Characterization of a double-hit mouse model of schizophrenia.

Focus on inflammatory and epigenetic processes, pharmacological modulation and sex bias

Doctoral thesis

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CHARACTERIZATION OF A DOUBLE-HIT MOUSE MODEL OF SCHIZOPHRENIA.

FOCUS ON INFLAMMATORY AND EPIGENETIC PROCESSES, PHARMACOLOGICAL MODULATION AND SEX BIAS.

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ABBREVIATION LIST

AC Adenylyl cyclase

AMPA α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate

ANOVA Analysis of variance

BBB Blood-brain barrier

BDNF Brain-derived neurotrophic factor

^{0}C Degrees Celsius of temperature

cAMP Cyclic adenosine monophosphate CAV-2 Canine adenovirus type 2

cm Centimeters

CNS Central nervous system

DA Dopamine

DLPFC Dorsolateral prefrontal cortex

DOI 2,5-dimethoxy-4-iodoamphetamine

dsRNA Double-stranded RNA

FEP First-episode psychosis

GABA Gamma-aminobutyric acid

GD Gestational day

GPCR G protein-coupled receptor

GWAS Genome-wide association study

HDAC Histone deacetylase

5-HT Serotonin

5-HT$_{2A}$R Serotonin 2A receptor

HTR Head-twitch response

IFN Interferon

IL Interleukin

lncRNA Long noncoding RNA

LPS Lipopolysaccharide

LSD lysergic acid diethylamide

mGluR metabotropic glutamate receptor

MIA Maternal immune activation

miRNA microRNA

MK801 (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten- 5,10-imine hydrogen maleate) or Dizocilpine

NAC N-acetyl cysteine

NF-κB Nuclear factor-kappa B

NMDA N-methyl-D-aspartate

NORT Novel object recognition test

OFT Open field test

PCP Phencyclidine

PET Positron emission tomography

PFC Prefrontal cortex

PND Postnatal day

Poly (I:C) Polyinosinic:polycytidylic acid

PPI Prepulse inhibition

SAHA Suberoylanilide hydroxamic acid

SI Social isolation

sncRNA Short noncoding RNA

SPT Social preference test

THC Δ^9-tetrahydrocannabinol

TLR3 Toll-like receptor 3

TNF-α Tumor necrosis factor alpha

YMSAT Y-maze spontaneous alternation test

INDEX

1. INTRODUCTION

1.1. SCHIZOPHRENIA

1.1.1. GENERAL CONSIDERATIONS

Schizophrenia literally translates as “shattered mind” (Möller et al., 2015). Schizophrenia is a complex, heterogeneous behavioral and cognitive syndrome, with a lifetime prevalence of 1% (Jauhar et al., 2022; Marder & Cannon, 2019; Owen et al., 2016). The traditional idea that the disorder affects about one in a hundred people continues to be broadly supported by systematic reviews reporting a mean lifetime morbid risk of 11.9 per 1000, with a median of 7.2 per 1000 (McGrath et al., 2008). Schizophrenia is among the world’s top 10 causes of long-term disability, affecting about 19.8 million people worldwide (Choudhury & Lennox, 2021). The high rates of unemployment and social maladjustment associated to schizophrenia, as well as the limited efficacy of currently available therapies, mean that this disorder constitutes a major public health problem (Owen et al., 2016). In addition, schizophrenia is associated to poor dietary habits, weight gain, smoking, and comorbid substance use, leading to a substantial reduction in life expectancy (10-20 years of potential life lost) (Chesney et al., 2014; Hjorthøj et al., 2017). Consensus on the lifetime risk of suicide in schizophrenia patients is a rate of approximately 5% (Barak et al., 2004; Carlborg et al., 2008; Limosin et al., 2007; Ran et al., 2007). Risk factors linked to later suicide in schizophrenia patients include being young, male, having a high level of education, having prior suicide attempts, suffering from depressive symptoms, having active hallucinations/delusions, family and comorbid substance use; whereas adherence to effective treatment acts as a protective factor for suicide in schizophrenia patients (Hor & Taylor, 2010).

1.1.2. SYMPTOMATOLOGY

Schizophrenia has classically been perceived as a single illness with mixed manifestations. However, nowadays it is best conceptualized as a clinical syndrome: a combination of individual component symptom complexes with distinct course, pathophysiology and treatment response. Thus, schizophrenia symptoms can be classified in three main clusters: positive (e.g. hallucinations, delusions, thought disorder), negative (e.g. asociality, alogia, anhedonia) and cognitive symptoms (e.g. impaired attention, memory and executive functions) (Ibrahim & Tamminga, 2011) (**figure 1.1**).

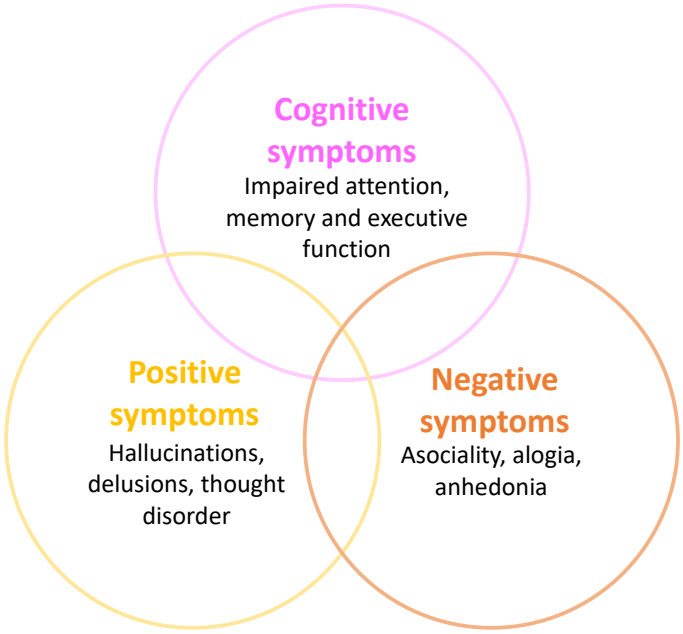


Figure 1.1: Component symptom complexes in schizophrenia. Originally created by Natalia Cordero Ruiz for this work, adapted from Ibrahim & Tamminga, 2011.

Although traditionally, positive psychotic symptoms have received the biggest attention, negative and cognitive symptoms represent a major concern in the clinical evolution of schizophrenia patients. On the one hand, negative symptoms are debilitating and chronic in nature, difficult to treat and contribute to poor functional outcomes (Goldsmith & Rapoport, 2020). Negative symptoms are responsible of much of the long-term morbidity and poor functional outcomes in schizophrenia patients, affecting their ability to live independently, perform activities of daily living, maintain personal relationships and participate in social, work and study activities (Weber et al., 2022). Moreover, although antipsychotic medications –the cornerstone of current schizophrenia pharmacotherapy– are effective for the treatment of positive psychotic symptoms, they have limited efficacy for treating negative symptoms (Erhart et al., 2006), as most antipsychotic medications provide only a small to moderate reduction in negative symptoms compared to placebo (Mizuno et al., 2020). On the other hand, cognitive deficits are a strong determinant of functional outcome and mortality in individuals with schizophrenia, they show high prevalence, and they are relatively stable over time and independent of psychotic symptoms (Gold, 2004; Lett et al., 2014). Cognitive decline in schizophrenia starts in adolescence and precedes the onset of the psychosis by more than a decade (Kahn, 2022; Kahn & Keefe, 2013). Several cognitive dimensions are affected in schizophrenia, including speed of information processing, attention span, working memory, verbal and visual learning, executive functions of reasoning and problem solving, and social

learning. Of these, working memory is the best-studied cognitive domain, which is defined as the ability to hold and manipulate a limited amount of information to guide thought or behavior (Lett et al., 2014). The prefrontal cortex (PFC) appears to be the main anatomical substrate of cognitive activities, especially working memory (D'Esposito et al., 2000). The PFC is able to inhibit irrelevant information by adjusting cortical connectivity to the demands of each task, thus organizing thoughts, behavior, emotions, inhibiting inappropriate conducts and allowing future planning (Arnsten, 2009). In schizophrenia, alterations of the PFC and its connections have been reported, both in neuropathological studies and *in vivo* neuroimaging studies (Ellison-Wright & Bullmore, 2010; Lett al., 2014; Lewis & González-Burgos, 2008; Olabi et al., 2011). Antipsychotic treatment either impairs or does not improve cognitive domains in patients with schizophrenia (reviewed by Hagan & Jones, 2005; Lett et al., 2014; Spark et al., 2022).

