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A study of the behavioral, anatomical, metabolic and transcriptomic disturbances associated to cocaine addiction-like behaviour in a two-hit animal model of schizophrenia-related symptoms

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ABBREVIATIONS KEY

2-CSRTT: 2 choice serial reaction time task.
5-CSRTT: 5 choice serial reaction time task.
5-HT: 5-hydroxytryptamine or serotonin.
ACTH: Adrenocorticotropic hormone.
AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.
AMYG: Amygdala.
ANOVA: Analysis of variance.
ASD: Autism-spectrum disorder.
CCL2: C-C motif ligand 2 chemokine.
CCL7: C-C motif ligand 7 chemokine.
CD68: Cluster of Differentiation 68.
CNS: Central nervous system.
CPP: Conditioned place preference.
Cr: Creatine.
CRH: Corticotropin-releasing hormone.
CS: Conditioned stimulus.
CSPP: Cortico-striato-pallido-pontine.
CT: Computed tomography.
CXCL1: C-X-C motif ligand 1.
CXCL10: C-X-C motif ligand 10.
CXCL2: C-X-C motif ligand 2.
CXCL8: C-X-C motif ligand 8.
DA: Dopamine.
DISC1: Disrupted-in-schizophrenia 1.
DTI: Diffusion tensor imaging.
DTNBP1: Dystrobrevin-binding protein 1.
FA: Fractional anisotropy.
FDR: False discovery rate.
FGF: Fibroblast growth factor.
FOV: Field-of-view.
FR1: Fixed ratio 1.
FR3: Fixed ratio 3.
GABA: *γ*-aminobutyric acid.
GCs: Glucocorticoids.
GD: Gestational day.
GFAP: Glial fibrillary acidic protein.
GPC: Glycerylphosphorylcholine.
GPe: External globus pallidus.
GPi: Internal globus pallidus.
HE: Head entry.
HLA-DR: Human Leukocyte Antigen-DR isotype.
HPA: Hypothalamic-pituitary-adrenal axis.
IFN-γ: Interferon-γ.
IL-10: Interleukin-10.
IL-13: Interleukin-13.
IL-1β: Interleukin-1β.
IL-25: Interleukin-25.
IL-4: Interleukin-4.
IL-6: Interleukin-6.
ITI: Inter-trial interval.
ICSS: Intracranial self-stimulation.
LPR1: Lever 1 (active) presses.
LPR2: Lever 2 (inactive) presses.

LPS: Lipopolysaccharide.
LPTOR1: Lever 1 presses during the time out.
LPTOR2: Lever 2 presses during the time out.
MAM: Methylazoxymethanol.
MCP-1: Monocyte chemoattractant protein-1.
MD: Mean diffusivity.
MIA: Maternal immune activation.
MRI: Magnetic resonance imaging.
MRS: Magnetic resonance spectroscopy
MSNs: Medium spiny neurons.
NAA: *N*-acetylaspartate.
NAAG: *N*-acetyl-aspartyl-glutamate.
NAcc: Nucleus accumbens
NMDA: *N*-methyl-D-aspartate.
NPY: Neuropeptide Y.
NS: No stress.
PCh: Phosphocholine.
PCP: Phencyclidine.
PCR: Polymerase chain reaction.
PCr: Phosphocreatine.
PET: Positron-emission tomography.
PFC: Prefrontal cortex
PGE_2: Prostaglandin E2.
PND: Post-natal day.
Poly I:C: Polyinosinic:polycytidylic acid.
POMC: Pro-opiomelanocortin.
PPI: Prepulse inhibition.
PR: Progressive ratio.
PUS: Peripubertal unpredictable stress.
qPCR: Quantitative polymerase chain reaction.
RARE: Rapid Acquisition with Relaxation Enhancement.
RIN: RNA integrity number.
S: Stress.
S.A.: Self-administration.
SAL: Saline.
SD: Standard deviation.
SEM: Standard error of the mean.
SNc: Substantia nigra *pars compacta*.
SNr: Substantia nigra *pars reticulata*.
STN: Subthalamic nucleus.
TE: Echo time.
TGF-β: Transforming growth factor-β.
THAL: Thalamus.
TLR-3: Toll like receptor 3.
TLR-4: Toll like receptor 4.
TNF: Tumor necrosis factor.
TNF-α: Tumor necrosis factor-α.
TR: Repetition time.
VGLUT1: Vesicular glutamate transporter 1.
VGLUT2: Vesicular glutamate transporter 2.
VR10: Variable ratio 10.
VR5: Variable ratio 5.
VTA: Ventral tegmental area.
Zn: Zinc.
η^2p: Partial eta squared.

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ABSTRACT

Prenatal infections and traumatic experiences around puberty are risk factors for the development of schizophrenia in adulthood, predominantly in men. In addition, there is a high incidence of addictive disorders among schizophrenic patients, a fact that usually worsens the prognosis of the disease. As a consequence, the characterization of the disturbances underlying this comorbidity is essential to implement more effective therapies in these patients. In the present study, we used a two-hit animal model of schizophrenia-related symptoms to study the presence of behavioral, anatomical, metabolic and transcriptomic alterations associated with cocaine addiction. To this end, we intraperitoneally injected 100 µg/kg/ml of lipopolysaccharide (LPS) or saline on gestational days 15 and 16 to pregnant rats. Their male offspring was then subjected to five episodes of stress or handling on alternate days during postnatal days (PND) 28 to 38. Once the animals reached adulthood (PND70), we evaluated prepulse inhibition (PPI), working memory and social interaction. At PND90, motor impulsivity and different parameters of cocaine addiction self-administration were analyzed to look for cardinal features of addiction. Magnetic resonance imaging (MRI, DTI) and spectroscopy (*in/ex vivo* H^1MRS), positron emission tomography (PET), and RNA massive sequencing (RNA-Seq) studies were employed to quantify potential anatomical, metabolic and transcriptomic changes in the brain. Pubertal stress reduced cocaine self-administration, an effect that was reversed in LPS-exposed animals. Conversely, LPS-exposed rats showed increased motivation for cocaine consumption. This intake pattern proved to be specific for cocaine, since no significant differences were observed between groups in pavlovian or instrumental conditioning programs with food as reinforcer. Moreover, no modulatory effects of motor impulsivity were observed. At the anatomo-metabolic level, prenatal LPS exposure reduced whole brain volume and increased hippocampal mean diffusivity in both hemispheres. Pubertal stress reduced right hippocampal volume and mesencephalic activity and increased cortical and dorsostriatal volumes, and hipocamapl activity. In addition, striatal glutamatergic neurotransmission was reduced by prenatal LPS exposure and increased after pubertal stress. These results suggest a complex interplay between the effects caused by prenatal immune activation and pubertal stress on neurodevelopment and call for more detailed studies of the comorbidity between schizophrenia and addiction.

RESUMEN

Las infecciones prenatales y las experiencias traumáticas en torno a la pubertad son factores de riesgo para el desarrollo de esquizofrenia en la edad adulta, sobre todo en hombres. Además, existe una alta incidencia de trastornos adictivos entre pacientes esquizofrénicos, lo que generalmente empeora el pronóstico de la enfermedad. Por tanto, la caracterización de las perturbaciones subyacentes a esta comorbilidad se vuelve esencial para implementar terapias más eficaces en dichos pacientes. En el presente estudio, empleamos un modelo animal de doble impacto cuyos síntomas se han relacionado con la esquizofrenia para estudiar la presencia de alteraciones conductuales, anatómicas, metabólicas y transcriptómicas asociadas con la adicción a la cocaína. Para ello, inyectamos intraperitonealmente 100 µg/kg/ml de lipopolisacárido (LPS) o solución salina los días gestacionales 15 y 16 a ratas preñadas. Sus descendientes machos fueron sometieron a cinco episodios de estrés o amansamiento en días alternos durante los días postnatales (PND) 28 a 38. Una vez que los animales alcanzaron la edad adulta (PND70), evaluamos la inhibición prepulso (PPI), la memoria de trabajo y la interacción social. En el PND90, se analizaron la impulsividad motora y diferentes aspectos de la autoadministración de cocaína en busca de rasgos cardinales de adicción. Estudios de imagen (MRI, DTI) y espectroscópicos (*in/ex vivo* H^1MRS) por resonancia magnética, de tomografía por emisión de positrones (PET) y de secuenciación masiva de ARN (RNA-Seq) fueron empleados para cuantificar posibles cambios anatómicos, metabólicos y transcriptómicos en el cerebro. Nuestros resultados mostraron que el estrés puberal redujo la autoadministración de cocaína, efecto que se vio revertido en animales expuestos a LPS, que también mostraron una mayor motivación por la droga. Este patrón de consumo resultó ser específico para la cocaína, ya que no se observaron diferencias significativas entre grupos en programas de condicionamiento pavloviano ni instrumental con comida. Además, no se encontraron efectos moduladores de la impulsividad motora. A nivel anatomo-metabólico, la exposición prenatal a LPS redujo el volumen total del cerebro y aumentó la difusividad media del hipocampo en ambos hemisferios. El estrés puberal redujo el volumen del hipocampo derecho y la actividad mesencefálica, al tiempo que aumentó el volumen cortical y dorsoestriatal, así como la actividad hipocampal. Además, la neurotransmisión glutamatérgica estriatal se redujo por la exposición prenatal a LPS y aumentó tras la combinación de estrés puberal probablemente. Estos resultados sugieren una compleja interacción entre los efectos provocados por la activación inmune prenatal y el estrés puberal sobre el neurodesarrollo, y llaman la atención sobre la necesidad de realizar estudios más detallados sobre la comorbilidad entre esquizofrenia y adicción a cocaína.

INTRODUCTION

1. A general overview of the problem of addiction among people suffering from mental diseases: the dual diagnosis conundrum.

Addiction and schizophrenia are two of the most debilitating and impairing mental disorders. There are mutual interactions between both conditions that influence the diagnosis and prognosis of the patients. Indeed, the high frequency of co-occurring substance use and abuse in schizophrenia patients has been linked to worse outcomes (Winklbaur, Ebner, Sachs, Thau, & Fischer, 2006). Both psychopathologies are closely interdependent; therefore, a particular treatment for schizophrenic patients with addiction comorbidity is needed in order to improve the prognosis of these individuals. The epidemiological data that we have to date do not allow to draw definite conclusions about the causal relationships between schizophrenia and addiction. In order to advance in our understanding of the psychobiology of dual diagnosis, the use of animal models arises as a crucial strategy. In this thesis, we will take advantage of a recent animal model of neurodevelopmental disorders that recapitulates several diagnostic features of schizophrenia, and that is based on two developmental challenges that have been documented in some patient cohorts: prenatal infections and stress experiences during adolescence. By using this approach, we will examine the proclivity of the animals to show cardinal features of addiction in a cocaine self-administration paradigm and we will analyze some behavioral, anatomical, transcriptional and metabolic alterations in the experimental animals to experimentally determine the causal relationships that are operating in the schizophrenia-addiction duality. In the following pages, we will begin by exploring the environmental factors affecting brain development in specific critical periods to then focus our discussion in the dual diagnosis issue and the experimental approaches to study the problem.

2. How does environment influence neurodevelopment?

The nervous system is an organized set of cells, involved in the conduction of electrical signals. With certain exceptions, it is common to the entire animal kingdom and requires a progressive maturation that allows it to carry out its function correctly. This maturation process is called "neurodevelopment" and in humans, as in the rest of mammals, begins at early gestation stages and continues until adulthood (Figure 1, Estes & McAllister, 2016). It is a process influenced by physical and chemical stimuli, internal or environmental, which in most cases are responsible for healthy development, but in some other cases can lead to the appearance of specific pathologies (D'Souza & Karmiloff-Smith, 2017), especially when the individual's genetic background predisposes to it (Hyman, 2000). Neurodevelopment vulnerability also depends on the life stage. Particularly, pregnancy and puberty are highly vulnerable periods to certain environmental stimuli. Next, we discuss it briefly.

2.1 Nervous system vulnerability.

2.1.1 During pregnancy:

Gestation begins with fertilization, giving rise to an egg or zygote, after which the embryonic stage begins. A rapid cellular multiplication is initiated as a blackberry-shaped aggregate, known as "morula", which later gives rise to the "blastula" after the formation of an internal cavity called blastocoel. The blastula is able to adhere and penetrate the wall of the uterine endometrium, after which the amniotic and vitelline cavities are separated by a structure called "neural plate". The blastula then undergoes a maturation process called "gastrulation", by which it is distributed in ectoderm, mesoderm and endoderm (Gilbert, 2005). Ectoderm is the layer that gives rise to the nervous system, as well as to the skin, hair or nails. The following maturational process is called "neurulation" and begins with the formation of the "neural tube" through the invagination of the neural plate. After experiencing a considerable lengthening and closure, the dorsal end of the neural tube will lead to the brain and the rest gives rise to the spinal cord and the peripheral nervous system. All this process would be impossible without the activation of numerous intracellular signaling pathways and without the secretion-reception of a large variety of cytokines capable of guiding each event. Thus, for example, during the neurulation phase, the Sonic Hedgehog signaling pathway has been shown to play a critical role in the regulation of cell proliferation and density in the neural tube, in addition to specifying the destination of these cells for the development of the cerebellum, the neocortex or the tectum, among other structures (Arias, Astudillo, Rayo,

Vallejo, & Moreno, 2016). Cytokines of the FGF family, TGF-β or retinoic acid have also been shown to participate in this process by forming a dorso-ventral gradient among the neural tube (J. L. Smith & Schoenwolf, 1997). The development of the immune system in this phase is still quite weak and has little influence on neurodevelopment (Figure 1, Estes & McAllister, 2016), but alterations can occur due to nutritional defects or the use of certain drugs. Thus, for example, a deficiency of folates, the use of drugs such as retinoic acid, the consumption of alcohol, caffeine or tobacco can produce defects in the neural tube and cause the appearance of malformations such as anencephaly, encephalocele or *spina bifida* (Copp & Greene, 2013).

From the eighth week of gestation, fetal development begins. No new organs or tissues are formed, but the maturation of existing ones takes place. At first, phenomena such as neurogenesis and neural migration predominate, in which neural stem cells differentiate into mature neurons and migrate to different structures of the nervous system (Figure 1, Estes & McAllister, 2016). Signaling pathways such as Notch (Kageyama, Ohtsuka, Shimojo, & Imayoshi, 2008) and proteins like Reelin (Lakatosova & Ostatnikova, 2012) guide this process. At the beginning of the second trimester of pregnancy, maturation, and differentiation of the fetal immune system begins (Figure 1, Estes & McAllister, 2016). It is essential to highlight this event since the immune system exerts a substantial effect on the nervous system and immune activation in the pregnant mother from this moment can influence more significantly on fetal neurodevelopment. In fact, infections such as those caused by *Candida spp*. produce a high rate of premature births in this phase (Holzer et al., 2017). At the end of this quarter, apoptosis, synaptogenesis and gliogenesis begin to occur and become predominant during the third trimester of pregnancy (Figure 1, Estes & McAllister, 2016). In this period, structural and functional maturation of the different neural circuits takes place (Tau & Peterson, 2010). Signaling pathways, growth factors and cytokines involved in this phase are increasingly numerous, as are environmental agents capable of generating disturbances in the development of these circuits. Thus, for example, maternal immune activation and exposure to drugs or stressful situations can influence circuits responsible for detecting and filtering sensory stimuli, for example, the connection between the anterior insula and the anterodorsal cingulate cortex (salience network) (Spann, Monk, Scheinost, & Peterson, 2018). Neuropsychiatric disorders such as schizophrenia (Brown, D., P.H., & Derkits, 2010), autism (B. K. Lee et al., 2015; Zerbo et al., 2015) or bipolar disorder (Canetta et al., 2014; Parboosing, Viro, Bao, & Shen, 2013) could appear at later stages, as a consequence.

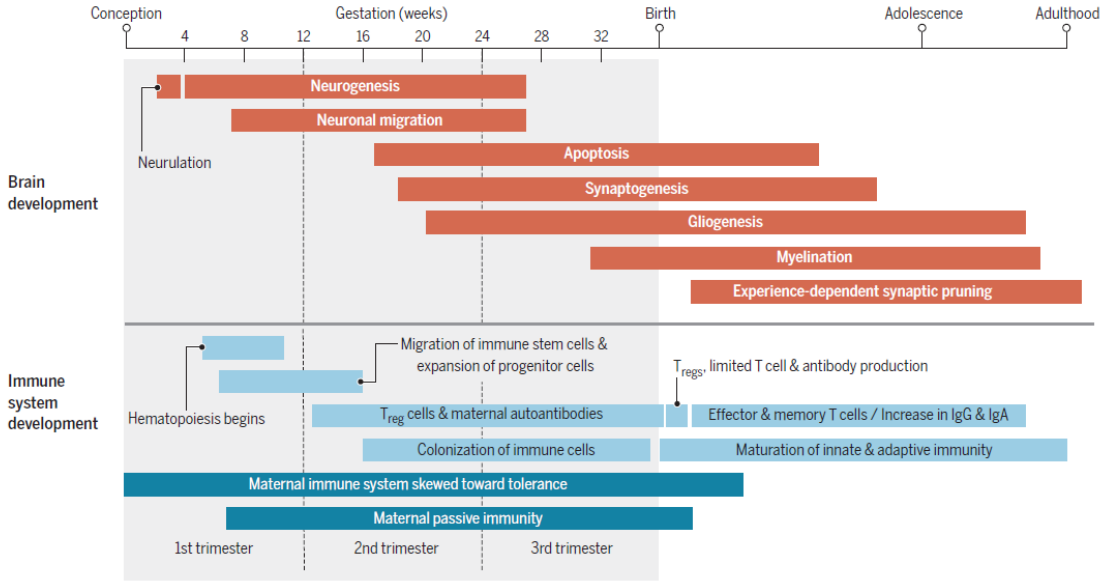


Figure 1: Timeline of significant events occurring in the brain and immune system development from conception to adulthood. (Modified from Estes & McAllister, 2016).

2.1.2 During puberty:

After birth, there is a remarkable and progressive development of body tissues and particularly, of the nervous system. Besides, there is a primary differential characteristic occurring during the postnatal period; the individual establishes direct contact with the environment that surrounds him or her. In addition to the innumerable internal mechanisms that enable its neurodevelopment, there are many environmental factors that can influence the individual, positively or negatively. Good nutritional or hygienic habits influence positively, while nutritional deficiencies (Gow & Hibbeln, 2014), infection (Karachaliou et al., 2017), or inflammation (John, Black, & Nelson, 2017) are major contributors to impaired neurodevelopment during childhood. Metabolically, apoptosis, synaptogenesis or gliogenesis processes still occur, and others such as myelination or synaptic pruning appear more frequently and remain until adulthood. The immune system, on the other hand, undergoes maturation and specialization processes (Figure 1, Estes & McAllister, 2016). The transition period from childhood to adulthood begins with puberty, which is characterized by the development of secondary sexual features. Neurodevelopment is entirely influenced by sex hormones, as well as by other environmental factors. Specific social skills and interpersonal relationships appear during this stage, for example. It is also important to highlight that synaptic pruning plays a fundamental role in neurodevelopment during this phase (Irwin Feinberg, 2017). Neuronal and synaptic loss allows the regional specialization of the nervous system.

Many environmental factors can negatively influence this process and facilitate the appearance of specific psychiatric-type pathologies (Paus, Keshavan, & Giedd, 2008). Exposure to traumatic experiences (Howes & McCutcheon, 2017) or the use of some drugs, such as cannabis (Lubman, Cheetham, & Yücel, 2015) for example, significantly increase the risk of developing mental disorders, such as schizophrenia (Howes & McCutcheon, 2017; Vaucher et al., 2018).

2.2 The influence of prenatal infections.

Infections have a decisive influence on neurodevelopment. Although this influence is important during childhood, it is during pregnancy where more evidence has been obtained about its relationship with other pathologies. Numerous studies relate infections by specific pathogens with the appearance of certain diseases. The term "maternal immune activation" (MIA) is used as an umbrella term for these infections during pregnancy in terms of their relationship with the future development of psychopathologies. We will now discuss the mechanisms that underlie the influence of MIA in fetal neurodevelopment and describe the main pathologies associated to maternal infection.

2.2.1 Mechanisms.

MIA is a phenomenon that triggers an important inflammatory response in the pregnant mother and can affect the fetus due to alterations in placental barrier permeability. Numerous processes are activated by this response (Figure 2, Boksa, 2010), but not all of them have the same ability to influence fetal neurodevelopment. In particular, it has been observed that a large part of the effect of MIA is based on its action on fetal microglia (Knuesel et al., 2014), which regulates the immune response and participates in neuronal maturation and differentiation processes. These processes are essential for synaptic pruning and neural circuits formation. Microglia function is modulated by numerous biomolecules such as neurotrophic and complement factors, neurotransmitters or cytokines (Bhat & Steinman, 2009; Glass, Saijo, Winner, Marchetto, & Gage, 2010). The latter are very varied and are secreted in significant quantities by monocytes, macrophages or lymphocytes in response to MIA. Among the main pro-inflammatory cytokines are CXCL8 (Ellman et al., 2010), TNF (Xu et al., 2006), IL-1β (Rousset et al., 2006) and IL-6 (S. E. P. Smith, Li, Garbett, Mirnics, & Patterson, 2007). This last cytokine, IL-6, seems to play a determining role and has been the most studied in the literature. Others, such as IL-10, play an anti-inflammatory role (U. Meyer et al.,

2008). The balance between pro and anti-inflammatory signaling pathways is what ultimately determines microglial impact, and has a vital impact on neurodevelopment. A favorable balance to pro-inflammatory pathways leads to a "priming" state of microglia characterized by an increase in its number, apparent changes in morphology and upregulation of cell surface antigens (V. H. Perry & Holmes, 2014). This state causes damage to the blood-brain barrier during neurodevelopment, and depending on the time and duration of the insult, it could result in loss (Stolp & Dziegielewska, 2009) or hypomyelination (Paintlia, Paintlia, Barbosa, Singh, & Singh, 2004) of vulnerable neurons and focal white matter injury (Theoharides, Kempuraj, & Redwood, 2009). Also, it induces a lower expression of reelin (Knuesel et al., 2009; Urs Meyer, Feldon, & Fatemi, 2009), a vital regulator of neuronal migration that has been linked to a decrease in neocortical and hippocampal thickness. Microglial priming among neurodevelopment has also been shown to induce persistent changes in glutamatergic neurotransmission (Roumier et al., 2008) and to trigger changes in synaptic plasticity and pruning during critical periods of brain development (Elmer, Estes, Barrow, & McAllister, 2013). All these factors could foster long-lasting impairments in brain function and behavior, which may lead to the appearance of certain disorders as we will discuss below.

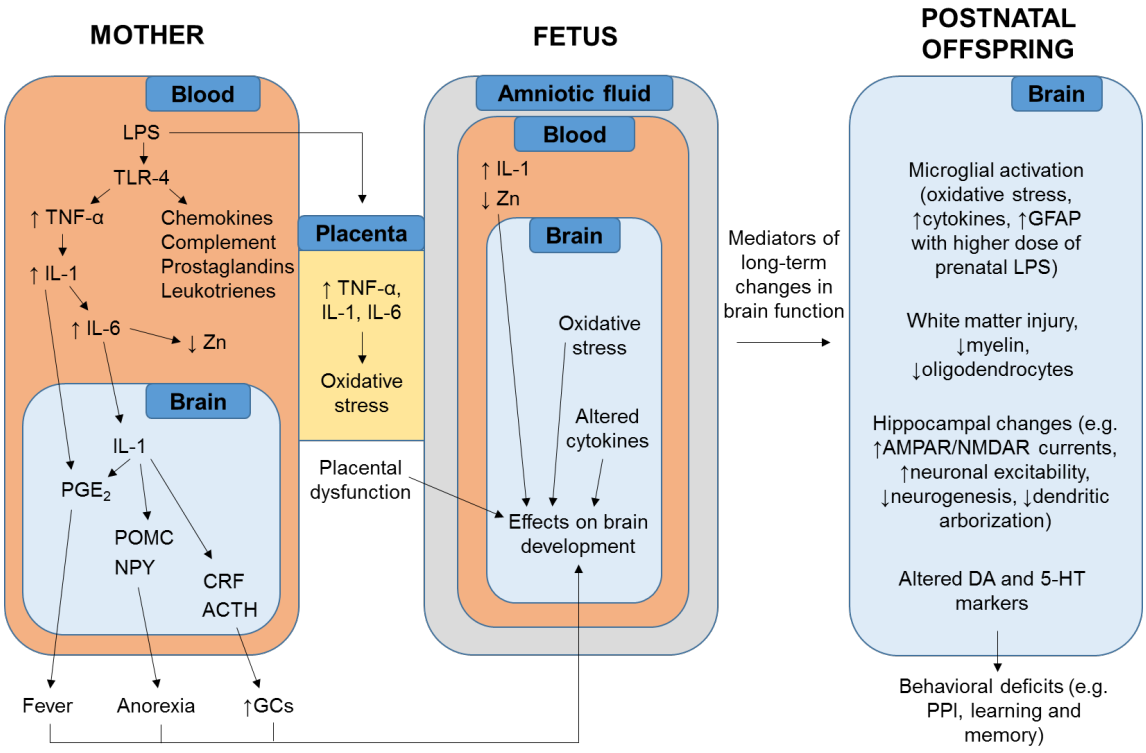


Figure 2: Potential mechanisms mediating effects of prenatal infection on brain function (modified from Boksa, 2010).

2.2.2 Neuropsychiatric disorders.

2.2.2.1 Schizophrenia.

Schizophrenia is a severe mental illness that usually onsets around the late adolescence or early adulthood. It affects men more than women (Picchioni & Murray, 2007) and is characterized by positive symptoms (such as hallucinations or delusions), negative symptoms (such as apathy, anhedonia or social withdrawal) and cognitive symptoms (such as disturbances in executive functions or working memory) (Turner, 1997). The exact etiopathogenesis of the disease is still unknown; however, a genetic predisposition (the estimation of heritability is around 80% (Cardno & Gottesman, 2000) or certain environmental stimuli exposure during neurodevelopment are risk factors that facilitate the emergence of the disorder. One of the primary environmental insults that behavioural been linked to schizophrenia is MIA (Knuesel et al., 2014). Epidemiological studies have found an association between prenatal infections by influenza virus (Alan S. Brown et al., 2004; Mednick & , Machon RA , Huttunen MO, 1988), *Toxoplasma gondii* (Torrey, Bartko, Lun, & Yolken, 2007; Torrey, Bartko, & Yolken, 2012) or Herpes simplex virus (Buka, Cannon, Torrey, & Yolken, 2008) and the development of the disease. Increased levels of C-reactive protein (Canetta et al., 2014) and cytokines such as IL-6, TNF or CXCL8 during pregnancy have also been correlated with an increased risk of developing schizophrenia by offspring (Anthony Stephen Brown et al., 2003; Buka et al., 2001). From a structural point of view, schizophrenic patients show a reduced volume of entorhinal and cingulate cortices, which has been associated with exposure to maternal elevations of CXCL8 (Ellman et al., 2010). At the molecular level, these patients show a greater microglial activation (Bayer, Falkai, & Maier, 1999) evidenced by increased levels of microglial markers CD68 and HLA-DR compared to healthy controls (Radewicz, Garey, Gentleman, & Reynolds, 2000).

2.2.2.2 Autism spectrum disorder (ASD).

ASD is a neurodevelopmental disorder that usually appears during the first three years of life. The main symptoms of this disorder are impairments in social communication and interaction, as well as restricted or repetitive behaviours, interests, or activities (Lauritsen, 2013). As in schizophrenia, the exact etiopathogenesis is unknown; however, an estimate of heritability of 60-91% has been obtained (Ronald & Hoekstra, 2011) and it has been linked to exposure to certain environmental stimuli during neurodevelopment, such as MIA. (Knuesel et al., 2014). Epidemiological studies have found an association between ASD and prenatal infections of herpes simplex virus, rubella or *Toxoplasma*

gondii, as well as other of bacterial type (Atladóttir et al., 2010). Increased levels of IL-6 (Parker-Athill & Tan, 2010) and C-reactive protein (A. S. Brown et al., 2014) during pregnancy have also been linked to raised risk of developing ASD by offspring. In addition, patients with ASD present higher expression of TNF (Chez, Dowling, Patel, Khanna, & Kominsky, 2007) and MCP-1 (Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005) cytokines in cerebrospinal fluid, as well as higher microglial activity in the cortex (Morgan et al., 2010; Suzuki et al., 2013), in comparison with healthy individuals.

2.2.3 Episodic and paroxysmal disorders: Epilepsy.

Epilepsy is a disease characterized by the appearance of recurrent seizures as a consequence of the abnormal, concurrent, and excessive activation of defined neuronal populations (Knuesel et al., 2014). MIA has been shown to play a fundamental role in the etiopathogenesis of this disease, as in schizophrenia or ASD. Thus, infections of the urinary, gastrointestinal or reproductive system accompanied by fever during early to mid-gestation increase the risk for the disease (Y. Sun, Vestergaard, Christensen, Nahmias, & Olsen, 2008; Yuelian Sun, Vestergaard, Christensen, & Olsen, 2011). Some of the typical features of MIA, such as elevated microglial activity, have also been observed in epileptic patients (Hirvonen et al., 2012).

2.2.4 Psychomotor disorders: Cerebral palsy.

Cerebral palsy affects psychomotricity and is usually accompanied by behavioral and cognitive impairments (Knuesel et al., 2014). Many factors can precipitate this condition such as placental abnormalities, intrauterine growth restriction, perinatal asphyxia or maternal infections (Mann, Mcdermott, Bao, & Bersabe, 2009; Ovali, 2010). As regards materal infections, MIA may generate inflammatory processes, such as chorioamnionitis and increase the risk of cerebral palsy in progeny (Patrick & Smith, 2002). As in schizophrenia, ASD or epilepsy, some of these processes may remain active in the offspring and cause neurotoxic effects. For example, the levels of inflammatory mediators derived from microglia or macrophages have been found to be elevated in the cerebrospinal fluid of newborn infants after birth asphyxia (Sävman, Heyes, Svedin, & Karlsson, 2013).

2.2.5 Neurodegenerative disorders: Alzheimer's and Parkinson's diseases.

Alzheimer's and Parkinson's diseases are the most common neurodegenerative disorders. Each of these diseases has its own symptoms; however, some as dementia, sleep disturbances, or impairments in autonomic and cognitive functions are common to both of them. Some specific environmental influences have been shown to facilitate the appearance of these diseases (Dosunmu, Wu, Basha, & Zawia, 2007; Monte, Lavasani, & Manning-bog, 2002); however, there is no documented epidemiological association between MIA and any of them. In spite of this, the suggested primary effector mechanism of MIA, microglial priming participates in both pathologies (Blaylock, 2017; J.-W. Li, Zong, Cao, Tan, & Tan, 2018) being a critical pathophysiological event that induces a dysfunction of specific immune system pathways (Knuesel et al., 2014). Such dysfunction is a vulnerability factor for Alzheimer's and Parkinson's diseases onset since many of these pathways could be involved in the phagocytosis of protein aggregates, in neuronal repair and myelination (Glass et al., 2010).

2.3 The influence of peripubertal stress.

2.3.1 Mechanisms.

Stress is a physiological response that starts when a particular stimulus, internal or external, is perceived as demanding or threatening. The emotional processing of this perception is carried out mainly in the amygdala (Öhman, 2005). This structure has direct connections with neurons of the paraventricular nucleus of the hypothalamus and initiates a complex neuroendocrine communication through the hypothalamic-pituitary-adrenal (HPA) axis (Gray, Carney, & Magnuson, 1989). These neurons release corticotropin-releasing hormone (CRH), which triggers the subsequent release of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH, in turn, leads to production and secretion of certain catecholamines and glucocorticoids by the adrenal cortex (figure 3), that, when acting on specific target organs, lead to a state of alertness characterized by an increase in heart rate, blood pressure or body temperature. Once the stressor disappears, feedback loops are activated and guide the system to the initial homeostatic point (Lupien, McEwen, Gunnar, & Heim, 2009). For example, glucocorticoid receptors in the hypothalamus or pituitary gland reduce CRH and ACTH secretion, respectively. Other structures, such as the hippocampus or frontal cortex, are also sensitive to glucocorticoids and contribute to a decrease in the secretion of these hormones (Lupien et al., 2009).

Although activation of the HPA axis is a ubiquitous process throughout life, the intensity and consequences of this activation vary considerably depending on the developmental stage. Mainly, puberty is a critical period for two reasons:

• During this stage, there is a higher baseline activity of the HPA axis (Gunnar, Wewerka, Frenn, & Griggs, 2009), probably due to large fluctuations in sex hormone levels (McCormick & Mathews, 2007).

• Not every brain structure matures at the same speed or in the same time interval. In humans, structures such as hippocampus reach complete maturation around two years of age; however, frontal cortex and amygdala experience significant maturing peaks during puberty (Figure 4, Lupien et al., 2009). Considering the enhanced activity of the HPA during puberty, and the increased sensitivity of the frontal cortex and amygdala to corticoids, these two structures are especially vulnerable to stress experiences during adolescence (Perlman, Webster, Herman, Kleinman, & Weickert, 2007).

We have already discussed that puberty is a period of high sensitivity to stress. Additional exposure to traumatic experiences during this period could further increase HPA axis hyperactivity and produce severe disturbances in the maturation of the frontal cortex and the amygdala. Moreover, as mentioned above, both structures are involved in the HPA axis (Figure 3), so that an inadequate cortical or amygdalar function could further alter HPA regulation. It has been proposed these altered feedback sequences may contribute to the appearance of certain psychiatric pathologies (Paus et al., 2008).

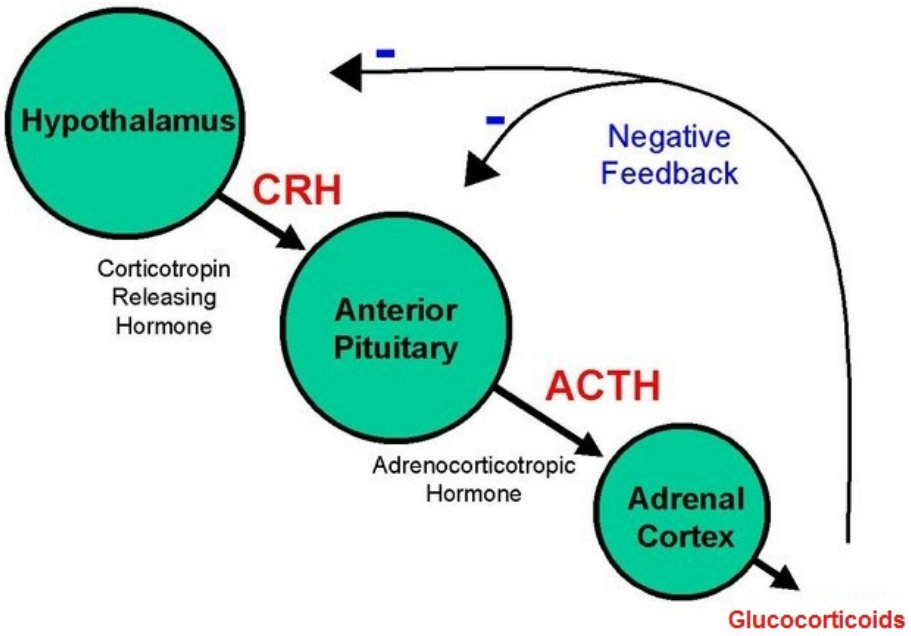


Figure 3: HPA axis diagram (modified from Xiao, 2015).

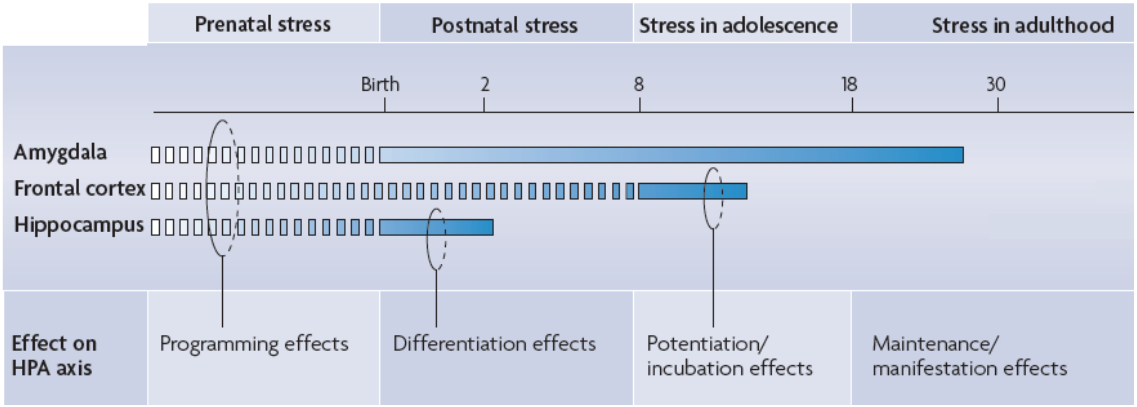


Figure 4: Developmental chronogram of the amygdala, frontal cortex and hippocampus in humans (modified from Lupien et al., 2009).

2.3.2 Associated disorders.

2.3.2.1 Schizophrenia.

Maturing processes can be increased in puberty in the face of a prolonged and intense stress response, contributing to certain neurodevelopmental diseases appearance such as schizophrenia (Paus et al., 2008). In healthy adolescents, a physiological decrease in the "delta-wave sleep periods" has been observed. These are sleep intervals in which the predominant electric waves are those of the delta type. Delta-waves are generated by the synchronic activity of specific cortical neurons. A decrease in their frequency and duration could be due to typical neuronal pruning of adolescence (I Feinberg, 1982). In schizophrenic patients, this decrease is accentuated and is thought to be due to excessive neuronal pruning (Keshavan, Anderson, & Pettergrew, 1994; Keshavan et al., 1998). Other studies have also found lower levels of membrane phospholipid precursors (Keshavan et al., 1994) and lower metabolic activity (Andreasen, 2011) in the prefrontal cortex of schizohrenia patients, as well as a reduction in the volume of the gray matter (Sporn et al., 2003) and in dendritic spines density (Garey et al., 1998) at the cortical level.

2.3.2.2 Affective and anxiety disorders.

Puberty is a stage in which episodes of emotional lability and anxiety abound, due in part to the already mentioned hypersensitivity to stress (determined, in part, by sex hormone levels) (McCormick & Mathews, 2007). It has been suggested that the neurosteroid allopregnanolone could play a key role. This steroid is secreted in response to stress and has been shown to exert an anxiolytic effect by the positive allosteric modulation of $GABA_A$ receptors in adult individuals (Freeman, Purdy, Coutifaris, Rickels, & Paul, 1993).

However, when acting on a specific subtype of these receptors, α4β2δ, prevents Cl^- flow through the channel and produces excitability. It is precisely during puberty when there is a higher expression of this type of receptor, mainly in the CA1 region of the hippocampus (Shen et al., 2007). This could explain, in part, the high rates of anxiety and affective disorders typical of this period, although further studies are necessary.

2.4 The two-hit hypothesis.

The etiopathogenesis of schizophrenia has been consistently linked to the joint influence of prenatal infections and peripubertal stress (Clarke, Tanskanen, Huttunen, Whittaker, & Cannon, 2009). The two-hit hypothesis proposes that both factors contribute synergistically to the onset of the disease by triggering an excessive microglial activation capable of producing permanent neuronal damage in certain neuroanatomical areas (Feigenson, Kusnecov, & Silverstein, 2014; Howes & McCutcheon, 2017). Broadly speaking, two pathways are distinguished in microglial activation, on one hand, the M1 pro-inflammatory pathway, stimulated by cytokines such as IFN-γ, IL-1β or TNFα and the anti-inflammatory M2 pathway, stimulated by cytokines such as IL-4, IL-13 or IL-25 (Colton, 2009). Predominance of M1 over M2 pathway can cause excessive neuronal and synaptic pruning at critical periods of neurodevelopment (Rao, Kellom, Kim, Rapoport, & Reese, 2012; Schafer et al., 2012). In addition, it has been observed that prenatal immune activation can induce priming state in the microglia that makes it much more sensitive to subsequent immunological insults (Diz-Chaves, Astiz, Bellini, & Garcia-Segura, 2013; Diz-Chaves, Pernía, Carrero, & Garcia-Segura, 2012; Sandra Giovanoli et al., 2013), causing an excessive response, with predominance of the M1 activation pathway. This effect is even more evident during puberty, since it is a high-sensitivity period to stress, which has been shown to influence the activity of microglia (Calcia et al., 2016). Although, normally, glucocorticoid secretion plays an anti-inflammatory role, the opposite effect can occur if there has been a previous immune challenge (Frank, Watkins, & Maier, 2015). Microglia cells express a large number of glucocorticoid receptors on their surface (Sierra, Gottfried-blackmore, Milner, Ewen, & Bulloch, 2008) and trigger a pro-inflammatory response if they have been previously primed. Pronounced elevations of glucocorticoids at puberty could increase the density and the activation state of primed microglia causing extensive neuronal damage, which could lead to permanent neurocognitive disturbances. In fact, by combining prenatal immune activation with peripubertal stress, an increase in the levels of activated microglia marker "Iba-I" has been observed, correlating with higher density of this type of cells (Sandra Giovanoli et al., 2013). In addition, neuronal damage itself feeds the process upon

glutamate release in the extracellular matrix, activating NMDA receptors of the microglial surface and promoting cell proliferation (Kaindl et al., 2012; Nair & Bonneau, 2006).

