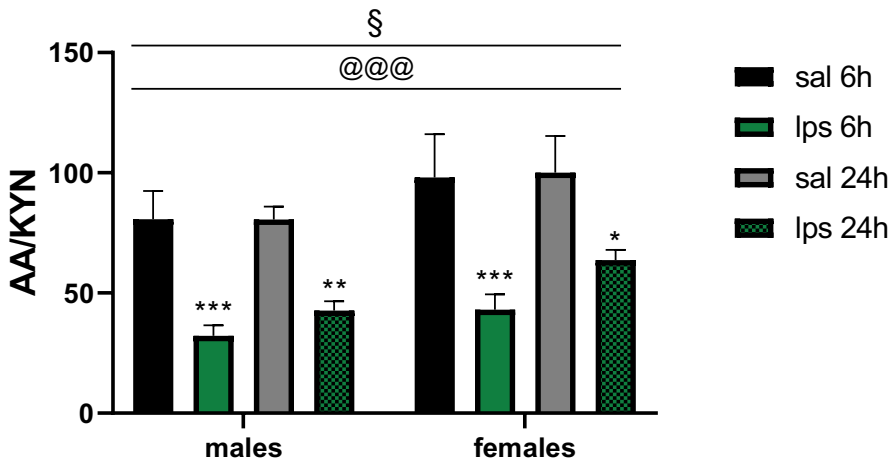

Figure 32. Effect of the treatment with LPS at PND35 on AA/KYN ratio in mice serum: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35 and then sacrificed after 6 or 24h (n=7-8 per group). HPLC-MS/MS was performed to evaluate the AA/KYN ratio. The ratio was expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§** = main effect of sex p=0.02, **@@@** = main effect treatment p<0.001. *Post hoc*: ******* = vs respective saline p≤0.001, ****** = vs respective saline p=0.01, ***** = vs respective saline p=0.017.

Three-way ANOVA revealed the main effects of sex ($F(1,63)=5.777$, $p=0.02$) and treatment ($F(1,63)=38.029$, $p<0.001$). Similarly to the KYNA/KYN ratio, Bonferroni *post hoc* demonstrated an LPS-dependent decrease of the AA/KYN ratio in males and females serum both 6 ($p<0.001$) and 24h ($p<0.05$) after the immune challenge.

4.2 COHORT 2

In Cohort 2 we evaluated the impact of an immune challenge (LPS (100 μg/Kg)) in juvenile (PND21) or adolescent (PND35) male or female mice on the molecular response to a second inflammatory hit (LPS, 830 μg/Kg) experienced later during adulthood (12 weeks).

Twenty-four hours after receiving either saline or LPS at 12 weeks of age, animals were sacrificed and we analyzed:

1) hippocampal gene expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), indoleamine 2,3-dioxygenase (IDO), microglial markers (CD11b, CD14, CD206) and fractalkine receptor (Cx3cr1);
2) TRP, 5-HT, and KP metabolite KYN concentrations and KYNA/KYN, AA/KYN ratios in serum.

4.2.1.1 Effect of an immune challenge on the body weight of male and female mice exposed to LPS or saline at PND21.

The weight of each animal was recorded each week starting from PND21 up to 12 wks of age. Male and female animals increased steadily their weight irrespective of the treatment received at PND21.

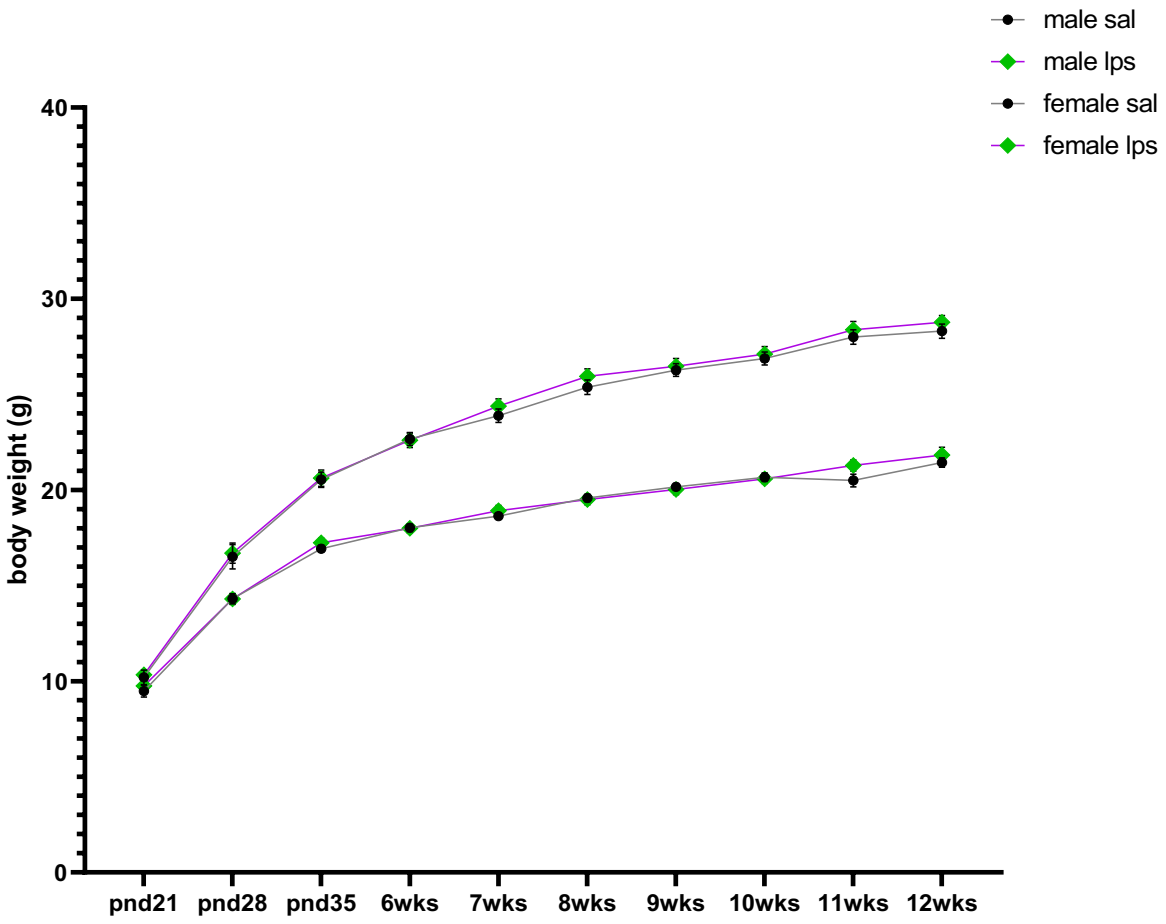

Figure 33. Weight of male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21 (n=14-20 per group). The body weight (g) was recorded each week starting from PND21 to 12wks. Data were expressed as mean (g) ± SEM (standard error of the mean). Statistical analysis was performed by repeated measure ANOVA (sex*juvenile treatment*adult treatment) followed by Bonferroni.

The body weight of the mice was comparable before the treatment at PND21. Repeated measures ANOVA revealed an interaction between the weight increase and the biological sex ($F(9;58)=36.436$, $p<0,0001$). As expected, the weight gain was higher in males with respect to females starting from PND28 to 12 wks of age. The treatment received at PND21 did not affect the weight trend in both male and female mice.

Following the immune challenge as an adult, the weight significantly decreased in mice exposed to LPS irrespective of the treatment received at PND21.

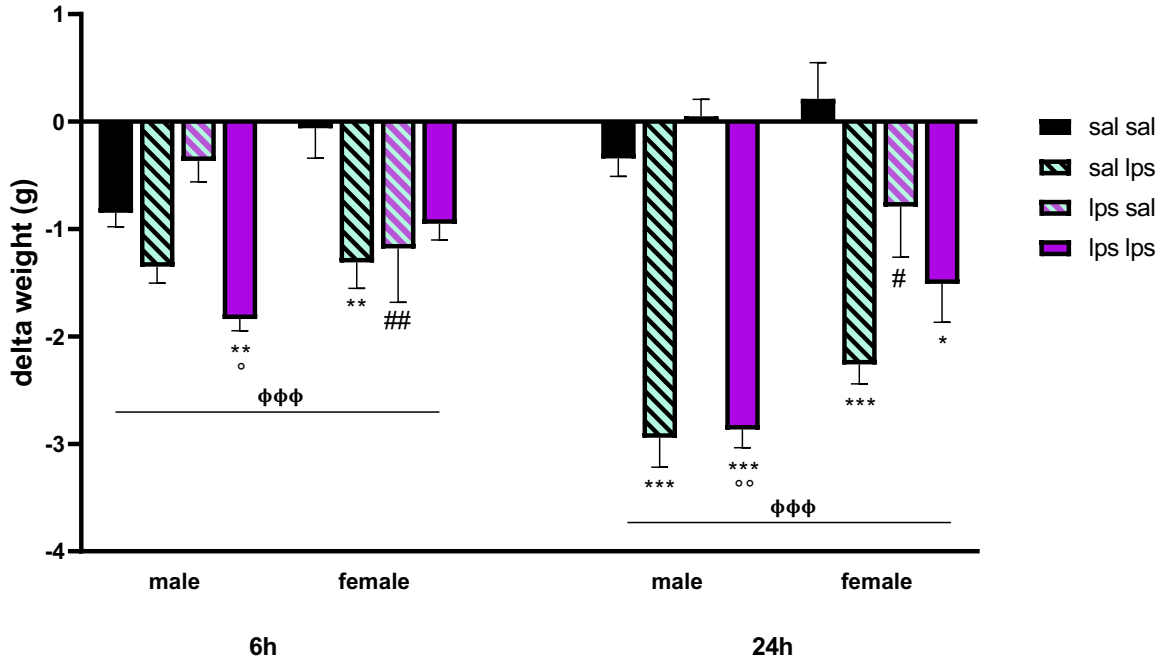

Figure 34. Effects of lipopolysaccharide (LPS) on body weight in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age. Animals' weight was checked 6 or 24h after the treatment and then mice were sacrificed after 24h (n=7-10 per group). Data were expressed as mean variation in body weight (g) and ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: **##** = vs juvenile saline p=0.006, **#** = vs juvenile saline p=0.029, ******* = vs adult saline p<0.001, ****** = vs adult saline p=0.002, ****** = vs adult saline p=0.003, ***** = vs adult saline p=0.048, **°°** = vs sex p=0.006, **°** = vs sex p=0.034.

Six hours after the treatment, the three-way ANOVA revealed the main effects of the adult treatment ($F(1,60)=12.317$, $p<0.001$) and the interaction between sex* juvenile treatment*adult treatment ($F(1,60)=8.269$, $p=0.006$).

Bonferroni *post hoc* analysis showed a significant decrease in weight for LPS/LPS males with respect to the group who received LPS only at PND21 ($p=0.002$) while LPS-treated females displayed a significant reduction in weight with respect to their saline-saline controls ($p<0.01$). Moreover, sex differences emerged, in fact, in females experiencing LPS two times, as juveniles and as adults, the weight loss was significantly reduced than their matching male counterparts ($p=0.034$).

At the 24h time-point, the three-way ANOVA revealed a main effect of the adult treatment ($F(1,69)=96.916$, $p<0.001$), the interaction between sex and adult treatment ($F(1,69)=6.663$, $p=0.012$) and the interaction of sex*juvenile treatment*adult treatment ($F(1,69)=4.477$, $p=0.038$).

Bonferroni's pairwise comparisons demonstrated that the immune challenge experienced as adults caused a significant decrease in body weight in both males ($p<0.001$) and females ($p=0.048$) with respect to their saline-exposed controls irrespective of the treatment received at PND21 ($p<0.001$

and p=0.048 for LPS/LPS vs LPS/SAL male and female animals respectively; and p<0.001 for SAL/LPS vs SAL/SAL).

Receiving LPS at PND21 also decreased the weight profile if compared to saline-saline controls (p=0.029) but only in females. As for the 6-hour time point, females re-exposed to LPS as adults were shown to be less sensitive if compared to the matching male counterparts (p=0.006): the decrease in body weight was significantly less in LPS/LPS female animals than the males receiving a matching treatment.

4.2.1.2.1 Effect of an immune challenge in adulthood on expression levels of inflammation-related targets in the hippocampus of male and female mice exposed to LPS or saline at PND21

The mRNA levels of the main pro-inflammatory cytokines analyzed, TNF-α and IL-1β, were significantly upregulated in the hippocampus of mice subjected to LPS only at 12 wks of age or both injected at PND21 and then in adulthood, while no induction was demonstrated for IL-6.

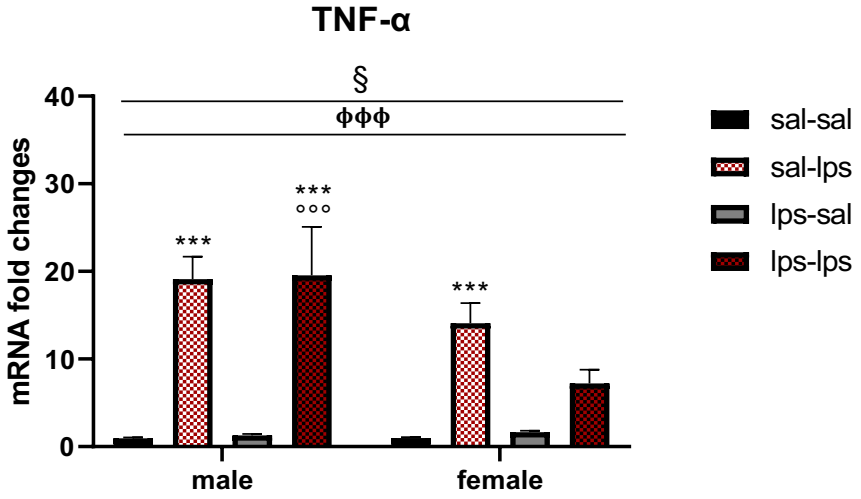

Figure 35. Effects on hippocampal TNF-α expression of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate TNF-α expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni: **§** = main effect of sex p=0.025, **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001, **°°°** = vs sex p<0.001.

Three-way ANOVA revealed for TNF-α hippocampal expression a main effect of sex ($F(1,56)=5.377$, $p=0.025$), adult treatment ($F(1,56)=56.696$, $p<0.001$) and a significant interaction between sex and adult treatment ($F(1,56)=5.892$, $p=0.019$).

Bonferroni's pairwise comparison demonstrated a significant upregulation of TNF-α mRNA in the hippocampus of males receiving LPS in adulthood with respect to their saline controls, irrespective

of the treatment received at PND21 (p<0.001). For female animals, the LPS-induced upregulation reached statistical significance only in animals exposed to saline (p<0.001) and not LPS at PND21.
LPS-LPS females showed two times lower levels of expression of TNF-α with respect to their matching male counterparts (p<0.001).

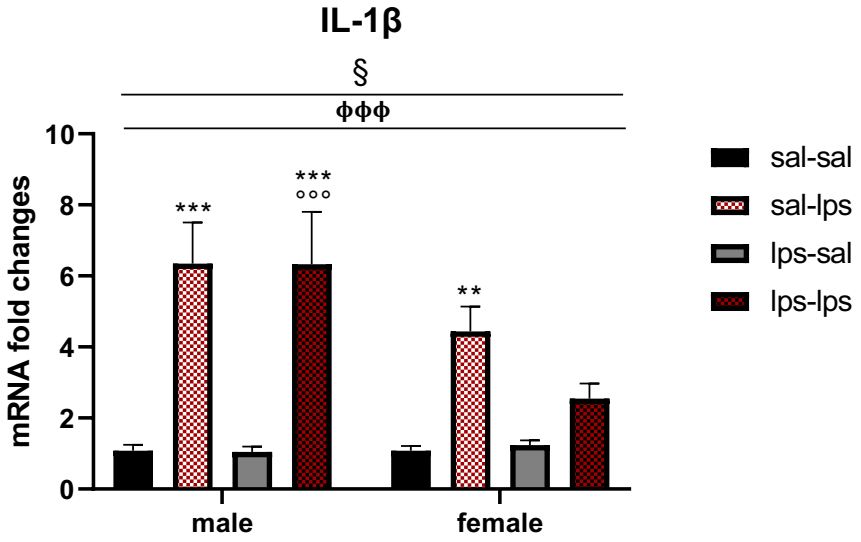

Figure 36. Effects on hippocampal IL-1β expression of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 μg/kg) or saline at PND21, re-exposed to LPS (830 μg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate IL-1β expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni: **§** = main effect of sex p=0.016, **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001, ****** = vs adult saline p=0.003, **°°°** = vs sex p<0.001.

Transcriptional effects of an immune challenge in the hippocampus of adult male and female mice receiving LPS or saline at PND35 for IL-1β were similar to TNF-α. Three-way ANOVA revealed the main effects of sex ($F(1,57)=6.274$, $p=0.016$), adult treatment ($F(1,57)=48.12$, $p<0.001$), and a significant interaction between sex and adult treatment ($F(1,57)=7.165$, $p=0.01$).
Following Bonferroni's pairwise comparisons showed a significant upregulation of IL-1β mRNA in the hippocampus of males injected with LPS in adulthood with respect to their saline controls, irrespective of the treatment received at PND21 (p<0.001). For female animals, the upregulation induced by the immune challenge reached statistical significance only in animals exposed to saline at PND21 (p=0.003) and not LPS.
Females injected with LPS in adulthood, exposed to LPS also at PND21, showed lower levels of expression of IL-1β with respect to their matching male counterparts (p<0.001).

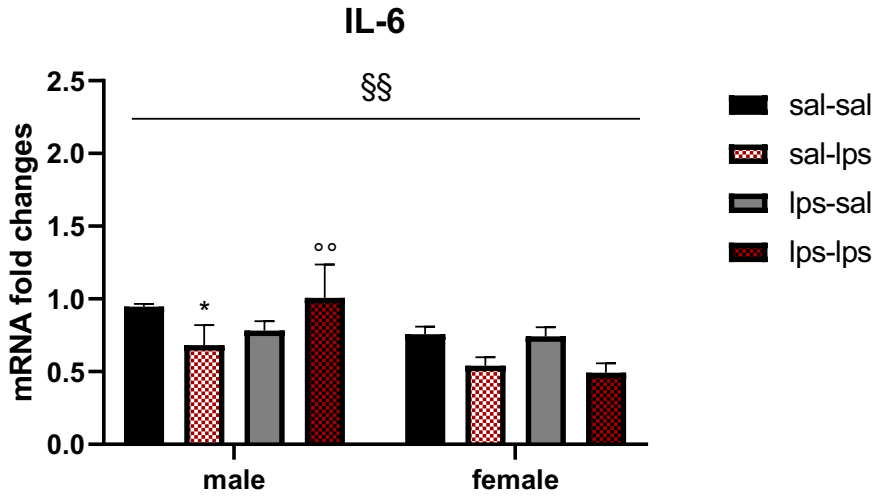

Figure 37. Effects on hippocampal IL-6 expression of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate IL-6 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*juvenile treatment*adult treatment) followed by Bonferroni: **§§** = main effect of sex p=0.004. *Post hoc*: ***** = vs adult saline p=0.04, **°°** = vs sex p=0.002.