Overall, while currently available antipsychotics are an effective therapy for the management of positive psychotic symptoms, they are not efficacious for neither the negative nor the cognitive dysfunctions. Hence, it is urgent to elucidate the neurobiological basis underlying negative and cognitive deficits, and to develop an effective pharmacological therapy for these "orphan symptoms" of schizophrenia. This knowledge could allow the development of drugs that improve the limitations of the current ones.

1.1.3. GENDER-RELATED DIFFERENCES

Schizophrenia typically develops in adolescence or early adult life and is modestly more common in men than in women (Jauhar et al., 2022). In this regard, evidence from a recent meta-analysis suggests a discreetly higher incidence in men comparing to women (male-to-female incidence rate ratio of 1.70) (Jongsma et al., 2019). Although the incidence of this disorder is only slightly higher in men than in women, gender-related differences play an important role in schizophrenia. Thus, various reports have suggested that the severity of psychosis outcomes is different between men and women (Abel et al., 2010; Ferrara & Srihari, 2021; Ochoa et al., 2012), and several environmental risk factors associated with schizophrenia are influenced by gender. These gender-influenced environmental factors include childhood adversity (Comacchio et al., 2019), migration (Cantor-Graae & Pedersen, 2013; Gayer-Anderson et al., 2015), urbanicity (Kelly et al., 2010), and birth season (Martínez-Ortega et al., 2011). In this sense, a recent systematic review further explored gender differences on the associations between environment and psychosis onset (for review see Pence et al., 2022). Childhood abuse has been reported to be more strongly associated with psychosis and with an earlier age at onset of illness

in women (Fisher et al., 2009; Gayer-Anderson et al., 2015; Toutountzidis et al., 2018). Childhood adversity has been associated with the severity of different symptom dimensions in men and women (Comacchio et al., 2019). In addition, growing up in an urban environment (Kelly et al., 2010) and immigration status (Cantor-Graae & Pedersen, 2013) have been more strongly associated with psychosis risk in men. Finally, despite a higher prevalence of comorbid substance use in men diagnosed with schizophrenia, association between substance use and psychosis risk may be stronger in women (Donoghue et al., 2014; Hodgins et al., 2016). In this regard, women who were treated for substance misuse had 7-fold risk to develop psychosis, whereas the risk for psychosis in men with substance abuse history increased around 4-fold (Hodgins et al., 2016). Furthermore, substance use, particularly cannabis use, may increase the risk for psychosis in women more than in men (Hernández-Ávila et al., 2004). Overall, a vast body of evidence demonstrates that there are gender-related differences in schizophrenia that should be taken into account in the treatment, diagnosis and prognosis of the disorder.

1.1.4. ALTERATIONS IN NEUROTRANSMISSION SYSTEMS

It has been classically considered that at least some of the clinical manifestations in schizophrenia might appear due to a neurochemical disturbance (Jauhar et al., 2022). Different neurotransmission systems have been implicated in the pathophysiology of schizophrenia. Bellow, the principal neurotransmitter hypothesis of schizophrenia are described: the dopaminergic, glutamatergic and serotonergic hypothesis.

Dopaminergic hypothesis of schizophrenia

The physiological actions of dopamine (DA) are mediated by five distinct but closely related G protein-coupled receptors (GPCRs) that are divided into two major groups: the D_1 and D_2 classes of DA receptors. This distinction was mostly based on their ability to modulate cAMP production (Kebabian & Calne, 1979). DA receptors are widely distributed within the brain, where they play critical modulator roles on motor functions, motivation and drive, as well as cognition (Martel & Mcarthur, 2020). Based on their structural, pharmacological and biochemical properties, DA receptors were classified as D_1-like (D_1R and D_5R) and D_2-like (D_2R, D_3R and D_4R) receptors (Andersen et al., 1990; Civelli et al., 1993; Niznik & Van Tol, 1992; Sibley and Monsma, 1992; Sokoloff et al., 1992; Vallone et al., 2000). It is commonly accepted that the D_1-class DA receptors activate the $G\alpha_{s/olf}$ family of G proteins to increase cAMP production by stimulating adenylyl cyclase (AC) activity, and have an exclusively postsynaptical location on DA-receptive cells. The

D_2-class DA receptors couple to the $G\alpha_{i/o}$ family of G proteins and thus induce inhibition of AC. In contrast to the D_1-class dopamine receptors, D_2R and D_3R are expressed both postsynaptically on DA target cells and presynaptically on DA neurons (Rankin et al., 2010; Rondou et al., 2010; Sokoloff et al., 2006). The D_1- and D_2-class DA receptors also manifest genetic differences, mainly because of the presence or absence of introns in their coding sequences. The D_1R and D_5R genes do not contain introns in their coding regions, but the genes encoding for the D_2-class DA receptors contain several introns on their sequences (six introns in the D_2R gene, five in the D_3R gene, and three in the D_4R gene) (Gingrich & Caron, 1993). Therefore, genetic differences in the sequence of D_2-class of DA receptors generates splice variants. Precisely, D_2R presents two major genetic isoforms, termed D_2S (D_2-short) and D_2L (D_2-long), depending on presence or absence of an additional 29 amino acids in the third intracellular loop (Giros et al., 1989; Monsma et al., 1989). As explained later (see **section 1.1.5.1**), therapeutic effect of typical antipsychotic drugs reducing positive symptoms depends on their ability to reduce DA function by blocking the dopamine D_2R postsynaptic receptors, suggesting that this receptors play a key role in the pathophysiology of schizophrenia (Creese et al., 1976; Johnstone et al., 1980; Peroutka & Synder, 1980; Seeman et al., 1976).

The dopaminergic hypothesis has been the most widely known theory concerning schizophrenia and the implication of the dopaminergic system on the pathophysiology of schizophrenia has been studied since more than 50 years ago (Bell, 1965; Creese et al., 1976; Seeman et al., 1976). The dopaminergic hypothesis of schizophrenia was formulated based in two main findings. First, several studies found that the therapeutic effect of classical (typical) antipsychotic drugs was based on their ability to decrease DA function by blocking postsynaptic dopamine D_2R receptors (Creese et al., 1976; Seeman et al., 1976). Second, that the use of the psychostimulant drug amphetamine (able to stimulate DA release), was able to resemble some schizophrenia symptoms in healthy volunteers (Bell, 1965). Thus, the original dopaminergic hypothesis of schizophrenia states that hyperactive DA transmission results in the psychotic symptoms of the disease. According to this classical dopaminergic hypothesis of schizophrenia, an aberrant functioning of midbrain DA projections to limbic regions is thought to provoke psychotic symptoms. However, more recent studies based on neuroimaging techniques point that DA dysfunction in schizophrenia is greatest within nigrostriatal pathways, implicating the dorsal striatum in the pathophysiology of the disease (McCutcheon et al., 2019). Currently, the DA imbalance hypothesis of schizophrenia proposes that enhanced activity in the mesolimbic dopaminergic system together with hypoactive mesocortical DA projections to the PFC contribute to the pathophysiology of the disease (Abi-Dargham & Moore, 2003; Winterer &

Weinberger, 2004). Accordingly, different results show correlation between the positive psychotic symptoms and the increase of DA release in the striatum (Laruelle et al., 1999). On the contrary, negative or cognitive dysfunctions of schizophrenia patients correlate with functional DA deficits in PFC (Davis et al., 1991; Slifstein et al., 2015).

Hence, the initial hypothesis of the excessive DA role in schizophrenia has been reformulated into a hyperactive DA transmission (*hyperdopaminergia*) in the mesolimbic areas and hypoactive DA transmission (*hypodopaminergia*) in the PFC of schizophrenia patients. However, its limitations in defining symptoms other than psychosis –such as cognitive and negative symptoms–, as well as the evidence of other neurotransmitters involved –glutamate, serotonin–, suggest a wider perspective of the disease.