All these phenomena precipitate the appearance of schizophrenia, especially when there is a genetic predisposition to the disease. Recently, an intracellular mechanism involving a specific signaling pathway with excessive microglial activation has been discovered, and it could explain how genetic background is related to the environmental factors discussed (Sekar et al., 2016). Schizophrenic patients showed a structural variation in the gene that codes for the C4 complement protein, which results in increased expression. C4 activates C3 complement protein, allowing it to bind to synapses. The latter molecule is recognized by microglia, which is activated and initiates synaptic phagocytosis. It is thought that convergence of environmental and genetic factors could produce functional disturbances on microglia, causing considerable damage in developing brain areas, all of which could facilitate the appearance of the disease (Cannon et al., 2015).

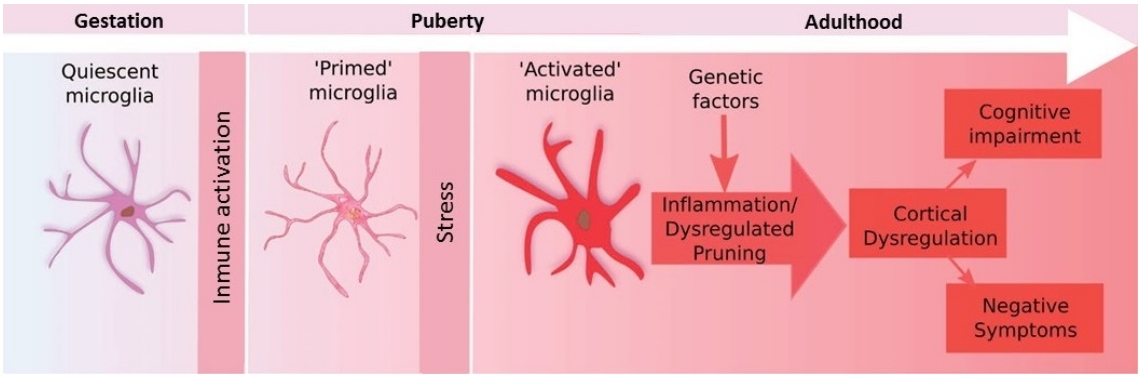


Figure 5: Graphic representation of the two-hit hypothesis (modified from Howes & McCutcheon, 2017). Prenatal immune activation leads to a primed state of microglia. Subsequent peripubertal stress triggers pathological overactivation, leading to cortical loss and psychiatric symptoms.

3. Why is it essential to study addiction in certain neurodevelopmental disorders?

3.1 The problem of drug addiction among psychiatric patients.

Epidemiological data show a higher rate of substance use disorders in psychiatric patients than in the rest of the population (Flynn & Brown, 2008; Herrero et al., 2008). The comorbidity between psychiatric and substance use disorders is known as "dual diagnosis" and it is an added difficulty when establishing the adequate therapy since the simultaneous treatment of both pathologies becomes necessary. Although dual diagnosis etiopathogenesis remains to be clarified, it has been proposed that disturbances in specific neurotransmission systems could increase the susceptibility of psychiatric patients to suffer an addictive disorder (Chambers, 2007; Chambers, Krystal, & Self, 2001).

3.2 Neurocircuitry involved in dual diagnosis.

Acute reinforcing effects of certain recreational drugs are due, among other processes, to direct or indirect stimulation of the mesolimbic pathway, composed of dopaminergic neurons that project from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) (Koob & Volkow, 2010). This nucleus is part, along with the olfactory tubercle, of the ventral striatum, and is composed by 95% of medium spiny neurons (MSNs), of GABAergic nature (Francis & Lobo, 2017). With some exceptions, the majority MSNs express a specific subtype of dopamine receptors on their surface, either D1 or D2, which determines their role in the activation of the direct or indirect pathway of the basal ganglia, respectively (Nishi, Kuroiwa, & Shuto, 2011).

It is thought that an imbalance in the activation of these pathways could underlie addictive-like behavioral patterns (Yager, Garcia, Wunsch, & Ferguson, 2015). Repeated exposure to certain recreational drugs can induce changes in the expression of D1 or D2 receptors in MSNs, causing alterations in connectivity with other structures such as the internal globus pallidus and the subthalamic nucleus (Yager et al., 2015) (Figure 6), involved in reward and punishment processing (Espinosa-Parrilla, Baunez, & Apicella, 2015; Howell et al., 2016). As an example of the importance of the D1/D2 differentiation, it has recently been proposed that therapies selectively targeted to D1 MSNs may help to prevent relapse in cocaine addicts (Calipari et al., 2016).

It is also important to note that dopaminergic inputs on MSNs are modulated by glutamatergic afferents from the prefrontal cortex, the hippocampus and the amygdala (Chambers, 2007; Yager et al., 2015) (Figure 6). Of note, these structures are highly influenced by prenatal immune activation and peripubertal stress. Functional disturbances due to these factors could lead to an inadequate modulation of the dopaminergic input on the MSNs in the nucleus accumbens, influencing the connectivity with other structures and contributing to drug addiction vulnerability.

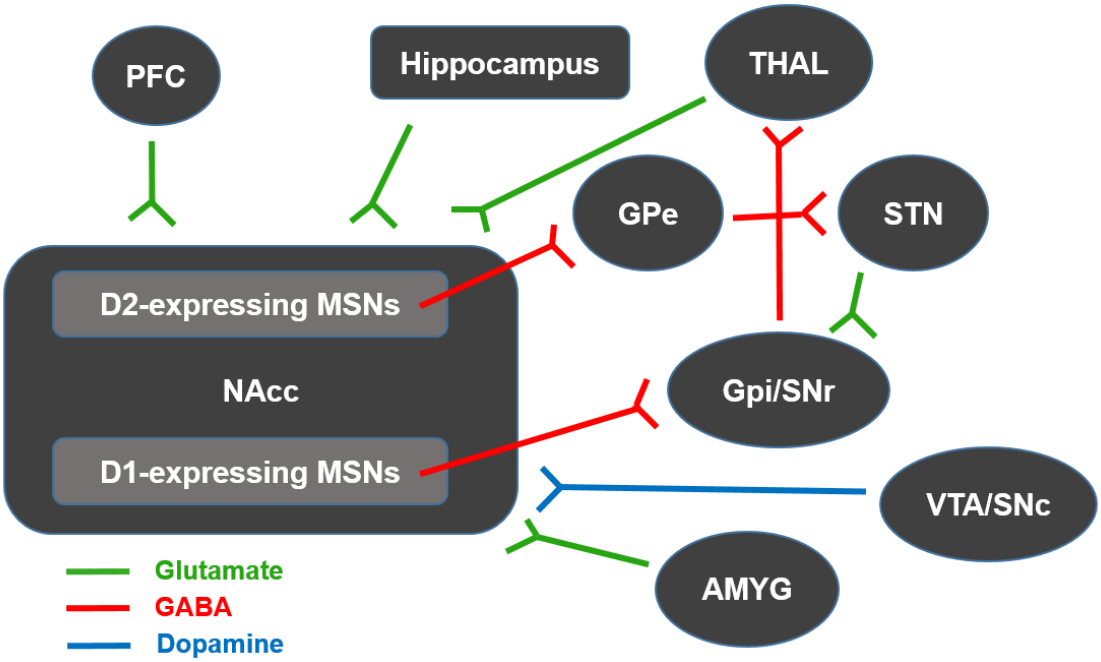


Figure 6: Simplified schematic view of inputs and outputs in the nucleus accumbens. This nucleus receives glutamatergic inputs (indicated in green) from the PFC, AMYG, Hippocampus and THAL, and dopaminergic (indicated in blue) from the VTA and the SNc. D1-expressing MSNs form the direct path projecting to GPi / SNr, while D2-expressing MSNs form the indirect path projecting to GPi / SNr via the GPe / VP and STN. Red lines denote GABAergic inhibitory projections. *NAcc: Nucleus accumbens; PFC: Prefrontal cortex; AMYG: Amygdala; GPe: External globus pallidus; GPi: Internal globus pallidus; THAL: Thalamus; SNr: Substantia nigra pars reticulata; STN: Subthalamic nucleus; VTA: Ventral tegmental area. SNc: Substantia nigra pars compacta.*

4. How to progress in the study of dual diagnosis? Animal models.

4.1 Animal models of schizophrenia.

Since the second half of the twentieth century, the use of increasingly effective antipsychotic drugs has significantly improved the life quality of schizophrenic patients, especially regarding their positive symptomatology. However, the treatment of cognitive and negative symptoms remains to be a challenge. A greater understanding of the neurobiological basis of the disease could lead to more effective treatments, whether prophylactic or therapeutic. Accordingly, the use of animal models is essential since they allow performing invasive or high-risk procedures that would not be possible in patients.

When validating an animal model of schizophrenia, it must meet three fundamental criteria (Jones, Watson, & Fone, 2011). On the one hand, it must show construct validity, relying on the theoretical hypothesis proposed for the disease. Secondly, it must evince face validity, namely symptomatic homology concerning schizophrenic patients. Finally, known treatments must generate an expected response in the animal model, what is known as predictive validity. Nowadays, most animal models of schizophrenia are developed in rodents (rats or mice) and can be classified into three categories:

4.1.1 Neurodevelopmental models.

During critical stages of neurodevelopment, animals are exposed to environmental or pharmacological insults capable of causing permanent and irreversible disturbances in the nervous system. The following stand out:

4.1.1.1 Models of prenatal administration of methylazoxymethanol (MAM).

MAM is a compound found in the seeds of cycad plants. It is an antimitotic agent, capable of specifically inhibiting neuroblasts proliferation by DNA methylation (Jones et al., 2011; Matsumoto & Higa, 1966). MAM administration to pregnant dams has been shown to affect fetal brain maturation, especially in those structures undergoing the most rapid development (Lodge & Grace, 2009). Thus, the gestational day (GD) of administration defines the type of anatomical and behavioral disturbances of the offspring (Fiore et al., 1999; Talamini, Ellenbroek, Koch, & Korf, 2006; Talamini, Koch, Ter Horst, & Korf, 1998). In particular, GD15 and 17 have been the most de prenatal days most extensively studied in the field. When MAM is administered at GD15, produces substantial morphological alterations and extensive volumetric reductions in structures such as the cortex, the hippocampus or the cerebellum (Cattabeni et al., 1989; Johnson et al., 2006),

which triggers significant behavioral disturbances that surpass those observed in schizophrenic patients (Shenton, Dickey, Frumin, & Mccarley, 2001). As a consequence, the apparent and construct validity of the model are significantly hindered. In contrast, MAM administration at GD17 has been shown to better induce typical alterations of the schizophrenic disease. For example, at the neuroanatomical level, it produces less aggressive volumetric reductions in the cortex and hippocampus. In this last area, it also generates morphological and structural changes similar to those observed in schizophrenic patients (Matricon et al., 2010; Moore, Jentsch, Ghajarnia, Geyer, & Grace, 2006).

At the behavioral level, MAM administration in GD17 enhances locomotor response to psychotomimetic drugs as amphetamine (Moore et al., 2006) or MK-801 (Le Pen et al., 2006), which is indicative of mesolimbic hyperactivity associated with positive symptoms of the disease (Flagstad et al., 2004). Cognitive symptomatology, such as sensorimotor gating deficits, has been observed in animals exposed to MAM at GD17, as revealed by the prepulse inhibition test (PPI) (Moore et al., 2006). These animals also showed working memory impairments in the Morris water maze (Hazane, Krebs, Jay, & Le Pen, 2009).

Accordingly, the MAM administration model in GD17 reports face validity in terms of positive and cognitive symptoms and construct validity in terms of neuroanatomical alterations. However, the evidence for face validity in terms of negative symptomatology and predictive validity is scarce.

4.1.1.2 Models of post-weaning social isolation.

Within a colony, animals establish social and hierarchical relationships capable of positively influencing neurodevelopment. Social isolation from the weaning age (~ postnatal day (PND) 21) has been shown to induce permanent anatomical and behavioral disturbances (Fone & Porkess, 2008). At the structural level, it produces a selective PFC volume reduction (Day-Wilson, Jones, Southam, Cilia, & Totterdell, 2006) accompanied by decreased dendritic spine density (Silva-Gómez, Rojas, Juárez, & Flores, 2003). Proteins involved in neuronal migration, such as reelin, also decrease in this brain area (A. W. Cassidy, Mulvany, Pangalos, Murphy, & Regan, 2010), as well as in the ventral hippocampus (Andrew W. Cassidy, Mulvany, Pangalos, Murphy, & Regan, 2010). Also, extensive neurochemical imbalances are observed in cortico-limbic regions, as well as in schizophrenic patients (Jones et al., 2011), conferring construct validity to

the model. Behaviorally, post-weaning social isolation gives rise to enhanced anxious states, characterized by increased locomotor activity and response to novelty (Fone & Porkess, 2008). The acute administration of antipsychotic drugs such as haloperidol, olanzapine or risperidone has been shown to revert isolation-induced hyperactivity, suggesting that this could be a consequence of mesolimbic hyperactivity and be related to positive symptoms of the disease (Fabricius, Helboe, Fink-Jensen, Wörtwein, & Steiniger-Brach, 2011). Sensorimotor gating deficits have also been observed in the PPI test (Cilia, Reavill, Hagan, & Jones, 2001; Schubert, Porkess, Dashdorj, Fone, & Auer, 2009; Varty & Geyer, 1998), although their reversal by different manipulations has been occasional and inconsistent (Jones et al., 2011).

Although its strength is limited, this model does not require physical intervention in animals and is simple to execute. Therefore, its combination with other models could be interesting in order to improve the reproducibility of typical features of the disease.

4.1.1.3 Models of prenatal immune activation.

Maternal exposure to either bacterial or viral infections during pregnancy increases pro-inflammatory cytokines secretion. Some of these cytokines (such as IL-6) are capable of crossing the placental barrier, as well as the fetal blood-brain barrier, causing a marked impact on offspring neurodevelopment (Boksa, 2010). This process can be induced and replicated in animal models by several agents; however, the most commonly used are lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (Poly I:C) (Boksa, 2010).

LPS is a structural component of the outer membrane of Gram-negative bacteria and is recognized by Toll-like receptors 4 (TLR4) located in the plasma membrane of various cell types, such as macrophages, dendritic cells or mast cells (Vaure & Liu, 2014). LPS administration triggers an inflammatory response characterized by an increase in cytokines, such as IL-1β, IL-6, TNFα, CXCL1, CXCL2, CXCL10, CCL2 and CCL7, as well as an increase in circulating corticosterone (Golan, Lev, Hallak, Sorokin, & Huleihel, 2005; Ortega, Jadeja, & Zhou, 2011; Urakubo, Jarskog, Lieberman, & Gilmore, 2001). The impact of such immunity challenge on adult offspring depends on multiple variables, such as the type of rodent used, injection timing, dose employed, or the route of administration (Kentner et al., 2018). In rat studies, the most used injection window is GD15-19 (i.p.), corresponding to first-to-second trimesters of human pregnancy (Clancy et al., 2007) and dosage usually vary from 50 to 500 µg/kg (Boksa, 2010; U. Meyer, 2006; Urs Meyer et al., 2009). At a morphological level, prenatal exposure to LPS reduces hippocampal neurogenesis (Cui, Ashdown, Luheshi, & Boksa, 2009), as well as

branch, length, and density of dendritic spines in the hippocampus and PFC (Baharnoori, Brake, & Srivastava, 2009). It also induces cell death in white matter regions (Paintlia, Paintlia, Contreras, Singh, & Singh, 2008) and at the neurochemical level, it has been shown to compromise the functionality of hippocampal NMDA receptors (Escobar et al., 2011). At the behavioral level, MIA induces sensorimotor gating deficits (M. E. Fortier, Luheshi, & Boksa, 2007; E. Romero, Guaza, Castellano, & Borrell, 2010; Santos-Toscano, Borcel, Ucha, Orihuel, Capellán, Roura-Martínez, Ambrosio, & Higuera-Matas, 2016; Simões et al., 2018; Swanepoel, Möller, Harvey, & Harvey, 2017; Waterhouse, Brennan, & Ellenbroek, 2017; Wischhof, Irrsack, Osorio, & Koch, 2015), enhanced locomotor response to amphetamine (M. È. Fortier, Joober, Luheshi, & Boksa, 2004; M. E. Fortier et al., 2007) and less spatial learning in water maze (Lanté et al., 2008, 2007). In addition, specific deficits have been reversed by antipsychotics like haloperidol (Eva Romero et al., 2007). All this gives the model excellent construct validity, predictive and face validity (mainly in terms of positive and cognitive symptomatology).

The administration of Poly I:C, a synthetic analogue of double-stranded RNA recognized by TLR3, has also been shown to increase the secretion of pro-inflammatory cytokines such as IL-1β, IL-6, TNFα or CXCL1 (Ballendine et al., 2015; Song et al., 2011) and activates the HPA axis (Milton, Hillhouse, & Milton, 1992). Like LPS, its effects on the offspring vary considerably according to the variables discussed (Kentner et al., 2018). In this regard, sensorimotor gating and social interaction deficits are more frequently observed when the injection(s) is (are) scheduled at an early developmental time (GD9-12). This, despite improving the face validity of the model in terms of cognitive and negative symptoms, separates the model from epidemiological studies that relate schizophrenia with immune activation at advanced gestational stages and hence reduces the construct validity of this approach. Other studies have also described increased sensitization to the locomotor effects of amphetamine (Zuckerman & Weiner, 2003) or deficits in working memory in water and T-mazes (Zuckerman & Weiner, 2005), increasing the face validity of this approach. Also, some of the cognitive impairments induced are reversed by the administration of antipsychotics such as clozapine (Ozawa et al., 2006; Zuckerman & Weiner, 2003), supporting the predictive validity of this model. In addition , there are also morphological alterations observed after MIA that should be mentioned, such as increases in the ventricular volume (Q. Li et al., 2009) and decrements in hippocampal neurogenesis (U. Meyer, 2006).

Thus, both agents solidly reproduce typical features of the disease and make this model a powerful tool to progress in the knowledge of the neurobiological processes underlying schizophrenia.

4.1.1.4 Models of neonatal ventral hippocampal lesion.

Neonatal (PND 7) ventral hippocampal lesion has been shown to cause substantial behavioural impairments that emerge after puberty and compromise the integrity of other developing structures on which it presents innervation, such as medial PFC and NAcc (Tseng, Chambers, & Lipska, 2009). Bilateral injection of ibotenic acid (Becker, Grecksch, Bernstein, Höllt, & Bogerts, 1999; B. K. Lipska, Jaskiw, & Weinberger, 1993), a non-selective glutamate receptor agonist, is the most commonly used procedure to induce such injury. Most behavioral impairments are evident from PND 56, which include enhanced locomotor sensitivity to psychotomimetics such as amphetamine (Beninger et al., 2009; Wan, Giovanni, Kafka, & Corbett, 1996) or MK-801 (Al-Amin, Weinberger, & Lipska, 2000), sensorimotor gating deficits (Le Pen et al., 2000), social interaction loss (Frank Sams-Dodd, Lipska, & Weinberger, 1997), and impaired spatial working memory in water (Beninger et al., 2009; Le Pen et al., 2000) and T-mazes (B. Lipska, Aultman, Verma, Weinberger, & Moghaddam, 2002). Antipsychotic administration prevents hyper-responsivity to amphetamine in injured animals, although in some cases, it is not able to reverse social interaction loss (Frank Sams-Dodd et al., 1997). Morphologically, lesioned rats show a reduction in spine density and dendritic length in both the NAcc and medial PFC (Flores et al., 2005). In this later brain area, they show reduced levels of N-acetylaspartate (a marker of neuronal integrity) (Jones et al., 2011), as well as increased $GABA_A$ receptors expression (Endo et al., 2007).

The construct validity of this model is reduced by its invasiveness, despite its excellent face validity. On the other hand, its predictive validity is mainly limited to positive symptoms of the disease.

4.1.2 Pharmacological models.

4.1.2.1 Amphetamine models.

One of the main theories that have been proposed to explain the etiopathogenesis of schizophrenia focuses on the dopaminergic hyperfunction at the mesolimbic level that is correlated with the positive symptoms of the disease, such as hallucinations or delusions (Murray, Lappin, & Di Forti, 2008). Indeed, amphetamine administration induces both effects in humans (Connell, 1957), and this approach could be a useful tool when replicating said symptoms in animal models. At the behavioural level, the chronic administration of amphetamine induces locomotor sensitisation, enhancing the hyperactivity caused by an acute amphetamine challenge (Robert E. Featherstone, Rizos, Kapur, & Fletcher, 2008; Robinson & Becker, 1986). Pre-administration of a low

dose of antipsychotics such as haloperidol or clozapine has been shown to prevent such sensitization (Meng, Feldpaush, & Merchant, 1998), providing predictive validity to the model in this regard. However, face validity is limited when it comes to replicate the negative symptoms of the disease. At the cognitive level, chronic amphetamine administration has been shown to produce sensorimotor gating deficits in the PPI test, albeit in a dose-dependent manner (R. E. Featherstone, Kapur, & Fletcher, 2007). In PFC-dependent cognitive tasks, such an attentional set-shifting task (Robert E. Featherstone et al., 2008) or five-choice serial reaction time task (Fletcher, Tenn, Sinyard, Rizos, & Kapur, 2007), chronic amphetamine has been shown to produce reversal learning deficits or omission increments, respectively. However, no effects on hippocampal-dependent cognition have been observed in the Morris water maze (Robert E. Featherstone et al., 2008; Russig, Durrer, Yee, Murphy, & Feldon, 2003), suggesting that the drug exerts a more considerable influence on prefrontal rather than on hippocampal function. At the morphological level, repeated amphetamine administration increases branch and density of dendritic spines in both the PFC and NAcc (Robinson & Kolb, 1999). In this latter area, it also reduces the expression of the GluR1 and GluR2 subunits of AMPA receptors (Lu & Wolf, 1999).

Therefore, this model accurately reproduces positive symptoms of the disease but has notorious limitations replicating cognitive and negative symptoms. Given that this drug has a strong effect on the dopaminergic system, the reversal of amphetamine-induced symptoms mainly responds to dopamine-acting antipsychotics.

4.1.2.2 Phencyclidine (PCP) models.

Blockade of NMDA receptors by non-competitive antagonists, such as PCP, is associated with the presence of positive, cognitive and negative symptoms similar to those found in schizophrenic patients (Krystal et al., 1994). The administration of this drug in rodents produces similar effects, allowing the study of typical symptoms of the disease. Like amphetamine, chronic administration of PCP provokes locomotor sensitisation, enhancing the hyperactivity caused by an acute PCP challenge (McLean, Woolley, & Neill, 2010). At a cognitive level, PCP reduces attention (Amitai & Markou, 2009), processing speed (Amitai, Semenova, & Markou, 2007), visual memory (Grayson, Idris, & Neill, 2007) and executive function (Rodefer, Murphy, & Baxter, 2005). However, the replication of cognitive symptoms is controversial since these impairments usually disappear after drug withdrawal. Also, it has been shown to decrease social interaction, replicating a specific subset of negative symptoms of the disease (F. Sams-Dodd, 1996).

Antipsychotic administration reverses positive (Phillips, Wang, & Johnson, 2001) and negative symptoms induced by PCP (Qiao, H.; Noda, Y.; Kamei, H.; Nagai, T.; Furukawa, H.; Miura, H.; Kayukawa, Y.; Ohta, T.; Nabeshima, 2001), but their efficacy is questionable regarding cognitive symptoms. At the neurochemical level, repeated administration of PCP decreases D_1 receptors in the striatum, increases 5-HT_{1A} receptors in the frontal cortex and alters GABAergic receptors expression in the NAcc, striatum and frontal cortex (Beninger et al., 2009; Choi, Snigdha, Shahid, Neill, & Tarazi, 2009). Also, PCP administration reduces NMDA receptors in the NAcc, hippocampus, thalamus and cortex (Newell, Zavitsanou, & Huang, 2007), as well as dopamine levels in the PFC (Jentsch, Tran, Le, Youngren, & Roth, 1997).

Although the model has both face and construct validity, the disappearance of the symptoms after drug withdrawal and the poor predictive validity evident by the failure of in the revearsal of some of the symptoms are its main limitations.

4.1.3 Genetic models.

4.1.3.1 DISC1 models.

Disrupted in schizophrenia 1 (DISC1) gene codes for DISC1 protein, which has been shown to play an essential role in neurodevelopment by participating in synaptogenesis, neuronal migration and synaptic plasticity (Jaaro-Peled, 2009). *DISC1* gene mutations resulting in DISC-1 protein hypofunction have been developed in mice models for schizophrenia research. Morphologically, these animals show enlarged lateral ventricles, reduced cortical thickness and lower cerebral volume (Jaaro-Peled, Ayhan, Pletnikov, & Sawa, 2010), as well as a decrease in the complexity, structure and density of hippocampal dendrites (Kvajo et al., 2008; W. Li et al., 2007). Behaviorally, some studies have reported sensorimotor gating deficits (Clapcote et al., 2007; Hikida et al., 2007), locomotor hyperactivity in the open field (Hikida et al., 2007), reduced social interaction (Clapcote et al., 2007; W. Li et al., 2007) and working memory impairments (Koike, Arguello, Kvajo, Karayiorgou, & Gogos, 2006). However, there is a great discrepancy in this regard since numerous studies have not observed such effects (Hikida et al., 2007; Koike et al., 2006; W. Li et al., 2007; Pletnikov et al., 2008). In addition, DISC1 model´s predictive validity is still limited, as there is not yet enough evidence supporting symptomatology reversal by antipsychotics administration.

The construct validity of this model is limited and the face validity studies provide discrepant results, although these problems are thought to be related to the method used

for the production of the transgenic mouse rather than to the *DISC1* gene mutation *per se*.

4.1.3.2 DTNBP1 models.

Dystobrevin-binding protein 1 (DTNBP1) gene codes for Dysbindin protein, which regulates exocytosis, vesicle biogenesis and receptor trafficking in glutamatergic synapses (Karlsgodt et al., 2011). *DTNBP1* gene mutations resulting in Dysbindin downregulation have been developed in mice models of schizophrenia mainly inspired in the reduced expression of this protein observed in post-mortem tissue of patients, specifically in PFC and hippocampus (Weickert, Rothmond, Hyde, Kleinman, & Straub, 2008; Weickert et al., 2004). Morphologically, mutant mice show narrower synaptic clefts in excitatory junctions and lower density of presynaptic vesicles in dendritic spines of the hippocampal CA1 region (Chen et al., 2008). Behaviorally, these animals show enhanced locomotor activity, reduced social interaction and working memory impairments (Feng et al., 2008; Papaleo et al., 2010), irreversible so far by antipsychotics.

It is a recent and promising model, although its predictive validity is limited, at the moment.

4.1.3.3 RELN models.

RELN gene codes for reelin protein, which is involved in synaptic formation and plasticity within the CNS (Borrell et al., 1999). *RELN* gene mutation resulting in reelin downregulation has been developed in mice models based of the fact that the expression of this protein is lower in the cerebellum, hippocampus and frontal cortex of schizophrenic patients (Jones et al., 2011). Morphologically, these mice show reduced dendritic spine density in the PFC and hippocampus (W. S. Liu et al., 2001), however, they do not present cognitive or social impairments (Krueger et al., 2006) and the presence of positive-like symptoms is inconsistent.

Although reelin may be involved in disrupted processes of schizophrenia, mutant mice use as a genetic model of the disease requires further validation.

4.1.3.4 NRG1 models.

NRG1 gene codes for neuregulin protein, which is involved in CNS development and function at multiple levels. *NRG1* gene mutation resulting in neuregulin hypofunction has been developed in mice models, although the diversity and lack of consistency of the effects, together with the absence of pharmacological studies, currently compromises their validity for the study of schizophrenia (Jones et al., 2011).

4.2 Animal models of drug addiction.

Animal models of drug addiction provide a powerful tool when studying neurobiological and behavioural processes involved in addiction, contributing to the identification of new therapeutic targets for its treatment. When developing such models, diagnostic manuals are of notable use (in spite of the debate that sorrounds them), since they define and classify accurately diagnostic criteria for the disease in humans. Escalation of drug use, impaired impulse control, resistance to extinction, increased motivation for drug-seeking or resistance to punishment are typical features of the human addictive process, which have been modelled in animals (Barry J Everitt, Giuliano, & Belin, 2018; Vanderschuren & Ahmed, 2013). Both the parameters to be analysed and the type of drug to be studied are variables to be taken into account when developing an adequate animal model. These are the most used:

4.2.1 Self-administration models.

These are models with high predictive validity, in which the animal self-administers the drug, intravenously or orally. Depending on the behavioural paradigm used, they can be classified as:

4.2.1.1 Operant learning models.

The animal must perform an operant behaviour, such as pressing a lever, to receive a dose of the drug. The intravenous route is the most used, mainly in rodents, for which it is necessary to implant an intravenous catheter by surgical operation. The oral route is an advantage in this sense; however, there is a time gap between the operant response and the rewarding effects of the substance, and sometimes a prior familiarisation with the drug is required (García Pardo, Roger Sánchez, de la Rubia Ortí, & Aguilar Calpe, 2017).

Generally, the procedure is divided into several phases, which allows the analysis of different features of the addictive process. In this sense, most protocols begin with an acquisition phase, which refers to the period necessary to achieve a stable rate of drug self-administration. Then, other phases can be introduced, depending on the topographies to analyse. For example, an extinction phase, followed by a reinstatement phase, can be useful when modelling relapse in drug-seeking. Extinction is defined as the progressive decrease in the operant response associated with the drug when it is not present (Stewart, 2000), while reinstatement refers to the ability of certain stimuli to restore the operant response learned initially (Shaham, Shalev, Lu, De Wit, & Stewart, 2003). Another feature to study could be motivation for the drug, using a progressive ratio schedule in which the animal has to perform a progressively higher number of responses to obtain the drug (Richardson & Roberts, 1996). The maximum number of operant responses that the animal is able to perform to obtain a reinforcer is called "breaking point" and assess how motivated th animal is to get the drug. Second-order conditioning programs are a powerful tool to measure, the strength of conditioned stimuli to induce drug-seeking in the animals (B. J. Everitt & Robbins, 2000). These paradigms link two different conditioning programs to obtain the reinforcer, so that to get a stimulus previously paired with the drug and for to obtain an infusion, the animals must perform a certain number of prefixed responses. This is intended to resemble consumer behavior in humans, in which the appetitive phase precedes the act of taking the drug. Compulsive behaviors, based on the maintenance of consumption despite the negative consequences derived from it, could be modelled by a phase in which there is an association of the conditioned stimulus, not only with the reinforcer but also with an electric foot shock (Deroche-Gamonet & Piazza, 2014). An extended access phase, on the other hand, could model the transition from moderate to abusive consumption (Edwards & Koob, 2013), since animals with access to the drug for a limited time (for example, 1-2 hours every day) show a stable consumption over time, unlike those with extended access to the drug (for example, 6 hours per day), which show an escalation in self-administration behavior. This escalation has been shown to progressively increase drug craving when the animal is exposed to a later extinction phase, which increases the risk of relapse (Belin, Balado, Piazza, & Deroche-Gamonet, 2009).

4.2.1.2 Free-consumption models.

In these models, unlike the previous ones, the drug is easily accessible to animals, which reduces learning and motivational processes to obtain it (García Pardo et al., 2017). However, they present certain advantages as they do not require prior training or surgical intervention in the animals. There are two outstanding models within this category, the "Two-bottle choice" and "Drinking in the dark".

In the two-bottle choice protocol, animals are allowed to choose in their home cage between the oral consumption of a drug, usually ethanol, and another non-addictive substance such as water, measuring the preference of one over the other (Tabakoff & Hoffman, 2000). In the drinking in the dark protocol (Rhodes, Best, Belknap, Finn, & Crabbe, 2005), water from the bottle of the home cage is replaced by a solution containing ethanol, for 2-4 hours in the dark cycle. Since rodents are nocturnal animals, they have great activity during this period so their drinking intake increase, and with it, plasma levels of ethanol. This model tries to simulate a pattern of binge drinking, although it is aimed at neurotoxic rather than addiction studies.

4.2.2 Conditioned place preference (CPP) models.

CPP is a paradigm that tries to evaluate the association between the reinforcing effect of a drug and the environmental context that surrounds it. Stimuli such as the colour/texture of the floor/walls can acquire appetitive properties when associated with a reinforcer (Aguilar, Rodríguez-Arias, & Miñarro, 2009; Tzschentke, 2007). Following this principle, animals are introduced in a box compound of two clearly differentiated compartments, separated by a neutral central platform. During the "pre-conditioning" phase, it is necessary to confirm that the animal has no innate preference towards any of the compartments. In the "conditioning" phase, the drug is administered in one of the compartments and vehicle in the other, which leads to the association between the reinforcing effect of the drug and the contextual cues of its compartment. Subsequently, in the "post-conditioning" phase, the preference of the animal for the drug-paired compartment is examined (Aguilar et al., 2009; García Pardo et al., 2017).

Processes such as the extinction of motivated behaviour and its reinstatement can be evaluated in the CPP model (Rodríguez-Arias, Castillo, Daza-Losada, Aguilar, & Miñarro, 2009; Roger-Sánchez, Aguilar, Rodríguez-Arias, Aragon, & Miñarro, 2012). If after the acquisition of CPP, the animal is repeatedly exposed to the compartment associated with the drug, but its administration is suppressed, the association between

the reinforcer and the contextual cues will be weakened or extinguished and the preference of conditioned place will disappear. Reinstatement refers to the recovery of the conditioned response after extinction and involves new associative learning between the reinforcing effect of the substance and the contextual cues. It may be induced by priming factors, such as re-administration of a low dose of the drug or by exposure to stressful situations (B. Wang, Luo, Zhang, & Han, 2000; Bin Wang, Luo, Ge, Fu, & Han, 2002). Also, increasing the number of conditioning sessions could assess the vulnerability to developing symptoms related to addiction or susceptibility to relapse, similar to the extended access phase in an operant self-administration model (Rodríguez-Arias et al., 2009). Another application of this paradigm could be to evaluate the opposite process, conditioned place aversion, observed at high doses or in abstinence from certain drugs (García-Carmona, Baroja-Mazo, Milanés, & Laorden, 2015; Parker & Mcdonald, 2000).

4.2.3 Intracranial self-stimulation (ICSS) models.

These models require the implantation of electrodes in brain areas related to reinforcement, using stereotactic surgery (Olds & Milner, 1954). In addition, it is necessary to adjust the frequency and amplitude of the electrical stimulation on these structures for each animal. Acute drug administration decreases ICSS threshold so that the animal needs less electrical stimulation to perceive the reinforcing sensation, as opposed to the abstinence, which increases it. This paradigm is useful to measure the reinforcing/aversive properties of certain drugs (Negus & Miller, 2014).

4.3 Animal models of dual diagnosis.

Up to this date, the use of animal models for dual diagnosis research is not widespread. Far from having developed a specific model for this purpose, the strategy used so far is based on generating a schizophrenic phenotype in the animals and then to analyse their drug consumption later on.

Neurodevelopmental models of schizophrenia are the most widely used in the context of dual diagnosis, specifically those employing prenatal MAM administration, prenatal immune activation and neonatal lesion of the ventral hippocampus.

Studies of prenatal MAM administration in dual diagnosis research show disparate results. Moore et al., 2006 observed a postpubertal increase in the locomotor responsiveness to amphetamine in GD17-MAM exposed animals which was not

observed before puberty. This effect has not been replicated with other stimulants such as cocaine. Robert E Featherstone et al., 2009 did not observe significant differences in the locomotor responsiveness to different dosages of cocaine between GD17-MAM exposed animals and controls. In addition, in this study, cocaine self-administration did not differ between groups neither in the acquisition, dose-response, progressive ratio, extinction, or in the reinstatement phases.

Models of prenatal immune activation present high validity for the replication of typical features of schizophrenia, which makes them a very porwerful tool for the study of dual diagnosis. Animals exposed to LPS during pregnancy showed higher ethanol intake and preference compared to controls (X. Liu et al., 2004). In addition, hyper-locomotion induced by acute amphetamine administration appeared to be increased in these animals, reflecting enhanced dopamine function at the mesolimbic level. However, no differences were observed in behavioural sensitization, a typical feature of the addictive process (Zager, Mennecier, & Palermo-neto, 2012). Also, results obtained in our laboratory (Santos-Toscano, Borcel, Ucha, Orihuel, Capellán, Roura-Martínez, Ambrosio, Higuera-Matas, et al., 2016) showed unaltered cocaine self-administration in LPS-exposed rats. Animals exposed to Poly I:C during pregnancy showed higher preference for amphetamine during the relapse phase in a CPP paradigm, although no differences were found regarding the control group in the acquisition phase (Richtand et al., 2012). However, Borçoi et al., 2015 did observe that prenatal exposure to poly I:C was able to potentiate both AMP-induced behavioural sensitization and CPP, as well as cocaine cross-sensitization.

Models of neonatal ventral hippocampal lesion for the study of dual diagnosis have focused mainly on stimulants. Chambers et al., 2001 observed that injured rats made more responses during the acquisition and maintenance phases of a cocaine self-administration program. In addition, they took longer to reach the extinction criterion, in comparison with sham rats (exposed to a simulated surgical intervention). In the same line, Karlsson, Kircher, Shaham, & Donnell, 2013 observed that it was harder for injured rats to extinguish self-administration behaviour. Moreover, these animals showed a higher rate of response during the cue-induced relapse phase of the cocaine self-administration program in comparison with control rats. In another study conducted with methamphetamine, lesioned rats showed faster self-administration and obtained a higher breaking point in the progressive ratio phase (Brady, Mccallum, Glick, & Donnell, 2008). With regard to nicotine, higher intake during acquisition, higher total consumption and increased drug-seeking were observed in such animals (Berg, Sentir, Cooley, Engleman, & Chambers, 2013). All these data seem to indicate that psychostimulants

consumption has a higher motivational value for injured animals than for controls. A recent study has also studied the effects of this model on alcohol consumption. A two-bottle choice paradigm showed that lesioned animals drunk more alcohol than sham animals and that latent inhibition level seems to be a predictor of consumption (Khokhar & Todd, 2017).

Models of post-weaning social isolation are not usually used for the study of dual diagnosis. However, traumatic experiences such as neonatal isolation or early maternal deprivation have been shown to induce also schizophrenic-like symptomatology as sensorimotor gating deficits (Ellenbroek, Kroonenberg, & Cools, 1998) or increased impulsive behavior (Marco, Adriani, Llorente, & Laviola, 2009) in the animals, which has allowed its use for dual diagnosis study. Different studies have shown that neonatal isolation and early maternal deprivation increases ethanol, stimulants and opiates self-administration (Neisewander, Peartree, & Pentkowski, 2012; Tesone-coelho et al., 2015). Another study observed that maternal deprivation enhances ethanol intake when combined with stressful events later in life (Peñasco, Mela, López-moreno, Viveros, & Marco, 2015). In addition, notable sexual differences have been found. Males exposed to neonatal isolation or early maternal deprivation are more sensitive to alcohol consumption (Gustafsson, Ploj, & Nylander, 2005; Roman, Hyytiä, & Nylander, 2003; Roman, Ploj, & Nylander, 2004), while females are more sensitive to cocaine consumption (Kosten, Sanchez, Yang, & Kehoe, 2004; Kosten, Zhang, & Kehoe, 2006).