Three-way ANOVA showed a main effect of sex ($F(1,57)=9.026$, $p=0.004$) for IL-6 hippocampal expression. Bonferroni's pairwise comparisons failed to demonstrate any effect on IL-6 expression induced by treatment except for the SAL/LPS males in which the target resulted significantly downregulated with respect to their SAL/SAL control group (p=0.04). The target was differentially expressed between sexes, in fact, females exposed to LPS both at PND21 and in adulthood showed significantly lower levels of expression of IL-6 mRNA with respect to their matching male counterparts (p=0.002).

4.2.1.2.2 Effect of an immune challenge in adulthood on the expression levels of microglial activation markers in the hippocampus of male and female mice exposed to LPS or saline at PND21

Considering the main microglial markers, CD11b, CD14, and Cx3cr1 mRNA levels of expression in the hippocampus were specifically regulated by the immune challenge in adulthood. CD206 mRNA expression levels were shown to be differentially expressed between sexes.

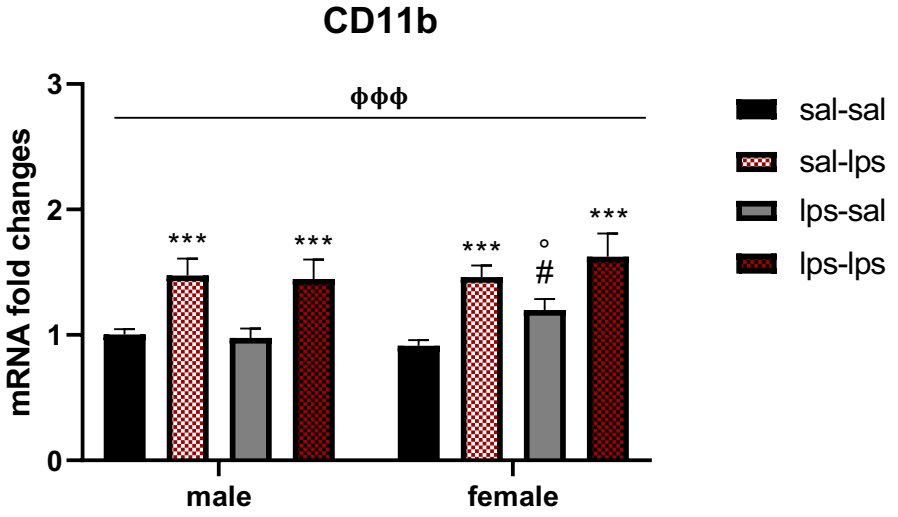

Figure 38. Effects on hippocampal CD11b expression of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate CD11b expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*juvenile treatment*adult treatment) followed by Bonferroni: **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001, **#** = vs juvenile saline p=0.044, **°** = vs sex p=0.045.

Three-way ANOVA revealed for CD11b mRNA levels a main effect of adult treatment ($F(1,57)=61.122$, $p<0.001$).

Bonferroni's pairwise comparison demonstrated a significant upregulation of CD11b mRNA in the hippocampus of males and females exposed to LPS in adulthood with respect to their saline matching-controls irrespective of the treatment received at PND21 ($p<0.001$). Moreover, higher levels of the target were found in LPS/SAL females with respect to SAL/SAL females ($p=0.044$). Considering the expression levels of this target between sexes, females treated with LPS at PND21 and with saline in adulthood showed different levels of expression of CD11b mRNA with respect to their matching male counterparts ($p=0.045$).

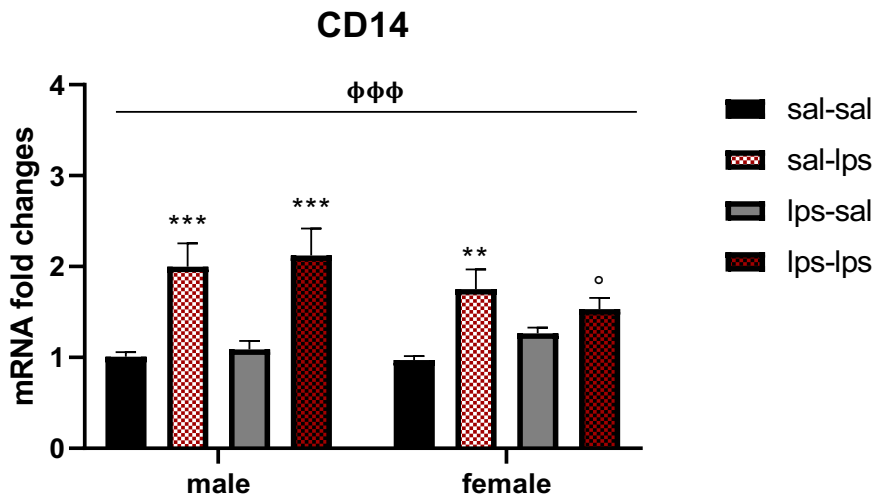

Figure 39. Effects on hippocampal CD14 expression of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate CD14 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*juvenile treatment*adult treatment) followed by Bonferroni: **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001, ****** = vs adult saline p=0.003, **°** = vs sex p=0.017.

When analyzing CD14 mRNA hippocampal expression, three-way ANOVA revealed a main effect of adult treatment ($F(1,59)=38.115$, $p<0.001$).

Bonferroni's pairwise comparisons demonstrated that the immune challenge experienced as an adult resulted in a significant increase of the CD14 mRNA in the hippocampus of male ($p<0.001$) mice irrespective of the juvenile treatment received, while in female animals this effect was present only in animals exposed to saline at PND21 ($p=0.003$).

We found a significant difference in the expression levels of this target between sexes: females treated with LPS both at PND21 and at 12wks of age showed significantly lower levels of expression of CD14 mRNA with respect to their matching male counterparts ($p=0.017$).

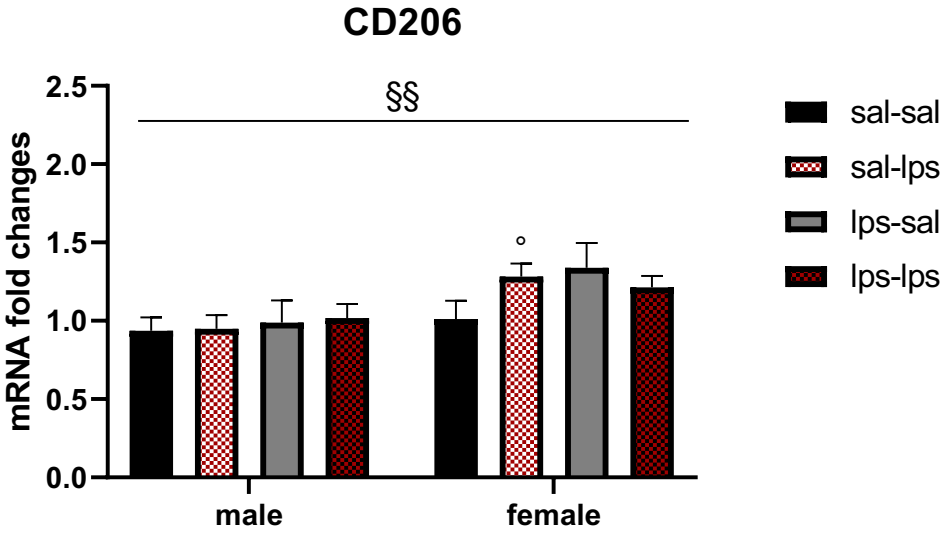

Figure 40. Effects on hippocampal CD206 expression of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate CD206 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*juvenile treatment*adult treatment) followed by Bonferroni: **§§** = main effect of sex p=0.005. *Post hoc*: **°** = vs sex p=0.047.

Considering CD206 mRNA expression, three-way ANOVA showed a main effects of sex ($F(1,56)=8.586$, $p=0.005$). Bonferroni *post hoc* analysis revealed no changes in CD206 mRNA expression in male animals in our experimental conditions except for the SAL/LPS group in which we observed that CD206 expression was higher in female animals with respect to their matching male counterparts (p=0.047).

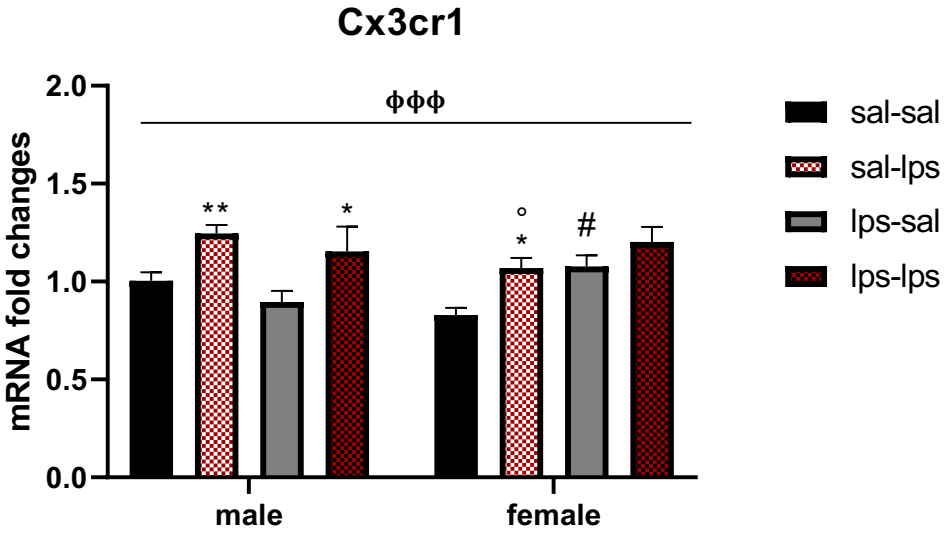

Figure 41. Effects on hippocampal Cx3cr1 expression of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate Cx3cr1 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*juvenile treatment*adult treatment) followed by Bonferroni: **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ****** = vs adult saline p=0.005, ***** = vs adult saline p=0.036, ***** = vs adult saline p=0.031, **°** = vs sex p=0.048, **#** = vs juvenile saline p=0.03.

Three-way ANOVA revealed a main effects of adult treatment ($F(1,59)=21.256$, $p<0.001$) and the interaction between sex and juvenile treatment ($F(1,59)=8.641$, $p=0.005$) for Cx3cr1 mRNA hippocampal expression.

Bonferroni *post hoc* analysis showed a significant upregulation of Cx3cr1 mRNA in the hippocampus of males (p=0.005) and females (p=0.031) receiving LPS in adulthood and saline at PND21 with respect to their controls. Cx3cr1 expression was significantly upregulated in LPS/SAL females with respect to the SAL/SAL control group (p=0.03). When re-exposed to LPS as adults, the expression of the fractalkine receptor was differentially regulated in the hippocampus of male and female animals. In males that experienced LPS as juveniles, the immune challenge as adults significantly increased the expression levels of Cx3cr1 (p=0.036), this effect was not elicited in females. Moreover, in SAL/LPS males Cx3cr1 mRNA was increased with respect to the matching female counterparts (p=0.048).

4.2.1.2.3 Effect of an immune challenge in adulthood on the expression levels of kynurenine pathway limiting enzyme, indoleamine 2,3-dioxygenase, in the hippocampus of male and female mice exposed to LPS or saline at PND21

IDO mRNA expression was significantly downregulated in females following the immune challenge only when exposed to LPS as adults.

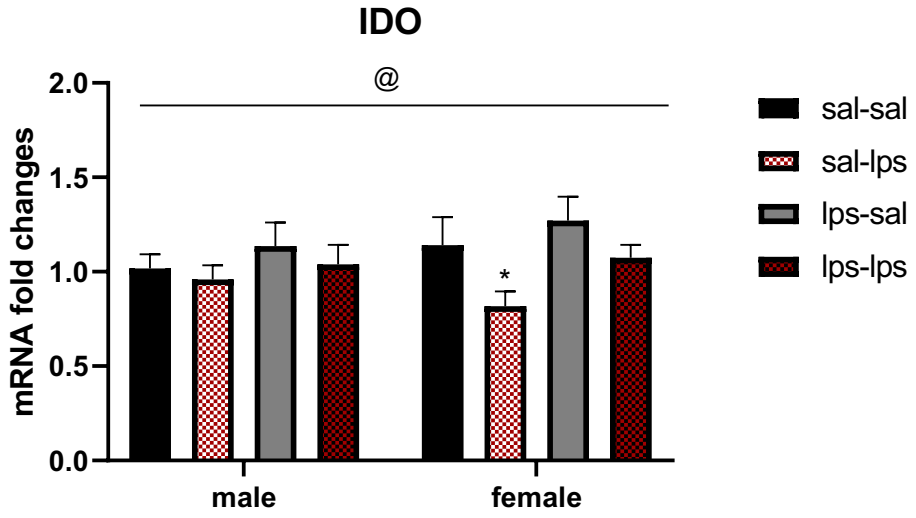

Figure 42. Effects on hippocampal IDO expression of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate IDO expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*juvenile treatment*adult treatment) followed by Bonferroni: **@** = main effect of juvenile treatment p=0.019. *Post hoc*: ***** = vs adult saline p=0.019.

For IDO hippocampal expression, three-way ANOVA revealed a main effect of juvenile treatment ($F(1,60)=5.883$, $p=0.019$) and a significant interaction between sex and juvenile treatment ($F(1,60)=6.076$, $p=0.017$).

4.2.2.1 Effect of an immune challenge in adulthood the levels of KP metabolites and related products in the serum of male and female mice exposed to LPS at PND21

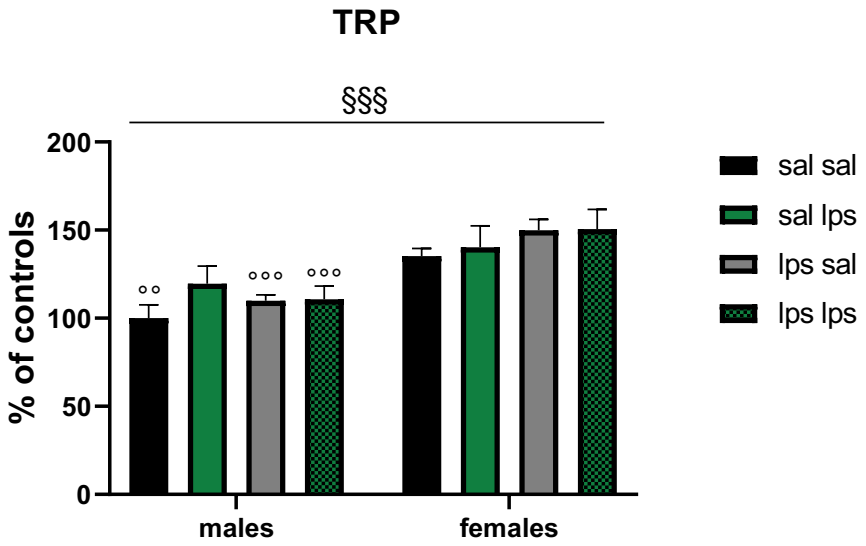

Figure 43. Effect on serum TRP concentration of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). HPLC-MS/MS was performed to evaluate TRP metabolite levels. Concentrations (µM) were expressed as a percent of controls (male sal sal) and as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§§** = main effect of sex p<0.001. *Post hoc*: **°°°** = vs sex p=0.001, **°°** = vs sex p=0.006.

When considering tryptophan levels in the serum of adult animals, three-way ANOVA showed only a main effect of sex ($F(1,45)=33.126$, $p<0.001$). After Bonferroni *post hoc*, no differences were demonstrated irrespective of the treatment that animals received in the juvenile stage and adulthood when compared to their respective controls. However, males displayed overall lower levels of TRP in their serum with respect to matching female groups (SAL/SAL m vs f, p=0.006; LPS/SAL and LPS/LPS m vs f, p=0.001 for both).

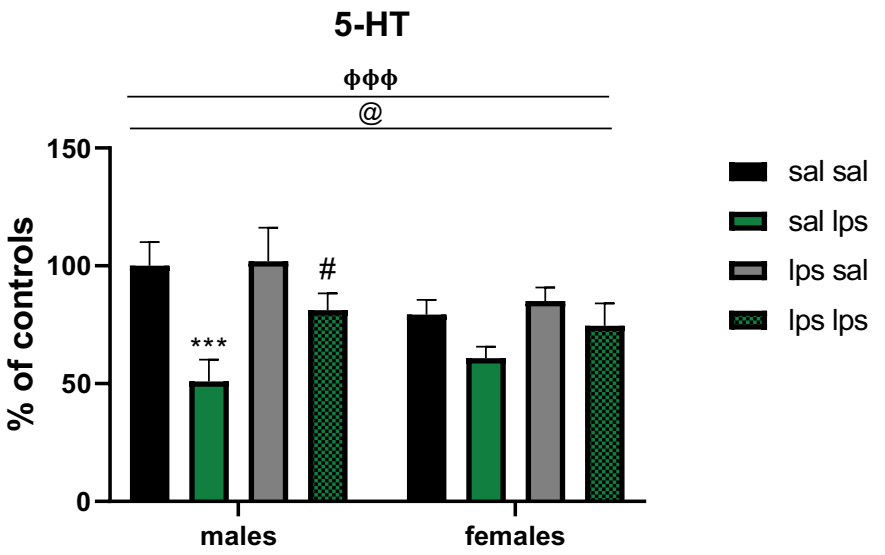

Figure 44. Effect on serum 5-HT concentration of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). HPLC-MS/MS was performed to evaluate 5-HT metabolite levels. Concentrations (µM) were expressed as a percent of controls (male sal sal) and as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **ϕϕϕ** = main effect of adult treatment p<0.001, **@** = main effect of juvenile treatment p=0.046. *Post hoc*: ******* = vs adult saline p=0.001, **#** = vs juvenile saline p=0.024.

Three-way ANOVA revealed the main effects of juvenile treatment ($F(1,45)=4.248$, $p=0.046$) and adult treatment ($F(1,45)=15.567$, $p<0.001$) for serum 5-HT levels in adult mice. Bonferroni's pairwise analysis showed decreased levels of serotonin in males receiving LPS as adults and saline at PND21 with respect to their control group ($p<0.001$) and with respect to males injected with LPS both at 12wks and previously as juveniles ($p=0.024$). Females did not show any serum 5-HT variations in our experimental conditions.