Glutamatergic hypothesis of schizophrenia

Glutamate is the major excitatory neurotransmitter in the brain. Glutamatergic neurotransmission is modulated by two types of receptors: the ionotropic and the metabotropic glutamate receptors. Glutamate ionotropic receptors include N-methyl-D-aspartate (NMDA), Kainate, and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) receptor subtypes. Heterotrimetric G protein-coupled metabotropic glutamate receptors (mGluRs) are divided in groups I (mGlu1, mGlu5), II (mGlu2, mGlu3) and III (mGlu6, mGlu4, mGlu7, mGlu8), and activate G protein-coupled signal transduction. Class II mGlu2 and mGlu3 receptors are coupled predominantly to inhibitory $G_{i/o}$ proteins, which mediate the downstream inhibition of adenylyl cyclase activity (Nakanishi, 1992; see Muguruza et al., 2016 for review).

The glutamatergic hypothesis of schizophrenia first emerged with the observation that healthy subjects under the effect of phencyclidine (PCP) and the dissociative anesthetic ketamine –two non-competitive NMDA receptor antagonists– developed prolonged psychotic states that could mimic schizophrenia symptoms (Farber, 2003; Javitt & Zukin, 1991; Olney & Farber, 1995; Stone et al., 2008). It was later demonstrated that non-competitive NMDA receptor antagonists, besides inducing positive psychotic-like symptoms, were also able to resemble negative and cognitive schizophrenia symptoms in healthy volunteers (Driesen et al., 2013, Krystal et al., 2005).

A large body of evidence indicates dysfunctional local circuitry in schizophrenia, particularly gamma-aminobutyric acid (GABA) deficits within the PFC and its connections with the limbic system (Lewis et al., 2005; Tamminga et al., 1992). In this regard, recent findings suggest that NMDA receptor hypofunction causes dysfunctional GABAergic circuitry locally within the PFC

and disruptions to the incoming and outgoing connections with other brain regions (Avery & Krichmar, 2017; Nakazawa et al., 2017). Thus, NMDA receptor hypofunction is thought to be involved on schizophrenia pathogenesis. The dysfunction of the NMDA receptor on GABAergic interneurons induces disinhibition of the excitatory pyramidal neurons, resulting in hyperexcitation, more release of glutamate, and increased release of striatal dopamine, resulting in psychotic symptoms (Howes et al., 2015; Kesby et al., 2018; Lisman et al., 2008; Uno & Coyle, 2019). Thus, glutamatergic hypothesis is closely related to the previously described limbic hyperdopaminergic and cortical hypodopaminergic states. Inhibition of NMDA receptors in GABAergic interneurons in mesolimbic dopaminergic areas would impair the inhibitory tonic state, resulting in a higher synthesis and release of DA in those areas, and explaining positive psychotic symptoms. In contrast, NMDA hypofunction in cortical projections could drive a hypoactive mesocortical DA state, and could be linked to cognitive and negative symptoms (Javitt, 2010).

Consequently, non-competitive NMDA antagonists (PCP, ketamine, MK-801) are routinely used to mimic schizophrenia-related phenotypes in animal models, including behaviors such as hyperlocomotion and prepulse inhibition deficits (PPI) –behavioral model that measures sensorimotor gating– (Moreno & González-Maeso, 2013). Additionally, agonists of the mGlu2/3 receptor and positive allosteric modulators (PAMs) of mGlu2 receptors have been tested as therapeutic options for schizophrenia treatment (Marek, 2004; Moghaddam & Adams, 1998; Muguruza & Callado, 2022). Moreover, genome-wide association studies (GWAS) have shown that genes involved in glutamatergic neurotransmission and synaptic plasticity –including, mGluR3 (*GRM3*), glutamate ionotropic receptor NMDA type subunit 2A (*GRIN2A*), serine racemase (*SRR*), glutamate ionotropic receptor AMPA type subunit 1 (*GRIA1*) or neurogranin (*NRGN*)– are linked to the risk of developing schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Stefansson et al., 2009). Finally, *postmortem* and neuroimaging studies have revealed that various components of the glutamatergic signaling system are altered in subjects with schizophrenia (Gao et al., 2000; Stone, 2009).

Serotonergic hypothesis of schizophrenia

Serotonin (5-HT), which was known to be the principal vasoconstrictor substance found in serum, was identified as 5-hydroxytryptamine and first isolated by Maurice Rapport back in 1948 (Rapport et al., 1948). Effects of 5-HT on smooth muscle and other peripheral organs were long appreciated, but it wasn't until its structural similarity to lysergic acid diethylamide (LSD) was

described that 5-HT and its receptors were linked to neurotransmission (Wooley and Shaw, 1954).

5-HT receptors are classified into seven major families: 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7 receptors. At a structural level, all 5-HT receptors are GPCRs, excepting the 5-HT_3 receptor, which is a ligand-gated ion channel. 5-HT_1 family has five members (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F}), while the 5-HT_2 family has three (5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}). Each member of the 5-HT_2 family displays distinct brain and tissue distributions. Particularly, the 5-HT_{2A} receptor (5-HT_{2A}R) is abundantly expressed in the PFC, and is highly enriched in pyramidal neurons in layer V of the human cerebral cortex, where it mediates the actions of atypical antipsychotic drugs (see **section 1.1.6.1**) (Meltzer & Roth, 2013; Santana et al., 2004).

The 5-HT_{2A}Rs are the key players in the serotonergic hypothesis of schizophrenia. On the one hand, hallucinogenic drugs such as LSD, psilocybin (and its active compound psilocin), 2,5-dimethoxy-4-iodoamphetamine (DOI) and N,N-dimethyltryptamine (DMT) are agonists/partial agonists at 5-HT_{2A}Rs, and its administration to healthy volunteers resembles some of the core symptoms of schizophrenia (Carhart-Harris et al., 2012; reviewed in González-Maeso & Sealfon, 2009). Indeed, studies in healthy volunteers have demonstrated a good correlation between 5-HT_{2A}R binding affinities and potencies of hallucinogenic drugs (Glennon et al., 1984). On the other hand, second generation or atypical antipsychotic drugs have a higher affinity for 5-HT_{2A}Rs as compared to D_2 receptors (reviewed in González-Maeso & Sealfon, 2009; Moreno and González-Maeso, 2013) (see **section 1.1.6.2**). Thus, hallucinogenic drugs targeting the 5-HT_{2A}R have been repeatedly used to model schizophrenia-like behaviors in rodents (González-Maeso & Sealfon, 2009; Shah & González-Maeso, 2019).

1.1.5. ETIOLOGY

Schizophrenia is a complex, heterogeneous behavioral and cognitive syndrome that seems to originate from disruption of brain development caused by genetic, environmental factors, or combination of both. Hence, although its etiology still remains unknown, schizophrenia has been proposed to be a neurodevelopmental disorder, in which a series of genetic factors involved would be modulated by environmental factors —including infections during pregnancy, stress, nutritional deficiencies or drug use in adolescence, among others— leading to the disorder (Birnbaum & Weinberger, 2017; Owen et al., 2016).

1.1.5.1. THE GENETICS OF SCHIZOPHRENIA

It was back in the beginning of the 20th century, when studies of monozygotic and dizygotic twins and adopted-away offspring of mothers affected with schizophrenia, revealed that this disorder tends to run in families (Jauhar et al., 2022). Schizophrenia is reported as a highly heritable disorder, with heritability estimated at 60-80% (Owen et al., 2016), and monozygotic twin concordance at ~45% (Stilo & Murray, 2019). GWAS reveal hundreds or possibly thousands of genes associated with schizophrenia, stablishing that schizophrenia is a polygenic disorder (Trubetskoy et al., 2022), and involves both rare damaging variants (inherited *de novo*) that highly increase risk, as well as common variants, with small to moderate effects (Stilo & Murray, 2019). These common and rare genetic risk factors are thought to converge on the same underlying neuronal genes important to synaptic organization, differentiation and transmission relevant to schizophrenia pathogenesis (Singh et al., 2022; Trubetskoy et al., 2022).