The use of pharmacological models of schizophrenia in relation to dual diagnosis is limited mostly due to the fact that the schizophrenic phenotype-inducing drugs (amphetamine, PCP) are also drugs of abuse.

Among the main studies that have used genetic models of schizophrenia, Lipina et al., 2010 observed that *DISC1* L100P line mice (which contain a mutation related to schizophrenia-like behaviour) show enhanced locomotor activity induced by amphetamine administration than control animals, reflecting enhanced dopamine function at the mesolimbic level. Similarly, mutant *DTNBP1* mice have shown increased locomotor sensitisation to repeated five daily injections of amphetamine (Bhardwaj et al., 2009). On the other hand, male *NRG1* mutant mice show a higher sensitivity to the behavioural effects of Δ^9-tetrahydrocannabinol (Boucher, Arnold, & Duffy, 2007), although this is not observed in females (Long, Chesworth, Arnold, & Karl, 2010).

RESEARCH APROACH AND OBJETIVES

Even though prenatal exposure to infections is a risk factor for the development of schizophrenia, epidemiological data show some variability in terms of prevalence and age of onset. The global rate of prevalence of the disease only increases marginally (1 - 2.5%) after an influenza pandemic, although around 20-50% of the population was infected (Selten, Frissen, Lensvelt-Mulders, & Morgan, 2010). For this reason, it has been suggested that other factors, such as traumatic experiences in critical periods of development, could contribute to triggering this disease in vulnerable individuals (Bayer et al., 1999). The vast majority of psychopathologies follow a model of diathesis-stress, in which along with predisposing factors such as exposure to prenatal infections, triggering factors such as abandonment or abuse also participate (Carr, Martins, Stingel, Lemgruber, & Juruena, 2013; Varese et al., 2012).

In this study, we intend to extrapolate this diathesis-stress reality to animal models in order to examine the behavioural, metabolic and anatomical alterations that underlie the possible appearance of the disease. There are previous literature studies that made a first successful approach in this regard. Giovanoli et al., 2013 showed in mice that although Poly I:C (a synthetic viral analog of double-stranded RNA) administration produced moderate effects on behavioural parameters associated with certain symptoms of schizophrenia, the exposure to stress episodes during puberty unmasked clear symptoms associated with the disease. For example, at the behavioral level, sensorimotor gating deficits and sensitised locomotor hyperactivity were observed in response to amphetamine administration, while at the neurobiological level an increase in microglial activity and pro-inflammatory cytokines secretion were observed in the hippocampus. These authors also demonstrated that a treatment that decreases microglial activation during puberty was able to prevent the appearance of multiple behavioural abnormalities relevant to schizophrenia (S. Giovanoli et al., 2016).

In addition, it is essential to keep in mind that very often, schizophrenic patients suffer from an associated substance use disorder. Epidemiological data suggest a comorbidity of more than 70% between both diseases, which makes it essential to approach their research jointly. Moreover, the primary addiction hypothesis proposed by Chambers et al., 2001 states that the high comorbidity between schizophrenia and substance use disorder comes from a shared dysregulation of the same neural network. Our approach, therefore, not only focuses on the etiopathogenic signs that underlie schizophrenia in particular but also on the comorbidity with substance use disorder. As previously stated, operant intravenous self-administration programs allow the analysis of cardinal traits of addiction in animals. There is an exacerbated consumption of cocaine, among other drugs, in the schizophrenic population compared to the healthy population, so we

decided to focus on this drug for our comorbidity studies. Given that cocaine self-administration relies on operant learning, we also decided to study food-rewarded learning to rule out possible learning deficits in the acquisition of the operant response. We also examined Pavlovian learning for its role in cue-induced relapse and in conditioned reinforcement in general.

In addition to studying the influence of prenatal immune activation and stress during puberty on the possible cocaine addiction phenotype, it is worth asking about the mechanisms that mediate such phenotype. Impulsivity is a trait that has proven to be critical in the appearance and maintenance of addictive behaviours (Belin, Mar, Dalley, Robbins, & Everitt, 2008). At present, impulsivity is understood as a multifactorial construct composed of cognitive and motor components (Evenden. John L., 1999). The first of them is characterised by delay aversion and is usually analysed in animals by a delay discounting task, in which a choice must be made between obtaining an immediate reinforcement, a food pellet, or four, that are presented at progressively increasing delays. The second one is characterized by the inability to inhibit a response and is usually studied in animals by a 5-choice serial reaction time task (5-CSRTT). In this last task, the animal must introduce its head in one of five possible openings when a light is turned above them, to obtain a pellet of food. The lights are turned on randomly and the time between each test is increased between successive phases, considering as a measure of impulsivity the responses made between trials, termed premature responses (Bari, Dalley, & Robbins, 2008). Rats performing more premature responses in 5-CSRTT show higher response rates in progressive ratio, compulsivity, escalation in consumption and relapse schedules (Belin et al., 2008; Dalley et al., 2007; Economidou, Pelloux, Robbins, Dalley, & Everitt, 2009). On the other hand, impulsive rats in the delay discounting task show faster cocaine consumption acquisition (J. L. Perry, Larson, German, Madden, & Carroll, 2005; J. L. Perry, Nelson, & Carroll, 2008). In considering these data, it would be interesting to check whether prenatal immune activation potentiates impulsivity in the animals, facilitating the appearance of a cocaine addiction phenotype and whether this phenomenon could be modulated by stressful experiences during puberty.

Although the study of the behavioral processes underlying cocaine addiction phenotype as a consequence of prenatal immune activation and stressful experiences during puberty is crucial, the assessment of the neurological bases of this phenomenon is also important. Neuroimaging techniques such as magnetic resonance imaging (MRI) or positron emission tomography-computed tomography (PET-CT) are very useful tools to attain this goal. MRI allows measuring the volume of different brain structures, while

PET-CT allows analyzing the level of activation. By using these techniques we can study the potential anatomofunctional disturbances arising as a consequence of prenatal immune activation or peripubertal stress exposure that could underlie the greater vulnerability to cocaine addiction found in schizophrenic patients. In addition, an extension of MRI called diffusion tensor imaging (DTI), permits the examination the diffusion of water molecules in different brain structures and tracts analyzing two parameters, mean diffusivity (MD) and fractional anisotropy (FA) which reveal possible changes in the architectural solidity of the tracts or areas. Previous studies with MRI have observed, for example, that prenatal exposure to Poly I:C in animals leads to a decrease in the size of the hippocampus, PFC and striatum (Piontkewitz, Arad, & Weiner, 2011). Another advantage that magnetic resonance studies offer is the possibility of analysing, both *in vivo* and *ex vivo*, the proton spectrum of different brain areas, which allows to measure the levels of a large number of metabolites that may be altered due to prenatal immune activation or peripubertal stress exposure and which could underlie cocaine addiction vulnerability. A previous report on this by Vernon et al., 2015 observed in rats that prenatal exposure to Poly I:C altered the levels of glutathione and taurine in the adult PFC.

It should also be noted that the behavioural, structural and metabolomic alterations underlying vulnerability to addiction could be a result of the influence of prenatal immune activation and peripubertal stress exposure on the gene expression profile. Massive sequencing techniques such as "RNA-seq" offer the possibility of obtaining a quantitative profile of the genes that are being expressed at each moment. So far, the use of this technique in the field of drug addiction has been very scarce, but, for example, Enoch et al., 2012 analyzed the gene expression profile in the hippocampus of rats that showed high preference for ethanol consumption, finding altered expression of some genes related to the GABAergic system such as GABBR1. Regarding dual diagnosis, we are not aware of studies that have used RNA-Seq so far.

Based on all these data and on the previous study of Santos-Toscano et al., 2016, in which we observed unaltered cocaine self-administration in prenatally-exposed rats to LPS, we intend to analyze in the adulthood of these animals if an exposure to a second hit, unpredictable peripubertal stress, may unmask latent neuropathological alterations (Sandra Giovanoli et al., 2013) underlying vulnerability to cocaine addiction, for which we have set the following specific goals:

1. To analyse the presence of schizophrenia-related symptoms in early adulthood as a consequence of prenatal LPS exposure and/or unpredictable peripubertal stress in PPI, social interaction and Y-maze tests.

2. To examine the effects of prenatal LPS exposure and/or unpredictable peripubertal stress on certain features of the addictive behaviour pattern observed in humans (acquisition of consumption, motivation for obtaining the drug, compulsivity, escalation in consumption and incubation of craving to consume during abstinence) in a cocaine self-administration program.

3. To evaluate, based on the results obtained on the acquisition of cocaine consumption, the presence of altered pavlovian and instrumental conditioning processes that could help us understand these results, using food pellets as reinforcers.

4. To study the motor impulsivity underlying the addictive phenotype induced by prenatal LPS exposure and/or unpredictable peripubertal stress, by an adaptation of 5-CSRTT, 2-CSRTT (Hoang, 2010), in operant conditioning chambers.

5. To evaluate the volume, myeloarchitecture and metabolic activity of different brain structures and/or tracts that could be affected by prenatal LPS exposure and/or unpredictable peripubertal stress using MRI, DTI and PET-CT.

6. To measure, by using *in* and *ex vivo* proton magnetic resonance spectroscopy (H^1MRS), the levels of a wide variety of metabolites that could fluctuate in susceptible brain areas due to prenatal LPS exposure and/or unpredictable peripubertal stress.

7. To perform a study of massive RNA sequencing using RNA-Seq to examine possible alterations in gene expression patterns in brain areas related to addiction (nucleus accumbens and dorsal striatum), as a consequence of prenatal exposure to LPS and/or unpredictable peripubertal stress.

MATERIALS AND METHODS

1. Animals.

Experiments were performed on the male offspring of 14-week-old male and 12-week old female Sprague-Dawley rats obtained from Charles River (France). Animals were kept in a temperature and humidity-controlled environment (21 °C/50–60%), artificial light (12 h/12 h light/dark cycle, lights on at 8 pm), *ad libitum* access to food (commercial diet for rodents A04: Panlab, Barcelona, Spain) and tap water, unless otherwise specified. Rats were housed in transparent Plexiglas cages (48.3 cm length x 26.7 cm width x 20.3 cm height). All the procedures performed were compliant with European Union guidelines for the care of laboratory animals (EU Directive 2010/63/EU governing animal experimentation) and approved by the Bioethics Committee of UNED.

2. Experimental procedure.

Male and female rats were mated one week after arrival to the animal facility. Vaginal smears were taken daily from the breeder females, and pregnancy was determined by the presence of sperm in the vaginal smear (day 0 of pregnancy). LPS was intraperitoneally injected to pregnant rats at a dose of 100 µg/kg/ml on gestational days (GD) 15 and 16 (Boksa, 2010; M. E. Fortier et al., 2007; Wischhof et al., 2015). The control group consisted of pregnant rats submitted to the same treatment schedule with saline injection (1 ml/kg) instead of LPS. We imposed a limit of 12 pups per dam, culling the animal surplus. We marked the pups according to their prenatal treatment with a tattoo in the paw and ensured that each damn had an equal number of LPS and saline exposed pups. In doing so, we homogenized any potential effect of prenatal treatment on maternal behaviour that could affect the development of the offspring. Litters were left undisturbed until postnatal day (PND) 21 when they were weaned and grouped in sets of 2-3. Each set belonged to the same litter and treatment. Between PNDs 28 and 38, we exposed the male offspring to peripubertal unpredictable stress (PUS) (Figure 7). This stage of development is a critical period known to be highly sensitive to the disrupting effects of traumatizing events relevant to neuropsychiatric disorders (Sandra Giovanoli et al., 2013). The stress protocol included five distinct stressors, applied on alternate days: 1) Stress by agitation (30 minutes in an orbital shaker at 100rpm). 2) Stress by immobilization (45 minutes in a cylindrical restrainer under bright light). 3) Water deprivation for 16 hours. 4) 10 minutes of a forced swimming session in a water tank of 40 cm high x 18 cm in diameter at a temperature of 22 ± 1ºC and a depth of 30 cm. 5) Constant changes of the home cage (five cage changes, with new sawdust, during the dark cycle at random intervals). Non-stressed controls received handling by the same

researcher and on the same days as the stressed subjects. This protocol was adapted from the work of Sandra Giovanoli et al., 2013.

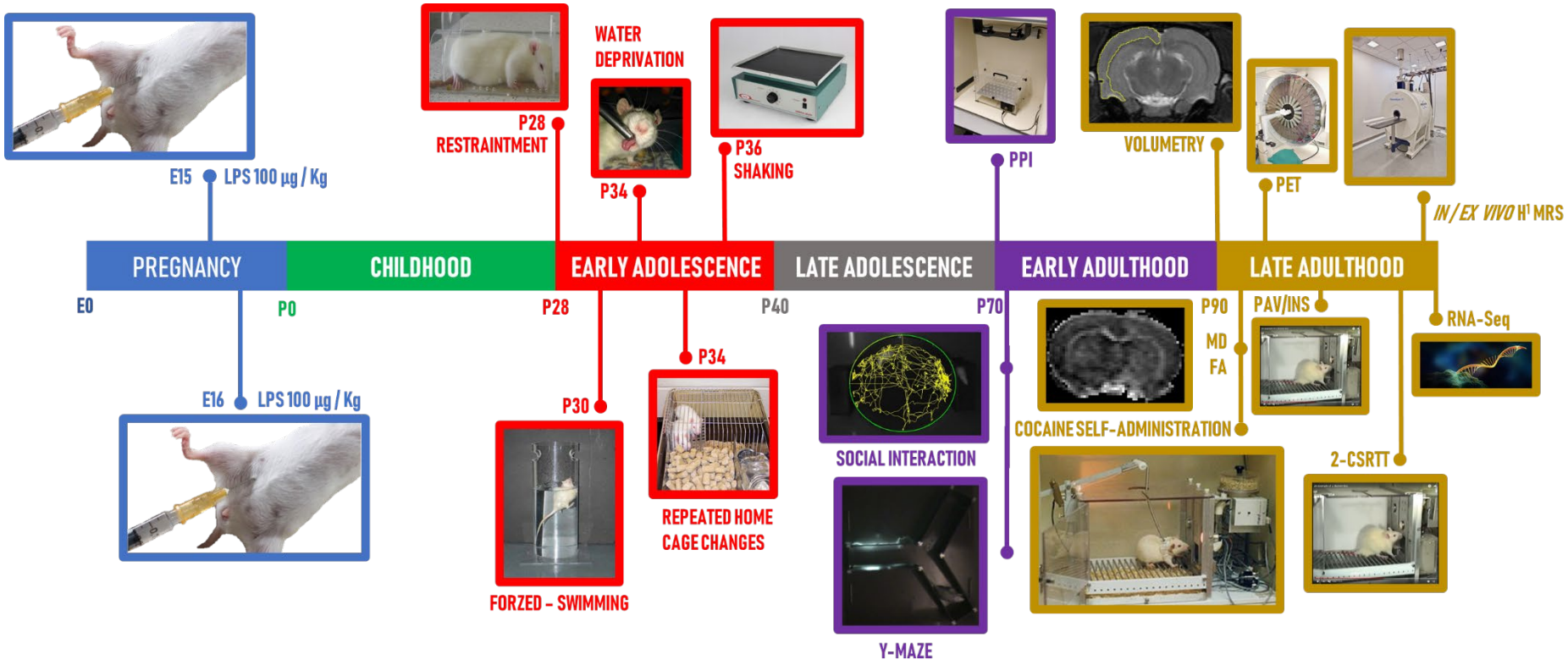


Figure 7: Experimental procedures outline. The sequence of peripubertal stressors shown in this figure responds to a representative example made for a better understanding of the outline. Under experimental conditions, different stress sequences were counterbalanced between groups.

Therefore, it is possible to distinguish four different experimental groups (Figure 8, A). From now on, a great variety of studies were carried out in independent batches of animals (as shown in Figure 8, B), except the PPI test, which was performed in all of them.

A

		Peripubertal unpredictable stress	
		NO	YES
Prenatal immune activation	NO	*SAL + NS*	*SAL + S*
	YES	*LPS + NS*	*LPS + S*

B

BATCH	EXPERIMENT	EARLY ADUTHOOD (≈ PND 70)	LATE ADULTHOOD (≈ PND 90)
6	Schizophrenia-related symptoms assessment	PPI → Social interaction → Y-maze	-
1, 4	Cocaine addiction-like behavior	PPI	Cocaine self-administration
3, 4, 5	Food reinforcement	PPI	Pavlovian and instrumental conditioning
3	Motor impulsivity	PPI	2-CSRTT
5	Neuroimaging	PPI	MRI and DTI or PET-CT
4	Brain metabolomics	PPI	*In/ex vivo* ^{1}H MRS
2	Brain transcriptomics	PPI	RNA-Seq

Figure 8: Groups (A) and batches (B) employed in the different experimental procedures of this study.

3. Behavioral assessment of schizophrenic-like symptoms.

3.1 Prepulse inhibition of the acoustic startle response (PPI).

Introduction to phenomenom.

An essential feature of schizophrenia is the inability to screen out irrelevant sensory input (Braff 1993). PPI provides a valuable tool to study this issue, also known as sensory-motor gating. PPI occurs when a relatively weak sensory event (the prepulse) is presented 30-500 ms before a strong startle inducing stimulus and reduces the magnitude of the startle response (Braff, Geyer, and Swerdlow 2001). Deficient PPI is consistently reported in schizophrenic patients (D. L. Braff et al., 2001; D. Braff et al., 1978; Kumari, Soni, & Sharma, 1999; Weike, Bauer, & Hamm, 2000) and in fact, antipsychotics normalize PPI deficits in these patients (Kumari et al., 1999; Weike et al., 2000). Rodent studies suggested that PPI is regulated by a cortico-striato-pallido-pontine (CSPP) circuitry including frontal and mediotemporal regions, ventral striatum, ventral pallidum, and pontine regions of the brainstem (Fendt, Li, & Yeomans, 2001). Within the CSPP circuit, several neurotransmitters have been demonstrated to be involved in PPI phenomenom such as dopamine, noradrenaline, serotonin, acetylcholine, glutamate, and γ-aminobutyric acid (Geyer, Krebs-Thomson, Braff, & Swerdlow, 2001; Koch, 1999).

Procedure.

PPI was measured at PND70-73 in a non-restrictive Plexiglas cage (28 × 15 × 17 cm) containing a vibration-sensitive platform (Cibertec) (Figure 9). Rats were habituated for 7 minutes with a background noise of 65 decibels (dB), which continued throughout the session. We exposed the rats to 6 pulse-alone trials at the beginning and the end of the session. This was done to stabilise the startle response and to calculate the habituation percentage (these pulses were not included in PPI calculations). The session was composed of 35 different trials: ten 120 dB pulse-alone trials, five null trials with no stimulus and twenty pulses preceded by a prepulse of 69- or 77-dB intensity (4 or 12 dB above the background noise, respectively) with an interval of 30 or 120 ms (Santos toscano et al., 2016). The duration of the test was 20 min, approximately. Prepulse inhibition is expressed as the % PPI and calculated using the following formula: 1 − [startle amplitude on prepulse + pulse trial / mean startle amplitude on pulse-alone trials]) × 100. The percentage of habituation is expressed as 100 × [(Mean of first pulse-alone block − Mean of last pulse-alone block)／Mean of first 6 pulse alone block].

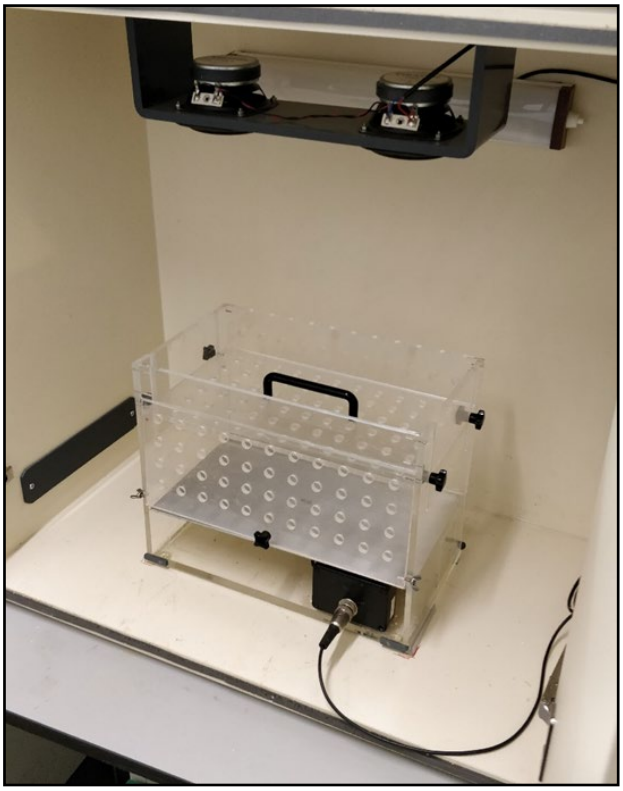


Figure 9: PPI of the startle response apparatus for rat.

3.2 Social interaction test.

Schizophrenia is typically associated with deficits in social interaction, constituting the core of the negative symptom cluster. At PND70, this feature was measured in a elliptical arena (95cm curved sides x 27cm straight sides) with two black opaque confinement cages that had perforated hatches (27cm wide x 25cm high x 15cm deep) (Figure 10). An experimentally naïve rat of the same age and sex was placed in one of the confinement cages, left or right, indistinctly. Next, the experimental rat was placed in the centre of the elliptical field and its behaviour was analyzed for 10 minutes in the dark. Particularly, sniffing time on the perforated hatch adjacent to the confined animal was measured by the Registro Visual de Conducta (RVC) (Cibertec) program and the tracking of the rat movements on the elliptical field was performed by the Animal Tracker plugin of Image J program. Tracking data consisted on the distance travelled and the time spent by each animal in the proximal (adjacent to the confined animal) and distal (not adjacent to the confined animal) areas of the elliptical field.

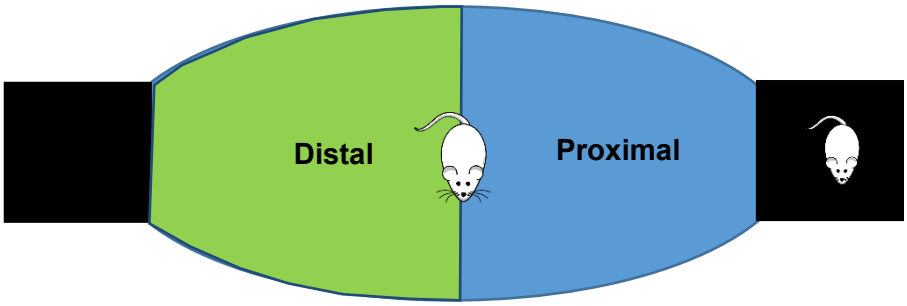


Figure 10: Social interaction testing arena.

3.3 Y-maze.

The Y-maze test is typically used for evaluating working memory in rats (Detrait, Brohez, Hanon, Ryck, & Alleud, 2010; Pulungan, Muttaqien, & Ginus, 2018). It consists of three symmetrical arms (A, B and C) (Figure 11), each with a length of 50 cm, a height of 20 cm and a width of 10 cm, which are placed at 120 ° to each other describing a "Y" shape. At PND70, each rat was placed in the distal part of a randomly selected arm. The rat was expected to move around the maze freely and was allowed to explore it for 8 min. The data on the sequence of arms the rat entered, total arm entries and the number of alternations was manually recorded. An entry to an arm was defined as the whole entry of all four of the rat's limbs into the arm. An alternation represents a sequence of three consecutive entries in three different arms. Alternation data were used to calculate % alternation, which is formulated as: % alternation = ((number of alternations) / (total arm entries – 2)) x 100. The maze was cleaned prior to the testing of each rat to ensure that no odour clues were left by previous rats.

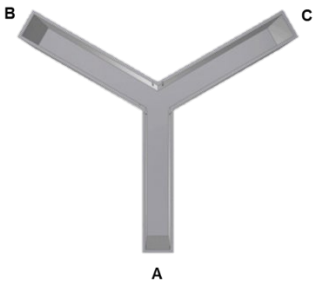


Figure 11: Y-maze.

4. Cocaine self-administration program.

At PND80 animals were underwent surgery to implant a jugular vein catheter that allowed intravenous cocaine self-administration. After this, they were single-housed in independent home cages. Surgeries were performed under isoflurane anesthesia (5% for induction and 1.5-2% during maintenance). An incision was made to implant a polyvinyl chloride catheter (0.064 i.d.) into the jugular vein at the atrium level. This catheter is implanted subcutaneously and reaches the exterior in the middle scapular region. Another incision is made on the back of the animal, where a mesh attached to a dental cement-made pedestal and a screw (Plastics One) is placed. The animals had a recovery time of ten days. From this moment and during the experiment, they were infused daily through the catheter with 0.5ml of a saline solution with heparin (1.5 IU/ml) and gentamicin (40mg/ml) to prevent possible infections and ensure catheter functionality.

On PND90, cocaine self-administration program began with six different phases as described below. All sessions (one per day) were carried out in operant conditioning boxes (Coulbourn Instruments) which had two levers, an “active” lever that was associated with the drug infusion and another “inactive” lever without programmed consequences. The active lever triggered a infusion pump that delived the infusion of ccocaine into the animal throughout a tubing that connects with the catheter previously implanted in the animal. The tubing reached the animal within a thin spring theter attached at the upper end of the conditioning box to prevent breakage. The end of the spring had a line that is attached to the screw that was implanted on the back of the animal. This system allowed the animal to move freely while keeping the infusion system connected, as shown in Figure 12. At the beginning of the sessions, a cue light was activated on the active lever, which is used as a conditioned stimulus (CS) to indicate the availability of the drug. When the animal obtained an infusion, a time out period of 7 seconds began (same length as the infusion) in which the CS disappeared, and the active lever presses had no consequence.

The first phase of the cocaine self-administration program, the Acquisition phase, consisted of 12 sessions, of 2 hours each in a FR1 program, in which an active lever press initiated the infusion of the drug by the pump. The second phase, where motivation for consumption was studied, consisted of six sessions of two hours each in a progressive ratio program, in which the lever presses required to obtain an infusion increased after each infusion according the following sequence: 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20 , 22, 24, 28, 32, 36, 40, 44, 48, 52, 56, 64, 72, 80, 88, 96, 104, etc...). In the third phase, Stabilization, the aim was to rebaseline the lever press, to do this, we

used three sessions of identical conditions as those used in the acquisition phase. The fourth phase, where cocaine compulsive consumption was measured, consisted of a single session of one hour in which under a FR3, the animals randomly received an infusion of 0.5 mg/kg or a mild plant shock of 0.5 mA for 0.5 seconds. The fifth phase, Extended Access, consisted of 10 days with sessions of 6 hours in FR1. At the end of this phase, a forced abstinence period was imposed, with the purpose of evaluating Cue-Induced Relapses, leading to the sixth phase. Drug-seeking sessions occured on days 1, 30, 60 and 100 after the beginning of abstinence, in the same conditions as those described in the Acquisition phase but without any solution flowing throughout the system.

The day before starting the self-administration program and after the last Extended Access session, catheter functionality was checked by a 0.1-0.2 mL infusion of a Thiopental solution (Northia, 20 mg/mL, NaCl 0.9%). The catheter was considered to be functional if the animal showed an immediate loss of consciousness or motor coordination impairment after the infusion.

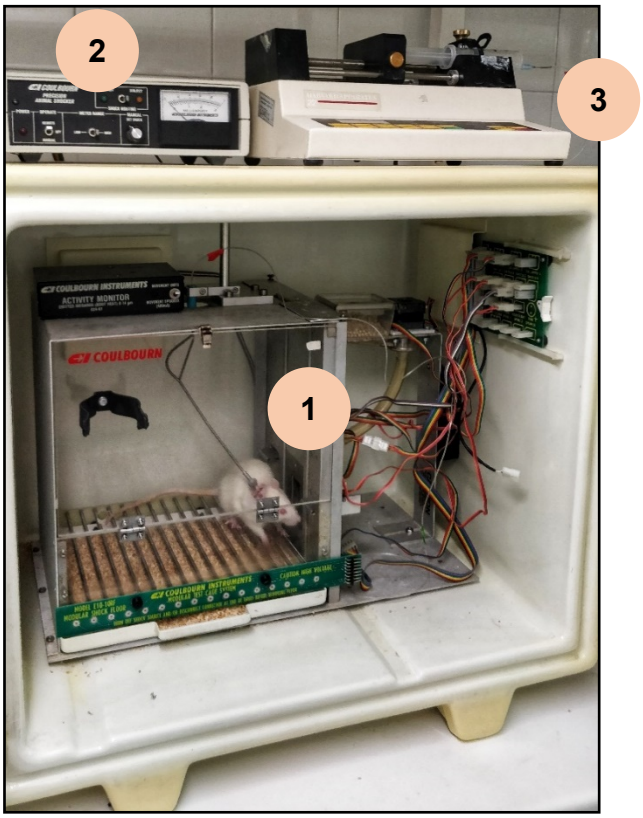


Figure 12: Skinner box (1), shock-generating device (2) and pump (3) used during the cocaine self-administration program.

5. Food-reinforcement by instrumental and pavlovian conditioning programs.

Once PND90 was reached, food was removed, and animal weight was controlled so that it remained between 90-95% of the free-feeding values. Food was provided after each experimental sessions. Experiments were performed in skinner boxes (Med Associates).

Pavlovian learning protocol consisted of eight daily sessions. Each session consisted of 4 cycles of 12 minutes. In each cycle, animals were exposed to 4 minutes of a continuous “tone” stimulus, 4 minutes of an intermittent “click” stimulus and 4 minutes in the absence of stimuli between both. During one of the auditory stimuli exposure (either tone or click), pellets were dropped according to a 5-second variable time program (different time intervals with an average time of five seconds). The conditioned stimulus "CS+", signalled cue-delivery periods whereas the absence of pellets was signalled by the "CS-" stimulus. The 4-minute interval between CS + and CS- was the ISI (inter-stimulus interval), where only the white noise produced by a fan designed to isolate the sound boxes from the outside was maintained. CS+ and CS- were counterbalanced between subjects to avoid attentional biases derived from the unconditioned excitatory capacity of each stimulus, as well as the order in which they were presented. During this protocol, Skinner boxes had no visible operating levers.

Instrumental learning protocol was performed afterwards and consisted of seven daily sessions. Animals had two levers available. Pressing on one of them produced pellet release so that this lever was called "active", while pressing the other one (“inactive”) had no consequence. Once the active lever was pressed, a 5-second timeout began with no pellets. Sessions ended after 30 minutes or after 30 pellets had been earned. Animals completed one fixed ratio 1 (FR1) session, three variable ratio-5 (VR5) sessions and three variable ratio-10 (VR10) sessions. No stimulus was presented during the sessions but white noise was kept to isolate the box from outside noise.

6. 2-CSRTT.

Once the PND90 was reached, food was removed, and body weight was controlled so that it remained between 90-95%. Food was provided after each experimental session.

2-CSRTT (2 Choice Serial Reaction Time Task) was an adaptation from 5-choice serial reaction time task protocol (Bari et al., 2008). We used Skinner boxes (Med Associates) equipped with a feeder with entry detectors, two levers on the sides of the feeder, and two small lamps above the levers. Before the beginning of the protocol, a brief training was implemented to associate light stimulus on each lever with one pellet release when

the lever was pressed. This training consisted of two sessions, one for each lever, with a 30 minute or 30 pellets limit.

The protocol consisted of 13 different stages, and each one was composed of 100 serial trials. Light stimulus duration was progressively reduced from 30 to 0.5 seconds. Response time (or time to respond since light stimulus appeared) was also diminished from 30 to 5 seconds, while inter-trial interval (ITI) (or time elapsed between trials) increased from 2 to 9 seconds, as shown in Table 1.

Trials started when the animal introduced its head inside the feeder, and the signalled lever was alternated randomly during these trials. Correct answers were rewarded with a food pellet, while incorrect answers, omissions (absence of response) and premature responses (lever presses before light stimulus presentation) were punished with a 5-second time-out, where the Skinner box remained in darkness, and no rewards were available. Each session ended after 100 trials were completed or after 30 minutes, whichever occurred first, and accuracy and omissions percentages were calculated to determine if the animal could progress to the next stage (Bari et al., 2008).

Once stage 12 was reached, animals remained in this phase until six consecutive sessions were successfully completed, to stabilise performance. The criterion applied in this phase was ≥75% accuracy and ≤20% omissions. Afterwards, three long ITI sessions (inter-trial interval was increased to 9 seconds) were performed, separated baseline sessions each. This was done to elicit impulsive behaviour (Belin et al., 2008). Between 25 and 45 sessions were carried out until all the animals reached this point. Long ITI data were used to divide animals in low impulsive (LI) and high impulsive (HI), according to premature responses.

Stage	Stimulus duration (s)	ITI (s)	Response time (s)	Criterion to move to next stage
1	30	2	30	≥ 30 Correct trials
2	20	2	20	≥ 30 Correct trials
3	10	5	10	≥ 50 Correct trials
4	5	5	5	≥ 50 Correct trials >80% Accuracy
5	2,5	5	5	≥ 50 Correct trials >80% Accuracy <20% Omissions
6	1,25	5	5	≥ 50 Correct trials >80% Accuracy <20% Omissions
7	1	5	5	≥ 50 Correct trials >80% Accuracy <20% Omissions
8	0,9	5	5	≥ 50 Correct trials >80% Accuracy <20% Omissions
9	0,8	5	5	≥ 50 Correct trials >80% Accuracy <20% Omissions
10	0,7	5	5	≥ 50 Correct trials >80% Accuracy <20% Omissions
11	0,6	5	5	≥ 50 Correct trials >80% Accuracy <20% Omissions
12	0,5	5	5	≥ 50 Correct trials >75% Accuracy <20% Omissions
Prolonged ITI	0,5	9	5	

Table 1: Overview of the stages involved in the 2-choice serial reaction time task (2-CSRTT). Modified from 5-choice serial reaction time task protocol (Bari et al., 2008).

7. Neuroimaging studies.

7.1 MRI and DTI.

At PND90, MRI and DTI studies were performed at the Biomedical Research Institute "Alberto Sols" (CSIC-UAM, Madrid, Spain).

Experiments were performed on a Bruker Pharmascan system (Bruker Medical Gmbh, Ettlingen, Germany) using a 7.0 Tesla horizontal-bore superconducting magnet, equipped with a ^{1}H selective quadrature 40 mm coil and a 90 mm-diameter gradient insert (36 G/cm maximum intensity). All data were acquired using a Hewlett-Packard console running Paravision 5.1 software (Bruker Medical Gmbh) operating on a Linux platform.

Rats were placed into the centre of the radiofrequency volume coil and positioned in the magnet under continuous inhalation anaesthesia via a nose cone. A respiratory sensor connected to a monitoring system (SA Instruments, Stony Brookk, NY) was placed under the abdomen to monitor respiration rate and depth. Animals were anesthetized with a 2% isoflurane-oxygen mixture in an induction chamber and the flow of anesthetic gas was constantly regulated to maintain a breathing rate of 50 +/- 20 beats per minute.

T2-weighted (T2-W) spin-echo anatomical images were acquired with a rapid acquisition with relaxation enhancement (RARE) sequence in axial and coronal orientations and using the following parameters:

- TR: 3000 ms.
- TE: 44 ms.
- RARE: factor 8.
- Averages: 3.
- FOV: 3,5 cm
- Acquisition matrix: 256 × 256 corresponding to an in-plane resolution of 136 × 136 μm^2.
- Slice thickness: 1,50 mm.
- Number of slices: 18 for axial and 8 for coronal images.

Diffusion-weighted images were acquired with a spin echo single shot echo planar imaging (EPI) pulse sequence using the following parameters:

- TR: 3500 ms.
- TE: 40 ms.
- Averages: 1.

- Diffusion gradient duration: 3,5 ms.
- Diffusion gradient separation: 20 ms.
- Gradient directions: 7.
- Acquisition matrix: 96x96 and zero filled in k-space to construct a 128 × 128 corresponding to an in-plane resolution of 273x273 μm^2.
- B values: 100 s/mm^2 and 1400 s/mm^2.
- Slices thickness 1,5 mm.

Fractional anisotropy, mean diffusivity, trace, eigenvalues and eigenvector maps were calculated with a homemade software application written in Matlab (R2007a). Values were extracted from maps using regions of interest (ROIs) with Image J software. The volumetric quantification of the different brain structures was normalized to the whole brain volume in each MRI section.

7.2 PET-CT.

Once PND90 was reached, PET-CT studies were performed at the Radioisotopes for Biomedicine research group of the Center for Energy, Environmental and Technological Research (CIEMAT) in Madrid, Spain, using a small-animal PET-CT (SEDECAL, Madrid, Spain).

PET (400–700 KeV energy window, 45 min static acquisition time) and CT studies (45 kV voltage, 150 μA current intensity, 8 shots, 360 projections, standard resolution) were performed 30 minutes after inoculation of 15.4±1.4 MBq of [^{18}F]-2-fluoro-2-deoxy-d-glucose (^{18}F-FDG) via the tail vein. Animals were anaesthetized by inhalation of 2–2.5% isoflurane in 100% oxygen. PET-CT images reconstruction was accomplished using a 2-dimension ordered subset expectation maximization (2D-OSEM) algorithm (16 subsets and 3 iterations), with random and scatter correction.

The relatively poor spatial resolution of PET-CT imaging and the difficulty in identifying anatomical regions was minimized by co-registering PET-CT images to same-subject MR images. Brain masks (corresponding to hippocampus, caudates, prefrontal cortex, cortex and whole body) were manually segmented on the RM template and applied to their corresponding PET-CT study. Image intensity was normalized to the brain average overall value (100%). Statistical Parametric Mapping (SPM) software was used for voxel-based analysis.

8. *In*/*ex vivo* ^{1}H MRS.

Immediately after conducting the MRI and DTI analysis, ^{1}H MRS studies were also performed in the Biomedical Research Institute "Alberto Sols" (CSIC-UAM, Madrid, Spain). Two brain regions were selected for these studies: cortex and striatum.

For the *in vivo* ^{1}H MRS, a Point-REsolved Spatially Spectroscopy (PRESS) was used, combined with a variable power radiofrequency (VAPOR) water suppression and employing the following parameters:

- TR: 3000 ms.
- TE: 35 ms.
- Averages: 128.
- Voxel volume: 3 mm^3.

First and second order shims were automatically adjusted with a fast, automatic shimming technique by mapping along projections (FASTMAP) in a large voxel (4 mm^3).

Ex vivo ^{1}H MRS was performed two days later. Rats were anesthetized and sacrificed by a brain fixation procudre using a microwave approach. Frontal cortex and dorsal striatuem were dissected out on ice. The samples were analysed on a Bruker Avance 11.7 Tesla spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with a 4 mm triple channel ^{1}H/^{13}C/^{31}P High Resolution Magic Angle Spinning (HR-MAS) resonance probe.

Samples (15mg) were introduced into a 50 µl zirconia rotor (4mm OD) with 50 µl D_2O and spun at 5000Hz at 4ºC to prevent tissue degradation. Two types of monodimensional proton spectra were acquired using a water-suppressed spin echo Carr-Purcell Meiboom-Gill (CPMG) sequence with 36ms and 144ms echo time and 128 scans. Data were collected into 64k data point using a 10kHz (20 ppm) spectral width and water presaturation during 2 s relaxation delay. Total acquisition time was 16 minutes.

In and *ex vivo* spectra were automatically analysed using LCModel software (Provencher, 2001), 6.2-OR version (Oakville, ON; Canada). Only the peak concentrations obtained with a standard deviation lower than 20% were accepted.

9. RNA-Seq

Reached PND90, animals were decapitated for the dissection of different brain structures, under isoflurane anaesthesia. All the dissection material was autoclaved and treated with RNase*Zap*™ (Invitrogen) to avoid RNA degradation by RNases. In addition,

water and saline solution containing diethyl pyrocarbonate (Sigma-Aldrich) (1:500) were used to wash the dissecting material and tissue, respectively. An acrylic brain matrix for a 300g-600g rat and razor blades were used for brain slicing, using (Paxinos & Watson, 2007) as a reference. The entire procedure was carried out cold to prevent tissue degradation. The dissected samples were preserved in RNAlater™ Stabilization Solution (Invitrogen) for one day at -20°C and subsequently stored at -80°C. RNA extraction was performed using the RNeasy Mini Kit (Qiagen).