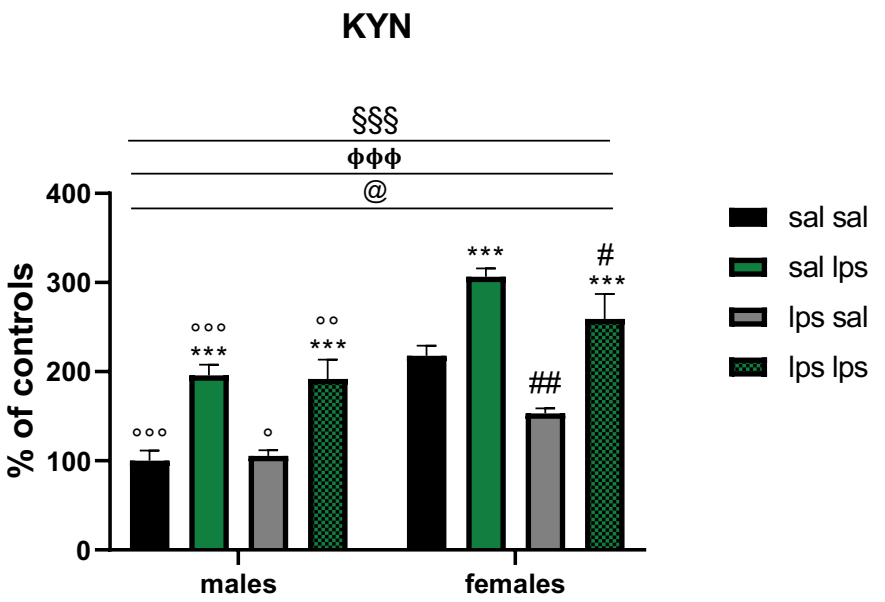

Figure 45. Effect on serum KYN concentration of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). HPLC-MS/MS was performed to evaluate KYN metabolite levels. Concentrations (µM) were expressed as a percent of controls (male sal sal) and as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§§** = main effect of sex p<0.001, **@** = main effect of juvenile treatment p=0.016, **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001, **°°°** = vs sex p<0.001, **°°** = vs sex p=0.003, **°** = vs sex p=0.032, **##** = vs juvenile saline p=0.004, **#** = vs juvenile saline p=0.033.

The main effects of sex (F(1,45)=61.623, p<0.001), juvenile treatment (F(1,45)=6.418, p=0.016), adult treatment (F(1,45)=74.382, p<0.001) and the interaction of sex*juvenile treatment (F(1,45)=6.717, p=0.013) were revealed for KYN serum concentration in adult mice after three-way ANOVA. Kynurenine concentration was upregulated after the adult LPS injection irrespective of the treatment received as juveniles in both male and female animal serum (p<0.001) after Bonferroni *post hoc.* Females exposed to LPS on PND21 showed decreased serum levels of KYN with respect to their juvenile saline exposed counterparts (p=0.004 LPS/SAL vs SAL/SAL) and (p=0.033 LSP/LPS vs SAL/LPS) groups. Moreover, sex differences emerged: male animals showed lower levels of this metabolite in their serum with respect to the respective matching female counterparts (p<0.001 SAL/SAL m vs f; p<0.001 SAL/LPS m vs f; LPS/SAL m vs f, p=0.032; LPS/LPS m vs f, p=0.003).

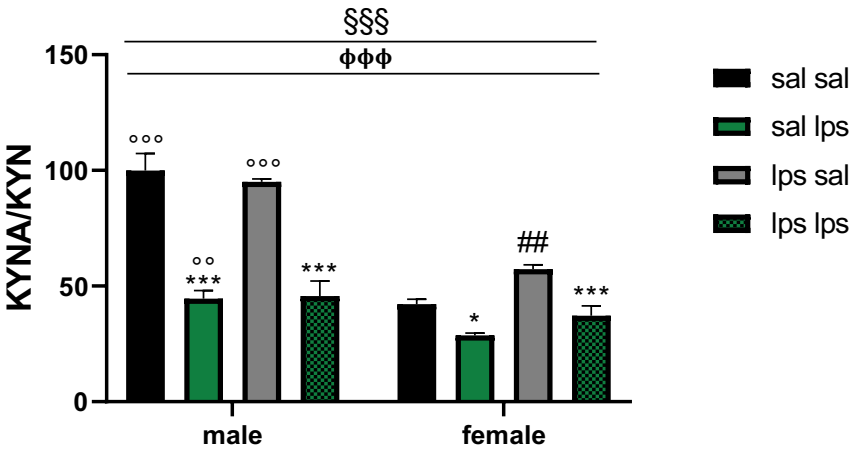

Figure 46. Effect on serum KYNA/KYN ratio of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). HPLC-MS/MS was performed to evaluate the KYNA/KYN ratio. The ratio was expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§§** = main effect of sex p<0.001, **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001, *** =** vs adult saline p=0.019**, °°°** = vs sex p<0.001, **°°** = vs sex p=0.009, **##** = vs juvenile saline p=0.009.

When analyzing KYNA/KYN ratio, the main effect of sex ($F(1,45)=113.558$, $p<0.001$), adult treatment ($F(1,45)=151.222$, $p<0.001$) and the interaction of sex*juvenile treatment ($F(1,45)=5.958$, $p=0.019$) and sex*adult treatment ($F(1,45)=39.846$, $p<0.001$) were demonstrated after three-way ANOVA. Bonferroni's pairwise analysis showed lower values of KYNA/KYN ratio in both male and female mice receiving the LPS injection at 12wks of age irrespective of the treatment received as juveniles ($p<0.05$) with respect to their respective control groups. Females experiencing the immune challenge at PND21 showed significantly higher levels of the ratio with respect to the female saline-saline control group ($p=0.009$). Considering the sex-based differential values of KYNA/KYN, Bonferroni *post hoc* demonstrated higher values of the ratio in SAL/SAL ($p<0.001$), SAL/LPS ($p=0.009$) and LPS/SAL ($p<0.001$) males with respect to matching female groups.

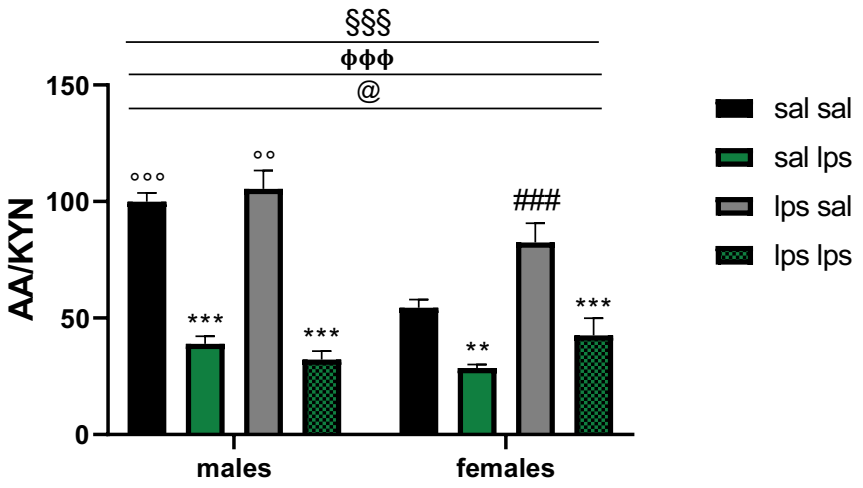

Figure 47. Effect on serum AA/KYN ratio of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 μg/kg) or saline at PND21, re-exposed to LPS (830 μg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). HPLC-MS/MS was performed to evaluate the AA/KYN ratio. The ratio was expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§§** = main effect of sex p<0.001, **ϕϕϕ** = main effect of adult treatment p<0.001, **@** = main effect of juvenile treatment p=0.015. *Post hoc*: ******* = vs respective saline p<0.001, ****** = vs respective saline p=0.002, **°°°** = vs sex p<0.001, **°°** = vs sex p=0.005, **###** = vs juvenile saline p<0.001.

For AA/KYN ratio, three-way ANOVA showed the main effects of sex ($F(1,45)=18.550$, $p<0.001$), juvenile ($F(1,45)=6.513$, $p=0.015$) and adult ($F(1,45)=157.543$, $p<0.001$) treatment and the interactions between sex and juvenile treatment ($F(1,45)=7.409$, $p=0.01$) and sex and adult treatment ($F(1,45)=18.516$, $p<0.001$). Similarly to the KYNA/KYN ratio, Bonferroni *post hoc* demonstrated decreased values of AA/KYN ratio in both male and female mice injected with LPS at 12wks of age irrespective of the treatment received at PND21 ($p<0.01$) when compared to their respective control groups. Again, female mice receiving LPS at PND21 and saline as adults showed higher levels of this ratio with respect to saline-saline females ($p<0.001$). Sex differences were present for AA/KYN values which were higher in the serum of SAL/SAL ($p<0.001$) and LPS/SAL ($p=0.005$) males with respect to matching female groups.

4.2.2.1 Effect of an immune challenge on body weight of male and female mice exposed to LPS or saline at PND35.

Male and female animals were weighed weekly starting from PND35 until 12 wks of age. Mice growth was regular regardless of the treatment received in adolescence.

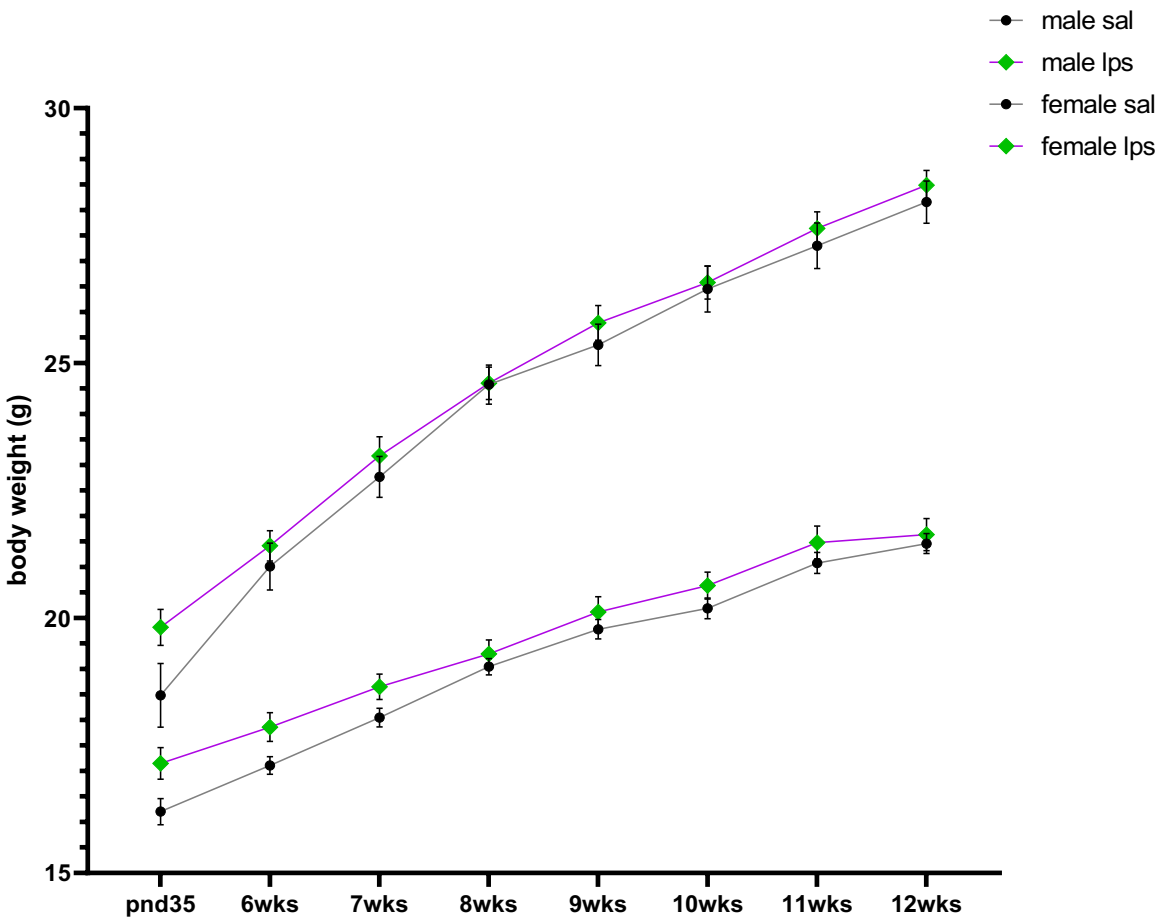

Figure 48. Effects on weight of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35 (n=14-20 per group). The body weight (g) was recorded each week starting from PND35 to 12wks. Data were expressed as mean (g) ± SEM (standard error of the mean). Statistical analysis was performed by repeated measure ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni.

The body weight of male and female mice was significantly different before the treatment at PND35. Repeated measures ANOVA revealed an interaction between the weight increase and the biological sex ($F(7;68)=23.762$; $p<0,0001$). As expected, the weight gain was higher in males with respect to females starting from PND42 to 12 wks of age. The treatment received at PND35 did not affect the weight trend in both male and female mice.

The weight profile significantly decreased when mice were exposed to LPS as adults or both treated with LPS at PND35 followed by another immune challenge at 12 wks.

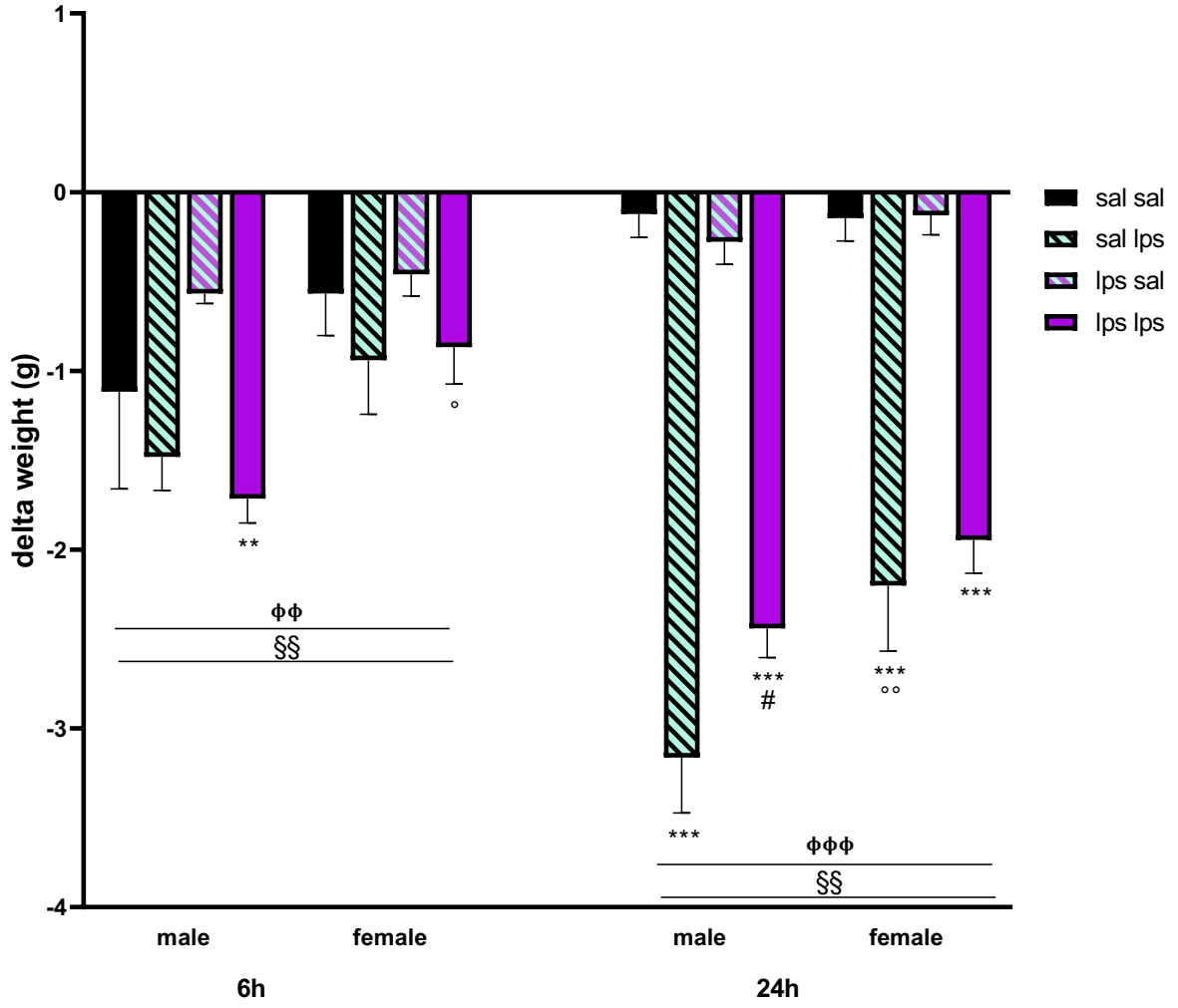

Figure 49. Effects on body weight of lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or saline at 12wks of age. Animals' weight was checked 6 or 24h after the treatment and then mice were sacrificed after 24h (n=7-10 per group). Data were expressed as mean variation in body weight (g) and ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **ΦΦΦ** = main effect of adult treatment p<0.001, **ΦΦ** = main effect of adult treatment p=0.005, **§§** = main effect of sex p=0.009, **§§** = main effect of sex p=0.01. *Post hoc*: ******* = vs adult saline p<0.001, ****** = vs adult saline p=0.003, **°°** = vs sex p=0.002, **°** = vs sex p=0.022, **#** = vs adolescent saline p=0.021.

When analyzing the weight profile 6h after receiving LPS or saline in adulthood, the three-way ANOVA demonstrated the main effects of sex ($F(1,45)=7.255$, $p=0.01$) and adult treatment ($F(1,45)=9.112$, $p=0.005$).

Bonferroni's pairwise comparisons showed a significant weight decrease in males LPS-treated as adults and at PND35 with respect to the group who received LPS only in adolescence ($p=0.003$). No effect was revealed for females in our experimental conditions, however, sex differences emerged: LPS/LPS females showed reduced weight loss if compared to the matching male counterparts ($p=0.022$).

For the weight profile of adult animals treated and sacrificed after 24h, the three-way ANOVA revealed the main effects of sex ($F(1,77)=7.286$, $p=0.009$), adult treatment ($F(1,77)=239.738$, $p<0.001$) and of the interaction between sex and adult treatment ($F(1,77)=5.172$, $p=0.026$).
Bonferroni *post hoc* analysis showed a significant weight decrease in males and females injected with LPS only in adulthood with respect to their respective controls ($p<0.001$) as for LPS/LPS males and females with respect to animals who received LPS only at PND35 ($p<0.001$). Moreover, in males, the weight decreased significantly in the animals who received LPS only at 12 wks of age with respect to LPS/LPS males ($p=0.021$). Weight loss was differentially induced by LPS between sexes: female animals were shown to be less sensitive if LPS-treated only at 12wks of age compared to the matching male counterpart ($p=0.002$) resulting in reduced weight loss.