GWAS have revealed a considerable number of genes as candidates underlying genetic risk of schizophrenia: neuregulin-1 (*NRG-1*), catechol-O-methyl-transferase (*COMT*), brain-delivered neurotrophic factor (*BDNF*), disrupted in schizophrenia-1 (*DISC-1*), NMDA receptor subunit (*NR1*), among others (Lee et al., 2012; Henriksen et al., 2017). Beyond the rare, highly penetrant mutations (for example, *DISC-1*), epistatic –epistasis describes how genes can affect a phenotype– interactions between more common, less penetrant variations may yield higher predictions of risk than our current list. Of course, the 50% concordance rate of homozygous twins reminds that genomics will not predict all forms of risk. Identifying environmental factors, detecting critical epigenetic modifications, or mapping neural circuit differences may provide more insight about the etiology of the disorder (Insel, 2010). Specially, it is thought that environmental factors may contribute to the disorder's etiology partly through epigenetic processes (see **section 1.3**).

1.1.5.2. NEURODEVELOPMENTAL HYPOTHESIS OF SCHIZOPHRENIA

Weinberger was the first author formulating the "Neurodevelopmental hypothesis of schizophrenia", claiming that early brain development disruption is able to increase the risk of later emergence of clinical symptoms of this disorder (Weinberger, 1986). Since then, accumulated evidence supports this hypothesis, which states that specific alterations in brain development induced by endogenous or exogenous factors might be key in schizophrenia onset (Lewis & Levitt, 2002; Rapoport et al., 2012; Schmidt & Mirnics, 2015). According to this theory, psychosis is not the start of the disorder, but rather a consequence of a developmental path

towards schizophrenia that could possibly be prevented by early intervention (Insel, 2010). A ‘‘double-hit’’ theory of schizophrenia (discussed later in **section 1.1.5.4.**) works within the framework of the neurodevelopmental hypothesis, and defends that certain insults occurring during two critical time points (early brain development and adolescence) have synergistic effects in order to drive the schizophrenia onset (Fatemi & Folsom, 2009).

As already mentioned, schizophrenia first emerges in the adolescence or early adulthood (**figure 1.2 a**), when the PFC is still developing. This late stage of brain maturation involves a careful calibration of excitatory–inhibitory balance in the cortex, with the PFC being the last region to mature (Insel, 2010). Thus, human brain development lasts two decades, from embryonic patterning *in utero* to synaptic pruning in adolescence, as showed in **figure 1.2 b**.

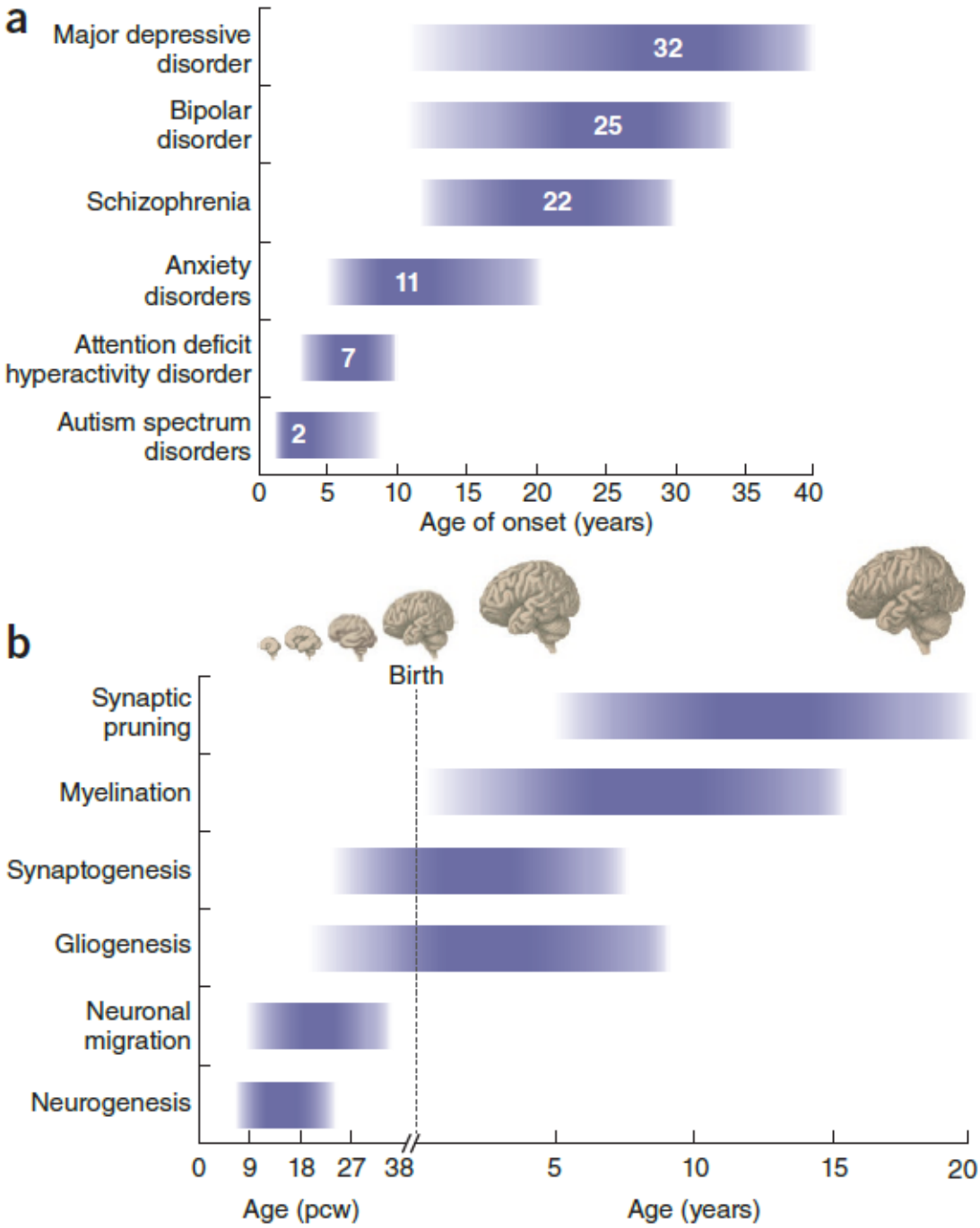


Figure 1.2: Age of diagnosis for several neuropsychiatric disorders in relation to key processes in human neurodevelopment. **Figure 1.2 a**: Schizophrenia is typically diagnosed in late adolescence or early adulthood. **Figure 1.2 b**: Timeline of human development during prenatal (in post-conception weeks, pcw) and postnatal (in years) life, in which the horizontal bars represent the approximate timing of key neurobiological processes and developmental milestones. Marin, 2016.

Normal cortical development, involves proliferation, migration, arborization (circuit formation) and myelination. While proliferation and migration occur mostly during prenatal life, arborization and myelination continue through the first two postnatal decades. Grey-matter volume changes during normal brain development are the result of combination of neuronal arbor pruning and myelin deposition. Underneath this observed general reduction, local changes are far more complex. Data from human and nonhuman primate brain indicate increases in inhibitory and decreases in excitatory synapses in the PFC during adolescence and early adulthood, hence, in the prodromal period and emergence of psychosis (Fair et al., 2008; Insel, 2010; Paus et al., 2008) (**figure 1.3 a**). However, in people developing schizophrenia, reduced elaboration of inhibitory pathways and excessive pruning of excitatory pathways leading to altered excitatory–inhibitory balance in the PFC is suggested to occur (Insel, 2010; Lewis & González-Burgos, 2008). Moreover, reduced myelination would alter connectivity. In this sense, it has been suggested that detection of prodromal neurodevelopmental changes could allow early intervention with potential prevention or preemption of psychosis (**figure 1.3 b**) (Insel, 2010; Lewis & González-Burgos, 2008).