RNA-Seq analysis was carried out in the Genomics Unit of the Madrid Science Park. RNA integrity number (RIN) and concentration were evaluated by employing an Agilent 2100 Bioanalyzer using a RNA 6000 nano LabChip kit. Libraries were prepared according to the "NEBNext Ultra Directional RNA Library Prep kit for Illumina" (New England Biolabs) instructions. "Chapter 1: Protocol for use with NEBNext Poly(A) mRNA Magnetic Isolation Module" indications were followed. Before starting the protocol, total RNA input yield was 1 µg. A 14-cycle PCR was used to obtain the library amplification included in the mentioned protocol. Libraries were validated and quantified by an Agilent 2100 Bioanalyzer using a DNA7500 LabChip kit. An equimolecular pool of libraries were titrated by quantitative PCR using the "Kapa-SYBR FAST qPCR kit forLightCycler480" (Kapa BioSystems) and a reference standard was used for quantification. The pool of libraries was denatured prior to be seeded on a flowcell at a 2,2pM density, where clusters were formed and sequenced using a "NextSeq™ 500 High Output Kit", in a 1x75 single read sequencing run on a NextSeq500 sequencer.

Once the sequencing process was finished, the Illumina Analysis Space tool was used to map and locate the different sequences in the reference genome, generating alignment files in ". bam" format. These files were used to perform a differential expression analysis using the CUFFDIFF tool, which counted the RNA expression of each gene, normalized by its size and by the global RNA expression of each sample, and made a comparison between groups applying a False Discovery Rate (FDR) correction. We then used Panther to analyse the enrichment in specific gene ontologies for each comparison.

10. Statistical analysis.

Data were analysed with IBM SPSS Statistics 24 for Windows. All results are expressed as the mean ± standard error of the mean (SEM) in the graphs or mean ± standard deviation (SD) in the tables. Outliers were identified by SPSS using the interquartile range criterion and a value of $p < 0.05$ was considered to represent a statistically

significant difference. Square root, Neperian logarithmic and inverse transformations were applied when appropriate to correct the skewness in the distribution of the data and the lack of homogeneity of variances. All results were analyzed by two-way ANOVA considering “Prenatal immune activation” (saline or LPS) and “PUS exposure” (no stress or stress) as between-subject factors. The within subject “session” factor was considered in cocaine self-administration, 2-CSRTT and instrumental/pavlovian conditioning schedules, due to repeated measures designs. The non-parametric Kruskal-Wallis H test was used if ANOVA assumptions were not met. We analysed interactions by simple effects analysis. F-value, effect sizes (partial eta square, η^2_p) and degrees of freedom are also reported when appropriate.

RESULTS

1. Behavioral assessment of schizophrenic-like symptoms.

1.1 Global PPI was not altered in LPS-treated animals or in those exposed to PUS, despite finding subtle variations in independent experimental batches.

We found a trend in LPS-exposed animals to show impaired PPI in the "12dB_30ms"condition (see Table 2). Other than this, there were no significant global effects observed due to prenatal immune activation or PUS exposure in any of the experimental conditions used in the PPI test (Figure 13). Negative values (reflecting prepulse facilitation) were not included in the analysis and global results obtained in the statistical tests are detailed in Table 2. Only in the experimental batches used for cocaine self-administration and RNASeq did we find a reduction in PPI in the "12dB_30ms" and "4dB_30ms conditions, in LPS-exposed animals ($F_{1,102}$ = 0.655, p= 0.012, η^2_p= 0.060) (Table 4) and RNA-Seq ($F_{1,42}$= 6.337, p= 0.016, η^2_p= 0.131) (Table 8), respectively. PUS, in contrast, we found an increased "4dB_30ms" PPI in the RNA-Seq experimental batches ($F_{1,42}$ = 4.400, p= 0.042, η^2_p= 0.095) (Table 8).

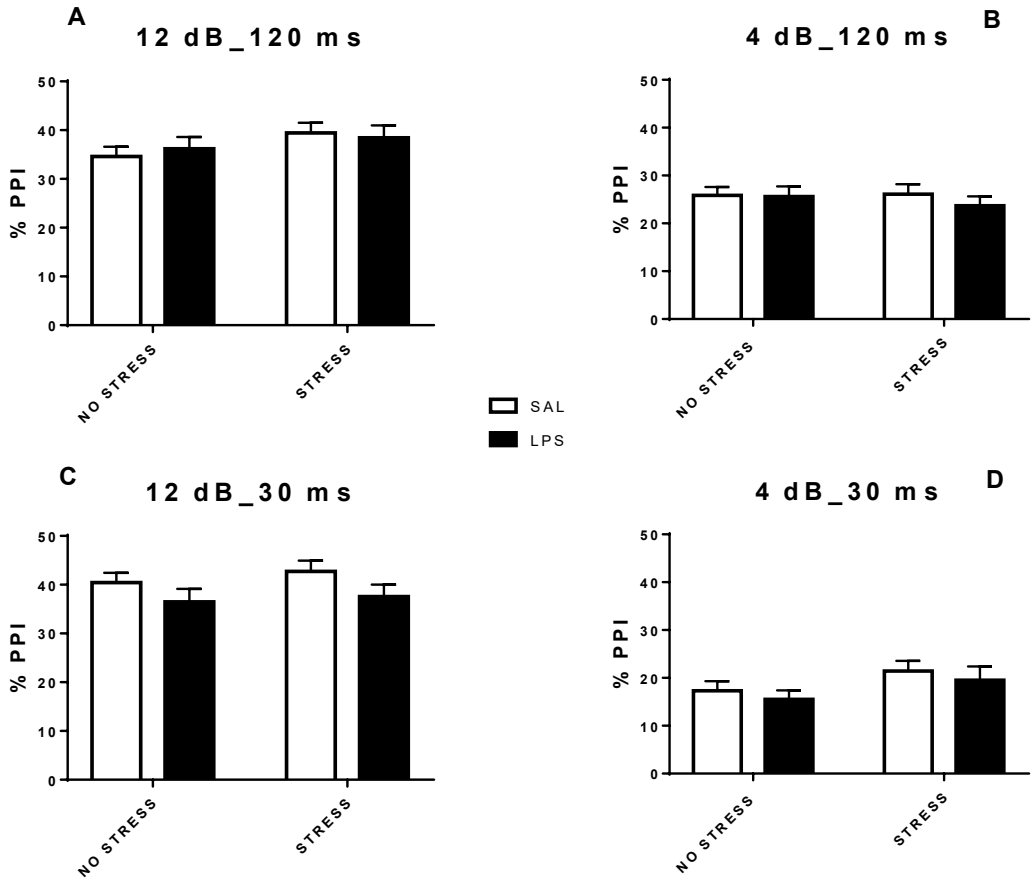


Figure 13: Effects of prenatal LPS treatment or PUS in % PPI. Figure shows % PPI at (A) 12 dB prepulse intensity and 120 ms interval (SAL+ NO STRESS: n=82; SAL + STRESS: n=82; LPS + NO STRESS: n=56; LPS + STRESS: n=55); (B) 4 dB prepulse intensity and 120 ms interval (SAL + NO STRESS S: n=69; SAL + STRESS: n=69;

LPS + NO STRESS: n=50; LPS + STRESS: n=44); (C) 12 dB prepulse intensity and 30 ms interval (SAL + NO STRESS: n=80; SAL + STRESS: n=83; LPS + NO STRESS: n=59; LPS + STRESS: n=52). (D) 4 dB prepulse intensity and 30 ms interval (SAL + NO STRESS: n=50; SAL + STRESS: n=46; LPS + NO STRESS: n=33; LPS + STRESS: n=27).

Habituation was not altered either by prenatal immune activation or PUS exposure (Figure 14).

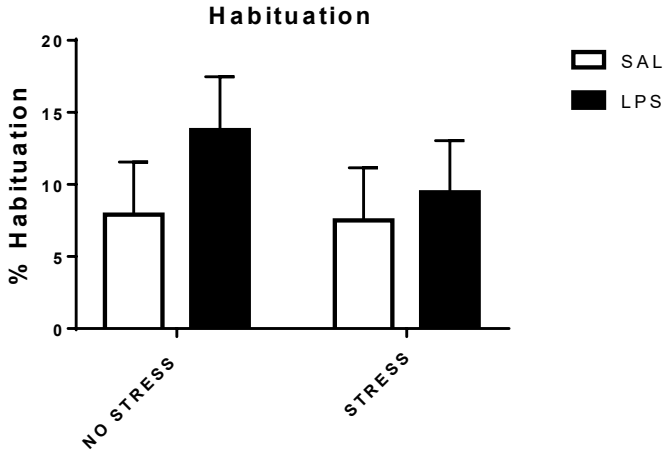


Figure 14: Effects of prenatal LPS treatment or PUS in % Habituation. (SAL + NO STRESS: n=83; SAL + STRESS: n=86; LPS + NO STRESS: n=62; LPS + STRESS: n=55).

Global		
PPI Condition	**F-value and degrees of freedom**	**P-value**
12_120		
Prenatal immune activation	**F (1, 271) = 0.019**	**0.891**
Peripubertal unpredictable stress	**F (1, 271) = 2.412**	**0.122**
Interaction	**F (1, 271) = 0.325**	**0.569**
4_120		
Prenatal immune activation	**F (1, 228) = 0.423**	**0.516**
Peripubertal unpredictable stress	**F (1, 228) = 0.160**	**0.689**
Interaction	**F (1, 228) = 0.279**	**0.598**
12_30		
Prenatal immune activation	**F (1, 270) = 3.678**	***0.056***
Peripubertal unpredictable stress	**F (1, 270) = 0.514**	**0.474**
Interaction	**F (1, 270) = 0.068**	**0.795**
4_30		
Prenatal immune activation	**F (1, 152) = 0.658**	**0.418**
Peripubertal unpredictable stress	**F (1, 152) = 3.195**	***0.076***
Interaction	**F (1, 152) = 0.001**	**0.972**
Habituation to main stimulus		
Prenatal immune activation	**F (1, 282) = 0.707**	**0.401**
Peripubertal unpredictable stress	**F (1, 282) = 0.176**	**0.675**
Interaction	**F (1, 282) = 0.576**	**0.447**

Table 2: Summarized statistical results of the PPI test at the global level.

Social int. and working mem.		
PPI Condition	**F-value and degrees of freedom**	**P-value**
12_120		
Prenatal immune activation	**F (1, 21) = 0.049**	**0.828**
Peripubertal unpredictable stress	**F (1, 21) = 1.741**	**0.201**
Interaction	**F (1, 21) = 0.471**	**0.500**
4_120		
Prenatal immune activation	**F (1, 19) = 0.014**	**0.908**
Peripubertal unpredictable stress	**F (1, 19) = 0.337**	**0.568**
Interaction	**F (1, 19) = 3.938**	***0.062***
12_30		
Prenatal immune activation	**F (1, 20) = 0.109**	**0.745**
Peripubertal unpredictable stress	**F (1, 20) = 0.192**	**0.666**
Interaction	**F (1, 20) = 0.027**	**0.871**
4_30		
Prenatal immune activation	**F (1, 14) = 1.617**	**0.224**
Peripubertal unpredictable stress	**F (1, 14) = 0.002**	**0.962**
Interaction	**F (1, 14) = 1.827**	**0.198**
Habituation to main stimulus		
Prenatal immune activation	**F (1, 21) = 0.496**	**0.489**
Peripubertal unpredictable stress	**F (1, 21) = 0.160**	**0.693**
Interaction	**F (1, 21) = 0.377**	**0.546**

Table 3: Summarized statistical results of the PPI test in the experimental batches used for the study of social interaction and working memory.

Cocaine self-administration		
PPI Condition	**F-value and degrees of freedom**	**P-value**
12_120		
Prenatal immune activation	**F (1, 103) = 0.535**	**0.466**
Peripubertal unpredictable stress	**F (1, 103) = 0.464**	**0.497**
Interaction	**F (1, 103) = 0.882**	**0.350**
4_120		
Prenatal immune activation	**F (1, 81) = 2.043**	**0.157**
Peripubertal unpredictable stress	**F (1, 81) = 0.644**	**0.425**
Interaction	**F (1, 81) = 2.019**	**0.159**
12_30		
Prenatal immune activation	**F (1, 102) = 0.655**	***0.012*** **(↓)**
Peripubertal unpredictable stress	**F (1, 102) = 1.333**	**0.251**
Interaction	**F (1, 102) = 2.587**	**0.111**
4_30		
Prenatal immune activation	**F (1, 52) = 0.360**	**0.551**
Peripubertal unpredictable stress	**F (1, 52) = 0.685**	**0.412**
Interaction	**F (1, 52) = 0.279**	**0.600**
Habituation to main stimulus		
Prenatal immune activation	**F (1, 106) = 0.159**	**0.691**
Peripubertal unpredictable stress	**F (1, 106) = 0.113**	**0.738**
Interaction	**F (1, 106) = 1.080**	**0.301**

Table 4: Summarized statistical results of the PPI test in the experimental batches used for the study of cocaine self-administration.

Food reinforcement		
PPI Condition	**F-value and degrees of freedom**	**P-value**
12_120		
Prenatal immune activation	**F (1, 167) = 0.457**	**0.500**
Peripubertal unpredictable stress	**F (1, 167) = 1.586**	**0.210**
Interaction	**F (1, 167) = 0.000**	**0.993**
4_120		
Prenatal immune activation	**F (1, 147) = 0.455**	**0.501**
Peripubertal unpredictable stress	**F (1, 147) = 0.292**	**0.590**
Interaction	**F (1, 147) = 0.455**	**0.501**
12_30		
Prenatal immune activation	**F (1, 166) = 1.653**	**0.200**
Peripubertal unpredictable stress	**F (1, 166) = 0.393**	**0.532**
Interaction	**F (1, 166) = 0.206**	**0.651**
4_30		
Prenatal immune activation	**F (1, 89) = 0.248**	**0.620**
Peripubertal unpredictable stress	**F (1, 89) = 0.416**	**0.521**
Interaction	**F (1, 89) = 0.093**	**0.761**
Habituation to main stimulus		
Prenatal immune activation	**F (1, 174) = 0.799**	**0.373**
Peripubertal unpredictable stress	**F (1, 174) = 0.532**	**0.467**
Interaction	**F (1, 174) = 0.026**	**0.872**

Table 5: Summarized statistical results of the PPI test in the experimental batches used for the study of food reinforcement.

Motor impulsivity		
PPI Condition	**F-value and degrees of freedom**	**P-value**
12_120		
Prenatal immune activation	**F (1, 42) = 0.577**	**0.452**
Peripubertal unpredictable stress	**F (1, 42) = 1.920**	**0.173**
Interaction	**F (1, 42) = 0.009**	**0.924**
4_120		
Prenatal immune activation	**F (1, 36) = 3.128**	**0.085**
Peripubertal unpredictable stress	**F (1, 36) = 2.079**	**0.158**
Interaction	**F (1, 36) = 1.191**	**0.282**
12_30		
Prenatal immune activation	**F (1, 45) = 0.525**	**0.472**
Peripubertal unpredictable stress	**F (1, 45) = 0.091**	**0.764**
Interaction	**F (1, 45) = 0.789**	**0.379**
4_30		
Prenatal immune activation	**F (1, 24) = 0.000**	**0.989**
Peripubertal unpredictable stress	**F (1, 24) = 0.091**	**0.765**
Interaction	**F (1, 24) = 1.109**	**0.303**
Habituation to main stimulus		
Prenatal immune activation	**F (1, 46) = 3.942**	***0.053***
Peripubertal unpredictable stress	**F (1, 46) = 0.379**	**0.541**
Interaction	**F (1, 46) = 0.805**	**0.374**

Table 6: Summarized statistical results of the PPI test in the experimental batches used for the study of motor impulsivity.

MRI, PET and in/ex vivo H^1MRS		
PPI Condition	**F-value and degrees of freedom**	**P-value**
12_120		
Prenatal immune activation	**F (1, 62) = 0.007**	**0.933**
Peripubertal unpredictable stress	**F (1, 62) = 0.501**	**0.482**
Interaction	**F (1, 62) = 0.000**	**1**
4_120		
Prenatal immune activation	**F (1, 55) = 3.614**	***0.063***
Peripubertal unpredictable stress	**F (1, 55) = 0.000**	**0.984**
Interaction	**F (1, 55) = 0.344**	**0.560**
12_30		
Prenatal immune activation	**F (1, 60) = 0.984**	**0.325**
Peripubertal unpredictable stress	**F (1, 60) = 0.191**	**0.663**
Interaction	**F (1, 60) = 0.077**	**0.783**
4_30		
Prenatal immune activation	**F (1, 29) = 0.164**	**0.689**
Peripubertal unpredictable stress	**F (1, 29) = 1.331**	**0.258**
Interaction	**F (1, 29) = 0.002**	**0.961**
Habituation to main stimulus		
Prenatal immune activation	**F (1, 63) = 0.059**	**0.808**
Peripubertal unpredictable stress	**F (1, 63) = 0.259**	**0.612**
Interaction	**F (1, 63) = 0.235**	**0.630**

Table 7: Summarized statistical results of the PPI test in the experimental batches used for MRI, PET and in/ex vivo H^1MRS studies.

RNA-Seq		
PPI Condition	**F-value and degrees of freedom**	**P-value**
12_120		
Prenatal immune activation	**F (1, 75) = 0.558**	**0.457**
Peripubertal unpredictable stress	**F (1, 75) = 0.121**	**0.729**
Interaction	**F (1, 75) = 1.986**	**0.163**
4_120		
Prenatal immune activation	**F (1, 54) = 0.106**	**0.746**
Peripubertal unpredictable stress	**F (1, 54) = 0.279**	**0.599**
Interaction	**F (1, 54) = 0.956**	**0.333**
12_30		
Prenatal immune activation	**F (1, 75) = 1.159**	**0.285**
Peripubertal unpredictable stress	**F (1, 75) = 0.231**	**0.632**
Interaction	**F (1, 75) = 1.922**	**0.170**
4_30		
Prenatal immune activation	**F (1, 42) = 6.337**	***0.016*** **(↓)**
Peripubertal unpredictable stress	**F (1, 42) = 4.400**	***0.042*** **(↑)**
Interaction	**F (1, 42) = 0.222**	**0.640**
Habituation to main stimulus		
Prenatal immune activation	**F (1, 80) = 0.000**	**0.986**
Peripubertal unpredictable stress	**F (1, 80) = 0.001**	**0.975**
Interaction	**F (1, 80) = 0.925**	**0.339**

Table 8: Summarized statistical results of the PPI test in the experimental batches used for the RNA-Seq study.

1.2 Social interaction was not altered by prenatal immune activation or by peripubertal unpredictable stress exposure.

No significant effects were found by prenatal immune activation, PUS exposure or their interaction in the social interaction parameters measured, either in the sniffing time at the occupied confinement cage (Figure 15), or in the time elapsed or distance travelled in the proximal area compared the global area (proximal + distal) (Figure 16, A and B respectively).

Figure 15: Sniffing time on the perforated hatch adjacent to the confined animal. (SAL+ NO STRESS: n=8; SAL + STRESS: n=9; LPS + NO STRESS: n=4; LPS + STRESS: n=4).

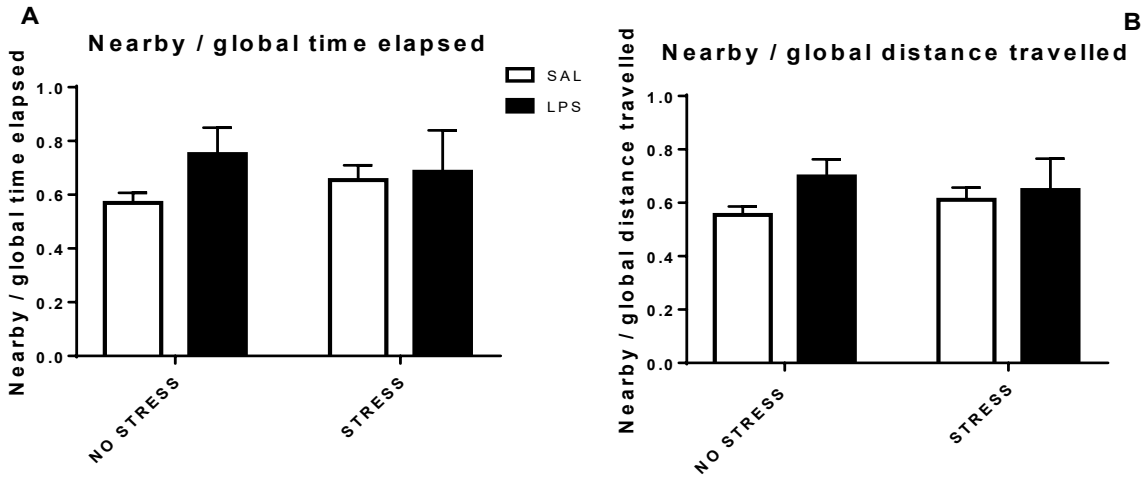


Figure 16: Time elapsed (A) and distance travelled (B) in the proxinal area compared to the global (proximal + distal) area. (SAL+ NO STRESS: n=8; SAL + STRESS: n=9; LPS + NO STRESS: n=4; LPS + STRESS: n=4).

1.3 Working memory was not altered by prenatal immune activation or by peripubertal unpredictable stress exposure.

Working memory, represented by the percentage of alternation in the Y-maze, was not significantly altered by prenatal immune activation, PUS exposure or their interaction (Figure 17).

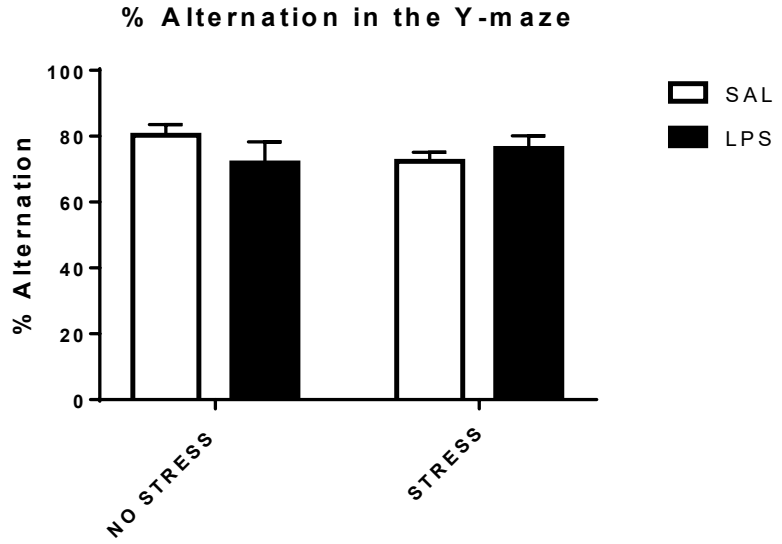


Figure 17: Percentage of alternation in the Y-maze. (SAL+ NO STRESS: n=8; SAL + STRESS: n=9; LPS + NO STRESS: n=4; LPS + STRESS: n=4).

2. Cocaine self-administration.

2.1 Peripubertal stress exposure prevented cocaine intake during the acquisition phase.

Significant effects were found due to the the Acquisition sessions factor ($F_{1,50}$=43.364; p=0.000; η^2_p=0.485) and the interaction between Acquisition sessions * PUS exposure ($F_{1,50}$=3.310; p=0.013; η^2_p=0.067) factors during this phase (Figure 18, A). In the 8th ($F_{1,50}$=4.428; p=0.041; η^2_p=0.086), 9th ($F_{1,50}$=8.021; p=0.007; η^2_p=0.146), 10th ($F_{1,50}$=8.077; p=0.007; η^2_p=0.147), 11th ($F_{1,50}$=5.086; p=0.029; η^2_p=0.098) and 12th ($F_{1,50}$=4.458; p=0.040; η^2_p=0.087) sessions of the acquisition phase, PUS exposure significantly reduced cocaine self-administration (Figure 18, A). No significant effects were found on inactive lever presses during this phase (Figure 18, B).

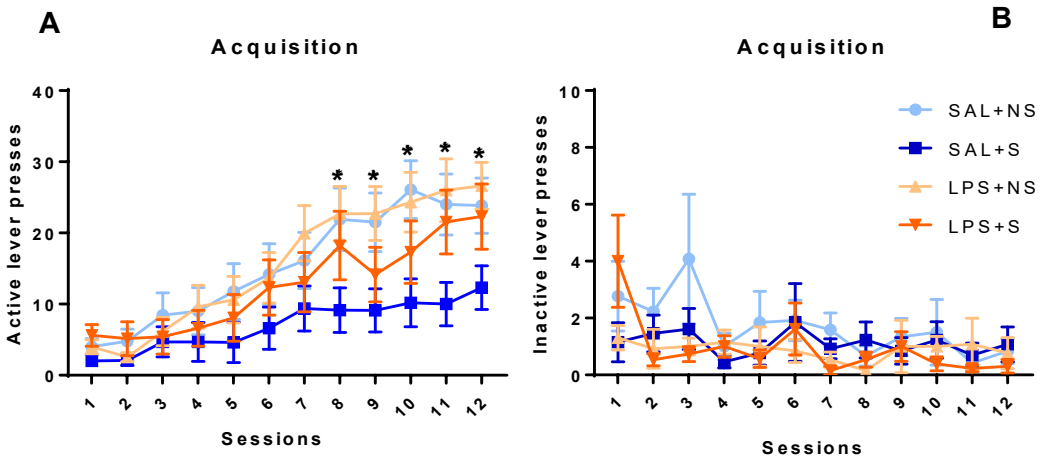


Figure 18: Acquisition phase of the cocaine self-administration program. Figure shows (A) active and (B) inactive lever presses under a 2-hours FR1 schedule. (SAL+NS: n=13; SAL+S: n=13; LPS+NS: n=13; LPS+S: n=15).

Discrimination between active and inactive lever presses was significant on SAL+NS ($F_{1,24}$=19.957; p=0.000; η^2_p=0.476), SAL+S ($F_{1,24}$=6.687; p=0.016; η^2_p=0.218), LPS+NS ($F_{1,24}$=33.525; p=0.000; η^2_p=0.583) and LPS+S ($F_{1,28}$=15.517; p=0.001; η^2_p=0.403) groups during the acquisition phase (Figure 19).

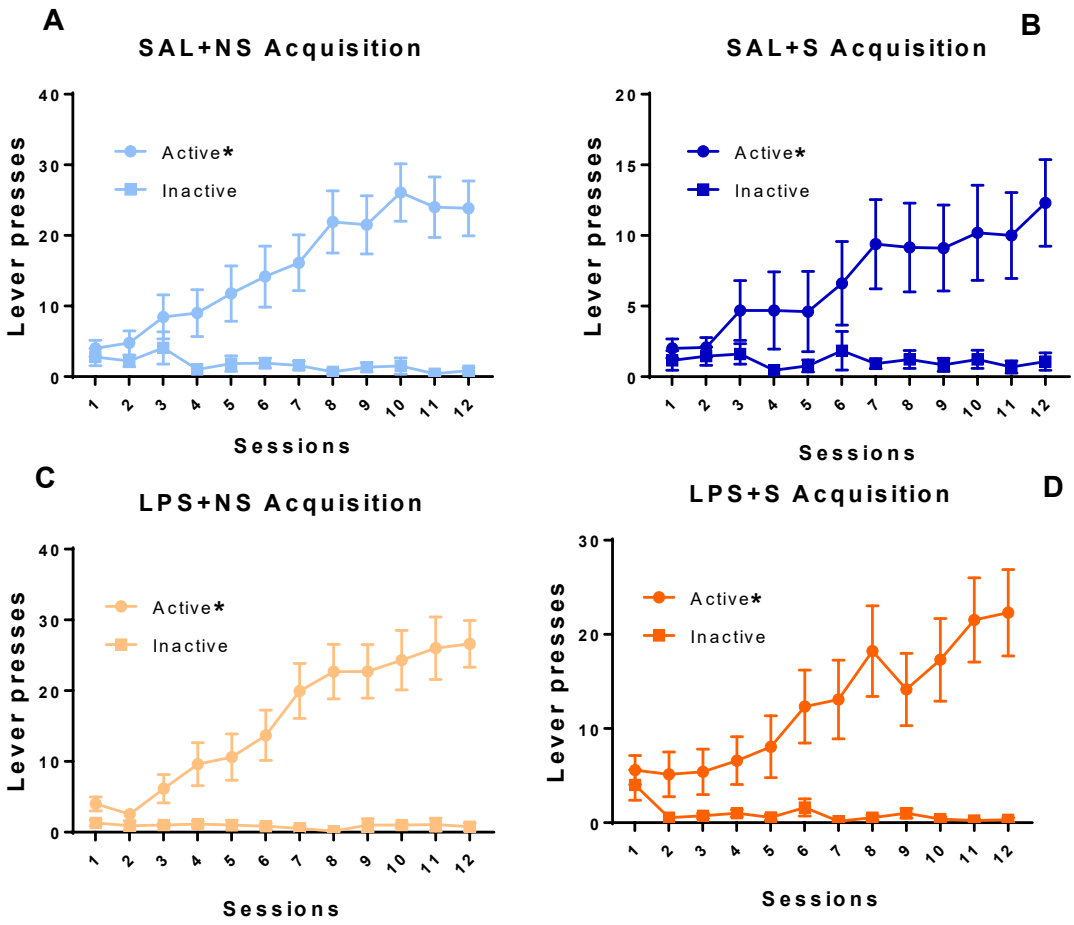


Figure 19: Discriminative analysis of active and inactive lever presses in the acquisition phase of the cocaine self-administration program. Figure shows active vs inactive lever presses in (A) SAL+NS, (B) SAL+S, (C) LPS+NS and (D) LPS+S animals under a FR1 schedule. (SAL+NS: n=13; SAL+S: n=13; LPS+NS: n=13; LPS+S: n=15).

To carry out a qualitative analysis of the acquisition phase (Figure 20), animals were categorized into:

- Normal acquisition: animals that showed a stable behaviour of cocaine self-administration after the first 12 sessions.
- Late acquisition: animals that took more than 12 sessions to present a stable behaviour of cocaine self-administration.
- Not acquisition: animals that never showed a stable behaviour of cocaine self-administration.
- Out of experiment: animals that, due to experimental problems (catheter obstruction, surgical area infection, etc.) did not complete the 12 sessions of the acquisition phase. This category was only included in the global qualitative analysis (Figure 20, A).

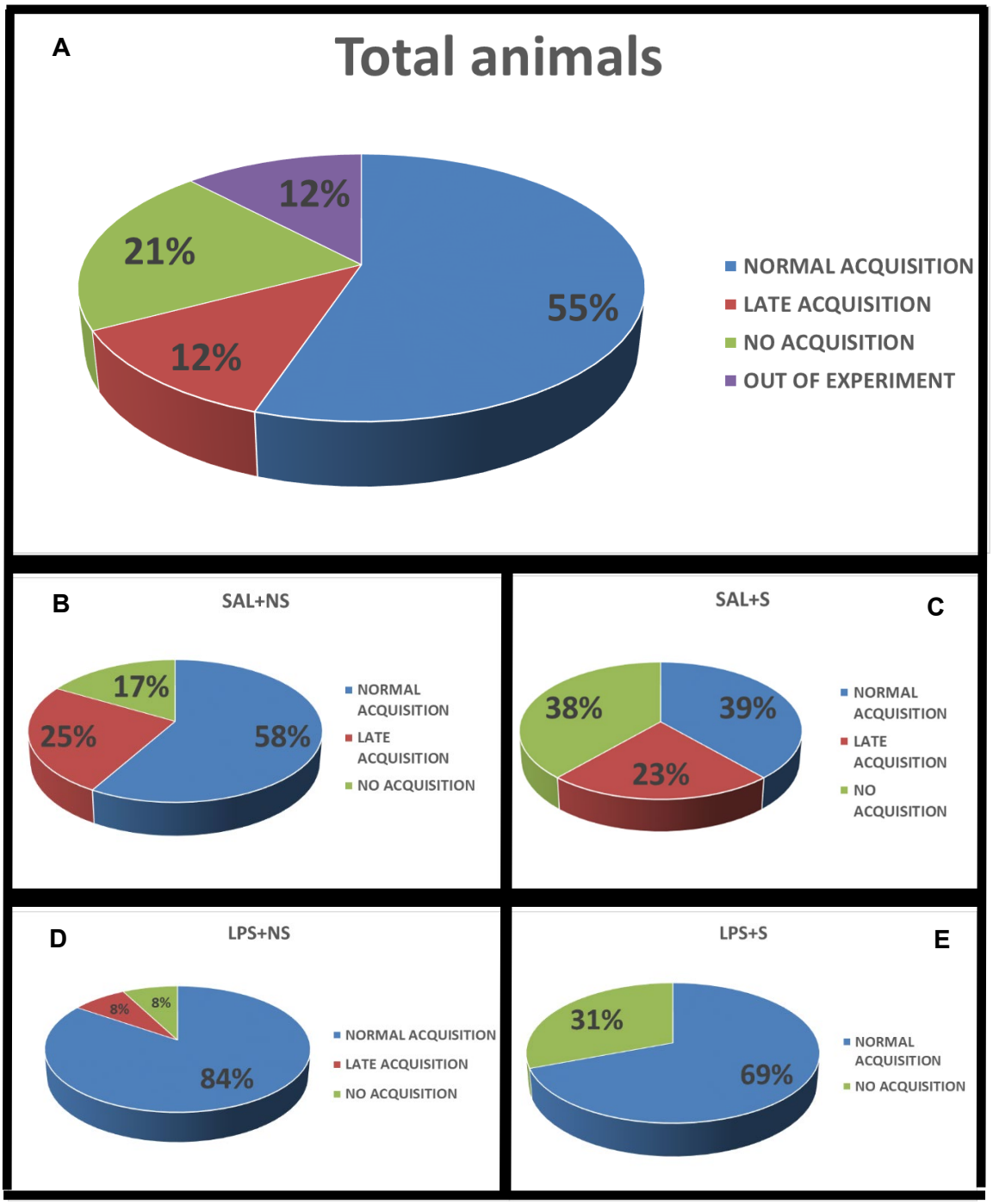


Figure 20: Analysis of the acquisition type during this phase of the cocaine self-administration program. Figure shows, globally (A) and by groups (B, C, D, E), the percentage of animals that represent each acquisition category. (Total animals: Normal acquisition = 32; Late acquisition = 7; No acquisition = 12; Out of experiment = 7. SAL+NS: Normal acquisition = 7; Late acquisition = 3; No acquisition = 2. SAL+S: Normal acquisition = 5; Late acquisition = 3; No acquisition = 5. LPS+NS: Normal acquisition = 11; Late acquisition = 1; No acquisition = 1. LPS+S: Normal acquisition = 9; Late acquisition = 0; No acquisition = 4.)

Pearson's chi-squared test showed no significant differences between groups (p = 0.145). No significant differences were found when analyzing the effect of PUS exposure (p = 0.163) individually but we found a trend in prenatal LPS-exposed animals (p = 0.053) to show facilitated acquisition of cocaine self-administration (higher percentage of normal acquisition in these animals).

2.2 LPS offspring showed higher motivation for cocaine consumption in the first session of the progressive ratio phase.

Greater motivation for cocaine consumption was observed in the first progressive ratio session (day 13) by LPS-exposed animals ($F_{1,34}$=6.628; p=0.015; η^2_p= 0.163) (Figure 21). Active lever presses values were normalized regarding the last session of the acquisition phase to rule out a possible influence of the consumption pattern exhibited in the previous phase.

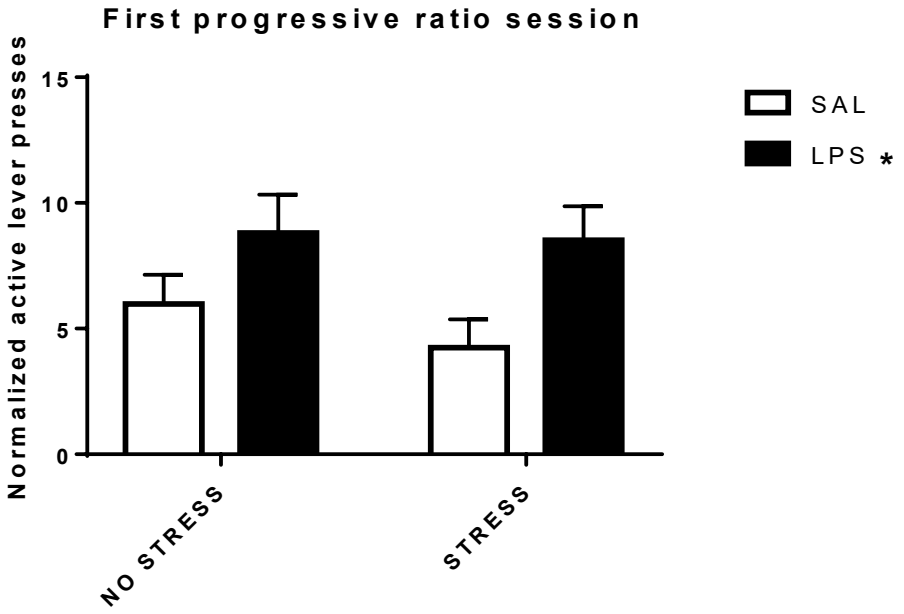


Figure 21: First progressive ratio session of the cocaine self-administration program. Figure shows normalized active lever presses (active lever presses in session 13/ active lever presses in session 12) under a 2-hours progressive ratio schedule. (SAL+NS: n=9; SAL+S: n=8; LPS+NS: n=12; LPS+S: n=9).

A significant effect of the Progressive ratio sessions factor ($F_{1,34}$=5.206; p=0.002; η^2_p=0.167) was found in breaking points executed during the progressive ratio phase, however, no significant differences were obtained between groups (Figure 22, A). Inactive lever presses were not significantly altered during this phase (Figure 22, B).

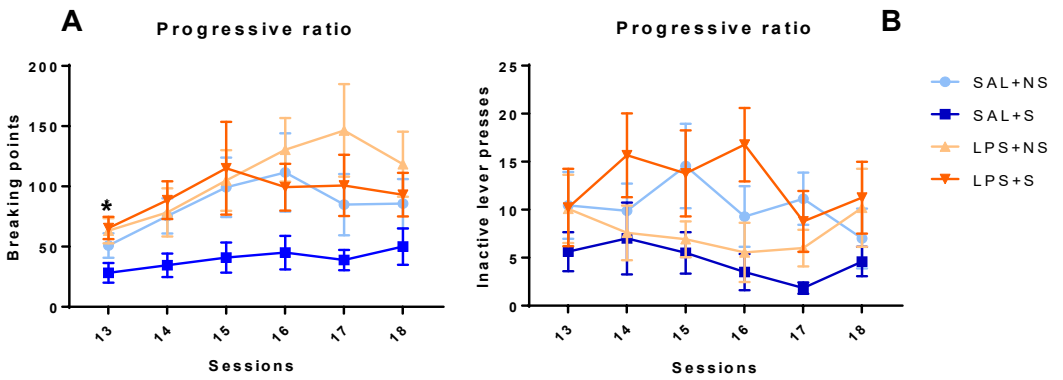


Figure 22: Progressive ratio phase of the cocaine self-administration program. Figure shows (A) breaking points and (B) inactive lever presses on a 2-hours progressive ratio schedule. (SAL+NS: n=9; SAL+S: n=8; LPS+NS: n=12; LPS+S: n=9).

Discrimination between active and inactive lever presses was significant on SAL+NS ($F_{1,16}$=12.105; p=0.005; η^2_p=0.502), SAL+S ($F_{1,14}$=7.322; p=0.027; η^2_p=0.478), LPS+NS ($F_{1,22}$=19.993; p=0.000; η^2_p=0.526) and LPS+S ($F_{1,16}$=14.640; p=0.002; η^2_p=0.511) groups during the progressive ratio phase (Figure 23).