4.2.2.2.1 Effect of an immune challenge in adulthood on expression levels of inflammation-related targets in the hippocampus of male and female mice exposed to LPS or saline at PND35

Similarly to what observed for PND21, the main pro-inflammatory cytokines TNF-α and IL-1β mRNA levels were significantly upregulated in the hippocampus when mice were exposed to LPS as adults or both treated with LPS at PND35 following by another immune challenge at 12 wks. In this case, an effect on IL-6 was revealed also.

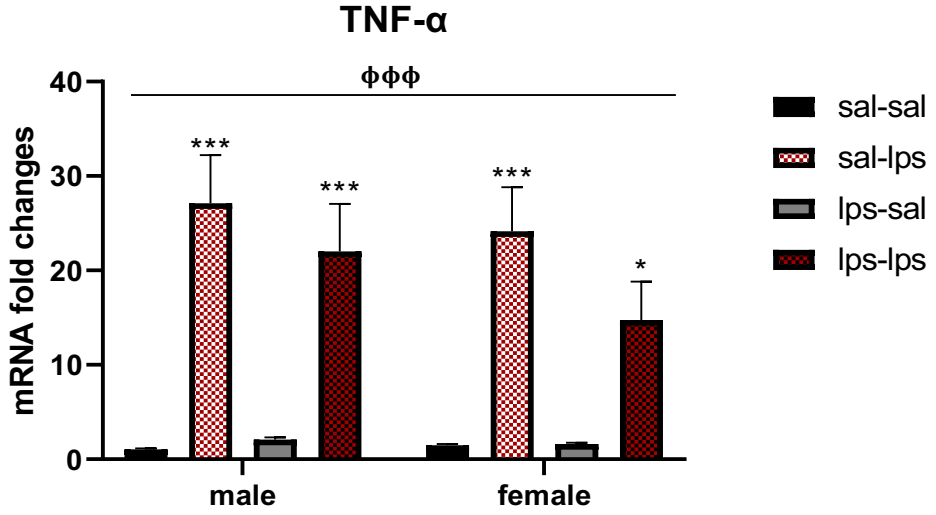

Figure 50. Effects on hippocampal TNF-α expression of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate TNF-α expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni: **ϕϕϕ** = main effect of adult treatment $p<0.001$. *Post hoc*: ******* = vs adult saline $p<0.001$, ***** = vs adult saline $p=0.012$.

Three-way ANOVA demonstrated for TNF-α hippocampal expression the main effect of adult treatment ($F(1,57)=66.993$, $p<0.001$).

Bonferroni *post hoc* demonstrated a significant upregulation of TNF-α mRNA in the hippocampus of males and females receiving LPS in adulthood with respect to their saline-controls, irrespective of the treatment received at PND35 (p<0.05).

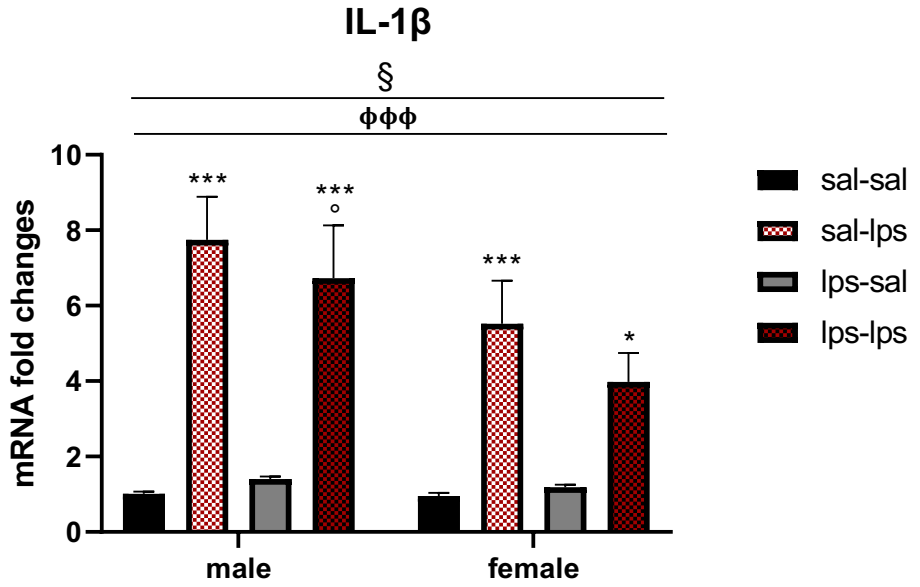

Figure 51. Effects on hippocampal IL-1β expression of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate IL-1β expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni: **§** = main effect of sex p=0.031, **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001, ***** = vs adult saline p=0.025, **°** = main effect of sex p=0.023.

Similarly to IL-1β gene expression in the hippocampus of PND21-12wks animals, in PND35-12wks three-way ANOVA revealed main effects of sex ($F(1,58)=4.921$, $p=0.031$) and adult treatment ($F(1,58)=67.298$, $p<0.001$).

Following Bonferroni's pairwise comparison demonstrated a significant upregulation of IL-1β mRNA expression in the hippocampus of males and females injected with LPS in adulthood with respect to their saline-controls, irrespective of the treatment received at PND35 (p<0.05). The target was differentially expressed between sexes: LPS/LPS males showed higher levels of IL-1β mRNA with respect to matching female counterparts (p=0.023).

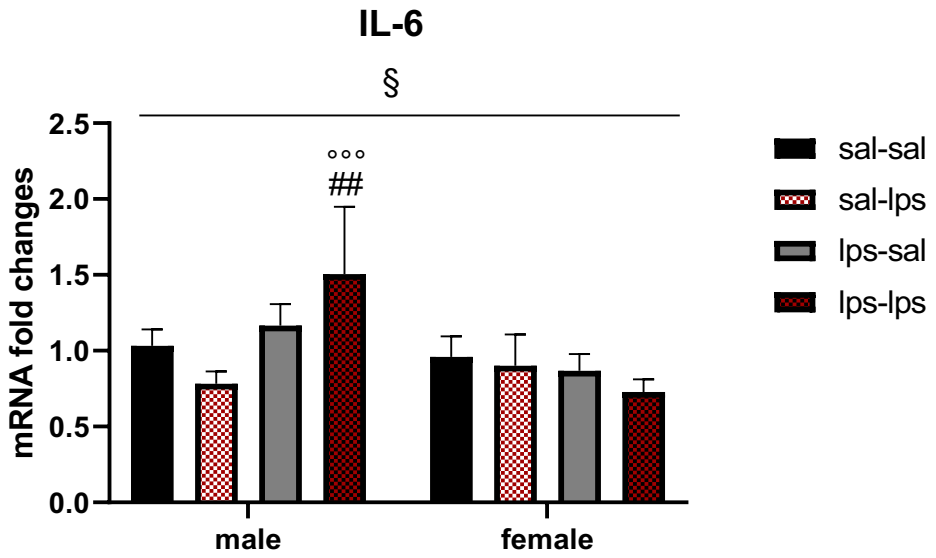

Figure 52. Effects on hippocampal IL-6 expression of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate IL-6 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni: **§** = main effect of sex p=0.018. *Post hoc*: **##** = vs adolescent saline p=0.007, **°°°** = vs sex p <0.001.

Three-way ANOVA showed a main effect of sex ($F(1,59)=6.021$, $p=0.018$) and a significant interaction of sex and adolescent treatment ($F(1,59)=7.768$, $p=0.007$) for IL-6 hippocampal expression levels. Bonferroni's pairwise comparisons failed to demonstrate any effect on IL-6 expression induced in our experimental conditions, with the sole exception of males who received LPS only in which the target resulted significantly upregulated if compared to the group injected with saline at PND35 and LPS-treated at 12wks of age ($p=0.007$). However, a difference between sexes emerged: females treated with LPS both at PND35 and at 12wks of age showed significant lower levels of expression of IL-6 mRNA with respect to their matching male counterparts ($p<0.001$).

4.2.2.2.2 Effect of an immune challenge in adulthood on expression levels of microglial activation markers in the hippocampus of male and female mice exposed to LPS or saline at PND35

Microglial markers CD11b, CD14, CD206 and Cx3cr1 mRNA levels were all significantly upregulated when animals experienced the immune challenge as adults.

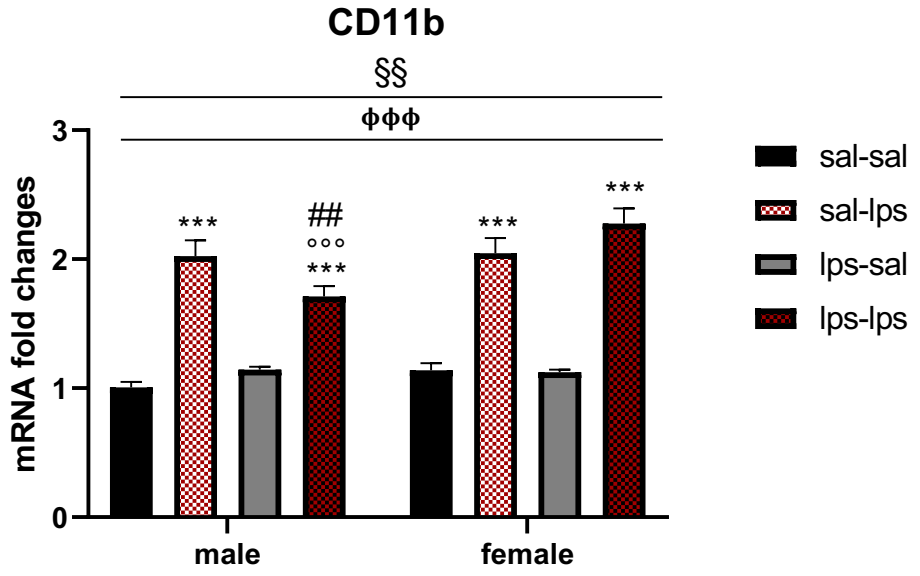

Figure 53. Effects on hippocampal CD11b expression of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate CD11b expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni: **§§** = main effect of sex p=0.005, **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001, **##** = vs adolescent saline p=0.01, **°°°** = vs sex p<0.001.

Three-way ANOVA revealed significant main effects of sex ($F(1,60)=8.694$, $p=0.005$) and adult treatment ($F(1,60)=234.205$, $p<0.001$).

Bonferroni's pairwise comparison showed a significant upregulation of CD11b mRNA in the hippocampus of males and females exposed to LPS in adulthood irrespective of the treatment received at PND35 (p<0.001). LPS/LPS males displayed also lower levels of this microglial marker with respect to males who experienced LPS only as adults (p=0.01). The target was differentially expressed between sexes: LPS/LPS males showed lower levels of the target with respect to the matching female group (p<0.001).

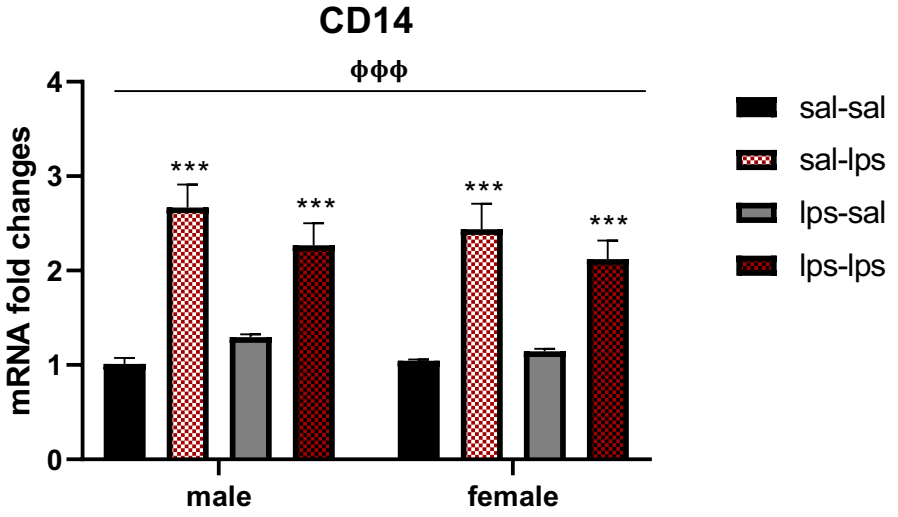

Figure 54. Effects on hippocampal CD14 expression of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate CD14 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni: **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001.

Considering CD14 mRNA hippocampal levels, three-way ANOVA revealed the main effects of adult treatment ($F(1,60)=102.341$, $p<0.001$) and the significant interaction between adolescent and adult treatment ($F(1,60)=4.938$, $p=0.031$).

Similarly to the target CD11b, Bonferroni's pairwise comparison showed a significant upregulation of CD14 mRNA in the hippocampus of males and females exposed to LPS in adulthood irrespective of the treatment received at PND35 ($p<0.001$). We did not find significant differences in the expression levels of CD14 mRNA between sexes.

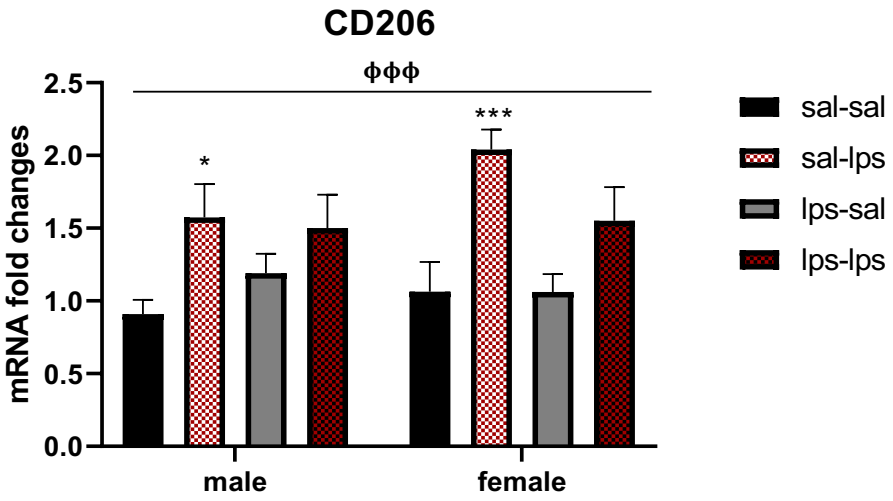

Figure 55. Effects on hippocampal CD206 expression of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 μg/kg) or saline at PND35, re-exposed to LPS (830 μg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate CD206 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni: **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001, ***** = vs adult saline p=0.016.

For CD206 mRNA expression, three-way ANOVA revealed a significant main effect of adult treatment ($F(1,57)=21.699$, $p<0.001$).

Bonferroni's *post hoc* analysis demonstrated a significant upregulation of CD206 mRNA in the hippocampus of males (p=0.16) and females (p<0.001) treated with saline at PND35 and then injected with LPS in adulthood with respect to their controls. The upregulation was stronger in females, but the difference failed to reach statistical significance if compared to the corresponding male group.

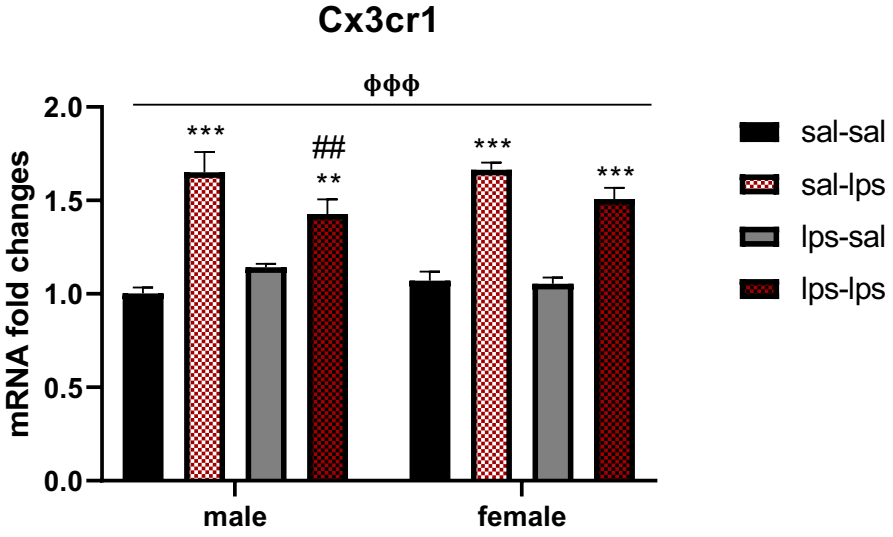

Figure 56. Effects on hippocampal Cx3cr1 expression of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 μg/kg) or saline at PND35, re-exposed to LPS (830 μg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate Cx3cr1 expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni: **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001, ****** = vs adult saline p=0.002, **##** = vs adolescent saline p=0.01.

Three-way ANOVA analysis revealed for Cx3cr1 mRNA the main effect of adult treatment ($F(1,58)=129.700$, $p<0.001$) and the significant interaction between adolescent and adult treatment ($F(1,58)=8.419$, $p=0.005$).

Bonferroni's pairwise comparison revealed a significant upregulation of Cx3cr1 mRNA in the hippocampus of males and females injected with saline at PND35 and exposed to LPS in adulthood with respect to their SAL/SAL controls (p<0.001). Cx3cr1 expression was significantly increased following LPS also in male (p=0.002) and female (p<0.001) animals that have already experienced the immune challenge in adolescence. LPS/LPS males also displayed lower levels of the fractalkine receptor with respect to their counterparts exposed to LPS only at 12 wks (p=0.01). Overall, Cx3cr1 expression levels were comparable between males and females in our experimental conditions.

4.2.2.2.3 Effect of an immune challenge in adulthood on expression levels of kynurenine pathway limiting enzyme, indoleamine 2,3-dioxygenase, in the hippocampus of male and female mice exposed to LPS or saline at PND35

IDO mRNA levels were significantly upregulated in the hippocampus of male mice exposed to LPS once as adults.

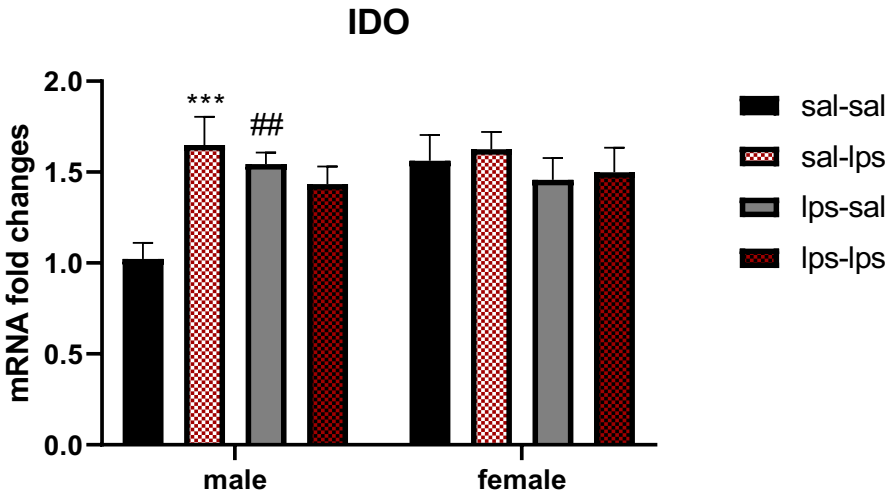

Figure 57. Effects on hippocampal IDO expression of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 μg/kg) or saline at PND35, re-exposed to LPS (830 μg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). Real-time PCR was performed to evaluate IDO expression levels: specific primers were used and $2^{-\Delta\Delta Ct}$ was applied with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni. *Post hoc*: *** = vs adult saline p<0.001, ## = vs adolescent saline p=0.002.