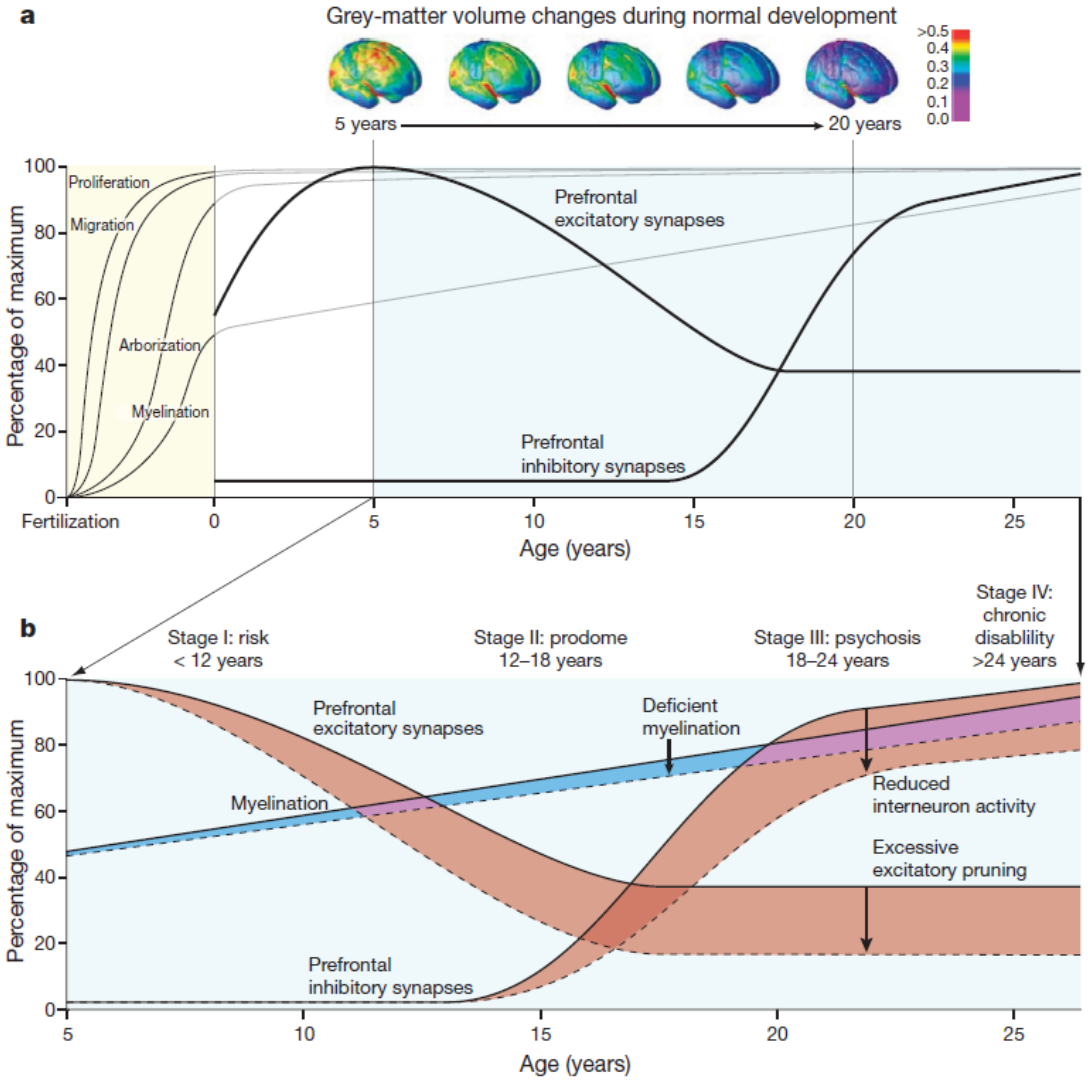


Figure 1.3: Neurodevelopmental model of schizophrenia. **Figure 1.3 a**: Normal cortical development occurs from prenatal stages until adolescence or early adulthood. During this period, grey-matter volume reductions occur. **Figure 1.3 b**: In subjects developing schizophrenia, altered excitatory–inhibitory balance in the PFC is proposed. Insel et al., 2010.

1.1.5.3. ENVIRONMENTAL RISK FACTORS

It has been well established that the interaction between an individual's genetic background and the environment plays an important role in the etiology of schizophrenia (Wahbeh & Avramopoulos, 2021). Thus, in addition to genetics, exposure to several environmental factors acting during different stages of the lifespan (prenatal life, perinatal life, adolescence, and adulthood) have been shown to contribute to the risk of developing schizophrenia (Stilo & Murray, 2019). Some of these environmental factors have cumulative/additive effects (Dean &

Murray, 2005; Stepniak et al., 2014) and each of these factors alone is usually not sufficient for schizophrenia onset (Stilo & Murray, 2010).

Multiple biological, psychological and social environmental risk factors have been associated with the risk of developing schizophrenia (Wahbeh & Avramopoulos, 2021). Particularly, risk factors during fetal and perinatal life have gained special attention. These include infections (e.g. herpes simplex, rubella, influenza or *Toxoplasma gondii*) (Børglum et al., 2014; Prasad et al., 2012), nutritional deficiencies (e.g., famine, folic acid, iron or vitamin D deficits) (Hoek et al., 1998; Susser et al., 1996), paternal age, fetal/neonatal hypoxia or other obstetric insults and complications, maternal stress and other exposures (e.g. Rh incompatibility) (Cannon et al., 2020; Dalman et al., 1999). Other putative neurodevelopmental determinants appearing later in life, during childhood, adolescence and/or early adulthood are also though to contribute in the onset of the disease. These environmental risk factors include cannabis and other drugs abuse, socioeconomic status, migration and urbanization status, childhood trauma, stressful events during adolescence, and infections during childhood and adolescence, among others (Brown, 2011; Wahbeh & Avramopoulos, 2021).

1.1.5.3.1. MATERNAL IMMUNE ACTIVATION

Epidemiologic studies have repeatedly implicated prenatal environmental factors, including maternal immune activation (MIA), in the etiology of neuropsychiatric illnesses such as schizophrenia (Brown et al., 2004; Brown & Derkits, 2010; Estes & McAllister, 2016).

Ecological studies were the first to link MIA via infection during pregnancy and increased schizophrenia risk in offspring (Choudhury & Lennox, 2021). Mednick and colleagues first stablished this link when they performed a study based in the 1957 influenza epidemic in Finland. They reported an increased rate of hospital diagnoses of schizophrenia among individuals exposed to a large type A2 influenza epidemic in their second trimester of fetal development (Mednick et al., 1988). Other authors reported that after the 1964 rubella pandemic, the incidence of schizophrenia rose from less than 1% in the unexposed population to about 20%, suggesting an association between clinically and serologically diagnosed prenatal viral infection and non affective psychosis later in adulthood (Brown et al., 2000). Subsequent studies associate historic outbreaks of flu, measles, mumps, chickenpox and polio, with schizophrenia (Reisinger et al., 2015). Other epidemiological reports have linked MIA and schizophrenia via other agents, such as bacteria causing pneumonia, sinusitis, tonsillitis, and parasites as *Toxoplasma gondii* (Knuesel et al., 2014; Patterson, 2009). However, there is some

controversy related to this topic, as not all the ecological studies have been able to replicate these associations (Brown et al., 2004; Selten et al., 2010). Consequently, MIA animal models have proved to be a necessary tool to stablish a causal relationship between maternal infection during pregnancy and schizophrenia-like abnormalities in the offspring (see **section 1.4.3.1**).

Potential mechanisms mediating the pathological effects of MIA on the developing organism *in utero* have been described, although the precise mechanisms remain to be determined (reviewed in Labouesse et al., 2015). In fact, different mechanisms induced by MIA may not be mutually exclusive, but rather interact with each other to affect various developmental processes that occur *in utero* (Labouesse et al., 2015). MIA induces a number of immune-related pathophysiological responses on the mother, such as production of soluble immune factors (including cytokines and reactive oxygen species) (Arsenault et al., 2014; Girard et al., 2010; Lanté et al., 2007; Lanté et al., 2008; Meyer et al., 2008; Smith et al., 2007), and soluble endocrine factors (including stress hormones) that affect the developing fetus (Seckl et al., 2007; Welberg & Seckl, 2008). These factors are thought to cross the placental barrier and induce fetal inflammation and oxidative stress, subsequently impairing the normal development of peripheral and central organs of the fetus, by modifying the differentiation, proliferation, and/or migration of target cells (Zaretsky et al., 2004). Ultimately, MIA could even induce inflammatory response in the placenta and cause placental insufficiency, which can lead to fetal hypoxemia (Labouesse et al., 2015). MIA can also cause states of nutritional deficiencies (macronutrient and micronutrient deficiencies), which are necessary for normal fetal development and growth (Daston et al., 1994; Kwik-Uribe et al., 2000; Lakshmy, 2013, Marques et al., 2015; Unger et al., 2007) (**figure 1.4**).