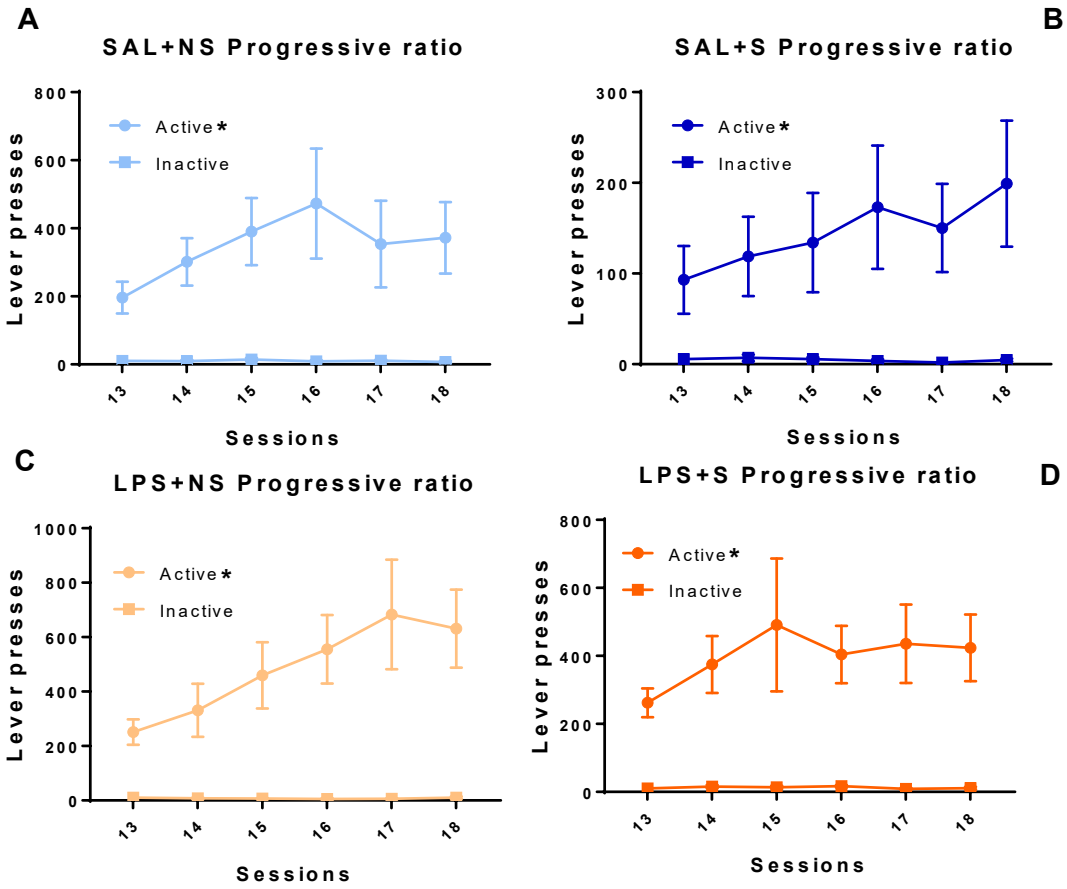


Figure 23: Discriminative analysis of active and inactive lever presses in the progressive ratio phase of the cocaine self-administration program. The figure shows active vs inactive lever presses in (A) SAL+NS, (B) SAL+S, (C) LPS+NS and (D) LPS+S animals under a progressive ratio schedule. (SAL+NS: n=9; SAL+S: n=8; LPS+NS: n=12; LPS+S: n=9).

2.3 Peripubertal stress exposure reduced cocaine self-administration, while prenatal LPS treatment reversed this effect, throughout the stabilization phase.

Significant effects were found due to the Stabilization sessions factor ($F_{1,30}$=7.432; p=0.001; η^2_p=0.204) and to the interaction between prenatal immune activation * PUS exposure ($F_{1,30}$=7.316; p=0.011; η^2_p=0.201) factors during the stabilization phase. Prenatal LPS-treatment reversed ($F_{1,32}$=6.21; p=0.018) the reduction in cocaine self-administration induced by PUS exposure ($F_{1,32}$=7.54; p=0.010) (Figure 24, A). No significant effects were found in inactive lever presses during this phase (Figure 24, B).

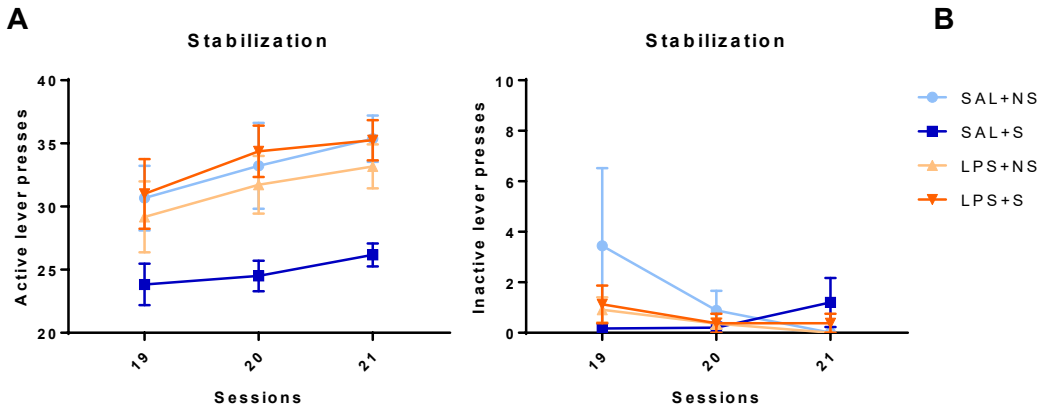


Figure 24: Stabilization phase of the cocaine self-administration program. Figure shows (A) active and (B) inactive lever presses under a 2-hours FR1 schedule. (SAL+NS: n=9; SAL+S: n=6; LPS+NS: n=11; LPS+S: n=8).

Discrimination between active and inactive lever presses was significant on SAL+NS ($F_{1,16}$=165.474; p=0.000; η^2_p=0.922), SAL+S ($F_{1,10}$=399.279; p=0.000; η^2_p=0.978), LPS+NS ($F_{1,20}$=196.732; p=0.000; η^2_p=0.908) and LPS+S ($F_{1,14}$=262.700; p=0.000; η^2_p=0.949) groups during the stabilization phase (Figure 25).

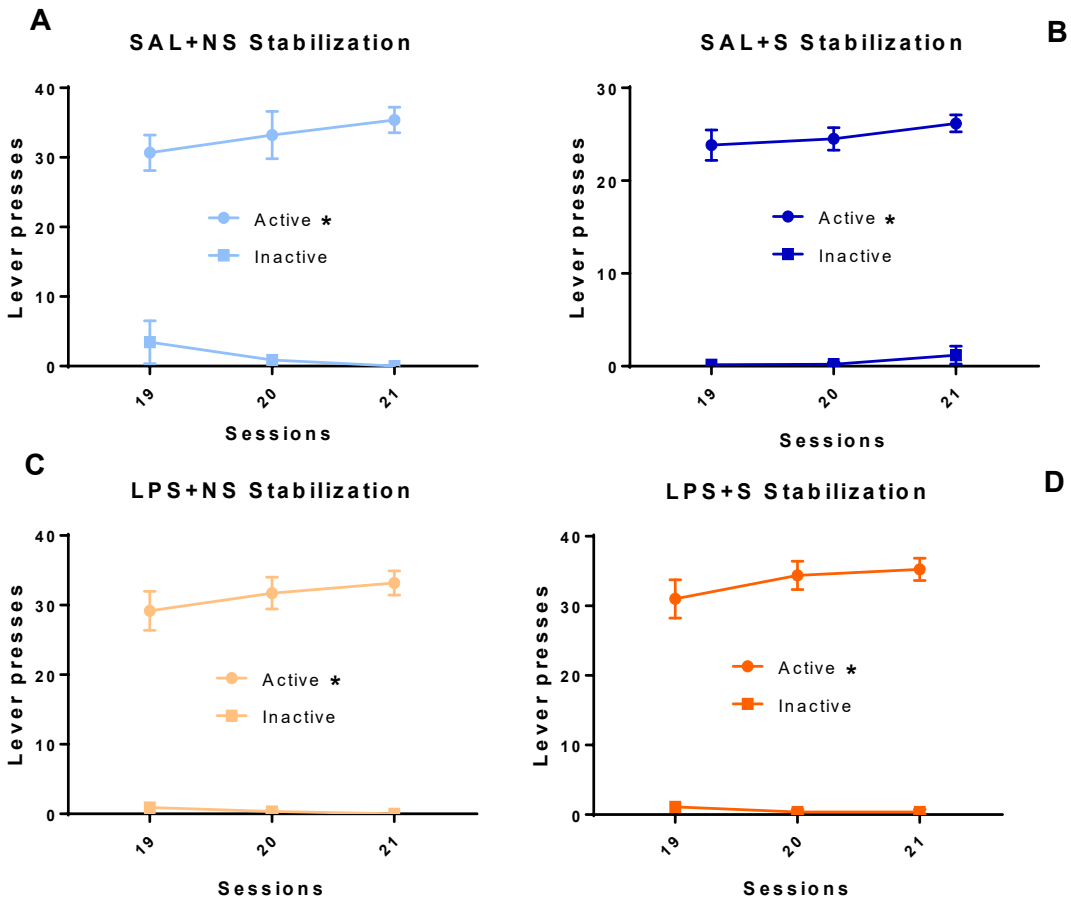


Figure 25: Discriminative analysis of active and inactive lever presses in the stabilization phase of the cocaine self-administration program. Figure shows active vs inactive lever presses in (A) SAL+NS, (B) SAL+S, (C) LPS+NS and (D) LPS+S animals under a FR1 schedule. (SAL+NS: n=9; SAL+S: n=6; LPS+NS: n=11; LPS+S: n=8).

2.4 Compulsive cocaine-seeking was not affected by prenatal immune activation or by PUS.

A significant effect of the Sessions factor ($F_{1,28}$=388.172; p=0.000; η^2_p=0.933) was found in the events (infusions) executed during the last stabilization phase session as compared to those observed in the compulsive-seeking phase (infusions/shocks), however, no significant differences were obtained between groups (Figure 26).

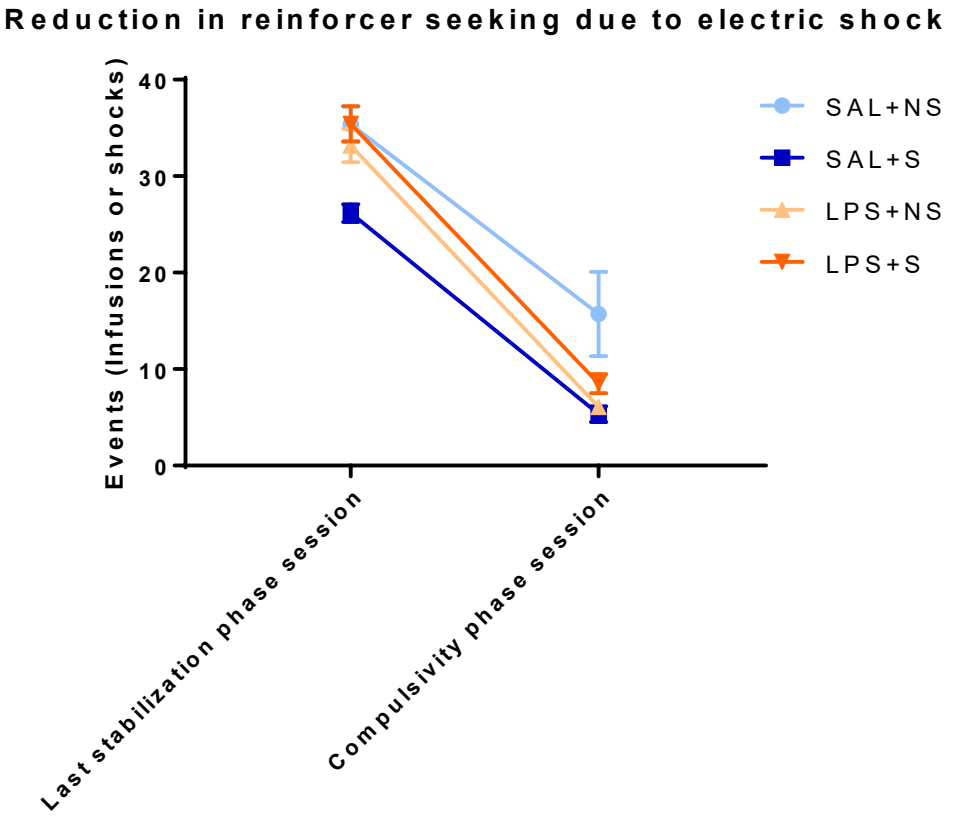


Figure 26: Reduction in reinforcer seeking due to electric shock. The figure shows the difference of events (infusions) between the last session of the stabilization phase and the compulsive seeing-phase (infusions or shocks) session. (SAL+NS: n=7; SAL+S: n=6; LPS+NS: n=11; LPS+S: n=8).

2.5 Prenatal LPS treatment increased cocaine self-administration among peripubertal-stressed animals, throughout the extended access phase.

Significant effects were found due to the Extended access sessions factor ($F_{1,28}$=4.215; p=0.008; η^2_p=0.161) and the interaction between prenatal immune activation * PUS exposure ($F_{1,28}$=5.423; p=0.029; η^2_p=0.198) factors during the extended access phase. Prenatal LPS-treatment increased cocaine self-administration in PUS-exposed animals ($F_{1,28}$=4.98; p=0.033) (Figure 27, A). No significant effects were found on inactive lever presses during this phase (Figure 27, B).

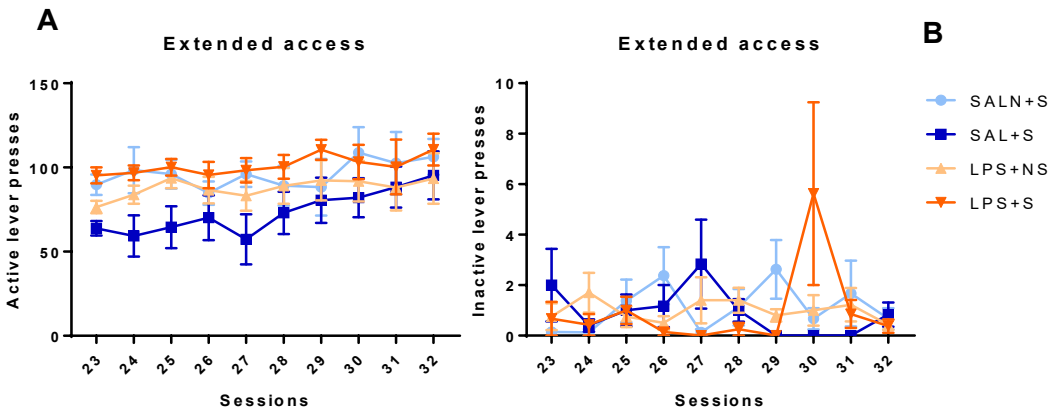


Figure 27: Extended access phase of the cocaine self-administration program. Figure shows (A) active and (B) inactive lever presses under 6-hours FR1 schedule. (SAL+NS: n=8; SAL+S: n=6; LPS+NS: n=11; LPS+S: n=8).

Discrimination between active and inactive lever presses was significant on SAL+NS ($F_{1,14}$=167.833; p=0.000; η^2_p=0.955), SAL+S ($F_{1,10}$=36.549; p=0.000; η^2_p=0.802), LPS+NS ($F_{1,20}$=14.050; p=0.007; η^2_p=0.667) and LPS+S ($F_{1,14}$=164.346; p=0.000; η^2_p=0.943) groups during the extended access phase (Figure 28).

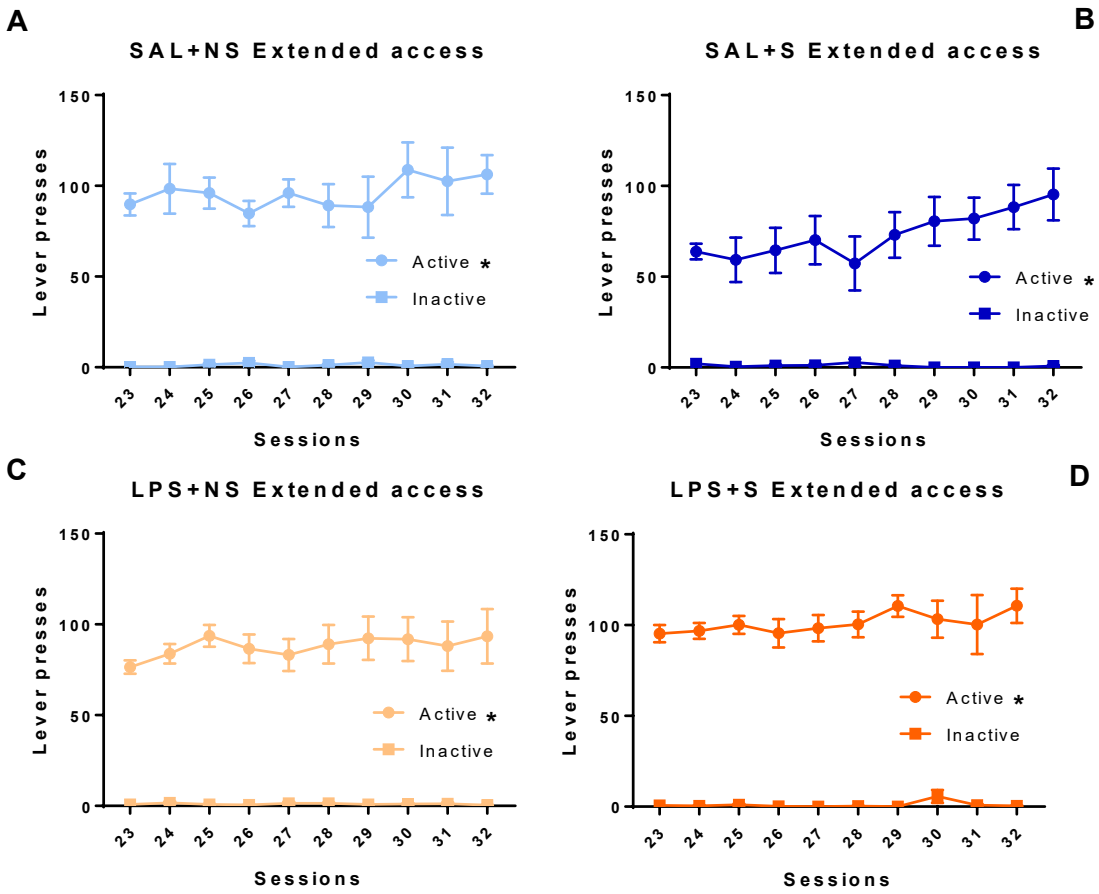


Figure 28: Discriminative analysis of active and inactive lever presses in the extended access phase of the cocaine self-administration program. Figure shows active vs inactive lever presses in (A) SAL+NS, (B) SAL+S, (C) LPS+NS and (D) LPS+S animals under a 6-hours FR1 schedule. (SAL+NS: n=8; SAL+S: n=6; LPS+NS: n=11; LPS+S: n=8).

2.6 Neither gestational LPS Treatment nor peripubertal stress exposure increased cue-induced cocaine seeking.

A significant effects of the Relapse sessions factor was found in active ($F_{1,24}$=10.989; p=0.000; η^2_p=0.314) (Figure 29, A) and inactive ($F_{1,24}$=6.790; p=0.002; η^2_p=0.221) (Figure 29, B) lever presses during the Relapse phase, however, no significant differences were obtained between groups.

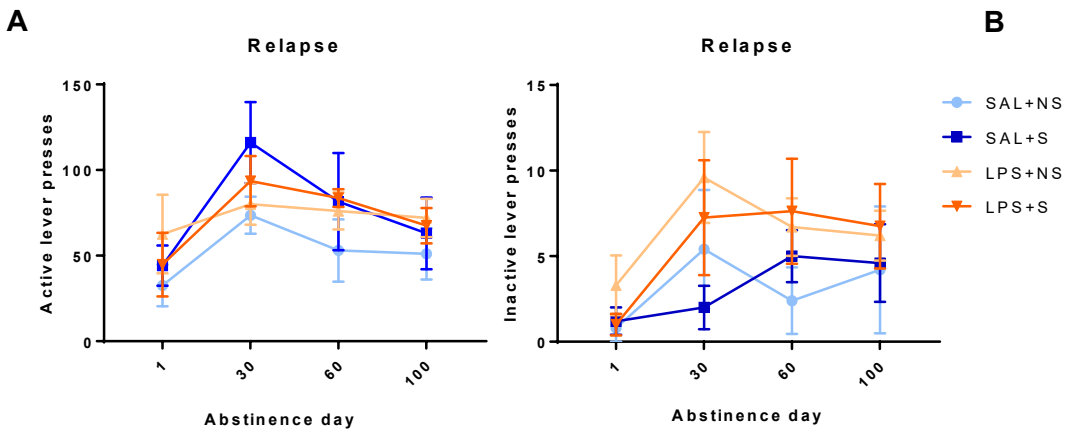


Figure 29: Cue-induced relapse phase of the cocaine self-administration program. Figure shows (A) active (LPR1+LPTOR1) and (B) inactive (LPR2+LPTOR2) lever presses under 2-hours sessions at abstinence days 1,30,60 and 100. (SAL+NS: n=5; SAL+S: n=5; LPS+NS: n=10; LPS+S: n=8).

Discrimination between active and inactive lever presses was significant on SAL+NS ($F_{1,8}$=19.335 p=0.002; η^2_p=0.707), SAL+S ($F_{1,8}$=14.776; p=0.005; η^2_p=0.649), LPS+NS ($F_{1,18}$=27.367; p=0.000; η^2_p=0.603) and LPS+S ($F_{1,14}$=42.701; p=0.000; η^2_p=0.753) groups during the cue-induced relapse phase (Figure 30).

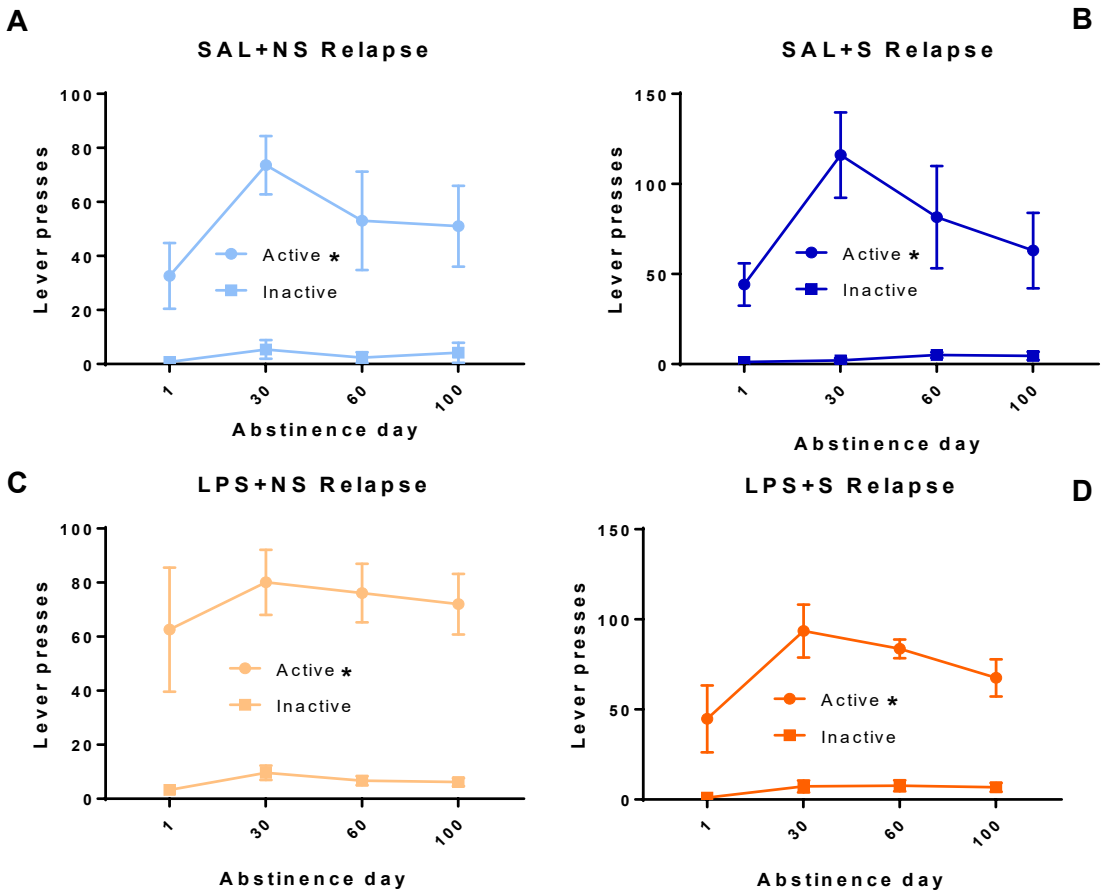


Figure 30: Discriminative analysis of active and inactive lever presses in the relapse phase of the cocaine self-administration program. Figure shows active vs inactive lever presses in (A) SAL+NS, (B) SAL+S, (C) LPS+NS and (D) LPS+S animals under 2-hours sessions at abstinence days 1,30,60 and 100. (SAL+NS: n=5; SAL+S: n=5; LPS+NS: n=10; LPS+S: n=8).

3. Food Reinforcement.

3.1 Pavlovian and instrumental conditioning programs did not reveal food-reinforced learning disturbances by prenatal immune activation or peripubertal unpredictable stress.

Significant effects of the Pavlovian ($F_{1,56}$=20.181; p=0.000; η^2_p=0.265) (Figure 31, A) and Instrumental ($F_{1,56}$=39.638; p=0.000; η^2_p=0.414) (Figure 31, B) sessions factors were found during the instrumental and pavlovian conditioning phases, however, no significant differences were obtained between groups.

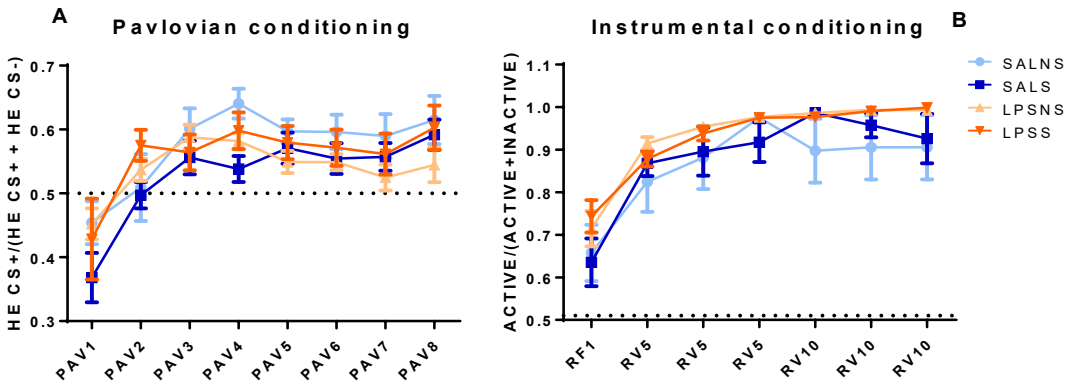


Figure 31: Food-reinforcement by pavlovian (A) and instrumental (B) conditioning programs. Figure A shows the normalized head entries (HE) ratio of the eight sessions of the pavlovian conditioning program, while Figure B shows the normalised active lever presses ratio of the RF1, VR5 and VR10 sessions of the instrumental conditioning program (SAL+NS: n=13; SAL+S: n=17; LPS+NS: n=19; LPS+S: n=11).

4. 2-CSRTT.

4.1 Motor impulsivity was not altered neither by prenatal immune activation nor by peripubertal unpredictable stress.

No significant effects were observed by prenatal immune activation, PUS exposure or their interaction in the number of sessions to reach stage 12 (Figure 32).

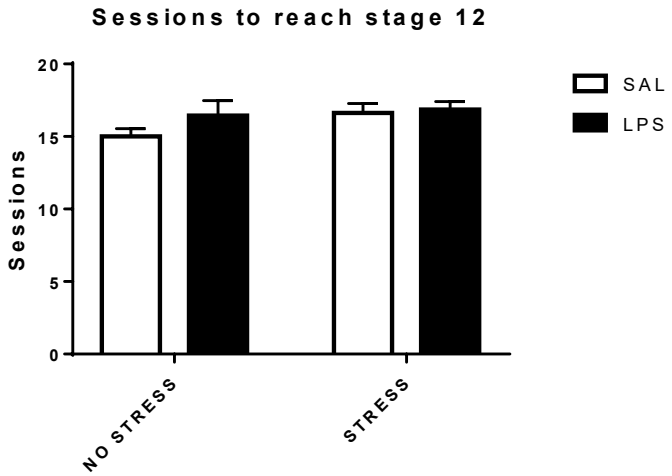
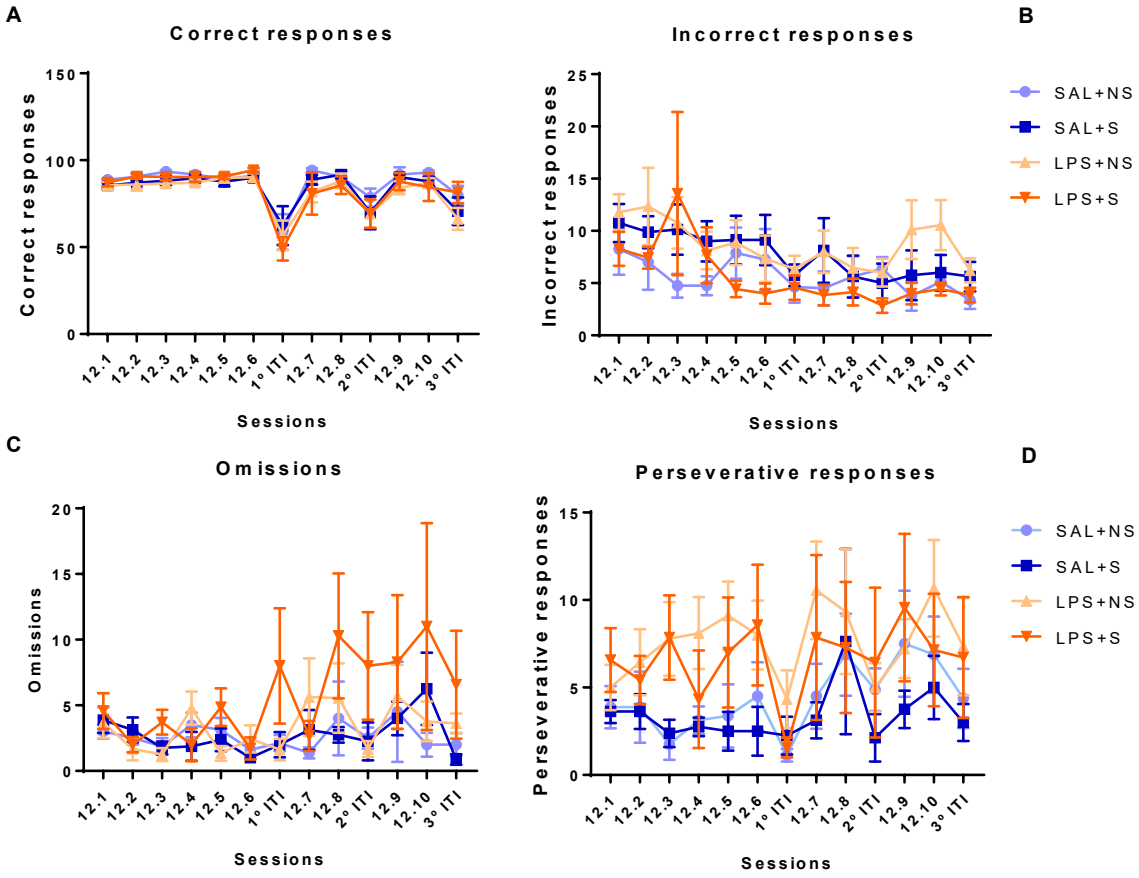


Figure 32: Number of sessions to reach stage 12 of the 2-CSRTT program. (SAL+NS: n=8; SAL+S: n=8; LPS+NS: n=9; LPS+S: n=7).

A significant effect of the Sessions factor was found in correct ($F_{1,28}$=20.177; p=0.000; η^2_p=0.428), incorrect ($F_{1,28}$=4.756; p=0.001; η^2_p=0.150), omitted ($F_{1,28}$=3.331; p=0.021;

η^2_p=0.118), perseverative ($F_{1,28}$=3.305; p=0.010; η^2_p=0.109), premature ($F_{1,28}$=33.707; p=0.000; η^2_p=0.555) and % premature ($F_{1,28}$=43.628; p=0.000; η^2_p=0.618) responses in stage 12 of the 2-CSRTT, however, no significant differences were obtained between groups in any of the sessions (Figure 33, A-H).

In figures G and H, premature responses values performed in the long-ITI sessions were normalised regarding the two previous sessions to rule out the possible influence of the baseline sessions.



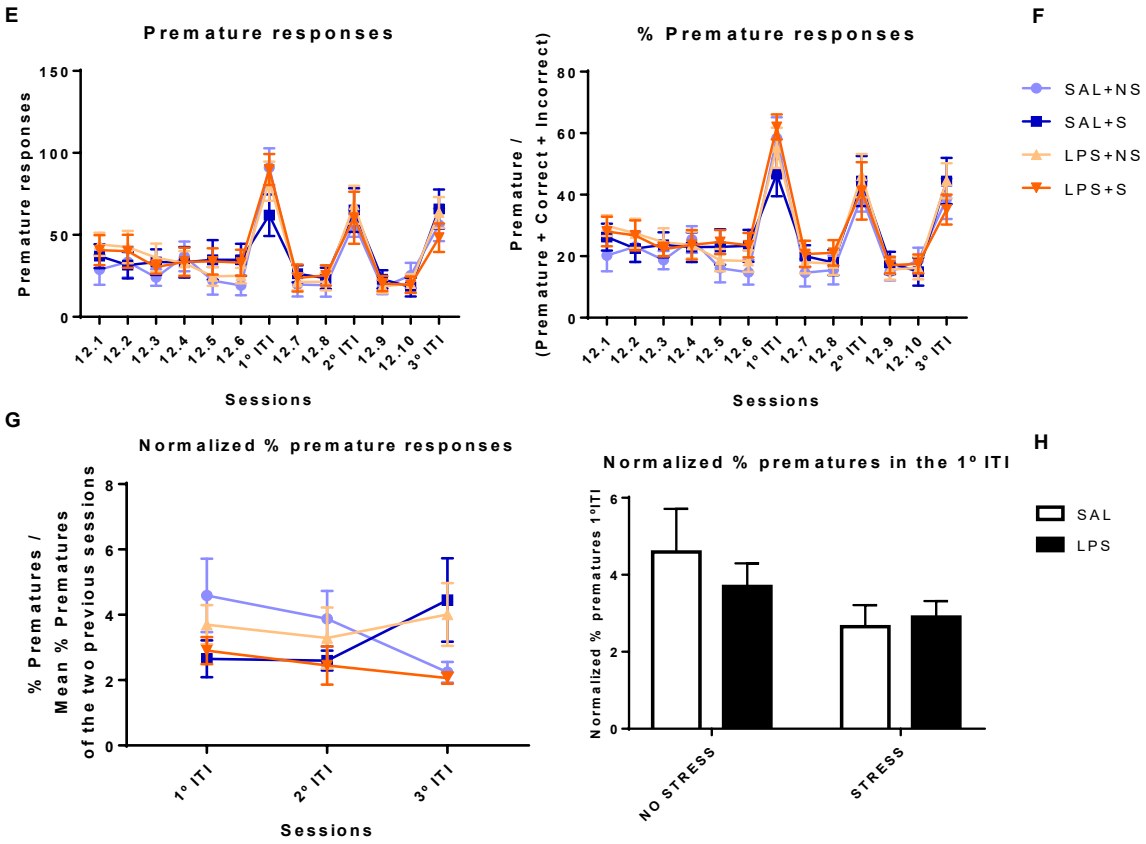
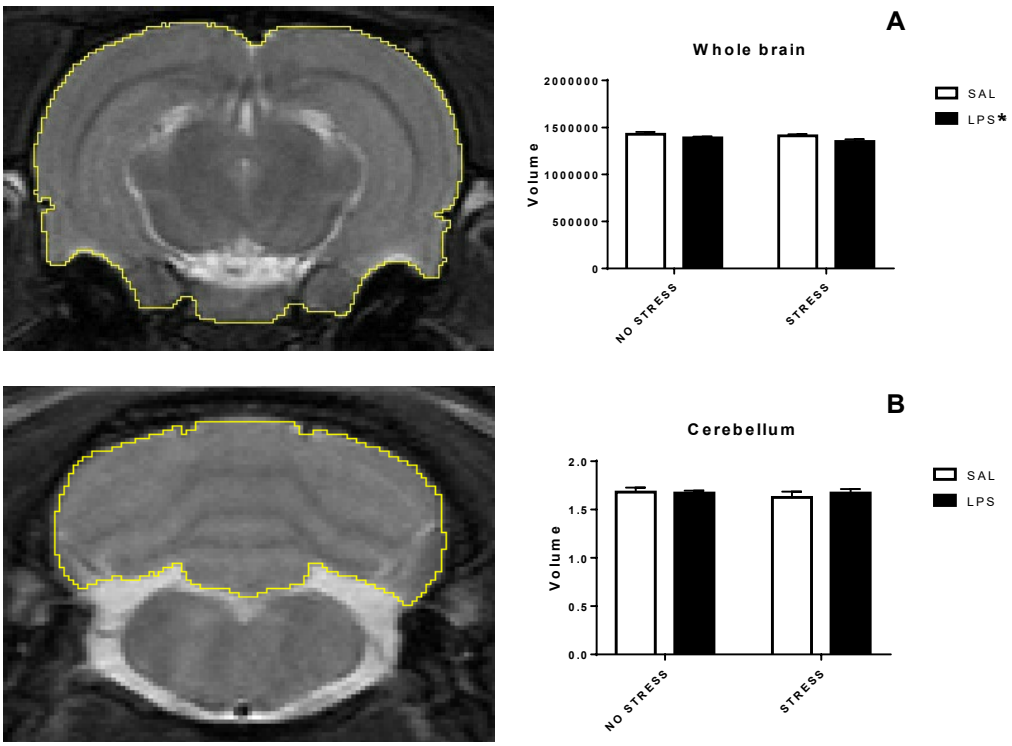


Figure 33: Parameters obtained in the different sessions of the 2-CSRTT program. Figure shows (A) correct, (B) incorrect, (C) omitted, (D) perseverative and (E) premature responses. % prematures regarding total responses (F) and its normalization in the ITI sessions (G, H) were also calculated. (SAL+NS: n=8; SAL+S: n=8; LPS+NS: n=9; LPS+S: n=7).

5. Neuroimaging studies.

5.1 Whole-brain and right hippocampal volumes were reduced by prenatal LPS exposure and peripubertal unpredictable stress, respectively. PUS increased the volume of the cortex and the dorsal striatum.

Whole brain volume was reduced as a consequence of prenatal immune activation ($F_{1,27}$ = 6.520; p = 0.017; η^2_p = 0.195) (Figure 34, A). On the other hand, PUS exposure increased the left cortical volume ($F_{1,27}$ = 5.709; p = 0.024; η^2_p = 0.175) (Figure 34, C), as well as that of the dorsal striatum bilaterally (Right: $F_{1,27}$ = 5.289; p = 0.029; η^2_p = 0.164 / Left: $F_{1,27}$ = 6.856; p = 0.014; η^2_p = 0.203) (Figure 34, F), but decreased the right hippocampal ($F_{1,27}$ = 4.417; p = 0.045; η^2_p = 0.141) (Figure 34, D). Prenatal LPS treatment * PUS exposure interaction increased right cortical volume ($F_{1,27}$ = 4.484; p = 0.044; η^2_p = 0.025) (Figure 34, C), although, surprisingly, no significant differences were observed in subsequent simple effects analysis. This interaction also increased the volume of the left dorsal striatum ($F_{1,27}$ = 6.105; p = 0.020; η^2_p = 0.184) (Figure 34, F), showing significant differences compated to non-stressed ($F_{1,27}$ = 12.519; p = 0.001) and saline-exposed ($F_{1,27}$ = 5.018; p = 0.034) controls (Figure 34, F).