Three-way ANOVA showed the interaction of adolescent treatment*adult treatment (F(1,60)=5.447, p=0.023) and of sex*adolescent treatment*adult treatment (F(1,60)=4.825, p=0.032) for IDO hippocampal mRNA level of expression. Bonferroni *post hoc* analysis revealed no variations of IDO mRNA between groups in our experimental conditions, except for, in this case, males subjected to LPS at PND35 only (p=0.002) as for males who experienced LPS only at 12wks (p<0.001) both with respect to their matching saline-saline control group.

4.2.2.3 Effect of an immune challenge in adulthood on the levels of KP metabolites and related products in serum of male and female mice exposed to LPS at PND35

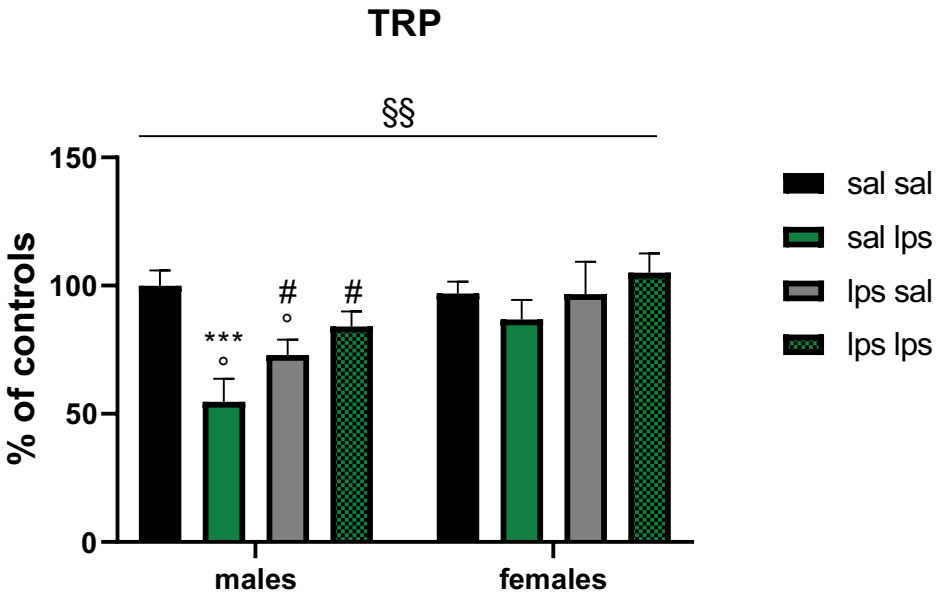

Figure 58. Effect on serum TRP concentration of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). HPLC-MS/MS was performed to evaluate TRP metabolites levels. Concentrations (µM) were expressed as percent of controls (male sal sal) and as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§** = main effect of sex p=0.002. *Post hoc*: **°** = vs sex p=0.014, **°** = vs sex p=0.032, *** = vs adult saline p<0.001, **#** = vs adolescent saline p=0.015, **#** = vs adolescent saline p=0.013.

In PND35-12wks animals, the main effects of sex (F(1,44)=11.021, p=0.002) and the interaction of adolescent treatment*adult treatment (F1,44)=11.338, p=0.002) were shown after three-way ANOVA for serum TRP concentration. Bonferroni's pairwise comparison demonstrated that male mice receiving LPS only in adulthood and not at PND35 showed a significant decrease in TRP serum levels with respect to the saline-saline control group (p<0.001). Following LPS exposure in adolescence, males receiving saline at 12wks showed lower concentrations of TRP with respect to the saline-saline counterparts (p=0.015) while higher levels of this target were observed in LPS/LPS

males when compared to the SAL/LPS group (p=0.013). Tryptophan serum concentrations were lower in males receiving LPS only in adolescence (p=0.032) or only as adults (p=0.014) with respect to matching female counterparts.

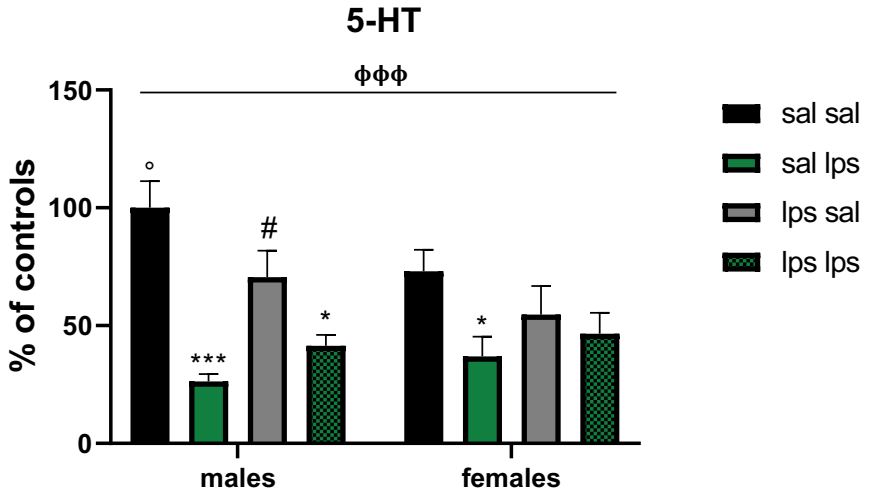

Figure 59. Effect on serum 5-HT concentration of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). HPLC-MS/MS was performed to evaluate 5-HT metabolite levels. Concentrations (µM) were expressed as per cent of controls (male sal sal) and as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001, ***** = vs adult saline p=0.026, ***** = vs adult saline p=0.014, **°** = vs sex p=0.039, **#** = vs adolescent saline p=0.025.

Three-way ANOVA revealed the main effects of adult treatment (F(1,43)=30.780, p<0.001) and the interaction of adolescent treatment*adult treatment (F(1,43)=7.489, p=0.01) in adult male and female mice serotonin concentration in the serum. After Bonferroni *post hoc,* lower 5-HT concentration was revealed in both males (p<0.001) and females (p=0.014) after receiving LPS at 12wks and not in adolescence with respect to their matching saline-saline control groups. This effect was present also in males exposed to the immune challenge both at PND35 and as adults with respect to their controls (p=0.026) while this downregulation failed to reach statistical significance in females. LPS in adolescence was associated with a lower concentration of this metabolite with respect to the saline-saline group only in males (p=0.025). SAL/SAL mice showed higher levels of 5-HT with respect to the corresponding female group (p=0.039).

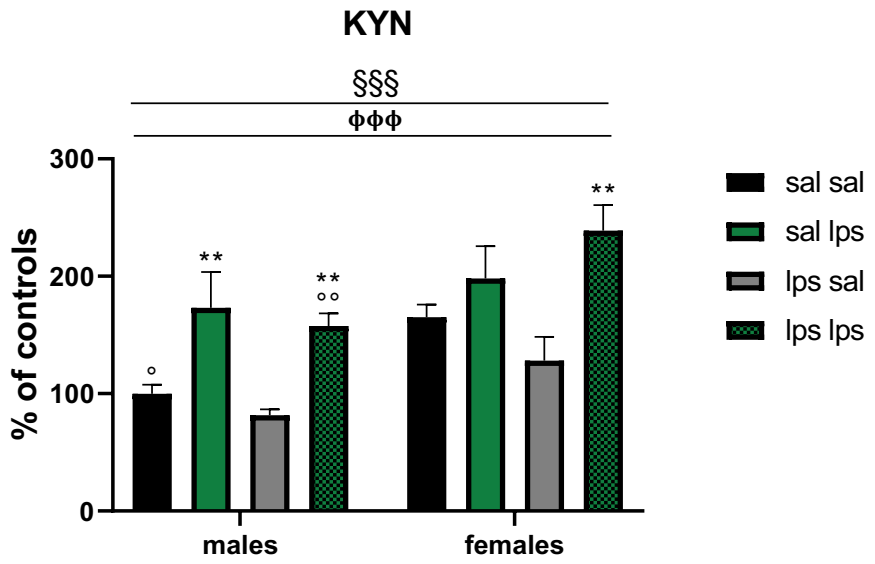

Figure 60. Effect on serum KYN concentration of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). HPLC-MS/MS was performed to evaluate KYN metabolites levels. Concentrations (µM) were expressed as percent of controls (male sal sal) and as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§§** = main effect of sex p<0.001, **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ****** = vs adult saline p<0.01, ****** = vs adult saline p=0.007, ****** = vs adult saline p=0.004, **°°** = vs sex p=0.002, **°** = vs sex p=0.012.

Three-way ANOVA showed the main effects of sex ($F(1,45)=18.603$, $p<0.001$) and adult treatment ($F(1,45)=33.640$, $p<0.001$) on KYN levels in the serum of adult mice receiving LPS or saline at PND35. Bonferroni's pairwise comparison revealed that the treatment with LPS in adulthood increased the levels of KYN in both males and females who experienced the immune challenge in adolescence ($p<0.01$) with respect to their matching controls receiving saline as adults. On the other hand, the same effect was observed after a single LPS injection administered only at 12wks and not at PND35 with respect to saline-saline group only in males ($p=0.007$). Other sex differences emerged: SAL/SAL males ($p=0.012$) or LPS/LPS males ($p=0.002$) showed lower KYN concentration when compared to matching female counterparts.

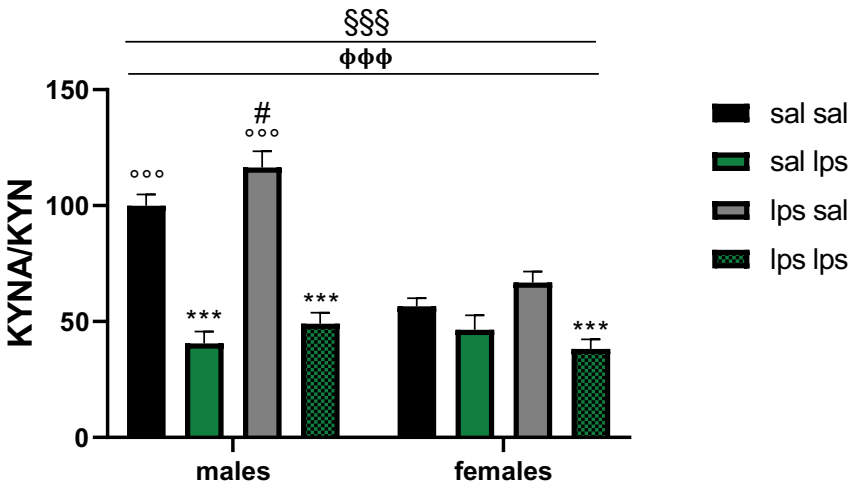

Figure 61. Effect on serum KYNA/KYN ratio of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). HPLC-MS/MS was performed to evaluate KYNA/KYN ratio. Ratio was expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **§§§** = main effect of sex p<0.001, **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001**, °°°** = vs sex p<0.001, **#** = vs adolescent saline p=0.024.

When considering the ratio KYNA/KYN in animals LPS- or saline-injected in adolescence, three-way ANOVA showed the main effects of sex (F(1,45)=46.230), adult treatment (F(1,45)=131.884, p<0.001) and the interaction between sex and adult treatment (F(1,45)=37.227, p<0.001). Decreased KYNA/KYN ratio was observed after the LPS injection in adulthood in males irrespective of the treatment received in adolescence (p<0.001) with respect to their matching SAL counterparts. In females, this effect was present only in animals injected with LPS at PND35 and re-exposed to the immune challenge as adults (p<0.001). Considering sex differences, males injected with saline at 12wks showed higher KYNA/KYN values with respect to matching female counterparts (p<0.001).

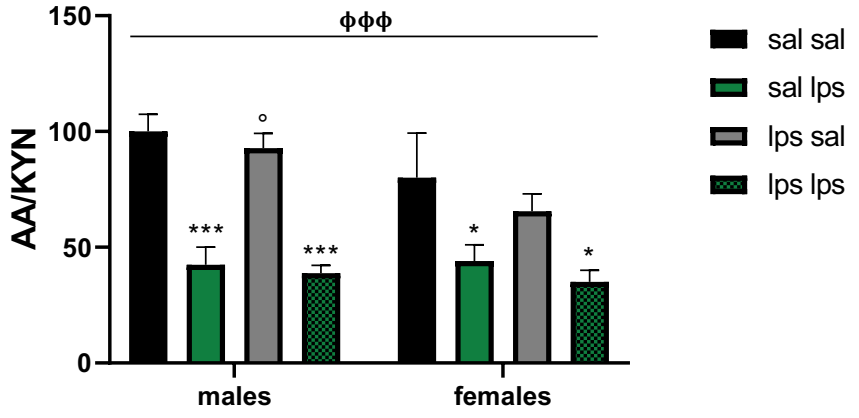

Figure 62. Effect on serum AA/KYN ratio of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or

saline at 12wks of age and then sacrificed after 24h (n=6-8 per group). HPLC-MS/MS was performed to evaluate AA/KYN ratio. Ratio was expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*treatment*time after treatment) followed by Bonferroni: **ϕϕϕ** = main effect of adult treatment p<0.001. *Post hoc*: ******* = vs adult saline p<0.001, ***** = vs adult saline p=0.011, ***** = vs adult saline p=0.023, **°** = vs sex p=0.041.

After three-way ANOVA, only the main effect of the adult treatment ($F(1,45)=45.464$, $p<0.001$) was showed for AA/KYN ratio in adult mice serum. Bonferroni's pairwise comparison showed a decreased ratio in both male and female mice exposed to LPS as adults, irrespective of experiencing LPS or saline at PND35 ($p<0.05$). In LPS/SAL groups different values of AA/KYN were observed according to sex, with males displaying higher levels of this ratio with respect to females ($p=0.041$).

4.3 COHORT 3

In Cohort 3 we evaluated the effect of an immune challenge (LPS, 100 µg/Kg) in juvenile (PND21) or adolescent (PND35) male or female mice on the behavioural responses to a second inflammatory hit (LPS, 830 µg/Kg) experienced in adulthood (12 weeks). Therefore, twenty-four hours after receiving either saline or LPS as adults, mice underwent a behavioural battery to assess locomotor activity (OF), anxiety-like behaviour (EPM), and cognition-like behaviour (YM) as described in Methods section.

4.3.1.1 Effect of an immune challenge in adulthood on locomotor activity of male and female mice exposed to LPS or saline at PND21

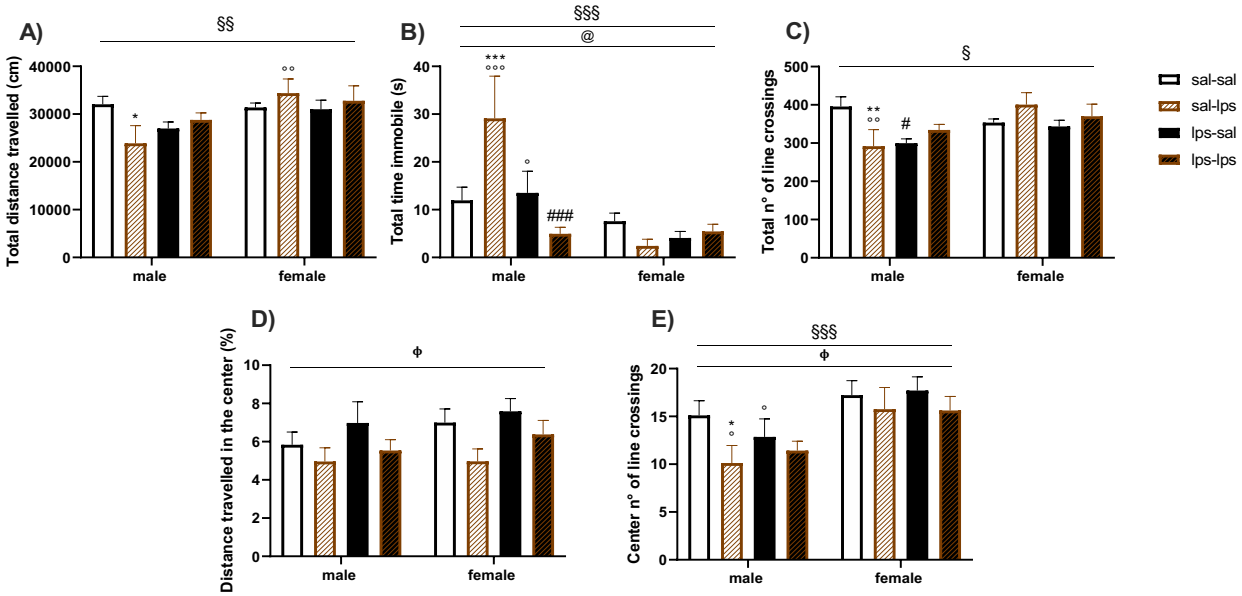

Figure 63. Effects on locomotor activity of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and tested after 23h (n=7-10 per group) in an OF arena. **A)** Total distance travelled (cm), **B)** total time immobile (s), **C)** total number of line crossings, **D)** distance travelled in the center (%) and **E)** number of line crossings in the center were recorded and expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*juvenile treatment*adult treatment) followed by Bonferroni: **A) §§** = main effect of sex p=0.008. *Post hoc*: ***** = vs adult saline, p=0.014, **°°** = vs sex p =0.002; **B) §§§** = main effect of sex p<0.001, **@** = main effect of juvenile treatment p=0.022. *Post hoc*: ******* = vs adult saline p=0.001, **°°°** = vs sex p<0.001, **°** = vs sex p=0.05, **###** = vs juvenile saline p<0.001; **C) §** = main effect of sex p=0.049. *Post hoc*: ****** = vs adult saline p=0.004, **°°** = vs sex p=0.005, **#** = vs juvenile saline p=0.011; **D) ϕ** = main effect of adult treatment p=0.011; **E) §§§** = main effect of sex p<0.001, **ϕ** = main effect of adult treatment p=0.038. *Post hoc*: ***** = vs adult saline p=0.035, **°** = vs sex p=0.021, **°** = vs sex p=0.043.