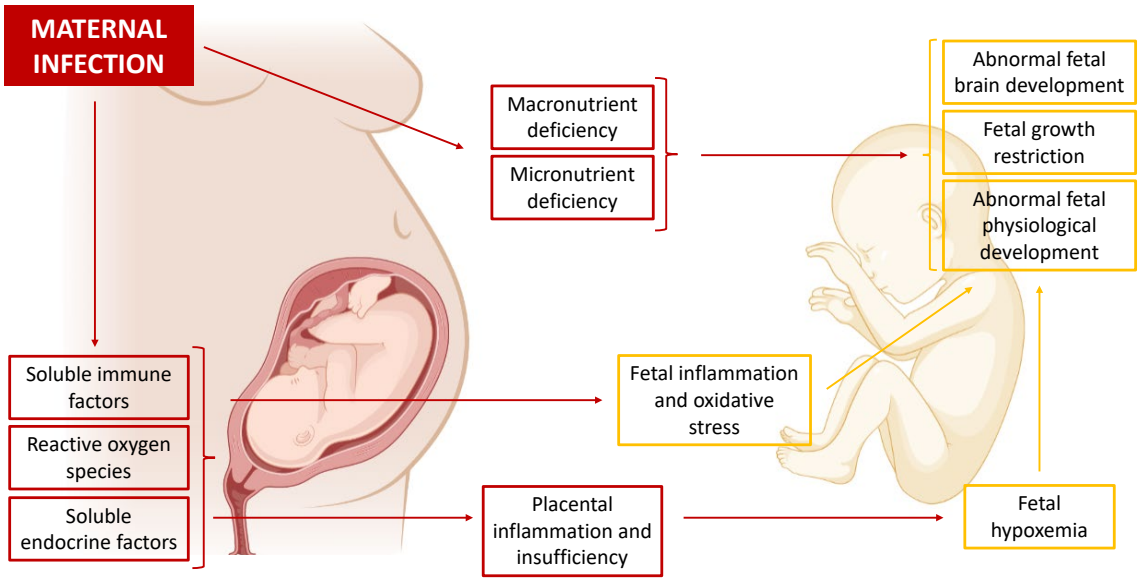


Figure 1.4: Potential mechanisms mediating the pathological effects of maternal infection on the developing organism *in utero*. Besides direct trans-placental actions by the pathogen (such as Cytomegalovirus or Zika virus, discussed below), cytokine-associated inflammatory events and downstream pathophysiological effects such as oxidative stress and (temporary) macronutrient and micronutrient deficiencies seem to be critical in mediating the adverse effects of maternal infection on the fetal system. These pathological processes can affect somatic cell development and change the offspring's neurodevelopmental trajectories, which in turn can lead to the emergence of behavioral and cognitive disturbances later in life. Originally created by Natalia Cordero Ruiz for this work, adapted from Labouesse et al., 2015.

Among pathogens that induce MIA, viruses have gained special attention. Viral infection during pregnancy can affect the brain development of the offspring by promoting the release of maternal pro-inflammatory cytokines, triggering neuroinflammation of the fetal brain, and/or directly infecting fetal neural cells. Specially, Zika virus (ZIKV), influenza A virus and cytomegalovirus (CMV), have strong tropism toward neural cells and have been linked to psychiatric disorders such as schizophrenia (reviewed in Elgueta et al., 2022). As discussed later (see section **1.4.3.1.1**), Poly (I:C) is a synthetic analog of double-stranded viral RNA, that is able to mimic these proinflammatory molecular responses and is used in animal models in order to produce MIA.

1.1.5.3.2. SOCIAL STRESS DURING ADOLESCENCE

In humans, adolescence is defined as the transition from childhood to adulthood. In this period, individuals gain sexual maturity, together with cognitive, emotional, and social skills needed to

establish independence from their parents (Spear, 2000). Adolescence is a period of increased exploration, including increased sensation seeking, risk taking, and drug use (Steinberg, 2004; Lipari & Jean-Francois, 2013). During adolescence, there is a qualitative change in the importance of social reward. In this period, adolescents become more sensitive to treatment by their contemporaries, and spent less time with their families (Ladd et al., 2014; Spear, 2000). In this sense, evidence suggests that social interactions during adolescence can influence the development and maintenance of maladaptive behaviors in adulthood (Patterson et al., 1992; Hankin et al., 1998).

Adolescence is associated with the emergence of psychiatric disorders such as schizophrenia, anxiety, depression and eating disorders (Kessler et al., 2005). It has been suggested that the increased sensitivity to stress during adolescence may contribute to the increased incidence of psychiatric illness at this stage in life (Grant, 1997; Grant & Dawson, 1998; Turner & Lloyd, 2004). In this regard, adolescence is proposed to be a key developmental window during stressful experiences may result in long-term –and even permanent– alterations in brain structure and function (Burke et al., 2017). Among environmental factors that appear in this period, social isolation and social defeat (social adversities, social trauma) have been linked with an increased risk of developing schizophrenia (Stowkowy & Addintong, 2012). Hence, research into the neurobiological basis of adolescence may provide a better understanding of social, emotional, reproductive, and cognitive development, as well as a better understanding of health risks and disorders that appear in this life stage (Walker et al., 2019). In this regard, animal models of stress in the peripubertal period could appear as valuable translational tools to provide insight in schizophrenia research (see **section 1.4.3.2.**).

1.1.5.4. THE DOUBLE-HIT HYPOTHESIS OF SCHIZOPHRENIA

As previously mentioned, schizophrenia is thought to have a multifactorial etiology that combines genetic susceptibility and prenatal and postnatal environmental risk factors (Modai & Shomron, 2016). However, one particular environmental factor alone seem to have a relatively weak impact on the individual and may not be sufficient to induce the development of schizophrenia (Guerrin, 2021; Jauhar et al., 2022). In this regard, the “double-hit hypothesis” of schizophrenia was first described as a combination of genetic susceptibility with a different developmental insult that could prime an individual for a later event ultimately leading to the onset of schizophrenia (Bayer et al., 1999; Maynard et al., 2001). The double-hit hypothesis of schizophrenia combines a “first hit”, defined as an early priming event that disrupts

neurodevelopment and establishes increased vulnerability, and a “second hit”, usually involving environmental insults occurring later in life. Nowadays, both genetic abnormalities and MIA are widely accepted as the “first hit” (Feigenson et al., 2014).

As previously mentioned, environmental exposures during critical developmental periods have been proven to play significant role in the emergence of the disorder (Brown, 2011). Therefore, prenatal infection, in combination with exposure to traumatizing experiences during peripuberty, has been associated with increased risk for neuropsychiatric disorders (Giovanoli et al., 2013). As already stated, MIA appears to be one of the most relevant environmental risk factors of neurodevelopmental brain dysfunctions in the offspring. Consequently, inflammation during pregnancy, induced by MIA, might act as a “priming event” to make the offspring more susceptible to the effect of stress later in life (Estes & McAllister, 2016).

Among “second hits” occurring later in life, during the adolescence or early adulthood, it is known that adolescence is a particularly critical stage of central nervous system (CNS) development, as previously stated. Men and women tend to first develop schizophrenia in the early and late 20s, respectively, suggesting that an event in adolescence or post-adolescence/early adulthood may be highly influential in the development of the disorder (Feigenson et al., 2014). Thus, exposure to traumatic or stressful stimuli during this period is considered an important etiological factor of vulnerability to suffering schizophrenia. In fact, the first psychotic episodes appear in this stage and debut in many cases after an environmental stimulus (Perälä et al., 2007; van Os & Kapur, 2009). In this period, brain remodeling is very active and is associated with specific morphological changes that may explain the brain's hypersensitivity to stressors. The fact that two etiopathogenic factors far apart in time, such as MIA and stress in adolescence, must come together to precipitate the disorder, could be the reason why it has been difficult to establish a causal relationship between prenatal factors as MIA and the onset of the disease. Once again, studies in double-hit animal models are a valuable tool to identify the importance of the first early priming stimulus and the second triggering stimulus on the onset of schizophrenia symptoms (**figure 1.5**).