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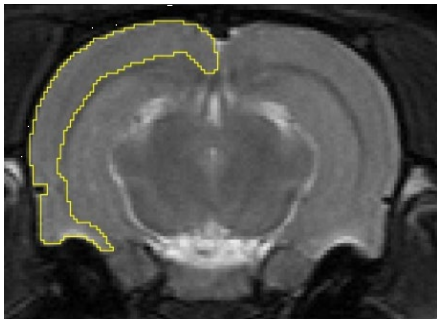
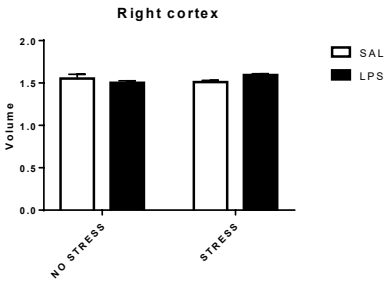
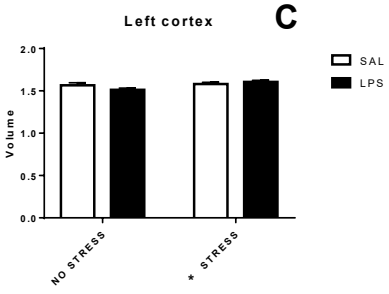
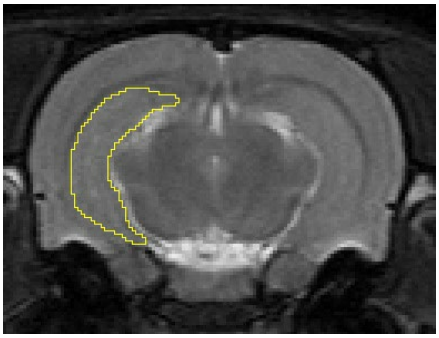
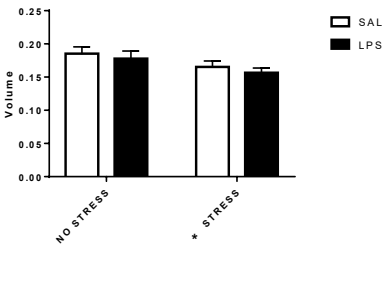



Right cortex
Volume
2.0
1.5
1.0
0.5
0.0
NO STRESS
STRESS
SAL
LPS

C


Left cortex
Volume
2.0
1.5
1.0
0.5
0.0
NO STRESS
* STRESS
SAL
LPS




Right hippocampus
Volume
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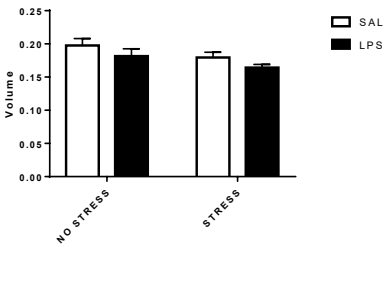
0.20
0.15
0.10
0.05
0.00
NO STRESS
* STRESS
SAL
LPS

D


Left hippocampus
Volume
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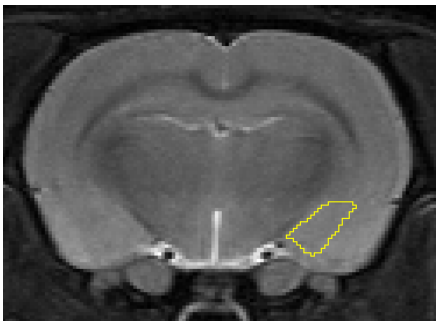
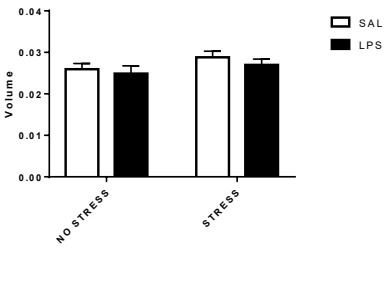
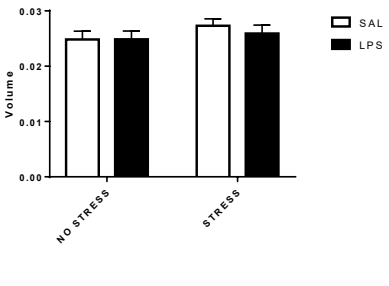
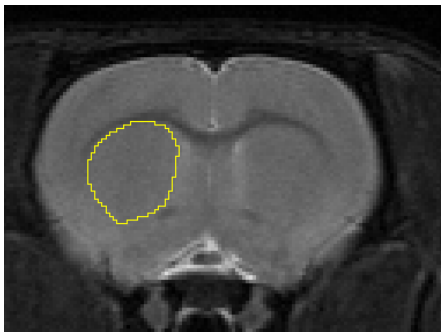
0.20
0.15
0.10
0.05
0.00
NO STRESS
STRESS
SAL
LPS

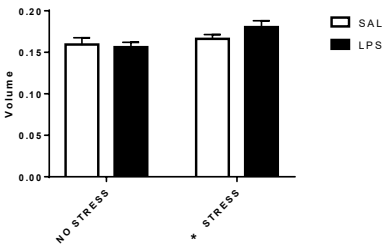



Right amygdala
Volume
0.04
0.03
0.02
0.01
0.00
NO STRESS
STRESS
SAL
LPS

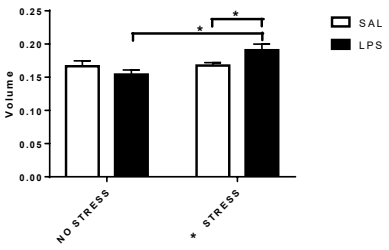
E

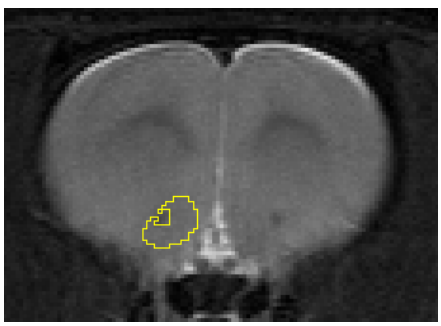

Left amygdala
Volume
0.03
0.02
0.01
0.00
NO STRESS
STRESS
SAL
LPS

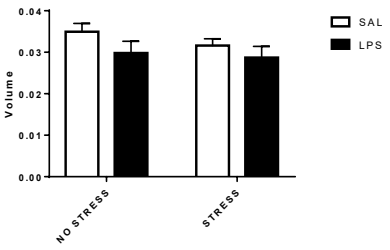



Right dorsal striatum
Volume
0.20
0.15
0.10
0.05
0.00
NO STRESS
* STRESS
SAL
LPS

F


Left dorsal striatum
Volume
0.25
0.20
0.15
0.10
0.05
0.00
*
*
NO STRESS
* STRESS
SAL
LPS




Right NAcc
Volume
0.04
0.03
0.02
0.01
0.00
NO STRESS
STRESS
SAL
LPS

G

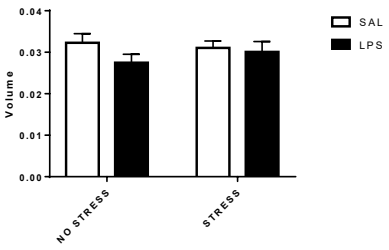
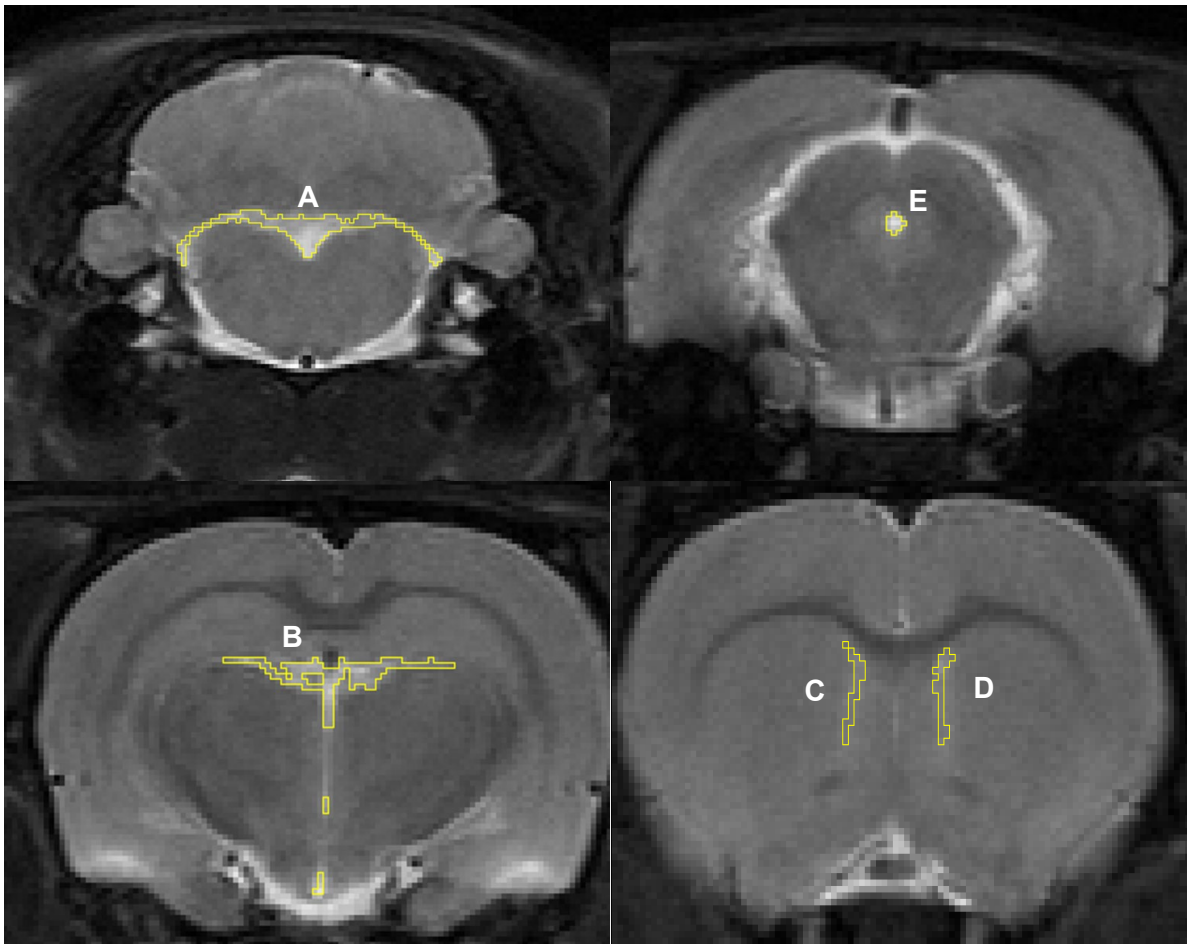

Left NAcc
Volume
0.04
0.03
0.02
0.01
0.00
NO STRESS
STRESS
SAL
LPS

Figure 34: Brain volumetric study. The figure shows the volume of whole brain (A), cerebellum (B), right and left cortex (C), right and left hippocampus (D), right and left amygdala (E), right and left dorsal striatum (F), right and left NAcc (G). The volumetric quantification of each brain structure was normalized by whole brain volume in the different MRI sections. (SAL+NS: n = 8; SAL+S: n = 8; LPS+NS: n = 8; LPS+S: n = 7). * p<0,005

In addition, the volume of the fourth ventricle, third ventricle, lateral ventricles, cerebral aqueduct and total ventricular (Figure 35, A-F) were not altered as a consequence of prenatal immune activation, PUS or their interaction.



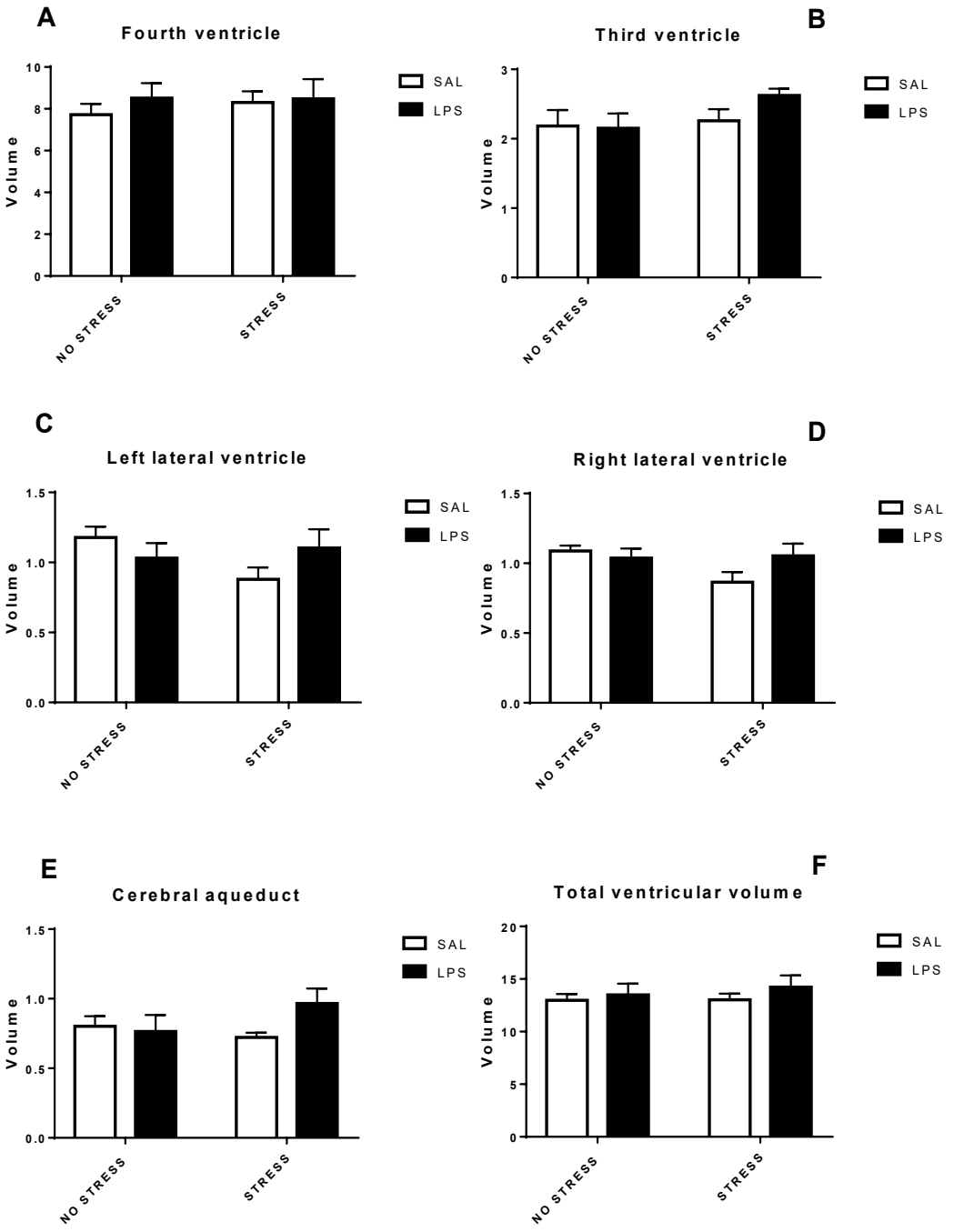


Figure 35: Cerebral ventricular volume. The figure shows the volume of the fourth ventricle (A), third ventricle (B), lateral ventricles (C, D), cerebral aqueduct (E) and total ventricular volumen (F). Volumetric quantification of each structure was normalized to the whole brain volume in the different MRI sections. (SAL + NS: n = 8; SAL + S: n = 8; LPS + NS: n = 8; LPS + S: n = 7).

5.2 Prenatal exposure to LPS increased hippocampal mean diffusivity in both hemispheres.

Mean diffusivity was significantly increased in the right ($H_{1,28}$ = 5.625, p = 0.0177, η^2_p = 0.280) and left ($H_{1,28}$ = 6.006, p = 0.0143, η^2_p = 0.235) hippocampus of LPS-exposed animals (Figure 36, C). Regarding this parameter, no significant differences were observed between groups in the cortex or in the dorsal striatum (Figure 36, A, B). There were also no changes in fractional anisotropy, in any of the structures mentioned (Figure 37, A, B, C) or in the main cerebral tracts (corpus callosum, internal capsule and anterior commissure) (Figure 38, A, B, C), as a consequence of prenatal immune activation and / or peripubertal exposure to unpredictable stress.

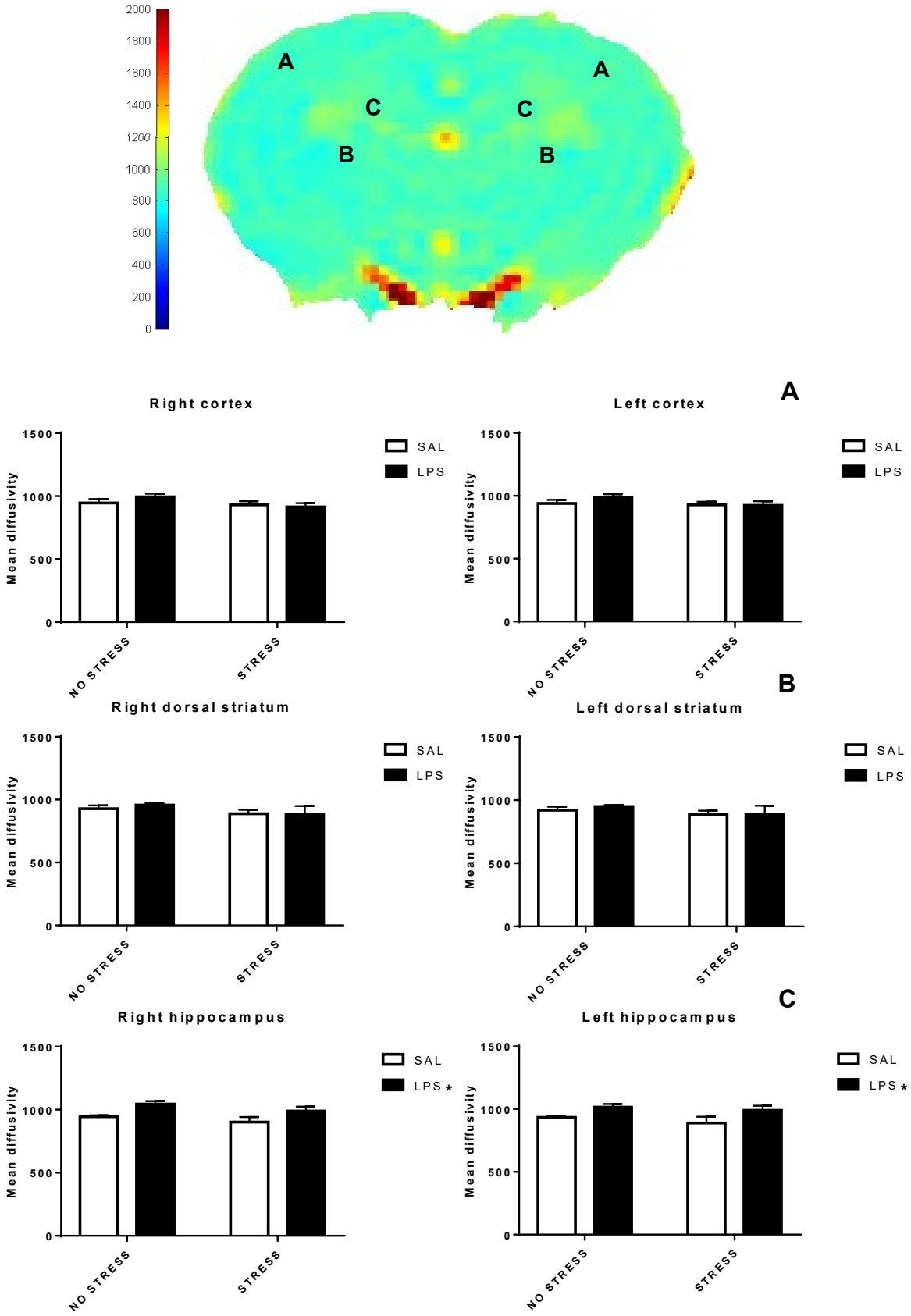


Figure 36: DTI study: mean diffusivity measurements in specific brain areas. Figure shows mean diffusivity values of the cortex (A), dorsal striatum (B) and hippocampus (C), in both hemispheres. (SAL+NS: n = 8; SAL+S: n = 8; LPS+NS: n = 8; LPS+S: n =8).

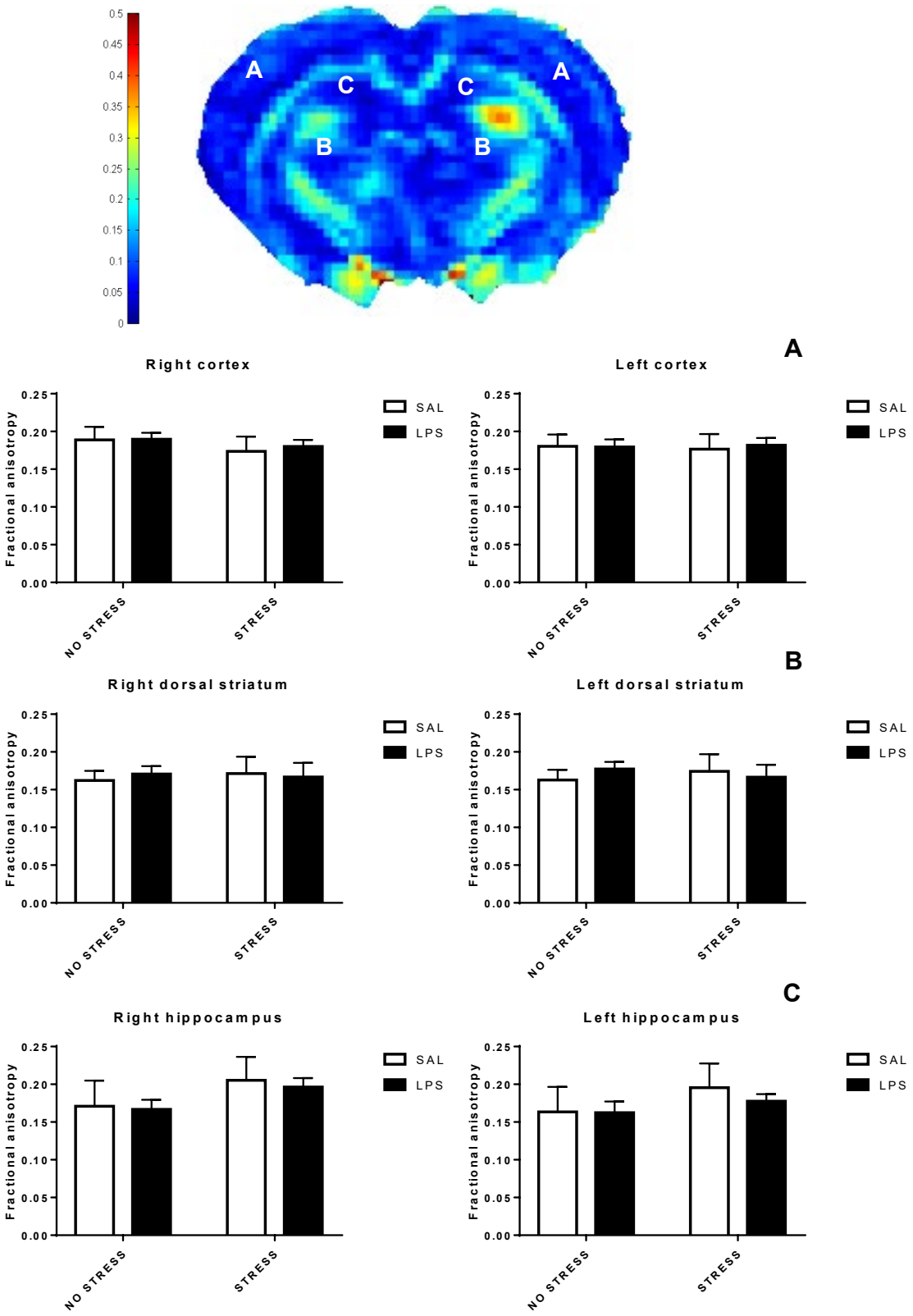


Figure 37: DTI study: fractional anisotropy measurements in specific brain areas. Figure shows fractional anisotropy values of the cortex (A), dorsal striatum (B) and hippocampus (C), in both hemispheres. (SAL+NS: n = 8; SAL+S: n = 8; LPS+NS: n = 8; LPS+S: n = 8).

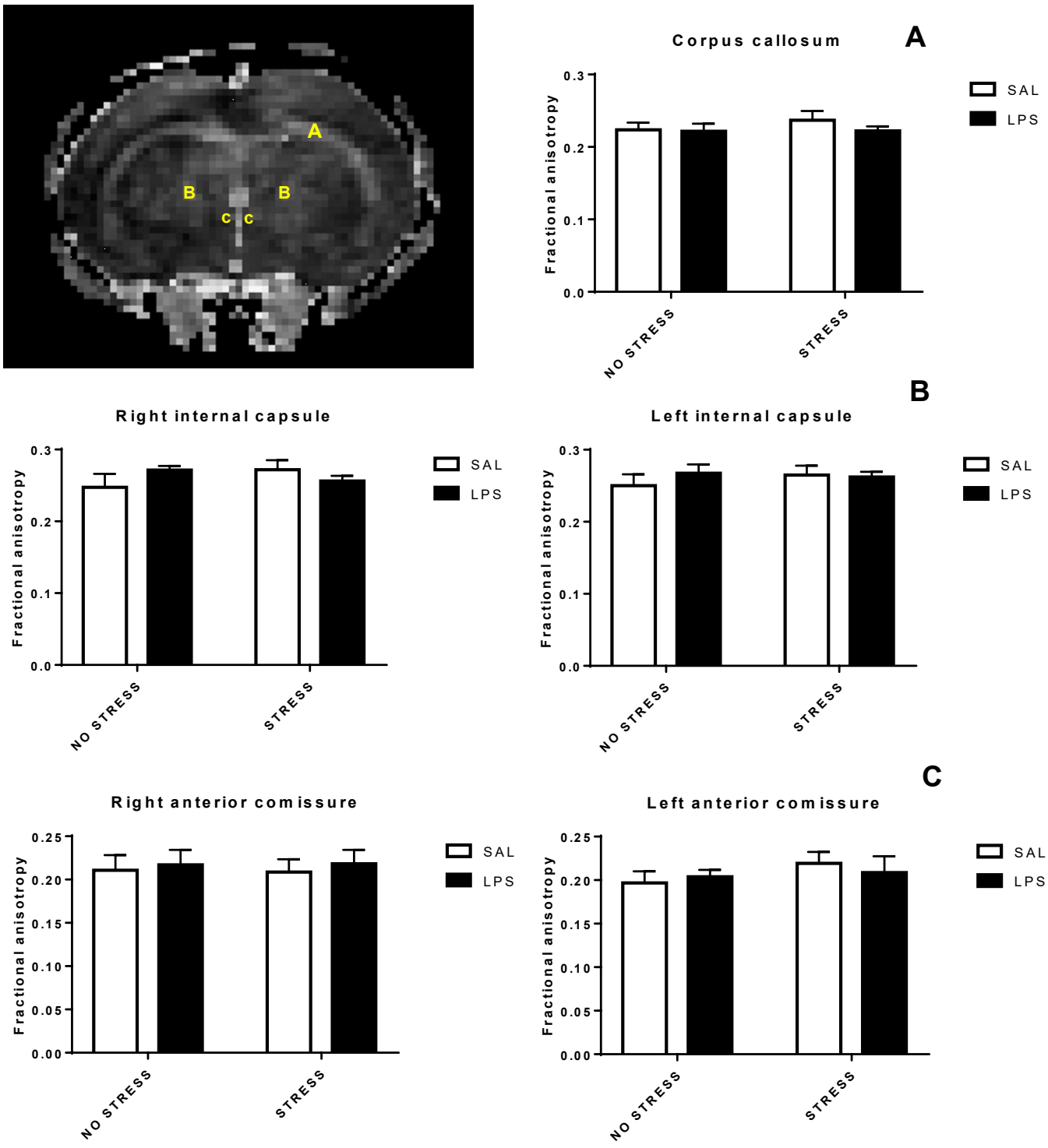


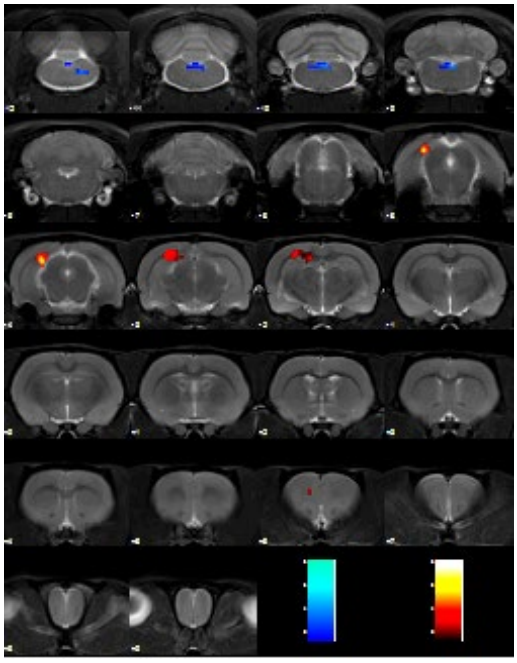
Figure 38: DTI study: fractional anisotropy measurements in the main cerebral tracts. The figure shows fractional anisotropy values of the corpus callosum (A), internal capsule (B) and anterior comissure (C), in both hemispheres. (SAL+NS: n = 8; SAL+S: n = 8; LPS+NS: n = 8; LPS+S: n = 8).

5.3 Unpredictable peripubertal stress increased the metabolic activity of the cortex and hippocampus at the dorsal level and reduced functional activity in brainstem regions.

There were no effects associated with LPS treatment and PUS did not change this resut (Figure 39, B, E). In general, PUS caused more profound changes than prenatal immune activation in the metabolic activity of specific brain areas, as observed by statistical parametric mapping (SPM). In particular, dorsal areas of the cortex and the hippocampus were hyperactivated by this factor, while others at the brainstem were hypoactivated. Interestingly, when the LPS factor was added to the analysis, the hyperactivation was displaced from the hippocampus to the cortex and the hypoactivation in pontine areas disappeared (Figure 39, A, C). This suggests that LPS modulated the effects of PUS on brain metabolism.

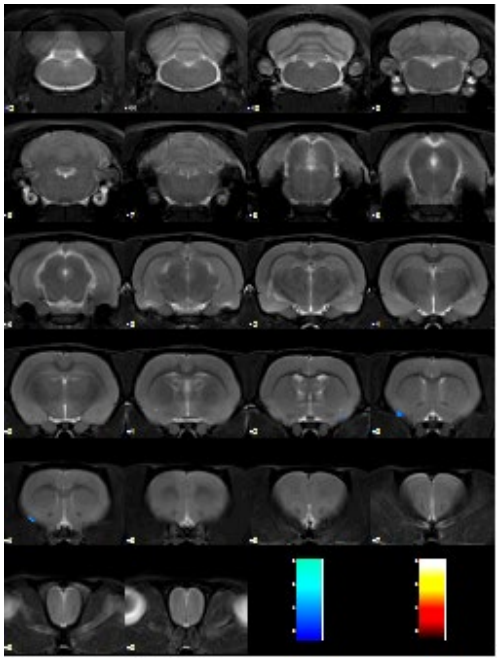
A

SAL+NS vs SAL+S



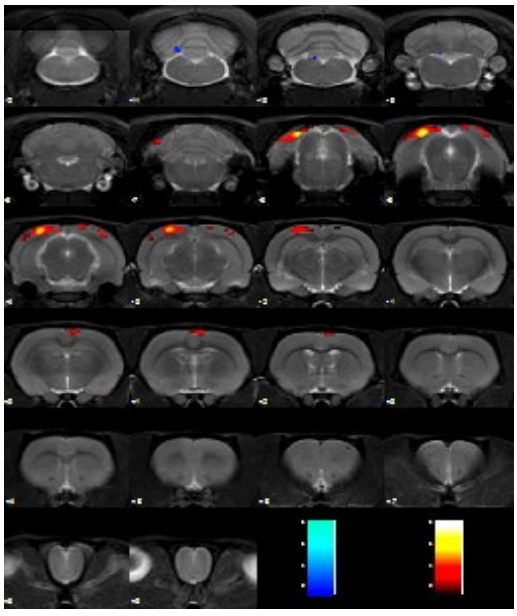
B

SAL+NS vs LPS+NS



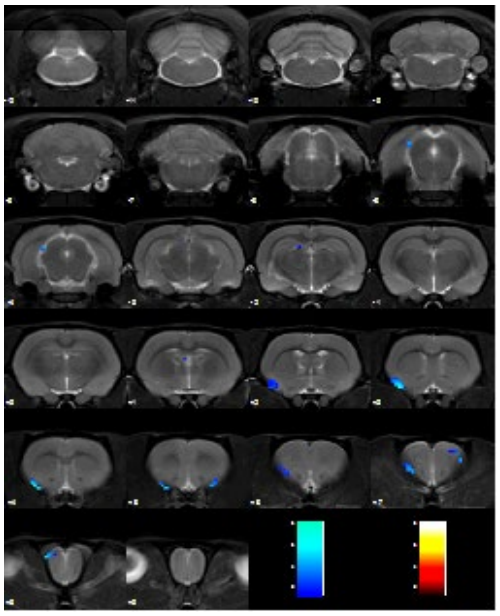
C

SAL+NS vs LPS+S



D

SAL+S vs LPS+NS



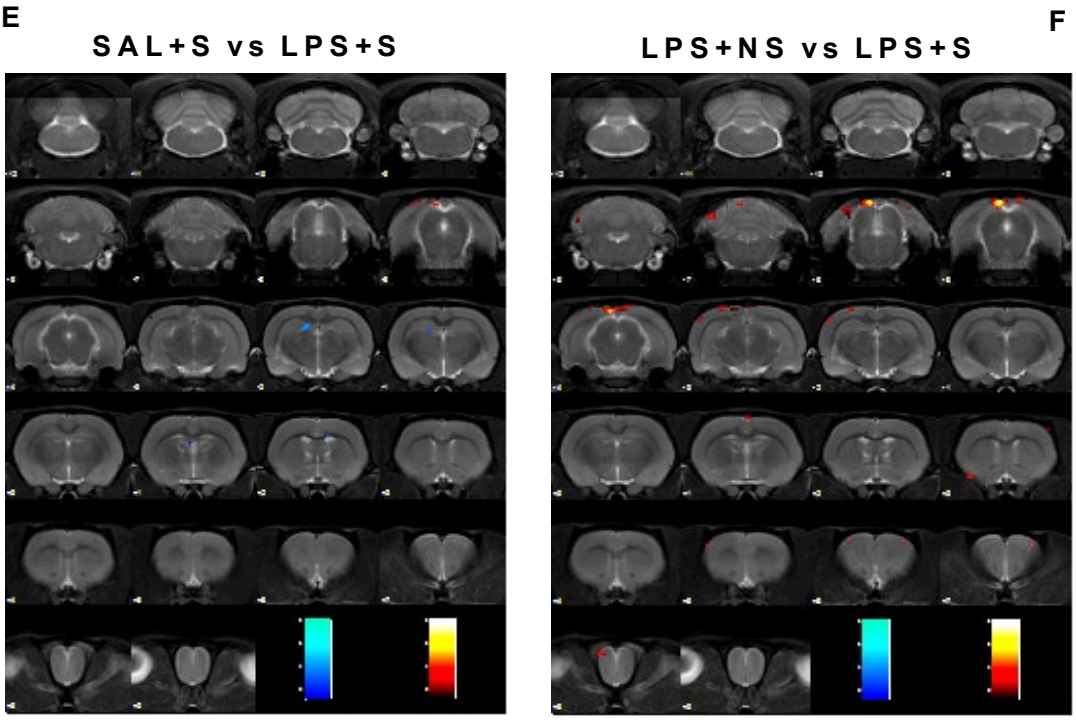


Figure 39: Superposition of MRI frames with PET-CT for metabolic activity quantification in different brain areas. Figure shows the different possible comparisons between groups (A-F), where warm or cold colors reflect the intensity of hyper- or hypo-activation of each area, respectively. The statistical significance threshold between groups was set at $p<0.01$, for a more delicate analysis.

6. Metabolomic studies.

All metabolite concentrations were normalized to creatine + phosphocreatine levels [Cr + PCr], due to its relatively constant concentration in the brain. There were more metabolites that did not pass the quality control (standard deviation below 20%) in the *in vivo* than in the *ex vivo* study. For this reason, every metabolic alteration found *in vivo* was graphically represented, unlike in the *ex vivo* situation, where only the most relevant effects are represented. Tables 1-4 of the supplementary material show the *ex vivo* statistical results summary of each metabolite and their mean ± SD in the left cortex, right cortex, left striatum and right striatum, respectively.

6.1 *In vivo* ^{1}H-MRS determinations.

6.1.1 Choline storage within the cytosol, represented by glycerophosphocholine (GPC) and phosphocholine (PCh) levels, was reduced in the cortex after PUS exposure.

A significant interaction was found between prenatal immune activation * PUS exposure ($F_{1,16}$ = 0.03, p = 0.018, η^2_p = 0.303) in the levels of [GPC + PCh] in the cortex. PUS reduced the levels of [GPC + PCh] ($F_{1,16}$ = 6.246, p = 0.024), but its combination with prenatal immune activation reversed this effect ($F_{1,16}$ = 6.481, p = 0.022) (Figure 40).

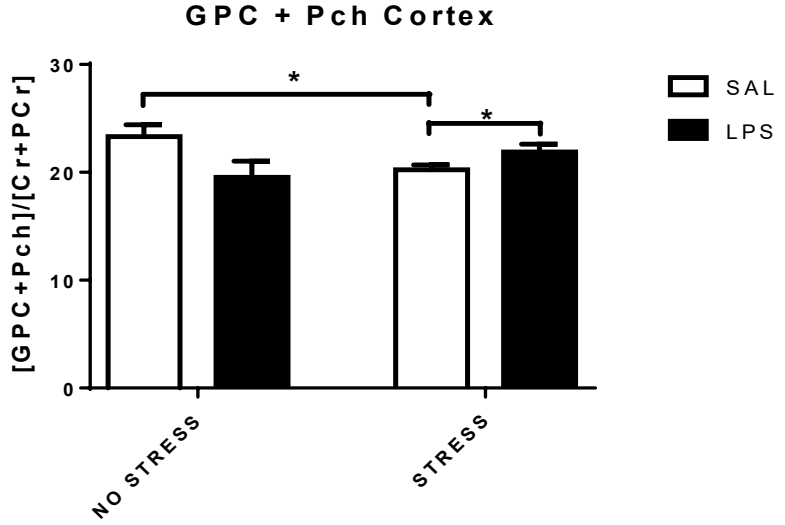


Figure 40: Normalized [GPC + PCh] levels in the cortex. (SAL + NO STRESS: n=6; SAL + STRESS: n=6; LPS + NO STRESS: n=4; LPS + STRESS: n=4).

6.1.2 The levels of N-acetylaspartylglutamic acid (NAAG) and its precursor N-acetylaspartic acid (NAA) were reduced by the combination of prenatal immune activation and PUS exposure.

A significant interaction was found between prenatal immune activation * PUS exposure ($F_{1,26}$ = 4.776, p = 0.038, η^2_p = 0.155) in the levels of [NAA + NAAG] in the striatum. PUS exposure in LPS-treated subjects reduced the levels of [NAA + NAAG] in that area ($F_{1,26}$ = 4.495, p = 0.044) (Figure 41).

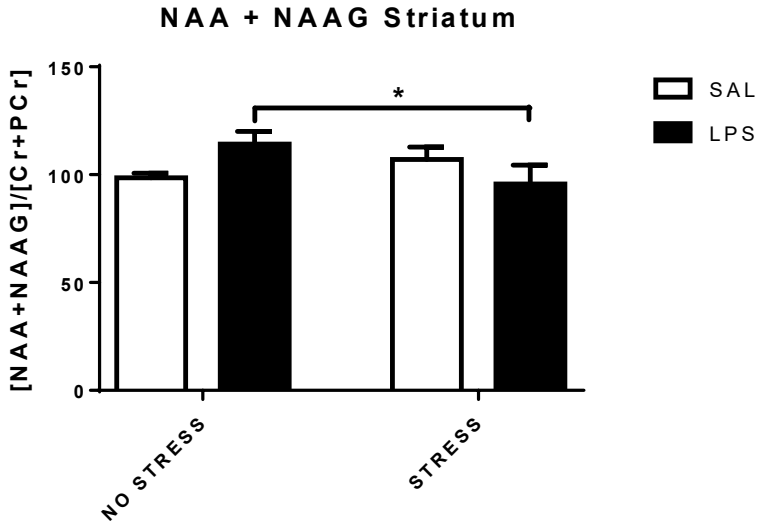


Figure 41: Normalized [NAA + NAAG] levels in the striatum (SAL + NO STRESS: n=7; SAL + STRESS: n=8; LPS + NO STRESS: n=8; LPS + STRESS: n=7).