When considering the whole OF apparatus (Fig.63 A; B; C), three-way ANOVA revealed a main effect of sex for total travelled distance, time immobile and number of line crossings (Fig.63 A: $F(1,66)=7.512$, $p=0.008$; Fig.63 B: $F(1,66)=16.692$, $p<0.001$; Fig.63 C: $F(1,66)=4.042$, $p=0.049$) and a main effect of juvenile treatment only for the time immobile ($F1,66)=5.563$, $p=0.022$). The

interaction of sex*juvenile treatment*adult treatment was showed for both time immobile and for the n° of line crossings (Fig.63 B: $F(1,66)=10.865$, $p=0.002$; Fig.63 C: $F(1,66)=4.788$, $p=0.033$) while the interaction of sex*juvenile treatment was revealed for the time immobile only (Fig.63 B: $F(1,66)=5.139$, $p=0.027$).

Bonferroni *post hoc* analysis demonstrated a general reduced locomotor activity in males. In fact, male mice travelled a shorter distance with respect to females ($p=0.002$) when exposed to LPS only as adults and with respect to their SAL/SAL controls ($p=0.014$). Reduced locomotion in SAL/LPS males was also suggested by the higher time spent immobile (Fig.63 B) when compared to the respective female counterparts ($p<0.001$) and to SAL/SAL-controls ($p=0.001$). The same effect was observed for the number of line crossings: male animals receiving saline at PND21 and LPS as adults performed significantly less crossing in the whole arena with respect to SAL/SAL males or their matching female counterparts (m SAL/LPS vs SAL/SAL, $p=0.004$ and m SAL/LPS vs f SAL/LPS, $p=0.005$). This group spent significantly more time immobile than males experiencing LPS both at PND21 and as adults ($p<0.001$). When male animals received LPS at PND21, they spent significantly more time immobile with respect to their female counterparts ($p=0.05$), while performing fewer total line crossings with respect to their matching controls belonging to the same sex ($p=0.011$).

When considering the central area of the OF (Fig.63 D; E), three-way ANOVA revealed a main effect of adult treatment for both the per cent distance travelled in the centre and the number of line crossings in this area (Fig.63 D: $F(1,65)=6.838$, $p=0.011$; Fig.63 E: $F(1,65)=4.486$, $p=0.038$) while a main effect of sex was present only for the number of line crossing ($F(1,65)=12.721$, $p=0.001$). No differences were revealed for the distance travelled in the centre in our experimental conditions, while the number of line crossings in the center were decreased in males injected with LPS only at 12 wks with respect to both their female counterparts ($p=0,021$) and their SAL/SAL controls ($p=0.035$). Males experiencing LPS at PND21 and saline as adults also showed lower values of this parameter with respect to LPS/SAL females ($p=0.043$).

4.3.1.2 Effect of an immune challenge in adulthood on anxiety-like behaviour of male and female mice exposed to LPS or saline at PND21

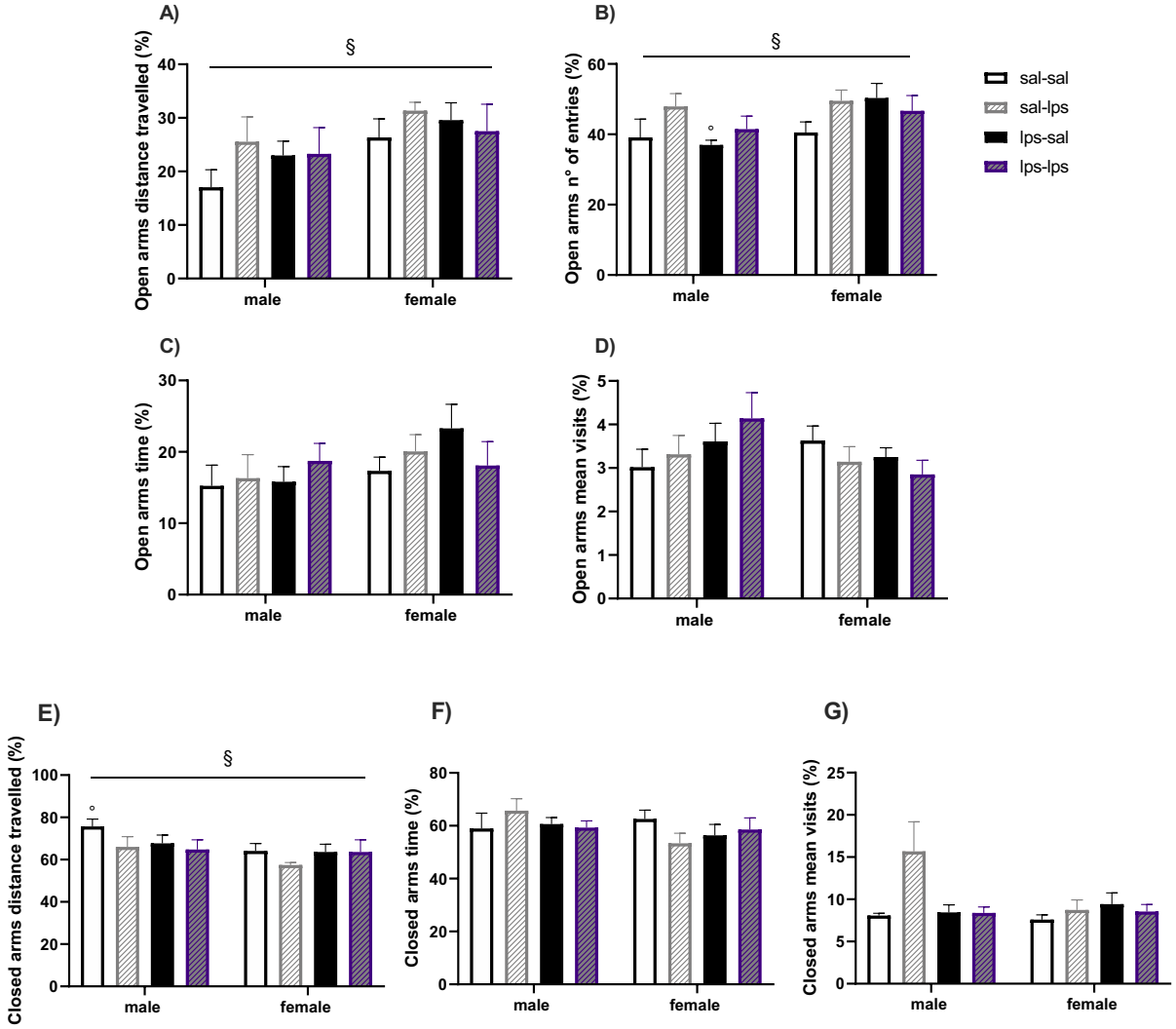

Figure 64. Effects on anxiety-like behaviour of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and tested after 25h (n=7-10 per group) in an EPM. **A)** Distance travelled in the open arms (%), **B)** number of entries in the open arms (%), **C)** time spent in the open arms (%), **D)** mean visits in the open arms (%), **E)** distance travelled in the closed arms (%), **F)** time spent in the closed arms (%) and **G)** mean visits in the closed arms (%) were recorded and expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*juvenile treatment*adult treatment) followed by Bonferroni: **A) § =** main effect of sex p=0.023; **B) §** = main effect of sex p=0.049. *Post hoc:* **°** = vs sex p=0.012; **E) §** = main effect of sex p=0.035. *Post hoc:* **°** = vs sex p=0.036.

In the EPM, the main effect of sex was demonstrated for the percent distance travelled in both the open and closed arms (Fig.64 A: F(1,62)=5.511, p=0.023; Fig.64 E: F(1,65)=4.667, p=0.035) and for the percent number of entries in the open arms (F(1,70)=4.042, p=0.049) in adult mice. Bonferroni *post hoc* revealed a higher percent distance travelled in the closed arms in SAL/SAL males with respect to females in the same conditions (p=0.036) while in the open arms, Bonferroni's pairwise comparison demonstrated a decrease in the percent number of entries in males treated with LPS at PND21 and not in adulthood if compared to their female counterparts (p=0.012).

4.3.1.3 Effect of an immune challenge in adulthood on spatial recognition memory of male and female mice exposed to LPS or saline at PND21

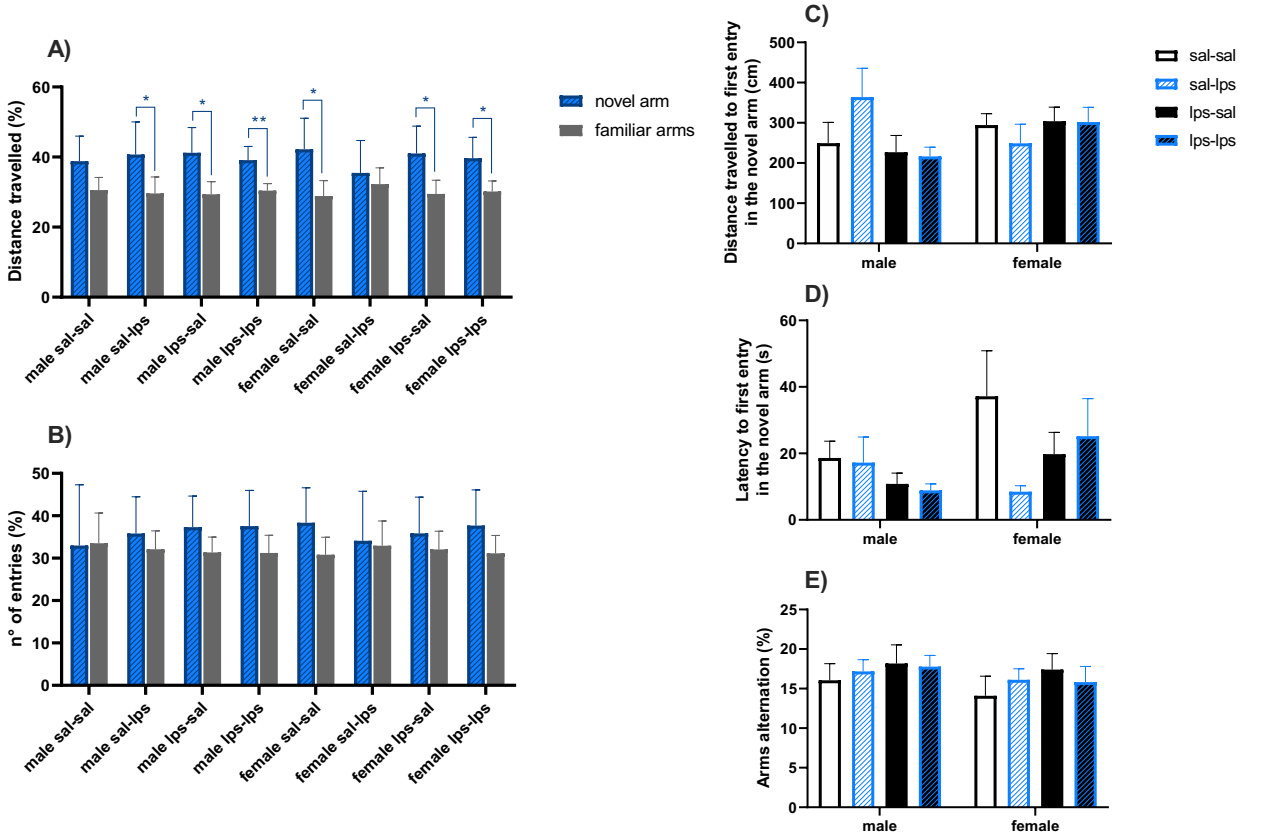

Figure 65. Effects on spatial recognition memory of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND21: Male and female mice were injected with LPS (100 µg/kg) or saline at PND21, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and tested after 27h (n=7-10 per group) in the YM. **A)** Total distance travelled in the novel arm vs familiar arms (%), **B)** number of entries in the novel arm vs familiar arms (%), **C)** distance travelled to first entry in the novel arm (cm), **D)** latency to first entry in the novel arm (s) and **E)** number of alternations (%) were recorded and expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by paired t test: **A)** Paired samples test: * = vs familiar arms p=0.045, * = vs familiar arms p=0.027, ** = vs familiar arms p=0.002, * = vs familiar arms p=0.016, * = vs familiar arms p=0.012, * = vs familiar arms p=0.02.

Paired samples t-test was performed to compare the distance travelled and number of entries between the "novel" arm and in the familiar arms in each group. While no significant difference was found for the number of entries in the novel or familiar arm, an increase in the distance travelled in the novel arm was observed in both males (SAL-LPS: t(8)=2.374, p=0.045; LPS-SAL: t(6)=2.905, p=0.027; LPS-LPS: t(8)=4.427, p=0.002) and females (SAL-SAL: t(8)=3.023, p=0.016; LPS-SAL: t(9)=3.117, p=0.012; LPS-LPS: t(7)=2.985, p=0.02), with the sole exception of SAL/SAL males for which the increase in the travelled distance in the NA failed to reach statistical significance. No differences were found for the alternation ratio among animals, distance or latency to first entry in the novel arm.

4.3.2.1 Effect of an immune challenge in adulthood on locomotor activity in an open field arena of male and female mice exposed to LPS or saline at PND35

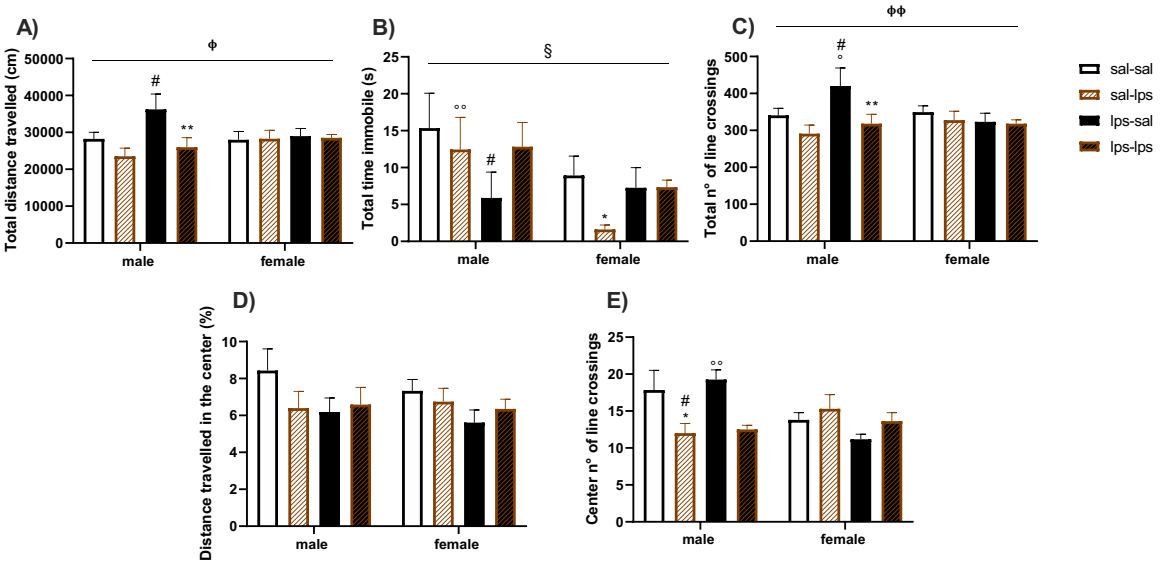

Figure 66. Effects on locomotor activity of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and tested after 23h (n=7-10 per group) in an OF arena. **A)** Total distance travelled (cm), **B)** total time immobile (s), **C)** total number of line crossings, **D)** distance travelled in the center (%) and **E)** number of line crossings in the center were recorded and expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni: **A) ϕ** = main effect of adult treatment p=0.028. *Post hoc*: **#** = vs adolescent saline, p=0.033, ****** = vs adult saline p =0.009; **B) §** = main effect of sex p=0.011. *Post hoc*: ***** = vs adult saline p=0.042, **°°** = vs sex p=0.007, **#** = vs adolescent saline p=0.046; **C) ϕϕ** = main effect of adult treatment p=0.01. *Post hoc*: ****** = vs adult saline p=0.01, **°** = vs sex p=0.011, **#** = vs adolescent saline p=0.035; **E)** *Post hoc*: ***** = vs adult saline p=0.015, **#** = vs adolescent saline p =0.015.

For adult animals exposed to LPS or saline at PND35, three-way ANOVA revealed a main effect of adult treatment for the total distance travelled and for the number of line crossings (Fig.66 A: F(1,60)=5.087, p=0.026; Fig.66 C: F(1,60)=7.078, p=0.01) while a main effect of sex was demonstrated for the time immobile (F1,60)=6.954, p=0.011). The interaction of sex and adult treatment was showed for total distance travelled and for both the total and number of line crossings in the central zone of the arena (Fig.66 A: F(1,60)=4.832, p=0.032; Fig.66 C: F(1,60)=4.512, p=0.038; Fig.66 E: F(1,59)=13.461, p=0.001) whereas the interaction of adolescent treatment*adult treatment was revealed for the time immobile only (Fig.66 B: F(1,60)=4.541, p=0.038). Bonferroni *post hoc* analysis revealed that males treated with LPS only at PND35 explored the arena significantly more: they travelled a higher total distance and performed an increased total number of line crossings with respect to the corresponding SAL/SAL (Fig.66 A: p=0.033; Fig.66 C: p=0.035) and to LPS/LPS group (Fig.66 A: p=0.009; Fig.66 C: p=0.015). Moreover, with respect to the matching females, male LPS/SAL showed a significant increase in both the total and centre number of line

crossings (Fig.66 C: (p=0.011; Fig.66 E: p=0.004). Supporting the higher locomotor activity of LPS/SAL males, a decrease in the total time immobile was revealed with respect to the SAL/SAL ctrl group (p=0.046).

In male animals the immune challenge experienced in adulthood caused a significant decrease in the number of crossings of the central zone with respect to the corresponding saline group, irrespective of the treatment received at PND35 (p=0.015 for both).