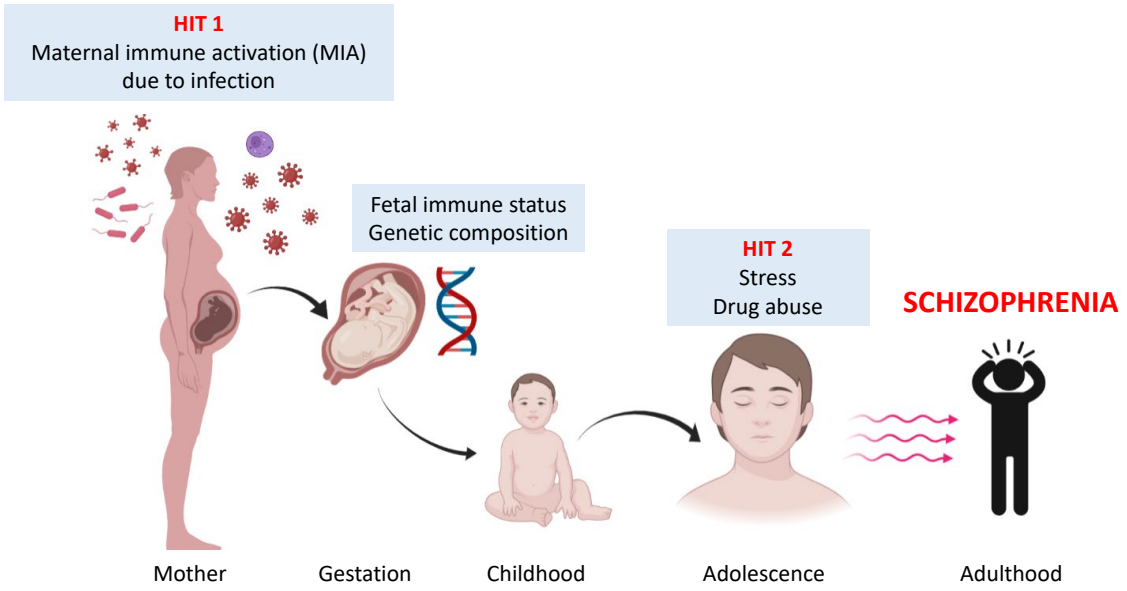


Figure 1.5: The double-hit hypothesis of schizophrenia, where MIA acts as a disease primer. Combination of genetic background and second hits during childhood and adolescence (including stress and drug abuse) associates with the consequences of MIA to increase the likelihood of offspring developing psychiatric disorders as adults. Originally created for this work by Natalia Cordero Ruiz adapted from Estes & MacAllister, 2016.

1.1.6. TREATMENT

Antipsychotic drugs are the first-line pharmacological approach for schizophrenia treatment. Nevertheless, these drugs have important therapeutic deficiencies. While antipsychotics are effective in controlling positive psychotic symptoms and in preventing relapses, their impact on the cognitive and negative deficits of schizophrenia is very limited (Conn et al., 2008; Haddad & Correll, 2018; Leucht et al., 2009). The response to antipsychotics is commonly incomplete, and between 20% and 30% of patients are resistant to treatment (Elkis, 2007). In addition, antipsychotics have major side effects, such as extrapyramidal symptoms of parkinsonism, acute dystonic reactions, akathisia and tardive dyskinesia, and other adverse effects such as sedation and weight gain (Owens, 2014).

Antipsychotic drugs can be divided in two main categories: first-generation or typical antipsychotics, and second generation or atypical antipsychotics.

1.1.6.1. TYPICAL ANTIPSYCHOTICS

Typical antipsychotics –chlorpromazine, haloperidol, fluphenazine, among others– base their therapeutic effect on the blockade of dopamine D_2R at the mesolimbic level, adequately reducing the positive symptoms of the disease in most cases (Kapur et al., 2000; Seeman, 1992; Marder et al., 1993). Despite providing an acceptable response in reducing positive symptoms, they hardly show clinical efficacy in reducing negative symptoms or cognitive impairment (Conley & Kelly, 2001; Legge et al., 2020). In addition, they have a wide range of adverse effects derived from the blockade of D_2R of the other dopaminergic pathways (fundamentally the nigrostriatal pathway), as well as from the blockade of other receptors (adrenergic, muscarinic and histaminergic receptors) (Miyamoto et al., 2008).

1.1.6.2. ATYPICAL ANTIPSYCHOTICS

Second generation or atypical antipsychotics are distinguished from the typical antipsychotics because they block 5-$HT_{2A}R$ as well as D_2 receptors, and have fewer motor side effects at standard doses (Richelson, 1996). Thus, atypical antipsychotics are characterized by an enhanced 5-$HT_{2A}R/D_2R$ affinity ratio (Meltzer et al., 1989). Moreover, atypical antipsychotics –including clozapine, risperidone, olanzapine, quetiapine, among others– present less study-defined relapse and less overall treatment failure and hospitalization than typical antipsychotics, having a modest but clinically relevant size effect (Kishimoto et al., 2013). The lower blockade of D_2R means that the risk of extrapyramidal symptoms is lower than when typical antipsychotics are used. However, this advantage is clouded by other side effects such as agranulocytosis (in the case of clozapine, which is the main limitation in its use) and metabolic syndrome (weight gain, hyperglycemia and dyslipidemia), among others. Moreover, as already mentioned, the efficacy on reducing negative and cognitive symptoms is very limited (Conn et al., 2008; Haddad & Correll, 2018; Leucht et al., 2009), and there is even the clinical suspicion that atypical antipsychotics could induce rather than alleviate schizophrenia cognitive deficits (Lett et al., 2014).

Clozapine was the first atypical antipsychotic drug to be identified and it is considered as the "gold standard" of second generation antipsychotics (Meltzer, 2012). Pharmacologically, clozapine demonstrated to have low affinity for D_2R and high affinity for 5-$HT_{2A}R$, which led to the hypothesis that its atypical nature was related to its high 5-HT receptor affinity (specifically 5-$HT_{2A}R$), relative to its low D_2R affinity (Meltzer, 1999). Nowadays, clozapine is known to have a complex pharmacological profile, with affinity for a large number of receptors, including

histaminergic, serotonergic, adrenergic, dopaminergic and cholinergic receptors (Coward, 1992; Nucifora et al., 2017). Clozapine blocks muscarinic M1, M2, M3 and M5 receptors, whereas it stimulates M4 receptor (Coward, 1992; Nucifora et al., 2017). Moreover, clozapine acts as a histaminergic antagonist, a mechanism related to its sedation side effects (Coward, 1992; Nucifora et al., 2017). Clozapine blocks adrenergic receptors, an action that is associated with hypotension and tachycardia side effects (Coward, 1992; Nucifora et al., 2017).

Clozapine is considered as the standard treatment for patients with refractory forms of schizophrenia. Unlike the typical antipsychotic drugs, clozapine does not produce parkinsonism, acute dystonic reactions, or tardive dyskinesia (Carbon et al., 2018). However, the main limitation of its use is the risk of neutropenia and agranulocytosis that occurs in a 3.8% of the cases. This side effect results severe in 0.9% of cases and fatal in 0.01% of cases, and mostly occurs in the first 3 months of treatment. Consequently, regular blood monitoring is necessary in patients under clozapine treatment (Myles et al., 2018).

Some authors (Samara et al., 2016) have questioned the superiority of clozapine compared with other second-generation antipsychotics in the management of refractory schizophrenia. In addition, it is no longer considered superior to other atypical antipsychotics –such as ziprasidone– in the treatment of cognitive impairment (Harvey et al., 2008).

Taking into account all the limitations of the currently available schizophrenia pharmacotherapy, there is an urgent need to develop new, more effective drugs, and to better understand the mechanism of action that gives rise to the different effects (beneficial and adverse) of the currently existing antipsychotics.

1.2. IMMUNE SYSTEM ALTERATIONS IN SCHIZOPHRENIA

A possible association between schizophrenia and the immune system was first postulated over a century ago (Menninger, 1919), and it has been later supported by epidemiological and genetic studies pointing to links with infection and inflammation (Brown & Derkits, 2010; Karlsson & Dalman, 2020; Khandaker et al., 2015).

1.2.1. PERIPHRERAL IMMUNE ALTERATIONS IN SCHIZOPHRENIA PATIENTS

A body of evidence links aberrant peripheral blood cytokine profile in patients with schizophrenia. Cytokines –including interleukins (ILs), chemokines, monokines and lymphokines– are small secreted proteins that are released by immune cells –mostly by macrophages and helper T cells– with proinflammatory or anti-inflammatory actions (Zhang & An, 2007).