6.2 *Ex vivo* ^{1}H-MRS determinations.

6.2.1 Glucose levels were decreased bilaterally in the striatum and the left cortex of LPS-exposed animals.

Prenatal immune activation diminished glucose levels in left ($F_{1,25}$ = 8.586, p =0.007, η_{2p} = 0.256) (Figure 42, A) and right ($F_{1,26}$ = 12.402, p = 0.002, η_{2p} = 0.323) (Figure 42, B) striatums, as compared to the saline groups. This effect was also observed in the left cortex ($F_{1,27}$ = 5.855, p = 0.023, η^2_p = 0.178) (Figure 42, C).

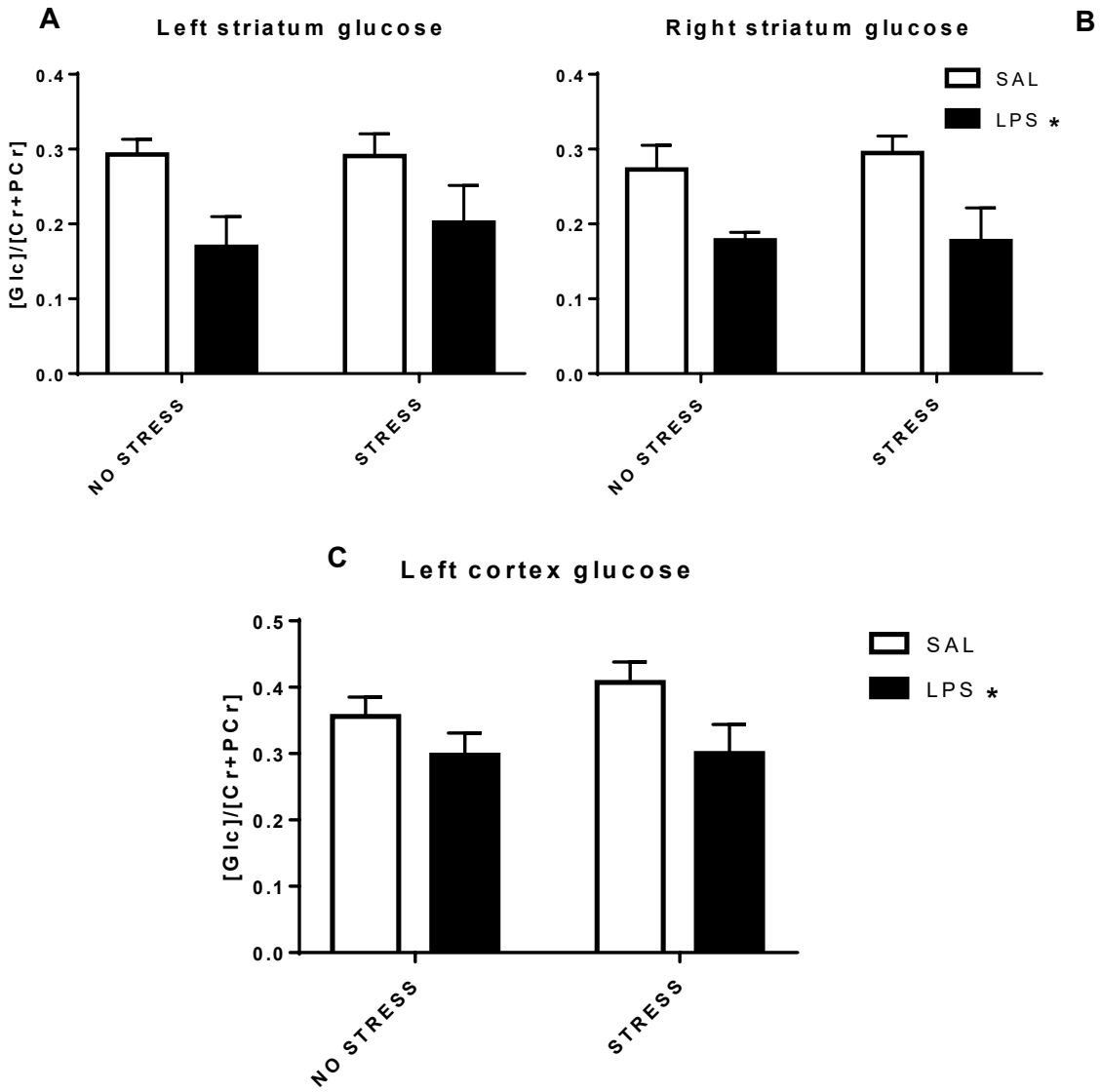


Figure 42: Normalized glucose levels in (A) the left striatum (SAL + NO STRESS: n=7; SAL + STRESS: n=8; LPS + NO STRESS: n=7; LPS + STRESS: n=7), (B) right striatum (SAL + NO STRESS: n=8; SAL + STRESS: n=8; LPS + NO STRESS: n=7; LPS + STRESS: n=7) and (C) left cortex (SAL+ NO STRESS: n=8; SAL + STRESS: n=8; LPS + NO STRESS: n=8; LPS + STRESS: n=7). * compared to saline groups.

6.2.2 Glutamate levels were increased in the left cortex and reduced in the left striatum of LPS-exposed animals. The combination with peripubertal stress increased glutamate levels in the striatum.

Higher glutamate levels ($F_{1,27}$ = 4.42, p = 0.045, η^2_p = 0.141) were obtained in the left cerebral cortex of LPS-exposed animals (Figure 43, A). Moreover, a significant interaction was observed between prenatal immune activation and PUS exposure ($F_{1,27}$ = 11.21, p = 0.002, η^2_p = 0.293) in the left striatum. In this area, LPS-exposed animals

showed lower glutamate levels in the absence of PUS ($F_{1,27}$ = 5.454, p = 0.027). However, in its presence, glutamate levels were increased ($F_{1,27}$ = 5.756, p = 0.024). Likewise, PUS increased glutamate levels only in LPS-exposed animals ($F_{1,27}$ = 12.219, p = 0.002) (Figure 43, B).

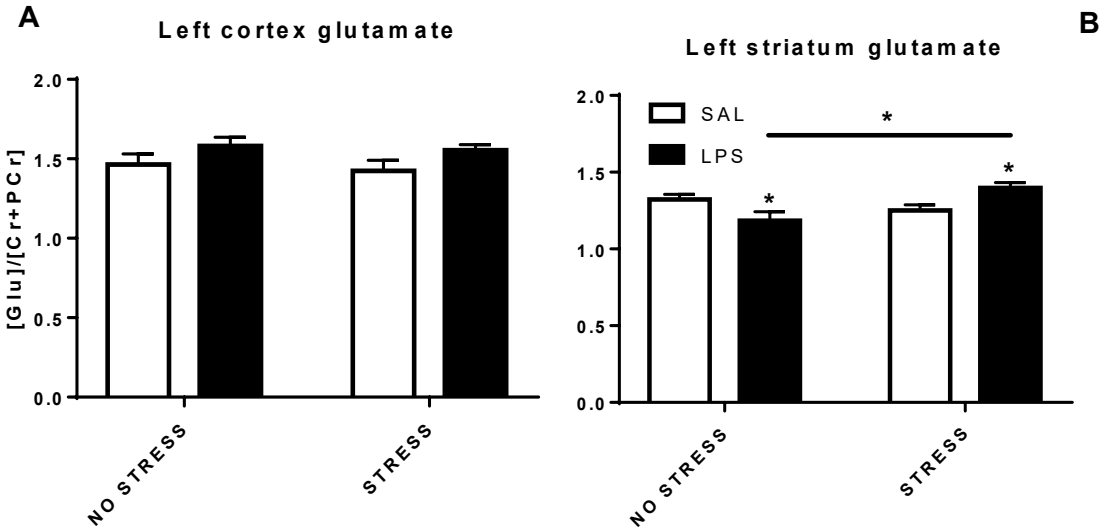


Figure 43: Normalized glutamate levels in the left cortex (A) (SAL + NO STRESS: n=8; SAL + STRESS: n=8; LPS + NO STRESS: n=8; LPS + STRESS: n=7) and left striatum (SAL + NO STRESS: n=8; SAL + STRESS: n=8; LPS + NO STRESS: n=8; LPS + STRESS: n=7).

6.2.3 Glutamine levels were reduced in the left striatum by prenatal immune activation and PUS exposure.

A significant interaction was found between prenatal immune activation * PUS exposure ($F_{1,27}$ = 6.155, p = 0.02, η^2_p = 0,186) in the levels of glutamine in the left striatum. In this area, LPS-exposed animals showed lower glutamine levels, in absence of PUS ($F_{1,27}$ = 5.641, p = 0.025). This effect was also obtained in peripubertally-stressed animals, but only in the absence of prenatal immune activation ($F_{1,27}$ = 4.411, p = 0.045) (Figure 44).

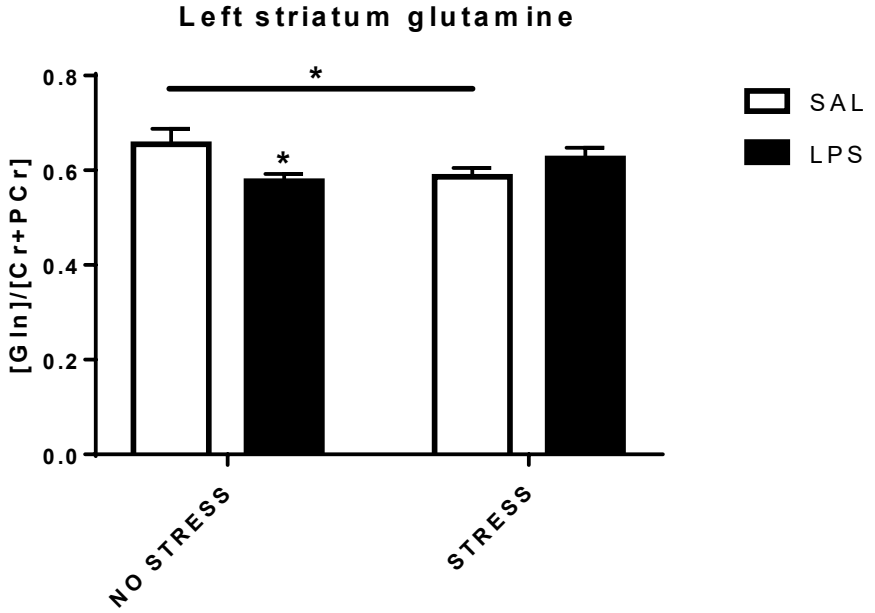


Figure 44: Normalized glutamine levels in the left striatum (SAL + NO STRESS: n=8; SAL + STRESS: n=8; LPS + NO STRESS: n=8; LPS + STRESS: n=7).

6.2.4 *N*-acetylaspartate levels were reduced in the right cortex of LPS-exposed animals.

LPS-exposed animals showed significantly lower *N*-acetylaspartate levels ($F_{1,27} = 4.438$, $p = 0.045$, $\eta_{2p} = 0.141$) in right cerebral cortex (Figure 45).

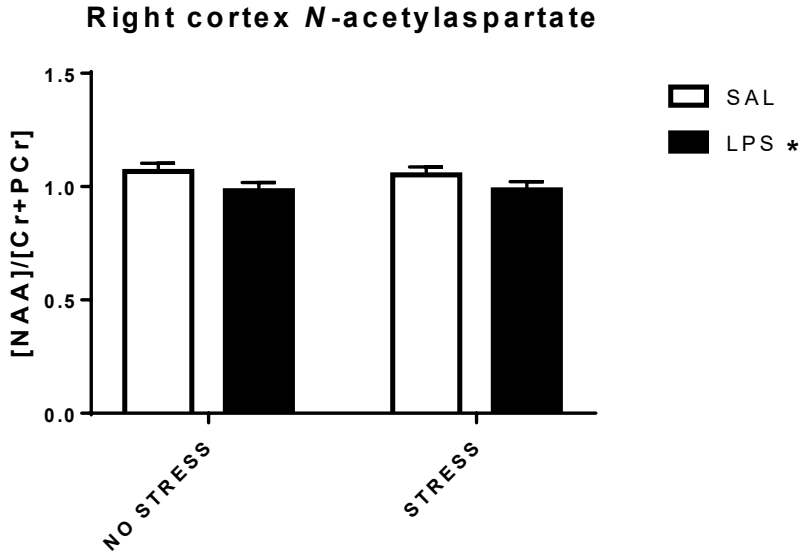


Figure 45: Normalized *N*-acetylaspartate levels in the right cortex (SAL + NO STRESS: n=8; SAL + STRESS: n=8; LPS + NO STRESS: n=8; LPS + STRESS: n=7).

7. RNA-Seq.

7.1 PUS altered the expression of nearly 2000 genes in the dorsolateral striatum, many of them involved in the regulation of the immune response. In the nucleus accumbens, this factor upregulated the expression of multiple genes involved in dual diagnosis etiopathogenesis.

Both integrity and concentration of the samples used were considered optimal by the Genomics Unit of the Madrid Science Park to carry out the RNA-Seq study (Table 9).

Brain area	Treatment	Sample	RIN	ng/µl
Dorsolateral striatum	SAL+NS	31	9,2	101
		32	8,9	129
		67	8,3	233
	SAL+S	35	8,9	161
		34	8,7	115
		49	8,6	124
	LPS+NS	38	9,3	63
		61	8,5	408
		81	8,1	149
	LPS+S	44	9	277
		41	8,4	206
		2	8,2	202
Nucleus accumbens	SAL+NS	32	8,8	165
		6	8,5	159
		5	8,9	132
	SAL+S	48	9,2	115
		49	9	119
		34	9,1	114
	LPS+NS	61	9,1	49
		39	9,5	86
		42	9,2	113
	LPS+S	77	9	88
		41	9	133
		40	9,3	165

Table 9: RNA integrity number (RIN) and RNA concentration (ng/µl) obtained in each sample used for the RNA-Seq study.

Illumina's RNA sequencing reports showed differential expression of 1938 genes in the dorsolateral striatum as a consequence of PUS while this effect was almost obliterated when PUS occurred in LPS-exposed animals. With regard to LPS effects, they were more restricted (only 69 genes were differentially expressed) and when PUS was added, these 69 genes were no longer differentially expressed. In the nucleus accumbens this

modulation of the LPS effects by stressed was not as evident (60 vs 53 genes); however, PUS alone did not affect the expression of any gene in this area. When the effects of PUS were analysed in PUS animals, the number of differentially expressed genes increased to 63 (Table 10). In general, it seems that the dorsal striatum is more vulberable to the effects of peripubertal stress than the nucleus accumbens but it is also more sensitive to the interactive effects of PUS and MIA.

Brain area	Dorsolateral striatum				Nucleus accumbens			
Comparison	SAL+NS vs LPS+NS	SAL+S vs LPS+S	SAL+NS vs SAL+S	LPS+NS vs LPS+S	SAL+NS vs LPS+NS	SAL+S vs LPS+S	SAL+NS vs SAL+S	LPS+NS vs LPS+S
Annotation Gene Count	17303	17303	17303	17303	17303	17303	17303	17303
Annotation Transcript Count	18752	18752	18752	18752	18752	18752	18752	18752
Assessed Gene Count	11428	11234	11352	11320	11771	11724	11757	11696
Assessed Transcript Count	11942	11713	11840	11817	12330	12274	12310	12238
Differential expression Gene Count	69	0	1938	15	60	53	0	63
Differential expression Transcript Count	65	0	1897	15	55	50	0	62

Table 10: RNA sequencing report attending to gene differential expression. Upregulation or downregulation of the different genes in each comparison was considered when $p < 0.01$. (SAL + NO STRESS: n = 3; SAL + STRESS: n = 3; LPS + NO STRESS: n = 3; LPS + STRESS: n = 3).

The RNA-Seq study allowed to assess the degree of differential expression of the genes that were evaluated in each comparison, based on its "fold change". This parameter analyzes, for each gene, the relationship between the amount of RNA expressed in an experimental and a reference group. Thus, positive fold change values would indicate upregulation in the expression of a certain gene, while negative values would indicate downregulation. In the present work, all those genes that showed over-1 or under-1 fold change values (that is, those that showed greater upregulation or downregulation) were selected to elaborate an ontological analysis in the PANTHER (protein analysis through evolutionary relationships) database to study their possible influence on common molecular, cellular or biological processes.

By doing this analysis, we observed that prenatal LPS exposure increased the expression of the *Sync* gene which codes for the syncoilin protein. Interestingly, a polymorphism in an enhancer sequence for this gene has been linked to nicotine dependence in a GWAS study. MIA also greatly increased the expression of the D3 dopamine receptor gene (*Drd3*) and the CD93 molecule (Cd93 gene), which is involved in intercellular adhesion and in the clearance of apoptotic cells. We also found reduced expression of genes that code for cytoskeletal proteins in the nucleus accumbens, as well as others implicated in axoneme assembly in the dorsolateral striatum (Table 11). Surprisingly, in this latter area, the combination of PUS in LPS-exposed animals increased the expression of most of these genes (Table 13). On the other hand, PUS increased in the nucleus accumbens the expression of several genes involved in the development of the limbic system and telencephalon, in cognitive functions such as eating behavior and memory, or in glutamatergic and neuropeptide neurotransmission, among other processes. In the dorsolateral striatum, PUS altered the expression of genes involved in ion transmembrane transport, cell proliferation and secretion of pro-inflammatory cytokines (Table 12).

Nucleus accumbens		
Upregulated genes	**Fold Change**	**Cellular component / Biological process**
Sync	1.70425	
Drd3	1.18354	
Cd93	1.14881	
Downregulated genes		
Car3	-3.74779	
Fcrl2	-2.47656	
Folr1	-1.89631	
Msx1	-1.58795	
Tnnt1	**-1.40661**	
Ropn1l	-1.39781	
Ak7	-1.37566	
RGD1310641	-1.25286	
Dynlrb2	**-1.22272**	
Lrrc34	-1.20103	
Aurkb	**-1.19877**	
Tnni3	**-1.19296**	
Ccdc153	-1.15702	
Armc3	-1.12309	
Mlf1	-1.09494	
Prss12	-1.08972	
Tcte1	-1.08864	
Rsph1	-1.08526	
Ccdc19	-1.07839	
P2rx6	-1.07327	
Ccdc114	**-1.05254**	
Ccdc77	**-1.04199**	
Odf3b	-1.02413	
Krt8	-1.00222	

Cytoskeleton

Microtubule associated complex

Outer dynein arm

Dorsolateral striatum		
Upregulated genes	**Fold Change**	**Cellular component / Biological process**
Grifin	3.12988	
Cort	2.24424	
Cbln2	2.03982	
Slc30a3	1.80534	
Cck	1.77234	
Nrn1	1.71856	
Rtn4r	1.69409	
Rprm	1.61582	
Neurod6	1.60511	
Hs3st2	1.41865	
Slc17a7	1.37965	
Necab3	1.36511	
Abcg2	1.30691	
Nr4a2	1.24159	
Ramp3	1.22932	
C3	1.06384	
Tmem178a	1.04837	

Gng13	1.04447	
Downregulated genes	**Fold Change**	
Aurkb	-3.69815	
Ak7	**-3.11873**	
Lrrc34	-2.99287	
Mlf1	-2.77694	
Cfap126	-2.73189	
Dnah1	**-2.65418**	
Ccdc19	-2.24642	
Ccdc114	**-2.21564**	
Drc7	-2.20892	
Itgb4	-2.20409	
Ppp1r32	-2.15251	
Wdr63	-2.14799	
P2rx6	-2.05102	
Krt8	-1.92704	
Rsph10b	-1.87174	
RGD1560672	-1.84818	
Top2a	-1.77931	
Ccdc153	-1.70806	
Dnaaf3	**-1.62388**	
Cdca7	-1.57999	
Foxj1	-1.34351	
Npas4	-1.28453	
Mapkbp1	-1.07178	
Fras1	-1.02507	
Myo1b	-1.01168	

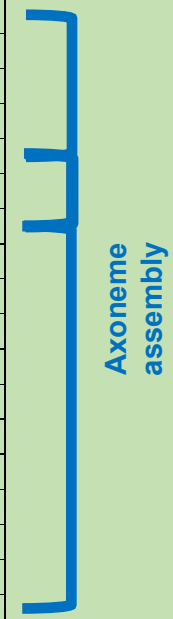
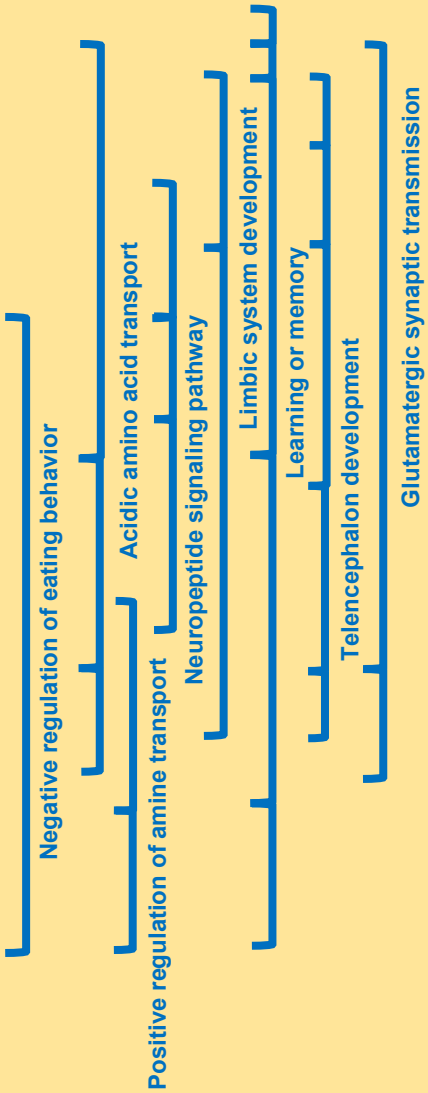


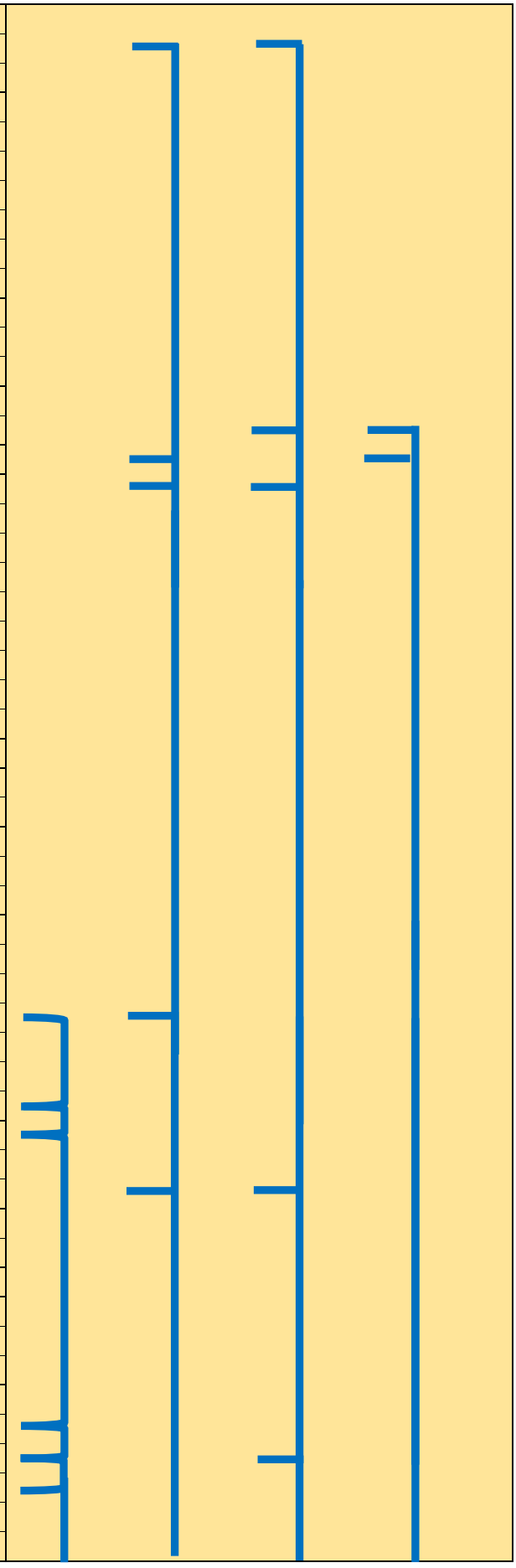
Table 11: Analysis of differential expression and ontologies of the SAL+NS vs LPS+NS comparison. Cytoskeleton ontology: FDR=1.18E-02. Microtubule-associated complex ontology: FDR=5.03E-02. Outer dynein arm ontology: FDR= 1.53E-02. Axoneme assembly ontology: FDR=3.84E-02.

Nucleus accumbens		
Upregulated genes	**Fold Change**	**Cellular component / Biological process**
Fam111a	3.21996	
Ndst4	2.68749	
Klhl14	2.31056	
Cyp26a1	1.94062	
Neurod2	**1.93784**	
Slc17a7	**1.87573**	
Tbr1	**1.86254**	
Sidt1	1.85186	
Rtn4rl2	**1.75435**	
Nppa	**1.71761**	
Wnt9a	1.70249	
Neurod6	**1.68088**	
Scube1	1.61581	
Npsr1	**1.58148**	
Hs6st3	1.55597	
Frzb	1.50611	
Nmbr	**1.42634**	
Bdnf	**1.41805**	
Rtn4r	**1.41643**	
Syt17	1.41331	
Kcnk9	1.38965	
Chrna4	**1.32425**	
Sstr3	**1.29333**	
Slc17a6	**1.23665**	
Rprm	1.19627	
Mas1	**1.19379**	
Grm2	**1.18701**	
Vip	**1.17839**	
Nov	1.13181	
Slc30a3	1.09753	
Slc6a7	1.04889	
Cck	**1.04838**	
C1ql3	1.04447	
Downregulated genes		
Car3	-2.79988	
Aurkb	-1.97407	
Mx1	-1.68686	

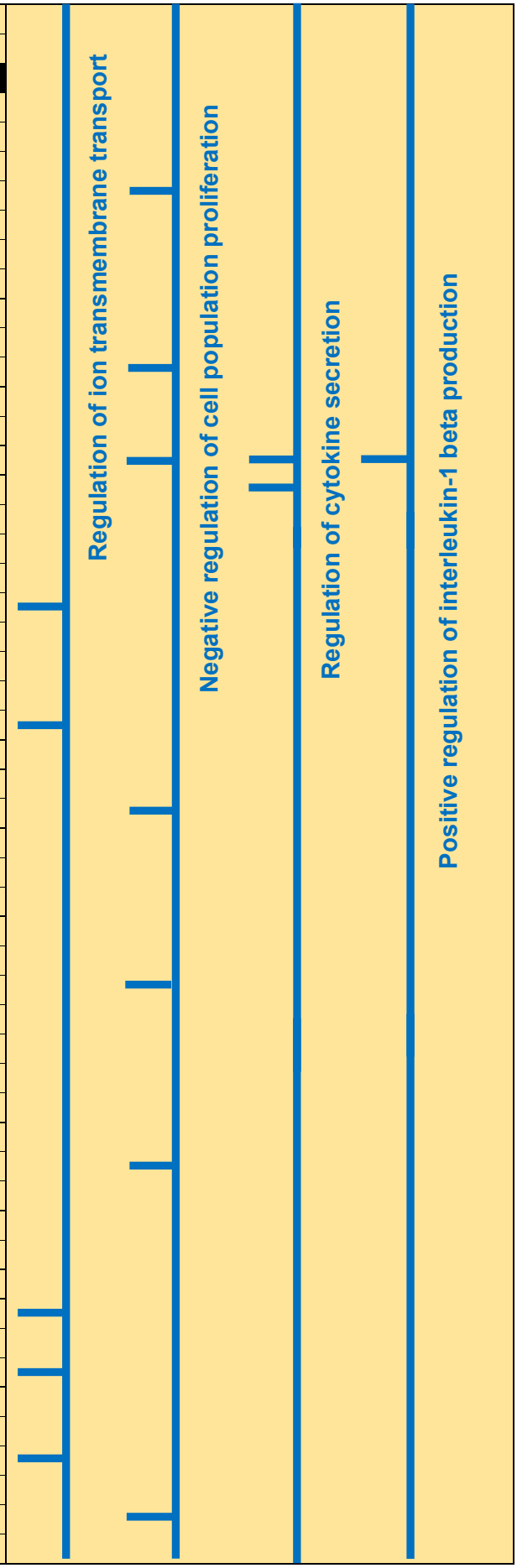


Dorsolateral striatum		
Upregulated genes	**Fold change**	**Cellular component / Biological process**
Grifin	3.14115	
Fam19a4	1.87251	
Krt17	1.87107	
Nanos3	1.72754	
Rpl41	1.57992	
Klf1	1.45753	
Asgr1	1.39401	
Gng13	1.37518	
Mt3	1.37388	

Ndufb7	1.36685
RT1-Db1	**1.36399**
Fam212a	1.33815
Hbb	1.33131
Atp5e	1.33108
Pfn3	1.32217
Enho	1.29378
Psmb10	1.27883
Dmrta2	1.26049
Snhg4	1.25257
Rps28	1.24988
Klk7	1.23124
Cort	1.21453
Rps29	1.19096
Ccl19	**1.18363**
Tyrobp	**1.17663**
Tnfrsf9	**1.17273**
Pspn	1.17107
Mx1	1.16867
Rpl9	1.16102
Snrpg	1.15764
RGD1566239	1.13407
Nrn1l	1.12645
Ppp1r14a	1.11268
Pomc	1.11263
Ephx2	1.11096
Cox6b2	1.10996
St14	1.10978
Ccdc28b	1.10746
Gng8	1.10463
Tmem256	1.09356
Selv	1.08359
Rps14	1.07877
Inafm1	1.07663
Gal	**1.07613**
Abcg2	1.06327
Pvalb	1.05774
Cyba	**1.05555**
Dbi	**1.05214**
Tmem160	1.05118
Aif1	**1.04999**
Ndufa2	1.04857
Rpl13a	1.04569
Serpinf2	1.04099
Rps12	1.03828
S100a13	1.03611
Dnph1	1.02991
S100a4	1.02263
Catsper2	**1.02236**
Tmsb4x	**1.01965**
Sf3b5	1.01799
Atp5i	1.01303
Ube2l6	1.00773



Rpsa	1.00524
Selm	1.00109
Downregulated genes	**Fold. change**
Car3	-2.99948
Fam111a	-2.86115
St3gal1	-2.19854
Dlk1	**-1.89698**
Htr2a	-1.87133
Gpr101	-1.83343
Spata18	-1.82585
Plag1	-1.82446
Ntn1	-1.81388
Efnb2	**-1.80613**
Gabrb2	-1.79917
Sema3a	-1.76523
Fzd5	**-1.74712**
Mapkbp1	**-1.72344**
Dcp1a	-1.66837
Mbnl1	-1.62666
Rbm33	-1.60606
Gstm2	**-1.58194**
Flrt2	-1.57352
Zfp483	-1.54858
Marveld2	-1.54085
Grm5	**-1.54083**
Chst15	-1.51127
Spata13	-1.50917
Cxadr	**-1.50594**
Gabrb3	-1.46918
Edem3	-1.46186
Dnah1	-1.45849
Ncoa2	-1.45127
Fras1	-1.44661
Robo1	**-1.44645**
Srgap3	-1.41987
B3galt1	-1.41985
Fam126a	-1.41906
Ksr1	-1.41838
Ctdspl2	-1.40768
Gli3	**-1.40605**
Zfp597	-1.40182
Xiap	-1.39826
Stpg1	-1.39084
Foxo1	-1.38506
Kcnq2	**-1.38478**
Slc16a14	-1.38189
Shank2	**-1.37874**
Mbtps2	-1.37804
Slc7a2	-1.37306
Amigo1	**-1.37021**
Gpr63	-1.36306
Sulf1	**-1.35207**
RGD1565611	-1.34983



Zfp516	-1.34914
Frmpd4	-1.34419
Fnbp1l	-1.34153
Slc30a10	-1.33991
Scn8a	**-1.33576**
Tmem255a	-1.33445
Fndc3b	-1.32688
Isl1	**-1.31577**
Trim16	**-1.31393**
Osbpl8	-1.30632

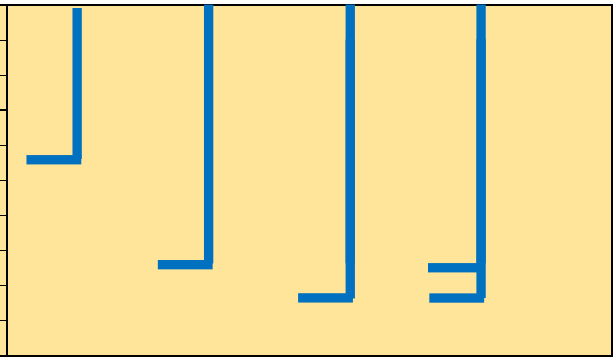


Table 12: Analysis of differential expression and ontologies of the SAL+NS vs SAL+S comparison. Negative regulation of eating behavior ontology: FDR=3.37E-02. Acidic amino acid transport ontology: FDR=5.91E-03. Positive regulation of amine transport ontology: FDR=4.21E-02. Neuropeptide signaling pathway ontology: FDR=1.98E-02. Limbic system development ontology: FDR=4.64E-02. Learning or memory ontology: FDR=1.38E-02. Telencephalon development ontology: FDR=1.36E-02. Glutamatergic synaptic transmission ontology: FDR=2.01E-02. Regulation of ion transmembrane transport ontology: FDR=2.47E-02. Negative regulation of cell population proliferation ontology: FDR=3.79E-02. Regulation of cytokine secretion ontology: FDR=2.29E-02. Positive regulation of interleukin-1 beta production ontology: FDR=2.28E-02

Nucleus accumbens		
Upregulated genes	**Fold Change**	**Cellular component / Biological process**
Slc16a5	1.86738	
Klhl14	1.78401	
Neurod2	1.61652	
Ndst4	1.59691	
Ephx2	1.37256	
Rprm	1.35536	
Slc17a7	1.31074	
Hs6st3	1.26279	
Tbr1	1.24018	
Sidt1	1.17868	
Ccdc77	1.11494	
Rtn4rl2	1.09737	
Nmbr	1.09387	
Nppa	1.09095	
Frzb	1.08686	
Slc30a3	1.05301	
Rtn4r	1.02992	
Sla	1.02326	
Downregulated genes		
Dorsolateral striatum		
Upregulated genes	**Fold Change**	**Cellular component / Biological process**
Aurkb	4.04473	
Mlf1	3.86546	
Cfap126	3.84011	
Ccdc19	3.17208	
Ccdc114	**2.99295**	∪ Axoneme assembly ∪ Axonemal dynein complex
Dnah1	**2.81849**	
Krt8	2.73678	
P2rx6	2.67838	
Ccdc153	2.67595	
Rsph1	**2.59463**	
RGD1560672	2.55296	
Rsph10b	2.39378	
Cdca7	2.24334	
Vim	1.27159	
Downregulated genes		

Table 13: Analysis of differential expression and ontologies of the LPS+NS vs LPS+S comparison. Axoneme assembly ontology: FDR=9.66E-04. Axonemal dynein complex ontology: FDR=1.16E-02.

DISCUSSION

1. Behavioral assessment of schizophrenic-like symptoms.

Under our specific experimental conditions, we did not observe a significant global effect of prenatal immune activation, PUS, or their combination on any of the behavioral parameters explored for the characterization of a possible schizophrenic endophenotype. The PPI test did not show synergic effects between these factors on the development of sensorimotor gating deficits, which contrasts with the study of (S. Giovanoli et al., 2013) who showed that PPI impairments in mice were only present when the animals were jointly exposed to both hits (using Poly I:C as an inducer of prenatal immune activation). However, significant PPI reductions were observed as a consequence of prenatal LPS exposure in independent experimental batches (Tables 4 and 8) and there was a trend which almost reached the traditional threshold for statistical significance in the "12dB_30ms" condition. Impaired PPI has consistently been documented in maternal immune activation models, especially those involving Poly I:C (see Urs Meyer, 2014 for a review) and also with LPS exposure (M. E. Fortier et al., 2007; E. Romero et al., 2010; Santos-Toscano, Borcel, Ucha, Orihuel, Capellán, Roura-Martínez, Ambrosio, Higuera-Matas, et al., 2016; Simões et al., 2018; Swanepoel et al., 2017; Waterhouse et al., 2017; Wischhof et al., 2015) although some variables such as the species used (Imai, Kotani, Tsuda, Nakano, & Ushida, 2018), the prenatal period at which exposure occurs (Waterhouse et al., 2017) or the age of testing (Basta-kaim et al., 2012) influence the magnitude or actual presence of PPI impairments. In the present study, we chose to evaluate PPI at a slightly earlier period of development as compared to a previous study by our group (PND 70-73 here, as compared to PND72-77 in Santos-Toscano et al., 2016 and did not include females. Moreover, as observed in Figure 13 and Table 8, PUS seems to be counteracting LPS effects to some extent. Therefore, a stronger effect of LPS exposure might be needed to obtain a main effect of the prenatal immune activation or an interaction between both factors. A similar effect was obtained in relation to social behavior and working memory, which were unchanged by the combination of prenatal immune activation and PUS. It is documented that both traits are reduced by prenatal immune activation (Kirsten, Taricano, Maiorka, & Bernardi, 2010; Urs Meyer, 2014b; Osborne, Solowij, Babic, & Weston-green, 2017; Shi, Fatemi, Sidwell, & Patterson, 2003), although the evidence of working memory impairments is generally supported by Poly I:C administration (Urs Meyer, 2014a). In fact, in the previous study of our group (Santos-Toscano, Borcel, Ucha, Orihuel, Capellán, Roura-Martínez, Ambrosio, Higuera-Matas, et al., 2016), male animals exposed to LPS did not show alterations in social interaction, which led us to test these traits by other experimental procedures such as the elliptical area with a confined conspecific (Figure 10) and the Y-

maze (Figure 11). However, it seems that more important variables such as the immunogen used, its dose, the timing of administration or the age of testing ultimately define the magnitude of the behavioral impairments (Boksa, 2010), independently of the experimental procedures employed.

2. Cocaine self-administration.

PUS diminised cocaine self-administration in adulthood, an effect that was reversed in LPS-exposed animals, which also showed increased motivation for consumption. Far from observing synergic effects between both factors towards greater cocaine consumption, we obtained antagonistic effects in certain phases of the program. During the acquisition phase, PUS reduced cocaine self-administration, an effect that was accentuated in its last sessions. A similar effect has been described in a recent study by Hofford, Prendergast, & Bardo, 2018, which found reduction of cocaine self-administration during the acquisition phase after exposing rats of the same strain to a single-prolonged-stress paradigm. Bolton and collegues also found that early-life adversity decreased the amount of self-administered cocaine under low-effort conditions (Bolton et al., 2018). Prenatal exposure to LPS, on the other hand, did not alter cocaine consumption in the acquisition phase, similarly to previous data obtained by our group (Santos-Toscano, Borcel, Ucha, Orihuel, Capellán, Roura-Martínez, Ambrosio, Higuera-Matas, et al., 2016). Other groups have also failed to obtain changes in cocaine self-administration in neurodevelopmental models of schizophrenia such as MAM administration on GD17 (Robert E. Featherstone et al., 2009) or even the NVHL model (Karlsson et al., 2013), questioning some of the previous results obtained using this model (Chambers & Self, 2002). However, it is during the progressive ratio phase (and particularly in the first session, where the lowest response cessation attributable to the progressive ratio schedule is observed (Richardson & Roberts, 1996)) where prenatal exposure to LPS seems to play a more important role, increasing one of the main features that characterize the addictive phenotype, motivation for consumption. This seems to be specific to the MIA model as one previous study using the MAM approach did not find any effect in the progressive ratio schedule of reinforcement for cocaine (Robert E. Featherstone et al., 2009). The effects of PUS on cocaine self-administration during the stabilization phase were lost in LPS-exposed animals, reflecting certain antagonistic effects between both factors. Surprisingly, these factors did not alter the compulsive cocaine intake, however, prenatal exposure to LPS increased consumption in stressed subjects during the extended access phase. Cue-induced relapse, one of the main problems found among addictive disorders, as not influenced either by prenatal

immune activation and / or PUS, contrary to other animal models of schizophrenia-like symptoms (Chambers & Self, 2002; Karlsson et al., 2013). All these data reveal that both factors exert a greater impact on early pathological characteristics of the cocaine addictive process, such as acquisition of consumption, motivation for drug seeking or escalation in drug use, than on later features such as resistance to punishment or incubation of drug craving during abstinence. Importantly, all the effects obtained in the cocaine self-administration paradimg cannot be attributed to general disruptions of operant or pavlovian learning processes as food-reinforced beahviour or conditioned responses to food were not altered by MIA, PUS or their combination.