4.3.2.2 Effect of an immune challenge in adulthood on anxiety-like behaviour assessed in an elevated plus maze of male and female mice exposed to LPS or saline at PND35

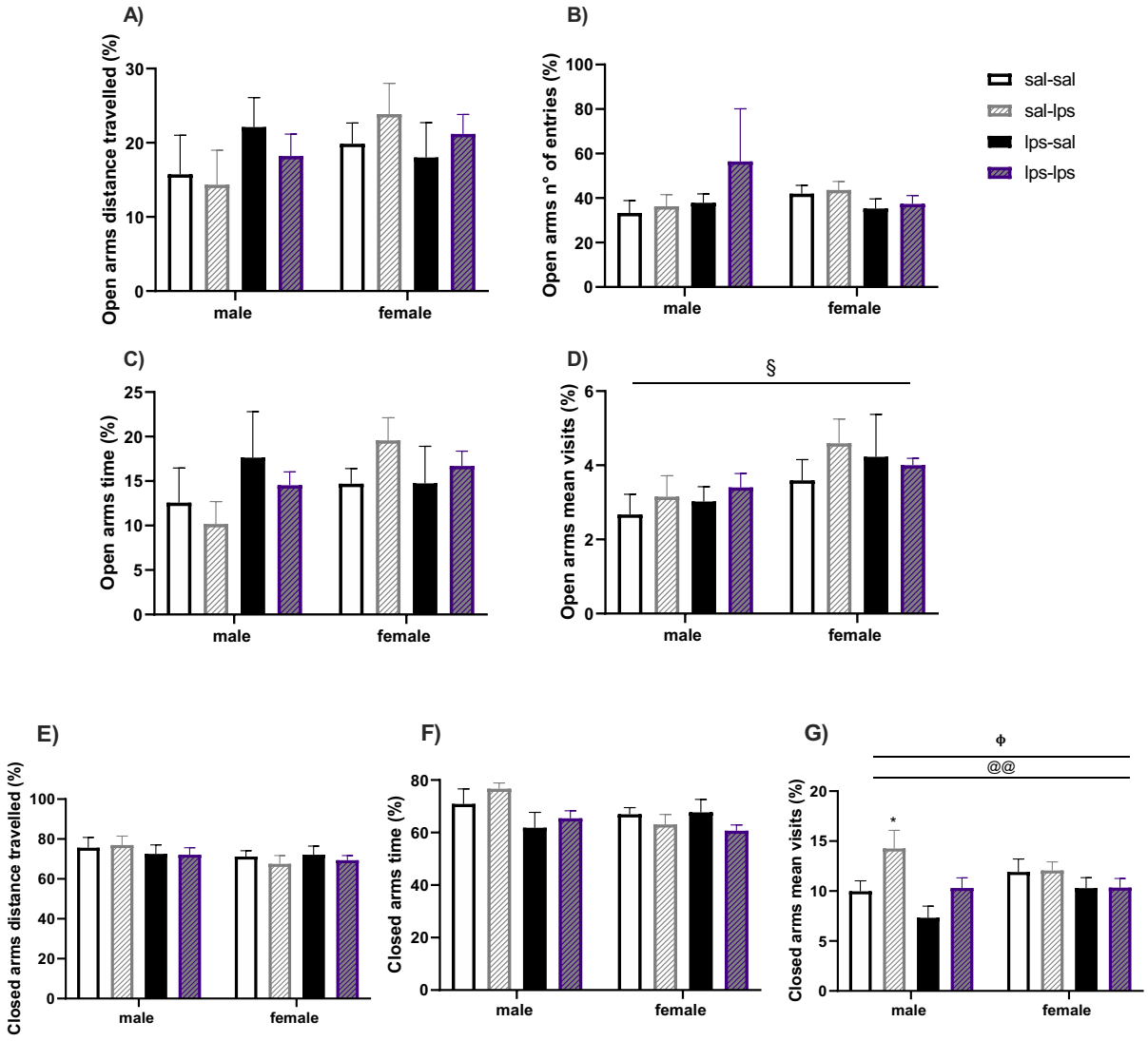

Figure 67. Effects on anxiety-like behaviour of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and tested after 25h (n=7-10 per group) in an EPM. **A)** Distance travelled in the open arms (%), **B)** number of entries in the open arms (%), **C)** time spent in the open arms (%), **D)** mean visits in the open arms (%), **E)** distance travelled in the closed arms (%), **F)** time spent in the closed arms (%) and **G)** mean visits in the closed arms (%) were recorded and expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by three-way ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni: **D) §** = main effect of sex p=0.029; **F)** Post hoc: ° = vs sex p=0.015; **G) @@** = main effect of adolescent treatment p=0.007, **ϕ** = main effect of adult treatment p=0.041. *Post hoc:* ***** = vs adult saline p=0.023.

In adult male and female C57BL/6J mice performing the EPM, three-way ANOVA demonstrated a main effect of sex for the open arms mean visits (Fig.67 D: F(1,60)=5.033, p=0.029) and the main effects of adolescent (F(1,58)=7.848, p=0.007) and adult treatment (F(1,58)=4.418, p=0.041) for the closed ones. The mean visits in the closed arm were increased in SAL/LPS males when compared to their controls (p=0.023).

4.3.2.3 Effect of an immune challenge in adulthood on spatial recognition memory in assessed in a Y maze of male and female mice exposed to LPS or saline at PND35

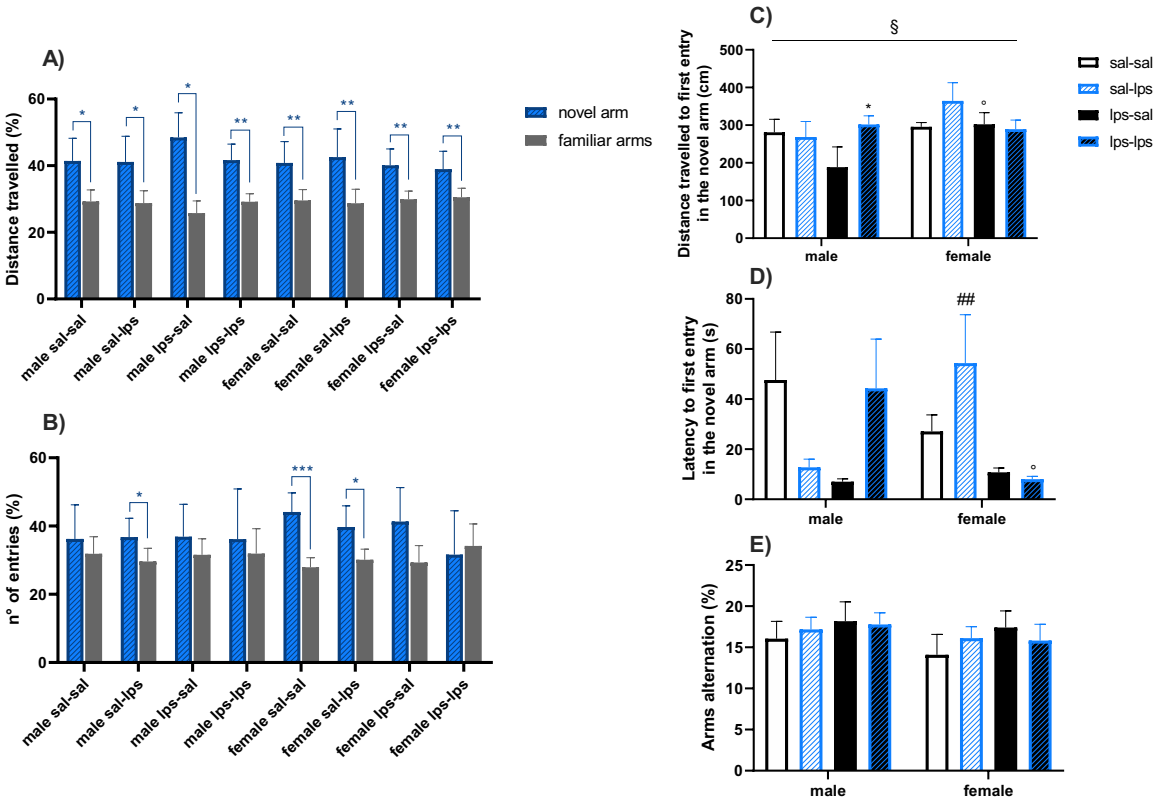

Figure 68. Effects on spatial recognition memory of a lipopolysaccharide (LPS) challenge in adult male and female mice receiving LPS or saline at PND35: Male and female mice were injected with LPS (100 µg/kg) or saline at PND35, re-exposed to LPS (830 µg/kg) or saline at 12wks of age and tested after 27h (n=7-10 per group) in the YM. **A)** Total distance travelled in the novel arm vs familiar arms (%), **B)** number of entries in the novel arm vs familiar arms (%), **C)** distance travelled to first entry in the novel arm (cm), **D)** latency to first entry in the novel arm (s) and **E)** number of alternations (%) were recorded and expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by paired t test: **A)** Paired samples test: * = vs familiar arms p=0.02, * = vs familiar arms p=0.026, ** = vs familiar arms p=0.01, ** = vs familiar arms p=0.008, ** = vs familiar arms p=0.003, ** = vs familiar arms p=0.007, ** = vs familiar arms p=0.006, ** = vs familiar arms p=0.009; **B)** Paired samples test: * = vs familiar arms p=0.017, *** = vs familiar arms p<0.001, * = vs familiar arms p=0.015, and by three-way ANOVA (sex*adolescent treatment*adult treatment) followed by Bonferroni: **C) §** = main effect of sex p=0.03. *Post hoc*: ***** = vs adult saline p=0.042, **°** = vs sex p=0.036; **D)** *Post hoc:* **##** = vs adolescent saline p=0.005, **°** = vs sex p=0.046.

By comparing the distance travelled and the number of entries between the "novel" and the familiar arm, significant differences were found in the distance travelled in the NA with respect to the FA in both males (sal-sal: t(6)= 3.138, p=0.02; sal-lps: t(6)= 2.905, p=0.026; lps-sal: t(4)= 4.598, p=0.01;

lps-lps: t(5)= 4.244, p=0.008) and females (sal-sal: t(10)= 3.843, p=0.003; sal-lps: t(9)= 3.464, p=0.007; lps-sal: t(7)= 3.936, p=0.006; lps-lps: t(9)=3.303, p=0.009) irrespective of the treatment received.

Differences were found also in the number of entries. Both males (t(6)=3.274. p=0.017) and females (t(8)=3.086, p=0.015) who experienced LPS only as adults and females saline-saline control group (t(8)=5.804, p<0.001) showed a higher number of entries in the NA with respect to the familiar arms.

For adult animals exposed to LPS or saline at PND35, the main effect of sex for the distance to first entry in the NA (F(1,54)=5.034, p=0.03) and the interaction of sex*adolescent treatment*adult treatment for both the distance (F(1,54)=4.871, p=0.032) and latency (F(1,57)=7.051, p=0.011) to first entry in the “novel” arm were revealed by a three-way ANOVA. LPS/SAL male's distance travelled before entering the NA for the first time was significantly less than their female counterparts (p=0.036) and the male LPS/LPS group (p=0.042). Adult females experiencing LPS both at PND35 and as adults showed a significantly reduced latency to the first entry in the NA with respect to both SAL/LPS counterparts (p=0.005) and the corresponding male group (p=0.046). As for PND21-12wks animals, no differences in the alternation ratio were revealed in our experimental conditions.

5. DISCUSSION

Infancy and adolescence emerged as crucial “critical time windows” of life in which stressors, like early life immune challenges, act altering brain maturity and neuroplasticity and associated outcomes may lead to the development of mental disease later in life, in adulthood through “programming”. In rodents, the peripheral systemic injection of LPS is the elective model used to mimic neuroinflammation by activating cytokine cascade, microglia and kynurenine pathway (KP), crucial for its involvement in tryptophan catabolism, linked to the development of several psychiatric disorders. Besides molecular imbalances, early-life exposure to LPS has been correlated to behavioural shifts as well as cognitive alterations in adulthood. This frame still lacks one topic: what are biological sex implications?

In the current study, we demonstrated that the effects evoked by an immune activation during critical windows of development, like juvenile, PND21, or adolescence, PND35, and the short-term and long-term molecular and behavioural consequences were differentially modulated in male and female C57BL6J mice, through three work-packages.

5.1 WORK-PACKAGE 1

We demonstrated body weight variations, central differential sex-based expression of neuroinflammatory-related targets as for peripheral tryptophan metabolism in male and female C57BL6J mice, 6 and 24h after receiving an i.p injection of 100 ug/Kg of LPS during juvenile (PND21) or adolescent (PND35) stage.

5.1.1 Impact of the immune challenge on body weight of male and female mice at PND21 and PND35

Body weight decrease observed in adult animals after an immune challenge can be strictly related to the sickness behaviour experienced, depending on the dose and serotype. In the early hours after the inflammatory hit, food and water intake are usually reduced, determining a transient decrease in body weight of about 10-15% (Danzer et al., 2008; Kane et al., 2017; Kinoshita et al., 2009). In contrast, in the juvenile animals, our data shows that the immune challenge determined a reduced weight gain in male mice and a moderate weight loss in females only in the late phase of the inflammatory process (after 24h). The weight variation was comparable between sexes, indicating that at this stage of life, body weight was not differentially influenced by sex dimorphism following an LPS-inflammatory hit. However, it is important to note that in mice, PND21 corresponds to weaning, thus the earliest stage in which animals begin to feed independently. The new housing

condition could potentially mask the autonomic effects of LPS. In adolescent animals, LPS caused a general weight loss in males at both 6 and 24h after LPS and for females only after 24h. Similar data were also demonstrated in the study of Ismail and collaborators (2013) but the LPS dose administered in mice was higher (1.5 μg/Kg) than ours as in the study of Yahfoufi et al. (2021) in 6wks old CD-1 males.

Notably, the present study showed that sex was a determining factor in modulating the effect of weight decrease: male mice were sensitised by the immune challenge more than females for this parameter specifically in the earlier phase of the infection (after 6h).

These findings suggest that the impact of an immune challenge on body weight is complex and varies with age, sex, and timing post-challenge. The early sensitivity in male adolescents could have implications for understanding sex-specific vulnerabilities to infections during developmental stages and may guide therapeutic interventions and nutritional support strategies during illness.

5.1.2 Sex-dependent modulation of inflammatory-related targets in the hippocampus of mice exposed to LPS at PND21 or PND35

The combined dialogue between pro-inflammatory and anti-inflammatory mediators pursued to protect the body against an exogenous agent is widely described in the literature in adult mice. In the case of LPS as a stimulus, the balance in favour of pro-inflammatory actors stands out in the earliest stage of the infection (Danzer et al., 2008) while decreasing in a time-dependent manner (Xu et al., 2017).

Our study highlights the differential hippocampal inflammatory response induced by systemic LPS injection during critical developmental windows. In particular, we found that the hippocampal inflammatory response induced following the systemic lipopolysaccharide injection during the juvenile critical time window of development resulted in the upregulation of pro-inflammatory cytokines, TNF-α and IL-1β, expression. In particular, we found their peaking at the very early stage of the inflammation, precisely after 6h as reported by Danzer and collaborators in 2008. Similarly to our results, Dinel and collaborators (2014) found a significant increase in the transcription of IL-1β, TNF-α and IL-6 in several CNS areas, including the hippocampus, in PND14 CD1 pups, thus in early-life, 3 hours after receiving LPS at the same dose and serotype employed in the present study.

Importantly, in our experimental condition, the inflammatory response of PND21 mice was differentially modulated between biological sexes. Females appeared to be more sensitive to the immune challenge, even after 24 hours, as the upregulation of IL-1β was still present at this time

point, the moment in which the pro-inflammatory mediators are supposed to go back to control levels (Xu et al., 2017). However, in the study of Couch and colleagues (2015) adult 8-week CD-1 mice injected i.p. with LPS displayed increased mRNA levels of IL-1β in the PFC but also TNF-α in both striatum and hippocampus. It should be taken into account that the dose administered was diff8-12 weeks0.5 mg/Kg, as the mice strain and associated age. Our data also showed an upregulation of IL-4 was upregulated after 24 hours, suggesting a balancing action of the anti-inflammatory system.

LPS induced a similar neuroinflammatory response also in adolescent mice with sex-dimorphism as a key factor in modulating this signalling as well: females showed the strongest hippocampal inflammatory response. In contrast to PND21 animals, but according to the activation of the anti-inflammatory signalling, IL-4 mRNA increased only in males treated with LPS and then sacrificed after 24h. IL-6 expression was not modulated by the LPS treatment in the hippocampus in both juvenile and adolescent male and female mice. In contrast to our findings, Dinel and collaborators (2014) reported a significant increase in the transcription also of IL-6. It is possible that the transcriptional effects on this cytokine may be differentially regulated in our conditions, in older animals of a different strain.

Overall, our findings underscore the importance of considering biological sex as a critical factor in modulating inflammatory responses and highlight the complex interplay between pro- and anti-inflammatory signalling in different developmental stages.

5.1.3 Biological sex influences microglial activation marker expression in the hippocampus of mice LPS-exposed at PND21 and PND35

The LPS-mediated immune challenge experienced as juveniles resulted in a mRNA upregulation of both the M1, CD11b and CD14 and M2, CD206, microglial markers of activation analysed in the hippocampus of both male and female mice. This effect was more prominent in the earlier phase of the neuroinflammatory process suggesting the expected shift of microglia from resting to activated phenotype in response to the infection.

The levels of expression of these markers were also modulated by LPS differentially in the two sexes, as only females showed higher levels of expression after 24h, together with Cx3cr1. Overall, the highest induction following LPS of the markers analysed was observed in females after LPS treatment. Considering the involvement of activated microglia in contributing to cytokine

production and their corresponding action, these data are our findings concerning the proinflammatory cytokines studied.

The immune challenge experienced in adolescence determined the mRNA upregulation of the microglial markers of activation regarding both M1 and M2 phenotypes in mice hippocampus. Among these, only CD206, the marker associated with the anti-inflammatory M2 phenotype, was differentially expressed between sexes, being nearly twice as high in females than in males, and only in the initial phase of the neuroinflammation. The other two targets, CD14 and CD11b were both induced, but their mRNA levels were comparable between males and females, indicating that sex did not modulate their expression in this stage of life. The same was observed also for Cx3cr1. Thus, our findings further corroborate the significant role of biological sex in modulating microglial activation and the inflammatory response. They highlight the complex interplay between pro- and anti-inflammatory signalling in different developmental stages, with females showing a more robust response to immune challenges.

5.1.4 Sex dimorphism differentially modulates the central expression of the KP rate-limiting enzyme IDO and serum peripheral shifts of TRP and KP after an immune challenge experienced at PND21 and PND35 in male and female mice

Our study reveals a critical role of sex differences in regulating Indoleamine 2,3-dioxygenase – the rate-limiting enzyme of the KP pathway - expression during key developmental periods under immune challenge. This regulation may have significant implications for understanding the broader impact of neuroinflammation on behaviour and development.

Literature reports its transcriptional peaking at 8h and enzymatic activity at 24h after LPS (10 ng/ml) in microglial cells obtained from <PND2 C57BL6J mice brains, increasing extracellular kynurenine (Wang et al., 2009). IDO activity after 24 hours is mainly associated with depressive-like behaviour (Danzer et al., 2008). Evidence from other studies also showed its mRNA induction 6h after the immune challenge as well in the hippocampus of adult mice of the same strain used in our study after an i.p. injection of LPS at a higher dose (0.83 mg/Kg) (Alboni et al, 2021). A similar effect was reported following an intracerebroventricular administration of 100 ng of LPS in 8-12wks C57BL6J mice (Fu et al., 2010). According to this, in the present research juvenile animals showed induced IDO expression 6h after the LPS injection. Its modulation was related to sex-based differences as, according to data concerning the other pro-inflammatory mediators studied, the target was induced in females. Consistent with the *milieu* promoted by the immune challenge, the hippocampal expression of this enzyme was induced under neuroinflammatory conditions also when experienced in mice adolescence. In contrast to juvenile animals, IDO was induced also in males both in the early

and late phases of the LPS-mediated acute inflammation. However, the process prompted more central IDO expression in females but only, again, after 6h. Altogether, these findings indicate that sex differences are crucial in regulating the expression of this enzyme when the immune challenge is experienced in this critical time window of development.