It is difficult to find a solid explanation for the developmental effects of stress on cocaine-reinforced behaviour, more so if the LPS actions are also to be considered. As we shall see in the RNASeq section, PUS affects the expression of several genes that are related to the brain development and eating behavior in the nucleus accumbens which could account for the decreased cocaine self-administration observed. In our PET study, the pervasive effects of PUS on hippocampal function were also reversed in animals exposed to LPS. Eventhough the hippocampus has not been traditionally attributed a causal role in the development of cocaine self-administration, it seems that lesions to the subicular complex affect performance in a cocaine self-administration paradigm (Black, Green-Jordan, Eichenbaum, & Kantak, 2004) so a parallelism might be suggested between cocaine self-administration and PET data that we have obtained.

In conclusion, it seems that there is no synergy but rather an antagonism between MIA and early stress experiences in terms of cocaine addiction suggesting that the increased rates of cocaine use among schizophrenic patients do not solely result from a biological predispositioned originated from these two factors and that societal and economic factors play a major role in humans suffering from schizophrenia and cocaine addiction. The long-term effects of stress that we have observed in cocaine intake are of notable importance and should be thoroughly studied in future experiments.

3. 2-CSRTT.

There were no significant effects on motor impulsivity, neither by prenatal immune activation, nor by PUS or by the combination of both factors. Previous studies showed that the effect of LPS exposure on impulsive behavior depends, to a large extent, on the stage of development and the context in which this exposure occurs. For example, when administered neonatally, LPS has been suggested to increase impulsive behavior upon reaching adolescence (Rico, Brufato, Ramalho-pinto, & Morato, 2010), however, 5-

CSRTT was not used to assess motor impulsivity. A recent study by Makinson, Lloyd, Grissom, & Reyes, 2019, which better fits the experimental conditions used in our work, showed that prenatal LPS exposure on GD15 did not alter the number of correct, incorrect, omitted or premature responses in the 5-CSRTT during adulthood, correlating exactly with the results obtained in our study. We can then conclude that there is no modulatory effects of impulsivity on the cocaine-addiction patterns that we have observed.

4. Neuroimaging studies.

The results obtained by MRI showed a reduction in whole brain volume as a consequence of prenatal exposure to LPS. On the other hand, PUS decreased the size of the right hippocampus. It is likely that both factors exerted these effects through a common neurobiological mechanism, the increase in the activation of microglia. As we mentioned in the introduction of this work, an over-activation of microglia could lead to excessive neuronal pruning in developing areas, causing cytoarchitectural and volumetric changes. Precisely, prenatal exposure to LPS has shown to increase mean diffusivity of water molecules in the hippocampus of our animals, which has been related to structural damage (Clark et al., 2011). The hippocampus is an area that, in rats, continues to develop until adulthood, unlike in humans where its maturation is completed around two years of age (Lupien et al., 2009). Structural disturbances could result in an inadequate functioning of this area, impacting on its connectivity with other structures. Our PET-CT studies showed hypocampal hyperactivation as a consequence of PUS, which could be the result of possible neuronal loss occurred during development that in adulthood would make impossible to achieve a normal metabolic balance capable of sustaining the different inputs and outputs that compose its neural connectivity. Volumetric reductions of the hippocampus, particularly in the right hemisphere (Janssen et al., 2004), have been widely documented in patients diagnosed with major depressive disorder (Janssen et al., 2007; Sheline, Sanghavi, Mintun, & Gado, 1999; Sheline, Wang, Gado, Csernansky, & Vannier, 1996; Sivakumar et al., 2018), a disease characterized by increased sensitivity to stress (Admon et al., 2014). The brainstem hypoactivation observed in our study of PET-CT as a consequence of PUS could also be related to typical peripheral features of this disease although the spatial resolution of the technique makes it difficult to locate specific nuclei and speculate in a more detailed fashion. All these results show how specific hits, especially stressful situations during puberty, are capable of producing important anatomical and functional changes in vulnerable areas,

mainly in the hippocampus, due to the high expression of glucocorticoid receptors the cells of this structure (López, Chalmers, Little, & Watson, 1998).

These results contrast with those obtained in the left cortex and the dorsal striatum of both hemispheres, which increased their size as a result of PUS. Throughout the literature most studies agree that chronic stress contributes to a reduction in left cortical volume, especially in the medial PFC (Czéh et al., 2007; Drevets et al., 1997; E. Lee et al., 2015). However, in the present study, limited resolution MR images led us to analyze the left cortical volume as a whole, which we believe could have masked this effect. As for the results obtained in the dorsal striatum, they could correlate with the study of Admon et al., 2014, which attributes to this structure a key role in the response to stress. Thus, mechanisms of plasticity and sensitization developed as a result of chronic stress could cause a volumetric increase in this structure. In favor of this hypothesis, Taylor et al., 2014 observed in Sprague-Dawley rats that chronic variable stress increased dendritic complexity in the lateral portion of the dorsal striatum.

5. Metabolomic studies.

In vivo ^{1}H-MRS showed that PUS reduced the levels of choline-containing compounds (GPC + PCh) in the cortex, as well as *N*-acetylaspartate-compounds (NAA + NAAG) in the striatum of prenatally LPS-exposed animals. Several studies have reported increases in the levels of these metabolites in relation to demyelination. Elevated choline-containing compounds have been interpreted as supportive of the "membrane hypothesis" of schizophrenia (Horrobin, Glen, & Vaddadi, 1994), suggesting that phospholipid disturbances and increased myelin degradation support to generalized membrane disorders in schizophrenic patients (Auer et al., 2001; Bustillo et al., 2002). *N*-acetylaspartate-compounds have high affinity for metabotropic glutamate receptors (Yan, Ishihara, Serikawa, & Sasa, 2003) and their levels are related to neuronal density and functionality (Demougeot et al., 2001). Aspartatoacylase is the enzyme responsible for its degradation and is located in oligodendrocytes (Nordengen & Heuser, 2015). Damage to these cells as a consequence of traumatic events in critical stages of neurodevelopment could lead to accumulation of these compounds and myelin deficit (Zhang et al., 2016). However, in the present study, far from observing an increase in the levels of the aforementioned compounds, they decreased. We believe that the observed effects would appear shortly after exposure to each hit, promoting a permanent microglial hyperactivation state capable of causing significant long-term neuronal damage. In this way, and given that the *in vivo* analysis was carried out in adulthood, it

is possible to understand the decrease found in the levels of choline and *N*-acetylaspartate compounds in the cortex and striatum.

Ex vivo, LPS-exposed animals showed lower glucose levels in the striatum, both in left and right hemispheres. In the cortex, this effect was only found in the left hemisphere. Given that glucose is the primary energetic substrate of the brain, we can speculate that a reduction in the glucose contents of these areas may be a consequence of a regional hypoactivation. In accordance with these results, PET studies in human schizophrenic patients have documented decreases in glucose metabolic rate in the striatum and the cortex (Hazlett, Vaccaro, Haznedar, & Goldstein, 2019) and, in animal models, rats with a subchronic exposure to MK-801 showed decreased glucose levels in the temporoparietal cortex, as assessed with ^{13}C-MRS (Eyjolfsson et al., 2011). As regards this, glucose metabolism seems to be altered in the striatum of schizophrenic patients. Indeed, the levels of the β subunit of pyruvate dehydrogenase were lower and levels of pyruvate and lactate were significantly higher in brain samples containing the striatum from subjects with a diagnosis of schizophrenia (Dean, Thomas, Scarr, & Udawela, 2016). In relation to the glutamatergic system, fascinating pattern results regarding glutamate and glutamine in the left striatum were obtained. While MIA per se was associated with a decrease in glutamate and glutamine levels, combination with PUS significantly increased glutamate levels above those seen in stressed animals without MIA. As regards glutamine, the pattern was almost identical: while MIA alone resulted in decreased levels of this amino acid, MIA + PUS tended to increase glutamine levels. This is in accordance to human studies where individuals at ultra-high-risk of schizophrenia and those with a first episode of psychosis that subsequently transitioned to the disease showed higher levels of glutamate/glutamine in the dorsal caudate nucleus (Wijtenburg, Yang, Fischer, & Rowland, 2015). On the other hand, glutamate levels were increased in the left cortex of rats exposed to MIA. There is no consensus in the literature with regard to glutamate levels in the cortex. across the different stages of the schizophrenic disorder (liability, ultra-high risk, first episode, chronicity, etc…). Some studies report no alterations in cortical glutamate in ultra-high-risk patients while others documented increases in the glutamate/glutamine peak (Wijtenburg et al., 2015). Increased glutamatergic tone in MIA rats could also be a consequence of increased oxidative stress in the cortex (Lin, Lane, & Lane, 2019). In support of this, Vernon et al., 2015 reported decreased cortical levels of the antioxidant glutathione in rats exposed to poly I:C during gestation. Lastly, *N*-acetylaspartylglutamate levels were decreased in the right cortical hemisphere. In their review of neurotransmitters and modulators involved in schizophrenia measured by magnetic resonance spectroscopy, Wijtenburg et al., 2015

conclude that "*the consistent finding from ^{1}H MRS studies in schizophrenia is reduced frontal and temporal lobe NAA […] plausibly reflecting neuronal dysfunction in these brain regions*" coinciding with the results that we report in our work.

6. RNA-Seq.

With regard to the RNA-Seq study, we observe that prenatal LPS exposure reduced the expression of genes implied in axoneme assembly in the dorsolateral striatum, as well as others coding for cytoskeletal proteins in the nucleus accumbens. Numerous authors have related alterations in the expression of these proteins with different psychiatric disorders, including schizophrenia (see Marchisella, Coffey, & Hollos, 2016 for a review), and even certain stabilizing agents thereof have been proposed as possible therapeutic targets (Bonini, Mastinu, Ferrari-Toninelli, & Memo, 2017; Varidaki, Hong, & Coffey, 2018). Moreover, in the nucleus accumbens, the remarkable increase observed in the expression of the *drd3* gene (which codes for the D3 dopamine receptor), could be especially relevant. It is located mainly in limbic areas such as the ventral striatum, the ventral pallidum, or the anteroventral nucleus of the thalamus (Joyce & Millan, 2005), whose levels have been shown to be increased in non-medicated schizophrenic patients (Gurevich et al., 1997). These results would be in accordance with our hypothesis, however, they should be taken cautiously since similar studies in animals have not shown such obvious results in other important brain regions. For example, a prior study by the group of Schahram Ackbarian showed that prenatal exposure to poly I:C did not significantly affect the cortical transcriptome of mice evaluated by microarray profiling (Connor et al., 2012). On the other hand, we observed a great influence of PUS on the transcriptomic profile of the nucleus accumbens and the dorsolateral striatum. In the nucleus accumbens, it increased the expression of several genes involved in biological processes relevant to schizophrenia, such as limbic system and telencephalon development or neuropeptide and glutamatergic neurotransmission. Hodes et al., 2015 found similar effects in C57BL/6J mice by performing an ontological analysis of RNA-seq data from the nucleus accumbens in which, although the specific genes that showed altered expression did not coincide with those of our study, most of them were involved in coincident biological processes such as "nervous system development", "cell proliferation" or "cell to cell signaling", among others. However, a key different between studies is the period and nature of the stress experience, as well as the species used. These procedimental differentes must be considered as factors that could explain the differences in the pattern of gene expression between both studies. In the dorsolateral striatum, we observed altered expression of genes involved in transmembrane transport,

cell proliferation and secretion of pro-inflammatory cytokines as a consequence of PUS. These processes can play an important role in the complex etiopathogenesis of schizophrenia. A recent study, by Rowson et al., 2019 also reported altered expression of a large number of genes as a result of chronic pubertal stress in the hippocampus of Wistar rats when reaching adulthood. Therefore, it seems that multiple brain areas are susceptible to alterations in gene expression as a result of PUS. It should also be noted that these studies agree that sex determines in a decisive way the effect of pubertal chronic stress on gene expression. Lastly, combination of prenatal immune activation and PUS reversed the pattern of expression generated by isolated prenatal immune activation in the dorsolateral striatum. This last factor reduced the expression of genes involved in axoneme assembly, but increased after combination of PUS, which may be due to neuroplastic changes underlying the transition from vulnerability to high risk for the onset of schizophrenia.

Thus, from a global point of view, we observed that exposure to prenatal immune activation and / or PUS (under our experimental conditions) has notorious and long-lasting effects on anatomical, metabolic and transcriptomic disturbances in the brain. These changes do not seem have a noticeable effect on some behavioural traits that are reminiscent of the schizophrenic disorder but they have disctinctic effects on cocaine (a trend to a facilitated acquisition and motivation for cocaine in the case of MIA and a significant decrease in cocaine selfa-administration across several stages of the program, which is reversed when animals have also been exposed to MIA). It is possible that these disturbances constitute in some extent the neural basis of a prodromal state, in which schizophrenia-related cognitive impairments would be incubating and only after exposure to other neurodevelopmental hits later in life would be unmasked.

CONCLUSIONS

1. No solid schizophrenia-related cognitive impairments were found in early adulthood as a consequence of prenatal LPS exposure and / or PUS through PPI, social interaction and working memory tests.

2. PUS reduced cocaine self-administration in adulthood, an effect that was reversed in LPS-exposed animals, which also showed increased motivation for consumption.

3. This intake pattern proved to be specific for cocaine, since no significant differences were observed between groups in pavlovian nor instrumental food-conditioning programs.

4. Motor impulsivity was not altered by prenatal LPS exposure and / or PUS.

5. Prenatal LPS exposure reduced whole brain volume and increased hippocampal mean diffusivity in both hemispheres. On the other hand, PUS reduced right hippocampal volume and mesencephalic activity, while increased cortical and dorsostriatal volumes and cortical activity.

6. Glutamate and glucose levels were reduced by prenatal LPS exposure in the striatum. Oppositely, PUS combination increased glutamate levels in this region.

7. PUS altered the expression of a large number of genes in the dorsolateral striatum, many of them involved in the regulation of the immune response. In the nucleus accumbens, this factor increased the expression of multiple genes especially relevant to dual diagnosis. Qualitatively, the influence of prenatal LPS exposure on the gene expression pattern of these structures was less evident.

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SUPPLEMENTARY MATERIAL

TABLE 1

RIGHT CORTEX

Metabolite	Treatment	F/t-value and degrees of freedom	P-value
Lactate	Maternal immune activation	F (1, 27) = 1.578	0.22
	Peripubertal stress	F (1, 27) = 0.214	0.647
	Interaction	F (1, 27) = 0.09	0.767
Myo-inositol	Maternal immune activation	F (1, 27) = 2.679	0.113
	Peripubertal stress	F (1, 27) = 0.009	0.926
	Interaction	F (1, 27) = 0.23	0.635
Glutamate	Maternal immune activation	F (1, 27) = 0.005	0.945
	Peripubertal stress	F (1, 27) = 2.127	0.156
	Interaction	F (1, 27) = 0.005	0.945
Glutamine	Maternal immune activation	F (1, 27) = 0.014	0.906
	Peripubertal stress	F (1, 27) = 2.144	0.155
	Interaction	F (1, 27) = 0.914	0.348
Glutamate/Glutamine	Maternal immune activation	F (1, 27) = 0.128	0.724
	Peripubertal stress	F (1, 27) = 0.296	0.591
	Interaction	F (1, 27) = 2.681	0.114
Lipid 13A	Maternal immune activation	F (1, 27) = 1.177	0.287
	Peripubertal stress	F (1, 27) = 1.539	0.225
	Interaction	F (1, 27) = 0.012	0.913
Lipid 09	Maternal immune activation	F (1, 27) = 0.39	0.538
	Peripubertal stress	F (1, 27) = 12.363	0.002
	Interaction	F (1, 27) = 1.67	0.207
Macromolecule 09	Maternal immune activation	F (1, 25) = 0.524	0.476
	Peripubertal stress	F (1, 25) = 0.25	0.621
	Interaction	F (1, 25) = 2.119	0.158
Lipid 20	Maternal immune activation	F (1, 26) = 0.001	0.982
	Peripubertal stress	F (1, 26) = 1.623	0.214

	Interaction	F (1, 26) = 0.021	0.885
Macromolecule 20	Maternal immune activation	F (1, 27) = 3.472	0.073
	Peripubertal stress	F (1, 27) = 0.316	0.579
	Interaction	F (1, 27) = 1.155	0.292
Macromolecule 12	Maternal immune activation	F (1, 27) = 0.013	0.91
	Peripubertal stress	F (1, 27) = 0.391	0.537
	Interaction	F (1, 27) = 0.013	0.911
Macromolecule 14§	Maternal immune activation	H(1, 26) = 1.269	0.26
	Peripubertal stress	H (1, 26) = 0.465	0.535
Macromolecule 17	Maternal immune activation	F (1, 27) = 1.586	0.219
	Peripubertal stress	F (1, 27) = 0.044	0.835
	Interaction	F (1, 27) = 0.157	0.695
Acetate	Maternal immune activation	F (1, 27) = 0.606	0.443
	Peripubertal stress	F (1, 27) = 3.07	0.091
	Interaction	F (1, 27) = 0.005	0.944
Alanine	Maternal immune activation	F (1, 27) = 1.148	0.294
	Peripubertal stress	F (1, 27) = 0.891	0.354
	Interaction	F (1, 27) = 0.108	0.745
Aspartate	Maternal immune activation	F (1, 27) = 0.997	0.327
	Peripubertal stress	F (1, 27) = 1.429	0.242
	Interaction	F (1, 27) = 3.252	0.082
Creatine	Maternal immune activation	F (1, 27) = 0.661	0.423
	Peripubertal stress	F (1, 27) = 0.32	0.576
	Interaction	F (1, 27) = 1.811	0.19
GABA	Maternal immune activation	F (1, 27) = 0.002	0.964
	Peripubertal stress	F (1, 27) = 0.042	0.84
	Interaction	F (1, 27) = 0.073	0.789
Glutamate/GABA	Maternal immune activation	F (1, 27) = 0.037	0.849
	Peripubertal stress	F (1, 27) = 0.656	0.425

	Interaction	F (1, 27) = 0.289	0.596
Glucose	Maternal immune activation	F (1, 27) = 3.675	0.066
	Peripubertal stress	F (1, 27) = 0.416	0.525
	Interaction	F (1, 27) = 0.277	0.603
Glycerophosphocholine	Maternal immune activation	F (1, 27) = 1.005	0.325
	Peripubertal stress	F (1, 27) = 0.142	0.709
	Interaction	F (1, 27) = 2.495	0.126
Glutathione	Maternal immune activation	F (1, 19) = 1.545	0.229
	Peripubertal stress	F (1, 19) = 0.633	0.436
	Interaction	F (1, 19) = 0.113	0.74
Iso-leucine	Maternal immune activation	F (1, 22) = 2.484	0.129
	Peripubertal stress	F (1, 22) = 0.122	0.73
	Interaction	F (1, 22) = 0.144	0.708
Leucine	Maternal immune activation	F (1, 27) = 2.198	0.15
	Peripubertal stress	F (1, 27) = 0.024	0.879
	Interaction	F (1, 27) = 0.009	0.925
***N*-acetylaspartate**	Maternal immune activation	F (1, 27) = 4.438	0.045
	Peripubertal stress	F (1, 27) = 0.028	0.869
	Interaction	F (1, 27) = 0.063	0.804
Phosphocholine	Maternal immune activation	F (1, 20) = 1.679	0.21
	Peripubertal stress	F (1, 20) = 0.447	0.511
	Interaction	F (1, 20) = 0.065	0.801
Phosphocreatine	Maternal immune activation	F (1, 27) = 0.661	0.423
	Peripubertal stress	F (1, 27) = 0.32	0.576
	Interaction	F (1, 27) = 1.811	0.19
Phosphatidylethanolamine	Maternal immune activation	F (1, 26) = 0.108	0.745
	Peripubertal stress	F (1, 26) = 2.777	0.108
	Interaction	F (1, 26) = 1.832	0.188

Taurine	Maternal immune activation	F (1, 27) = 2.261	0.144
	Peripubertal stress	F (1, 27) = 1.604	0.216
	Interaction	F (1, 27) = 0.154	0.698
Threonine	Maternal immune activation	F (1, 12) = 3.026	0.107
	Peripubertal stress	F (1, 12) = 0.75	0.404
	Interaction	F (1, 12) = 0.104	0.753

Supplementary table 1: Summarized statistical results for right-cortex metabolites. Two-way ANOVA analysis was applied to study the effects of maternal immune activation and peripubertal stress, as well as their possible interactions. Non-parametric statistics (**§**, Kruskal-Wallis H test) was used if ANOVA assumptions were not met.

LEFT CORTEX

Metabolite	Treatment	F/t-value and degrees of freedom	P-value
Lactate	Maternal immune activation	F (1, 27) = 1.596	0.217
	Peripubertal stress	F (1, 27) = 0.45	0.508
	Interaction	F (1, 27) = 0.013	0.91
Myo-inositol	Maternal immune activation	F (1, 27) = 2.851	0.103
	Peripubertal stress	F (1, 27) = 0.022	0.884
	Interaction	F (1, 27) = 1.224	0.278
Glutamate	Maternal immune activation	F (1, 27) = 4.42	0.045
	Peripubertal stress	F (1, 27) = 0.323	0.575
	Interaction	F (1, 27) = 0.013	0.911
Glutamine	Maternal immune activation	F (1, 27) = 0.661	0.423
	Peripubertal stress	F (1, 27) = 2.1	0.159
	Interaction	F (1, 27) = 0.025	0.876
Glutamate/Glutamine	Maternal immune activation	F (1, 27) = 1.875	0.182
	Peripubertal stress	F (1, 27) = 1.756	0.196
	Interaction	F (1, 27) = 0.195	0.662
Lipid 13A	Maternal immune activation	F (1, 27) = 1.742	0.198
	Peripubertal stress	F (1, 27) = 0.236	0.631

	Interaction	F (1, 27) = 0.134	0.717
Lipid 09	Maternal immune activation	F (1, 26) = 1.165	0.29
	Peripubertal stress	F (1, 26) = 1.482	0.234
	Interaction	F (1, 26) = 0.24	0.628
Macromolecule 09	Maternal immune activation	F (1, 27) = 0.223	0.641
	Peripubertal stress	F (1, 27) = 0.003	0.959
	Interaction	F (1, 27) = 0.131	0.72
Lipid 20	Maternal immune activation	F (1, 25) = 0.002	0.965
	Peripubertal stress	F (1, 25) = 0.096	0.759
	Interaction	F (1, 25) = 0.421	0.522
Macromolecule 20	Maternal immune activation	F (1, 26) = 0.175	0.68
	Peripubertal stress	F (1, 26) = 0.028	0.869
	Interaction	F (1, 26) = 0.111	0.742
Macromolecule 12	Maternal immune activation	F (1, 26) = 1.641	0.211
	Peripubertal stress	F (1, 26) = 0.257	0.617
	Interaction	F (1, 26) = 0.014	0.916
Macromolecule 14	Maternal immune activation	F (1, 27) = 1.667	0.208
	Peripubertal stress	F (1, 27) = 0.234	0.632
	Interaction	F (1, 27) = 0.041	0.841
Macromolecule 17	Maternal immune activation	F (1, 27) = 0.916	0.347
	Peripubertal stress	F (1, 27) = 0.115	0.737
	Interaction	F (1, 27) = 0.301	0.588
Acetate	Maternal immune activation	F (1, 27) = 0.176	0.678
	Peripubertal stress	F (1, 27) = 0.6	0.445
	Interaction	F (1, 27) = 1.35	0.256
Alanine	Maternal immune activation	F (1, 27) = 0.873	0.358
	Peripubertal stress	F (1, 27) = 0.034	0.856
	Interaction	F (1, 27) = 0.089	0.767
Aspartate	Maternal immune activation	F (1, 27) = 0.013	0.911
	Peripubertal stress	F (1, 27) = 2.905	0.1

	Interaction	F (1, 27) = 0.472	0.498
Creatine§	Maternal immune activation	H (1, 26) = 3.997	0.0456
	Peripubertal stress	H (1, 26) = 0.047	0.828
GABA	Maternal immune activation	F (1, 27) = 0.261	0.613
	Peripubertal stress	F (1, 27) = 0.087	0.771
	Interaction	F (1, 27) = 1.293	0.266
Glutamate/GABA	Maternal immune activation	F (1, 27) = 0.439	0.514
	Peripubertal stress	F (1, 27) = 0.010	0.923
	Interaction	F (1, 27) = 4.097	0.053
Glucose	Maternal immune activation	F (1, 27) = 5.855	0.023
	Peripubertal stress	F (1, 27) = 0.613	0.44
	Interaction	F (1, 27) = 0.508	0.482
Glycerophosphocholine	Maternal immune activation	F (1, 26) = 1.13	0.298
	Peripubertal stress	F (1, 26) = 2.168	0.153
	Interaction	F (1, 26) = 1.016	0.323
Glutathione	Maternal immune activation	F (1, 21) = 1.435	0.244
	Peripubertal stress	F (1, 21) = 2.049	0.167
	Interaction	F (1, 21) = 0.288	0.597
Iso-leucine	Maternal immune activation	F (1, 25) = 2.35	0.138
	Peripubertal stress	F (1, 25) = 2.816	0.106
	Interaction	F (1, 25) = 1.786	0.193
Leucine§	Maternal immune activation	H (1, 25) = 0.252	0.616
	Peripubertal stress	H (1, 25) = 0.44	0.596
***N*-acetylaspartate**	Maternal immune activation	F (1, 27) = 0.003	0.96
	Peripubertal stress	F (1, 27) = 0.594	0.448
	Interaction	F (1, 27) = 0.132	0.719
Phosphocholine	Maternal immune activation	F (1, 23) = 0.482	0.495
	Peripubertal stress	F (1, 23) = 0.998	0.328
	Interaction	F (1, 23) = 0.259	0.616

Phosphocreatine§	Maternal immune activation	H (1, 26) = 3.997	0.0456
	Peripubertal stress	H (1, 26) = 0.047	0.828
Phosphatidylethanolamine	Maternal immune activation	F (1, 27) = 0.116	0.736
	Peripubertal stress	F (1, 27) = 0.081	0.777
	Interaction	F (1, 27) = 4.744	0.038
Taurine	Maternal immune activation	F (1, 27) = 1.065	0.311
	Peripubertal stress	F (1, 27) = 0.001	0.981
	Interaction	F (1, 27) = 0.065	0.8
Threonine	Maternal immune activation	F (1, 13) = 0.027	0.871
	Peripubertal stress	F (1, 13) = 1.718	0.213
	Interaction	F (1, 13) = 0.002	0.962

Supplementary table 2: Summarized statistical results for left-cortex metabolites. Two-way ANOVA analysis was applied to study the effects of maternal immune activation and peripubertal stress, as well as their possible interactions. Non-parametric statistics (**§**, Kruskal-Wallis H test) was used if ANOVA assumptions were not met.

RIGHT STRIATUM

Metabolite	Treatment	F/t-value and degrees of freedom	P-value
Lactate	Maternal immune activation	F (1, 27) = 0.001	0.97
	Peripubertal stress	F (1, 27) = 0.053	0.82
	Interaction	F (1, 27) = 0.01	0.921
Myo-inositol	Maternal immune activation	F (1, 26) = 1.102	0.304
	Peripubertal stress	F (1, 26) = 1.081	0.308
	Interaction	F (1, 26) = 0.11	0.743
Glutamate	Maternal immune activation	F (1, 27) = 0.307	0.584
	Peripubertal stress	F (1, 27) = 1.056	0.313
	Interaction	F (1, 27) = 2.556	0.122
Glutamine	Maternal immune activation	F (1, 27) = 0.699	0.411
	Peripubertal stress	F (1, 27) = 3.167	0.086
	Interaction	F (1, 27) = 0.654	0.426

Glutamate/Glutamine§	Maternal immune activation	H (1, 27) = 0.156	0.693
	Peripubertal stress	H (1, 27) = 0.625	0.429
Lipid 13A	Maternal immune activation	F (1, 26) = 0.16	0.693
	Peripubertal stress	F (1, 26) = 0.599	0.446
	Interaction	F (1, 26) = 0.682	0.416
Lipid 09	Maternal immune activation	F (1, 25) = 0.129	0.722
	Peripubertal stress	F (1, 25) = 0.906	0.35
	Interaction	F (1, 25) = 0.215	0.647
Macromolecule 09	Maternal immune activation	F (1, 27) = 1.21	0.281
	Peripubertal stress	F (1, 27) = 3.893	0.059
	Interaction	F (1, 27) = 0.67	0.42
Lipid 20	Maternal immune activation	F (1, 25) = 0.506	0.484
	Peripubertal stress	F (1, 25) = 1.887	0.182
	Interaction	F (1, 25) = 1.084	0.308
Macromolecule 20	Maternal immune activation	F (1, 27) = 8.636	0.007
	Peripubertal stress	F (1, 27) = 2.137	0.155
	Interaction	F (1, 27) = 2.706	0.112
Macromolecule 12	Maternal immune activation	F (1, 27) = 2.034	0.165
	Peripubertal stress	F (1, 27) = 7.063	0.013
	Interaction	F (1, 27) = 0.022	0.883
Macromolecule 14	Maternal immune activation	F (1, 27) = 3.17	0.086
	Peripubertal stress	F (1, 27) = 2.471	0.128
	Interaction	F (1, 27) = 2.234	0.147
Macromolecule 17§	Maternal immune activation	H (1, 26) = 0.757	0.384
	Peripubertal stress	H (1, 26) = 0.088	0.767
Acetate	Maternal immune activation	F (1, 27) = 0.787	0.383
	Peripubertal stress	F (1, 27) = 0.52	0.477
	Interaction	F (1, 27) = 0.075	0.786
	Maternal immune activation	F (1, 27) = 0.787	0.383

Alanine	Peripubertal stress	F (1, 27) = 0	0.992
	Interaction	F (1, 27) = 1.407	0.246
Aspartate	Maternal immune activation	F (1, 25) = 4.789	0.038
	Peripubertal stress	F (1, 25) = 1.649	0.211
	Interaction	F (1, 25) = 0.745	0.396
Creatine	Maternal immune activation	F (1, 27) = 3.023	0.093
	Peripubertal stress	F (1, 27) = 6.743	0.015
	Interaction	F (1, 27) = 3.138	0.088
GABA§	Maternal immune activation	H (1, 27) = 3.165	0.0752
	Peripubertal stress	H(1, 27) = 0.000	1
Glutamate/GABA§	Maternal immune activation	H (1, 27) = 2.5	0.114
	Peripubertal stress	H (1, 27) = 0.306	0.58
Glucose	Maternal immune activation	F (1, 26) = 12.402	0.002
	Peripubertal stress	F (1, 26) = 0.12	0.732
	Interaction	F (1, 26) = 0.147	0.704
Glycerophosphocholine	Maternal immune activation	F (1, 27) = 3.557	0.07
	Peripubertal stress	F (1, 27) = 0.632	0.434
	Interaction	F (1, 27) = 1.311	0.262
Glutathione	Maternal immune activation	F (1, 22) = 0.013	0.911
	Peripubertal stress	F (1, 22) = 0.394	0.536
	Interaction	F (1, 22) = 0.008	0.931
Iso-leucine	Maternal immune activation	F (1, 22) = 2.56	0.124
	Peripubertal stress	F (1, 22) = 2.075	0.164
	Interaction	F (1, 22) = 1.086	0.309
Leucine	Maternal immune activation	F (1, 26) = 0.512	0.481
	Peripubertal stress	F (1, 26) = 0.662	0.423
	Interaction	F (1, 26) = 0.177	0.677
***N*-acetylaspartate**	Maternal immune activation	F (1, 27) = 2.379	0.135
	Peripubertal stress	F (1, 27) = 0.046	0.832

	Interaction	F (1, 27) = 0.59	0.449
Phosphocholine	Maternal immune activation	F (1, 23) = 0.284	0.599
	Peripubertal stress	F (1, 23) = 0.009	0.925
	Interaction	F (1, 23) = 0.796	0.381
Phosphocreatine	Maternal immune activation	F (1, 27) = 3.023	0.093
	Peripubertal stress	F (1, 27) = 6.743	0.015
	Interaction	F (1, 27) = 3.138	0.088
Phosphatidylethanolamine§	Maternal immune activation	H(1, 25) = 0.189	0.664
	Peripubertal stress	H (1, 25) = 1.651	0.199
Taurine	Maternal immune activation	F (1, 27) = 0.089	0.767
	Peripubertal stress	F (1, 27) = 0.161	0.691
	Interaction	F (1, 27) = 0.195	0.662
Threonine	Maternal immune activation	F (1, 15) = 0.105	0.751
	Peripubertal stress	F (1, 15) = 0.071	0.794
	Interaction	F (1, 15) = 0.033	0.859
Guanine	Maternal immune activation	F (1, 17) = 0.128	0.725
	Peripubertal stress	F (1, 17) = 0.408	0.531
	Interaction	F (1, 17) = 0.164	0.691

Supplementary table 3: Summarized statistical results for right-striatum metabolites. Two-way ANOVA analysis was applied to study the effects of maternal immune activation and peripubertal stress, as well as their possible interactions. Non-parametric statistics (**§**, Kruskal-Wallis H test) was used if ANOVA assumptions were not met.

LEFT STRIATUM

Metabolite	Treatment	F/t-value and degrees of freedom	P-value
Lactate	Maternal immune activation	F (1, 27) = 0.138	0.713
	Peripubertal stress	F (1, 27) = 0.089	0.767
	Interaction	F (1, 27) = 0.17	0.683
Myo-inositol	Maternal immune activation	F (1, 27) = 0.124	0.728
	Peripubertal stress	F (1, 27) = 0.755	0.393

	Interaction	F (1, 27) = 0.112	0.741
Glutamate	Maternal immune activation	F (1, 27) = 0.011	0.919
	Peripubertal stress	F (1, 27) = 2.822	0.105
	Interaction	F (1, 27) = 11.21	0.002
Glutamine	Maternal immune activation	F (1, 27) = 0.671	0.42
	Peripubertal stress	F (1, 27) = 0.191	0.665
	Interaction	F (1, 27) = 6.155	0.02
Glutamate/Glutamine	Maternal immune activation	F (1, 27) = 0.598	0.446
	Peripubertal stress	F (1, 27) = 3.546	0.070
	Interaction	F (1, 27) = 0.448	0.509
Lipid 13A	Maternal immune activation	F (1, 27) = 1.148	0.293
	Peripubertal stress	F (1, 27) = 3.516	0.072
	Interaction	F (1, 27) = 0.891	0.354
Lipid 09	Maternal immune activation	F (1, 25) = 2.238	0.147
	Peripubertal stress	F (1, 25) = 1.099	0.304
	Interaction	F (1, 25) = 5.884	0.023
Macromolecule 09§	Maternal immune activation	H(1, 26) = 3.829	0.0504
	Peripubertal stress	H (1, 26) = 0.425	0.514
Lipid 20	Maternal immune activation	F (1, 25) = 0.589	0.45
	Peripubertal stress	F (1, 25) = 0.248	0.623
	Interaction	F (1, 25) = 0.535	0.471
Macromolecule 20	Maternal immune activation	F (1, 25) = 3.012	0.095
	Peripubertal stress	F (1, 25) = 4.057	0.055
	Interaction	F (1, 25) = 9.805	0.004
Macromolecule 12	Maternal immune activation	F (1, 26) = 1.766	0.195
	Peripubertal stress	F (1, 26) = 0.282	0.6
	Interaction	F (1, 26) = 0.726	0.402
Macromolecule 14	Maternal immune activation	F (1, 27) = 5.674	0.025
	Peripubertal stress	F (1, 27) = 0.363	0.552
	Interaction	F (1, 27) = 0.752	0.393

Macromolecule 17§	Maternal immune activation	H (1, 26) = 0.002	0.968
	Peripubertal stress	H (1, 26) = 0.400	0.527
Acetate	Maternal immune activation	F (1, 27) = 0.643	0.43
	Peripubertal stress	F (1, 27) = 0.523	0.476
	Interaction	F (1, 27) = 0.075	0.787
Alanine	Maternal immune activation	F (1, 27) = 1.121	0.299
	Peripubertal stress	F (1, 27) = 0.429	0.518
	Interaction	F (1, 27) = 0.093	0.763
Aspartate	Maternal immune activation	F (1, 27) = 3.222	0.084
	Peripubertal stress	F (1, 27) = 0.111	0.742
	Interaction	F (1, 27) = 1.4	0.247
Creatine	Maternal immune activation	F (1, 27) = 3.818	0.061
	Peripubertal stress	F (1, 27) = 4.296	0.048
	Interaction	F (1, 27) = 0.255	0.617
GABA	Maternal immune activation	F (1, 27) = 0.627	0.435
	Peripubertal stress	F (1, 27) = 0.093	0.762
	Interaction	F (1, 27) = 1.053	0.314
Glutamate/GABA	Maternal immune activation	F (1, 27) = 2.105	0.158
	Peripubertal stress	F (1, 27) = 0.012	0.914
	Interaction	F (1, 27) = 0.326	0.573
Glucose	Maternal immune activation	F (1, 25) = 8.586	0.007
	Peripubertal stress	F (1, 25) = 0.176	0.678
	Interaction	F (1, 25) = 0.222	0.642
Glycerophosphocholine	Maternal immune activation	F (1, 26) = 0.233	0.633
	Peripubertal stress	F (1, 26) = 0.055	0.817
	Interaction	F (1, 26) = 0.007	0.933
Glutathione	Maternal immune activation	F (1, 19) = 0.038	0.848
	Peripubertal stress	F (1, 19) = 0.199	0.661
	Interaction	F (1, 19) = 0.249	0.624

Iso-leucine	Maternal immune activation	F (1, 19) = 0.675	0.422
	Peripubertal stress	F (1, 19) = 0.272	0.608
	Interaction	F (1, 19) = 0.088	0.77
Leucine§	Maternal immune activation	H (1, 26) = 0.459	0.498
	Peripubertal stress	H (1, 26) = 1.202	0.273
***N*-acetylaspartate**	Maternal immune activation	F (1, 27) = 2.916	0.099
	Peripubertal stress	F (1, 27) = 0.519	0.478
	Interaction	F (1, 27) = 3.792	0.062
Phosphocholine	Maternal immune activation	F (1, 20) = 1.301	0.268
	Peripubertal stress	F (1, 20) = 0.755	0.395
	Interaction	F (1, 20) = 0.356	0.557
Phosphocreatine	Maternal immune activation	F (1, 27) = 3.818	0.061
	Peripubertal stress	F (1, 27) = 4.296	0.048
	Interaction	F (1, 27) = 0.255	0.617
Phosphatidylethanolamine	Maternal immune activation	F (1, 27) = 0.085	0.773
	Peripubertal stress	F (1, 27) = 0.731	0.4
	Interaction	F (1, 27) = 0.908	0.349
Taurine	Maternal immune activation	F (1, 27) = 0.657	0.425
	Peripubertal stress	F (1, 27) = 2.146	0.155
	Interaction	F (1, 27) = 1.283	0.267
Threonine	Maternal immune activation	F (1, 14) = 0.835	0.376
	Peripubertal stress	F (1, 14) = 1.497	0.241
	Interaction	F (1, 14) = 0.017	0.897
Guanine	Maternal immune activation	F (1, 12) = 0.025	0.878
	Peripubertal stress	F (1, 12) = 1.311	0.275
	Interaction	F (1, 12) = 0.009	0.925

Supplementary table 4: Summarized statistical results for left striatum metabolites. Two-way ANOVA analysis was applied to study the effects of maternal immune activation and peripubertal stress, as well as their possible interactions. Non-parametric statistics (**§**, Kruskal-Wallis H test) was used if ANOVA assumptions were not met.