IDO functioning is the main responsible for TRP catabolism (Wang et al., 2009). TRP is the main source for 5-HT production and only 5% of it is converted into serotonin in physiological conditions, the remaining percentage, which is the most, is used as a substrate to be converted into KYN through the activation of KP (Savitz et al., 2019). Under inflammatory conditions, proinflammatory cytokines shunt the metabolism of TRP towards KYN by upregulating IDO expression (Savitz et al., 2019). This makes tryptophan less available to be converted into serotonin with potential implications for mood disorders.

Thus, in the present study, we also investigated the peripheral modulation of TRP metabolism by LPS in both juvenile and adolescent animals.
After 24h, LPS injection determined a higher production of TRP only in females, suggesting a role for sex-dimorphism in the late phase of the acute inflammation on tryptophan metabolism at PND21, but not at PND35.
In PND35, the primary shifts following LPS began with changes in serotonin levels. Its serum concentration was decreased, as was found for juvenile LPS-treated animals. In adolescents, this effect was not influenced by sex-dimorphism or by the timing following i.p. injection while in PND21 lowered serum circulating serotonin levels were observed in the initial stage of the inflammatory process in both males and females, but in males, serotonin remained low even after 24h. No previous data are available on the effect of an immune challenge on serum levels of 5-HT in young animals. Considering that lower levels of both TRP and 5-HT correlate with depressive symptoms, together with the central expression of the inflammatory-related target, these peripheral findings may remark on the vulnerability of the female sex in both critical time windows of life.

Entering in KP, the first active metabolite produced after IDO action on tryptophan, KYN, was not influenced in our experimental conditions. Peripheral levels of kynurenic acid (KYNA), and anthranilic acid (ANA) were measured and then analysed as a ratio to kynurenine. These analyses provided an overview of the two alternative branches of the metabolic pathway to understand which among them was most activated during inflammation.

However, the immune challenge experienced at PND21 lowered only male levels of the KYNA/KYN ratio in the early stage of inflammation, while no effects were revealed for AA/KYN, and both the KYNA/KYN and AA/KYN ratio decreased in males and females.

This decrease may indicate a higher presence of kynurenine metabolite compared to kynurenic acid and anthranilic acid, suggesting a shift of the pathway toward the main branch, the quinolinic acid branch, which leads to the production of the more neurotoxic-associated KP metabolites.

These findings underscore the importance of sex differences in the regulation of TRP metabolism and the inflammatory response during critical developmental windows. The observed shifts in KP metabolites suggest a potential pathway towards neurotoxicity, particularly highlighting the heightened sensitivity and vulnerability of females to LPS-induced inflammation.

5.2 WORK-PACKAGE 2

The present study reports also the adult weight shifts induced by an i.p. injection of 830 ug/Kg of LPS at 12wks after a previous exposure at PND21 or 35 (100 ug/Kg), central hippocampal expression of the main pro-inflammatory-related targets and peripheral TRP metabolism in serum of male and female C57BL6J mice. By highlighting the enduring impact of early-life LPS exposure on adult weight regulation, neuroinflammatory responses in the hippocampus, and peripheral TRP metabolism within a sex-specific context, this study enhances our knowledge of developmental programming and vulnerability to inflammatory insults.

5.2.1 Experiencing an immune challenge as adults affects the body weight of mice exposed to LPS or saline at PND21 or PND35 according to sex-dimorphism

The weight checked weekly followed a trend which was almost completely overlapping between animals regardless of whether they received the LPS both as juveniles or as adolescents suggesting no effects of experiencing the immune challenge as a stressful event on animals' growth until adulthood. However, experiencing the immune challenge as adults impacted the weight gain of animals according to sex-based differences, with the great weight loss observed primarily in male mice. In particular, in the initial stage of the adult inflammation, males exhibited weight shifts, only if previously sensitised through an homotypic stimulus both at PND21 or PND35 suggesting a synergic role of LPS/LPS affecting majorly this sex. Adult females were shown to be sensitive to weight shifts when subjected to LPS only as juveniles and a comparable weight decrease was observed when LPS-injected only at 12 weeks.

In the late stage of inflammation, thus, 24h after the adult inflammatory hit, although the inflammation in adulthood evoked a weight decrease in both males and females irrespective of the

treatment received at PND21 or 35, females displayed a reduced weight decrease when previously exposed to LPS as males showed the greater decrease. It is important to note that although the males were found to be more vulnerable to this parameter, this process seems to be mitigated when they were first exposed to LPS in adolescence and then again as adults.

Overall, while males appeared more vulnerable to weight changes induced by adult inflammation, early-life LPS exposure, especially during adolescence, seemed to mitigate this vulnerability. This highlights a potentially antagonistic role of early-life immune challenges in modulating stress responses and body weight regulation later in life. Further research into the mechanisms underlying these sex-specific responses is warranted to better understand developmental programming and resilience to inflammatory insults.

5.2.2 Sex-specific modulation of hippocampal inflammatory-related targets in adult mice exposed to LPS or saline at PND21 or PND35

The inflammation induced by the systemic injection of LPS as adults upregulated hippocampal mRNA levels of TNF-α and IL-1β in males irrespective of the treatment received at PND21 or 35. Differently, in females, the upregulation of these cytokines was present only in animals experiencing LPS as adults and in females previously exposed to LPS but only as adolescents and not at PND21. Adult males also showed hippocampal IL-6 downregulation only when experiencing the immune challenge as adults while when previously exposed as adolescents, this cytokine was strongly upregulated both with respect to LPS/SAL and to matching female counterparts.

Moreover, the immune challenge differentially modulated the expression levels of the pro-inflammatory cytokines studied according to sex only when the stimulus had been already experienced in a critical time window of development as demonstrated for both TNF-α (PND21-12wks) and IL-1β (PND21 or 35-12wks).

Similar data were observed in the study of Sharma and colleagues (2019) but in the PFC of CD-1 mice. Indeed, as male mice showed higher levels of expression of pro-inflammatory cytokines with respect to females, we can speculate on a synergic effect of LPS on potentiating the hippocampal male's neuroinflammation process in response to the homotypic stress later in life when previously experienced in a critical time window of development.

Our data suggest that LPS may synergistically potentiate neuroinflammatory processes in the hippocampus of males when exposed to homotypic stress later in life, particularly if they experienced LPS during critical developmental windows. This highlights the importance of understanding sex-specific responses to early-life immune challenges and their implications for

neuroinflammation and stress responses in adulthood. Further research into these mechanisms could elucidate potential therapeutic strategies for mitigating inflammatory responses and promoting resilience in vulnerable populations.

5.2.3 The immune challenge experienced during the critical time windows of development juvenile (PND21) and adolescence (PND35) programmed the microglial later in life response after a second exposure at adulthood in a sex-dependent manner

In the present study, we investigated the effects of adult LPS-mediated systemic inflammation on hippocampal microglial marker expression in both male and female C57BL6J mice. Our analysis focused on CD11b, CD14, Cx3cr1, and CD206, markers associated with microglial activation and phenotype. Interestingly, we found that exposure to LPS during critical developmental windows—specifically in juvenile and adolescent stages—resulted in distinct patterns of microglial marker expression in adulthood. This effect was present also for CD11b in both sexes when previously exposed to the inflammatory hit both in the juvenile and adolescent critical time windows of development. This effect was not present for CD14 and Cx3cr1 in PND21-12wks females while it was elicited in PND35-12wks. The mRNA expression of the marker CD206, associated with the M2 alternative microglial phenotype, remained unaltered after LPS irrespective of the moment in which animals experienced the immune challenge. In contrast, in PND35-12wks animals, the adult LPS injection induced CD206, but the modulation was not regulated by previous exposure to the homotypic stress or by differences between sexes.

It is important to notice that our findings showed that experiencing an immune challenge previously in adolescence differentially modulated the later-in-life microglial response by upregulating its expression marker of M1 phenotype, CD11b, namely the one related to promoting the inflammatory process but in an attenuated way in contrast to animals who experienced the immune challenge only once, thus, only in adulthood. The same was observed for Cx3cr1 but only CD11b expression followed a sex-based modulation by manifesting also a greater antagonistic LPS/LPS effect in males with respect to the opposite sex.

In sum, females showed increased microglial activation after LPS when experienced only in the juvenile critical time window of development or when receiving LPS only as adults as demonstrated for CD11b, CD14, and Cx3cr1, while in the case of receiving homotypic stress experienced in the adolescent critical time window of development and again later in life sensitizes more female sex prompting their microglial response to the immune challenge more than in male sex.

Our findings underscore the critical role of developmental timing and sex in shaping microglial responses to immune challenges. Understanding these dynamics is crucial for elucidating neuroinflammatory mechanisms and exploring therapeutic strategies aimed at modulating microglial activation in various neurological conditions. Future research should delve deeper into the molecular pathways underlying these sex-specific responses to further inform targeted interventions and improve outcomes in neuroinflammatory disorders.

5.2.4 Sex dimorphism differentially modulates the central expression of the KP rate-limiting enzyme IDO and serum peripheral shifts of TRP and KP in adult LPS-treated mice after an immune challenge previously experienced at PND21 and PND35

The immune challenge lowered the KP limiting enzyme IDO mRNA only in adult females, indicating that its regulation was differentially modulated according to sex and not to a programmed response in PND21-12wks animals. On the other hand, LPS evoked a lasting effect on adult male mice after a prior immune challenge experienced during adolescence, leading to IDO induction. This upregulation occurred at a level comparable to that observed in males experiencing the stressful event only in adulthood. Sex played a role in modulating this effect as females did not exhibit a similar activation of the central KP under these conditions while the opposite was true for males, by hippocampal gene expression. Thus, these results further underscore the importance of considering sex-specific responses in neuroinflammatory processes and suggest avenues for further research into the molecular mechanisms underlying these differential regulatory patterns.

According to central IDO induction that emerged in our results, the effect of the LPS inflammatory insult affected the tryptophan metabolism, directly altering its levels in a sex-dependent manner, impacting mainly on males: experiencing the inflammatory hit only as adults evoked a decrease in their serum TRP concentration comparable to animals insulted only in adolescence, indicating that undergoing the inflammatory process during this critical stage of life has lasting effects, enduring until adulthood. However, when the insult was experienced again at 12 weeks, TRP reduction was attenuated, suggesting that prior exposure to homotypic stress can lead to a “more resilient” response later in life concerning this metabolic pathway within this sex. In contrast, males showed overall lower levels of this metabolite in their serum with respect to females in PND21-12wks animals but the immune challenge did not evoke shifts of the mice serum tryptophan concentration.

Downstream-related processes related variations were profoundly linked to experiencing the stressful inflammatory hit. Exposure to LPS as adults resulted in a reduction in serum serotonin concentration. This effect was differentially modulated between sexes, as only males, not previously exposed to the immune challenge, showed lower levels with respect to their controls. These data are in agreement with studies showing a decrease in 5-HT levels measured centrally, however, particularly in the hippocampus of adult male C57BL6/J strain mice sacrificed twenty-four hours after intraperitoneal injection with LPS (0.5 mg/kg) (Phing et al., 2023).

Our data suggests that experiencing inflammation during critical time windows evokes lasting and additive effects on later life response to a homotypic stressor: the modulation of serotonin was sex-specific under these conditions, impacting primarily on males also in this case by decreasing 5-HT levels.

As reported in the literature, the adult immune challenge affected TRP catabolism toward KP activation (Savitz et al., 2019). Its first active metabolite, KYN, was augmented after the adult LPS injection in males irrespective of the treatment received at PND21 or 35. This increase is concordant with previous studies stating that, under neuroinflammatory conditions, TRP shunt occurs from the pathway leading to 5HT synthesis to KYN formation. Differently, in females, KYN levels increased only in animals experiencing LPS as only as adults only if receiving saline at PND21, and when previously exposed to LPS both as juveniles or as adolescents. According to a sex-differential modulation, after the adult immune challenge, KYN increase was significantly higher in females than in males even when previously exposed at PND21 or 35.

Importantly, although experiencing the inflammatory hit once as adults when previously exposed to LPS at PND21 increased kynurenine levels in female mice serum compared to their controls, KYN concentration was found to be decreased with respect to females experiencing LPS only at 12wks underling a potential antagonistic effect of LPS/LPS within female sex. We can speculate on the KYN trend suggesting that females previously exposed to LPS as juveniles attenuated the peripheral KYN response in adulthood to a homotypic inflammatory stress later in life.

Given these findings, it is interesting to note that TRP metabolism downstream-related processes are so differentially modulated between sexes to “run” two distinct paths: females seem to respond to the inflammation with greater KP activation while males exhibit a shift toward TRP-serotonin signalling.

Additionally, KYN-related ratio analysis showed a reversed trend with respect to KYN concentrations, indicating a higher presence of KYN with respect to the other metabolites of KP studied suggesting a potential preferential shift to the QA main branch of KP, responsible for producing the most neurotoxic metabolites. This was observed both in PND21- or 35- 12 weeks animals.

5.3 WORK-PACKAGE 3

Lastly, we also demonstrated behavioural impairments induced by an i.p. injection of 830 ug/Kg of LPS at 12 weeks after a previous exposure at PND21 or 35 (100 ug/Kg) in C57BL6J mice.

5.3.1 The immune challenge experienced in as juveniles and as adolescents programmed the later in life behavioural response of mice exposed to LPS as adults in a sex-dependent manner

Literature reports that systemic inflammation in the hippocampus is responsible for behavioural shifts with TNF-α and IL-1β as main actors (Dinel et al., 2011; Coach et al., 2015). Also, KP seems to be implicated with cognitive behaviour alteration, as described in the Review of Phing and colleagues (2023). According to sickness- and depressive-like behaviour, and consequent general host activity reduction induced by LPS, it is shown that the administration of this endotoxin at the specific dose of 0.83 mg/Kg in Swiss albino mice results in decreased locomotor activity in the open field 3 and 24h after the injection, as indicated by a reduction in both central and peripheral crossings, and increased anxiety-like in the EPM highlighted by a reduced time spent in the open arms (Jangra et al., 2016). Similar data were reported in Balb/C adult mice after LPS at the same dose in the study of Ekci et al. (2023). Findings also report impaired cognitive functions such as spatial memory in the Y-maze after an inflammation LPS-mediated at different doses, from 0.25 up to 0.75 mg/Kg in adult Swiss mice (Bahaidrah et al., 2022). Concerning the emerging concept of "programming", impairments in anxiety-like behaviour in the EPM and the OF or a novel environment were also observed in adult animals following juvenile stress in rats (Cymerblit-Sabba et al., 2015)

In the present study, data obtained in the EPM showed that anxiety-like behaviour was not induced in our experimental conditions. However, adult animals exposed to the immune challenge displayed impairments in different behavioural domains according to the "cytokine model of cognitive function" (Fourrier et al., 2019).

In the open field test, PND21-12wks male mice subjected to LPS as adults travelled a shorter total distance, spent higher total time immobile and performed fewer total and central crossings with

respect to their controls and matching female counterparts. Moreover, this group spent significantly a higher time immobile than males previously exposed to LPS at PND21. Similar data were obtained also in PND35-12wks males.

Considering that mice are curious animals with a natural predisposition to explore the environment, data obtained in this study demonstrate that experiencing the immune challenge as adults modulated the explorative behaviour of mice suggesting a reduction in the locomotor activity of both PND21- and 35-12wks males. Depending on the dose and serotype, sickness, as measured by decreased locomotion in the OFT, occurred at 2 h after LPS treatment and dissipated at 24 h in mice treated with a low dose of LPS. Animals treated with higher doses of LPS, however, still showed reduced locomotor activity at this point, indicating that sickness remained present in these mice (Biesmans et al., 2013)

Differences emerged when animals experienced LPS at PND21 and 35.

Adult males LPS-injected only as juveniles showed higher immobility time and reduced parameters associated with locomotion, also with respect to their female counterparts and nearly an opposite trend was displayed by males injected as adolescents.

This can suggest that concerning explorative behaviour, the single inflammatory exposition during a critical time window as juveniles or adolescents can determine a differential programmed response according to sex differences benefiting males previously exposed as adolescents. However, it is important to highlight that hyper-locomotion is also associated with anxiety-like behaviour, thus, further investigation to better distinguish these behavioural domains might help clarify these results.

Overall, PND21-12wks animals showed no impairments in terms of both memory and locomotion or exploration irrespective of the moment in which they received the immune stimulation in the Y maze after the early life exposure to an immune challenge and no enduring effects were evoked after a subsequent experience of homotypic stress in adulthood. It is important to consider that Y-maze requires minimal resources, as well as animal training and output. Therefore, it is widely used to study short-term memory. However, this test may not have brought out the complexity of the associated cognitive impairments in our experimental condition. A similar trend was shown in PND35-12wks although females experiencing LPS only as adolescents showed a higher distance travelled before entering the NA for the first time than their male counterparts. Despite being a sign of memory impairment, this parameter can also be an index of a higher anxiety-like behaviour shown in this sex.

5.4 Conclusions

Data that emerged in this study demonstrated that the exposure to stress, such as an immune challenge, experienced in a critical time window of development impacts the individual broadly. Indeed, molecular effects were evoked resulting in lasting marks associated with consequences on body response and different behavioural outcomes later in life following a homotypic stimulus, with sex-dimorphism as the main substrate. Altogether these results help to integrate the knowledge of biological mechanisms linking sex and vulnerability to the development of mental diseases and to progressively clarify the therapeutic approaches to be adopted to effectively address them.

5.5 Considerations and Future Perspectives

Considering the importance and complexity of psychiatric disorders and in light of the results obtained in this study in the hippocampus, it would be interesting to investigate the effect of a programmed response on inflammatory targets also in other brain areas such as the amygdala and prefrontal cortex. PFC is involved in decision-making and it is one of the last in reaching maturity later in life while the amygdala, belonging to the limbic system, is the main actor involved in the regulation of emotions. Both areas are sensitive to stress and are involved in the onset of brain diseases.

Moreover, deepening the effect of repeated stress in more than one critical time window starting from the prenatal and perinatal period should be a topic to be focused on as we found substantial differences in the central and peripheral inflammatory response in animals, modulated by sex differences even before reaching sexual maturity and by LPS-programming with enduring effect related to sex differences as well.

In the periphery, the micro-environment characterised by the gut microbiota is sensitive to stress. Notably, the gut is also considered our “second-brain”. Thus, considering its microbiome eventual variations in our experimental condition could integrate an important piece of the “puzzle” concerning the body's later-in-life response to early-life adverse events.

As in our study, the main behavioural shifts emerged in the field of locomotor activity and short-term memory. Despite being bound to cognitive impairments, psychiatric disorders are strictly related also to anxiety and related disturbs, thus deepening the anxiety-like behaviour through the use of even more specific behavioural tests such as resident intruder, light dark or marble burying

tests can help to deeply characterise the effects of a programmed response induced by an inflammatory hit repeated later in life on this behavioural domain.

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