Epidemiological prospective cohort studies link elevated inflammatory marker levels in childhood/adolescence with a higher risk of psychosis subsequently in adulthood (Perry et al., 2021). In this regard, a body of evidence reports an association between schizophrenia and disrupted peripheral cytokine milieu (Dickerson et al., 2013; Fernandes et al., 2016; Goldsmith et al. 2016; Khandaker et al., 2014; Miller et al., 2011; Potvin et al., 2008; Upthegrove et al. 2014). In a meta-analysis assessing 40 studies with patients with acute psychotic status, researchers reported that proinflammatory cytokines IL-1β, IL-6 and TGF-β (Transforming Growth Factor β) were raised in the acute phase of the illness and reduced with successful antipsychotic treatment (Miller et al., 2011). In addition, elevated levels of plasmatic IL-6 in children, measured years before the onset of psychosis, were associated with later schizophrenia onset in early-adulthood (Khandaker et al., 2014). In another meta-analysis including 40 studies, IL-6, sIL2r (soluble IL-2 receptor), IL-1RA (IL-1 receptor antagonist) and TNF-α (Tumor Necrosis Factor α) were all significantly raised in blood samples of patients with schizophrenia (Goldsmith et al. 2016). In a systematic quantitative review, analyzing data from 62 studies assessing blood samples of schizophrenia patients, increased levels of IL-1RA, sIL-2r and IL-6 were reported (Potvin et al., 2008). Increased serum levels of CRP (C-Reactive Protein) –an acute-phase protein which is elevated in response to proinflammatory cytokines, widely accepted as a marker of peripheral inflammation (Yap et al., 1991)– have also been reported in first-episode psychosis (FEP) and chronic schizophrenia, regardless antipsychotic medication (Dickerson et al., 2013; Fernandes et al. 2016).

Moreover, alterations of peripheral inflammatory signaling appear to be related with cognitive and negative symptomatology. In this sense, a body of evidence showed associations between different inflammatory markers and schizophrenia cognitive (Cabrera et al., 2016; Hope et al., 2015; Martínez-Cengotitabengoa et al., 2012; Martínez-Cengotitabengoa et al., 2014; North et al., 2021) and negative symptoms (Goldsmith et al., 2018; Goldsmith & Rapoport, 2020). In addition, it has been suggested that poor cognitive functioning in chronic schizophrenia patients could be associated with high CRP levels (Dickerson et al., 2007).

Interestingly, as the antipsychotic medication is known to influence the immune system by altering immune cell function (Drzyzga et al., 2006; Bian et al., 2008; Seki et al., 2013), several studies have focused on antipsychotic naïve patients for a better understanding of inflammatory cytokine alteration in schizophrenia. Thus, antipsychotic-naïve FEP patients are also associated with increased serum concentration of proinflammatory cytokines (Khandaker et al., 2015). A systematic review and meta-analysis including 14 different studies, reported increases of serum proinflammatory cytokines IL-1β, IL-6, sIL2r and TNFα of naïve FEP compared with controls, showing that the increase in these cytokines in FEP patients is unrelated to antipsychotic drugs (Upthegrove et al., 2014). In consequence, the increase in peripheral cytokines observed in patients with schizophrenia under antipsychotic medication could also be unrelated to the antipsychotic treatment itself.

Besides cytokines, other inflammatory markers and peripheral inflammatory signaling pathways have been shown altered in schizophrenia patients. Nuclear factor-kappa B (NF-κB) is a family of dimeric transcription factors best studied for its involvement in inflammation and immune responses and that has more recently been shown to regulate synaptic plasticity and brain function (Gutierrez & Davies, 2011). NF-κB is related to diseases in which inflammation plays an important role, and recently, dysregulation of the NF-κB pathway has also been linked to schizophrenia (Gandal et al., 2018; Murphy et al., 2020; Volk et al., 2019). In this regard, peripheral dysregulations of NF-κB pathway have been found on FEP drug-naïve schizophrenic subjects, that show higher levels of cytokines in serum and NF-κB activation (with increased *RELA* gene expression and RelA/p65 nuclear activity) in peripheral blood mononuclear cells (see below **section 1.2.2.1** for a detailed description of the NF-κB pathway) (Song et al., 2009).

Overall, a vast body of evidence shows a link between peripheral inflammation and schizophrenia, and some specific cytokines have been proposed as possible biomarkers. However, there is still none proinflammatory cytokine with enough evidence to be used in clinical practice as a biomarker in prodromal states of the disease, as a marker of disease

progression or response to treatment. Moreover, it remains unclear if patients with schizophrenia undergo a generalized inflammatory status that finally affects the CNS, or, in contrast, if the aberrant inflammatory activity starts in the CNS and later expands to the rest of the organism. Further research should be conducted to elucidate these unanswered questions.

1.2.2. NEUROINFLAMMATION IN SCHIZOPHRENIA PATIENTS

Contrary to the traditional perception of the brain as an immunologically privileged site protected by the blood–brain barrier (BBB), increasing evidence has reported complex interactions between the immune system, systemic inflammation and the brain, which could be related to changes in mood, cognition, and behavior (Khandaker et al., 2015). Thus, the study of human *postmortem* brain tissue has revealed evidence of neuroinflammation in several brain areas, including the PFC, the orbital frontal cortex and midbrain, in a high percentage of people with schizophrenia (Dean et al., 2013; Fillman et al., 2013; Fillman et al., 2014; Harris et al., 2012; Purves-Tyson et al., 2021; Volk et al., 2015; Volk et al., 2019; Zhang et al., 2016). In this regard, increased mRNA levels of several proinflammatory transcripts, such as IL-1β, IL-6 and IL-8 in *postmortem* brain samples of schizophrenia patients have been identified (Fillman et al., 2013; Fillman et al., 2014; Purves-Tyson et al., 2021; Zhang et al., 2016). Precisely, neuroinflammation in the PFC –given the structural and functional abnormalities associated to this brain area in schizophrenia– is thought to drive the neuropathology, and potentially symptoms, in some schizophrenia patients (Murphy et al., 2021). In this regard, there is evidence of alterations in the mRNA expression of the initial elements of the TLR4 signaling pathway –TLR4, Myeloid differentiation primary response gene 88 (MyD88) and NF-κB– in the PFC of schizophrenia patients (García-Bueno et al., 2016). Further, recent findings also suggest that some schizophrenia patients had altered BBB function, which could facilitate the trafficking of macrophages from blood to brain (Cai et al., 2020; Purves-Tyson et al., 2020). This was evidenced by increased endothelial expression of adhesion molecules that capture white blood cells and elevated transcript levels of macrophage marker CD163 mRNA in schizophrenia *postmortem* brain samples (Cai et al., 2020; Purves-Tyson et al., 2020).

Overall, all these findings suggest that aberrant neuroinflammatory processes could play a critical role in causing neuropathology in some schizophrenia patients. In this regard, excessive microglia activation and altered NF-κB expression have received especial attention in the pathophysiology of schizophrenia, as discussed below in **section 1.2.2.1.** and **section 1.2.2.2**.

1.2.2.1. NEUROINFLAMMATION AND MICROGLIA IN SCHIZOPHRENIA

Among the numerous cells and mediators possibly involved in the inflammatory processes of the CNS, microglia play a particularly relevant role, since they represent the main cells of innate immunity at the CNS level (Laskaris et al., 2016; Müller, 2018). Microglia are the first line of defense against CNS invading pathogens, and react to CNS injury or microenvironment changes, thus, playing a key role in central infections and inflammation (Gomez-Nicola & Perry, 2015; Hanisch & Kettenmann, 2007; Kettenmann et al. 2011). Microglia also contribute to the modulation and synchronization of neuronal activity, to information processing, and to structural and functional plasticity of neural networks (Dietz et al., 2020). Moreover, microglia are involved in synaptic pruning and neuronal remodeling (Howes & McCutcheon, 2017).

When a systemic infection occurs, there is a microglial activation of quiescent or resting microglia and a transition of this towards a proinflammatory M1 phenotype, with the consequent synthesis and release of various cytokines and proinflammatory mediators. Under normal physiological conditions, part of the microglia also changes towards an M2 state, which is more anti-inflammatory, so that a certain balance would be maintained (Howes & McCutcheon, 2017; Miller & Goldsmith, 2017) (see **figure 1.6**).

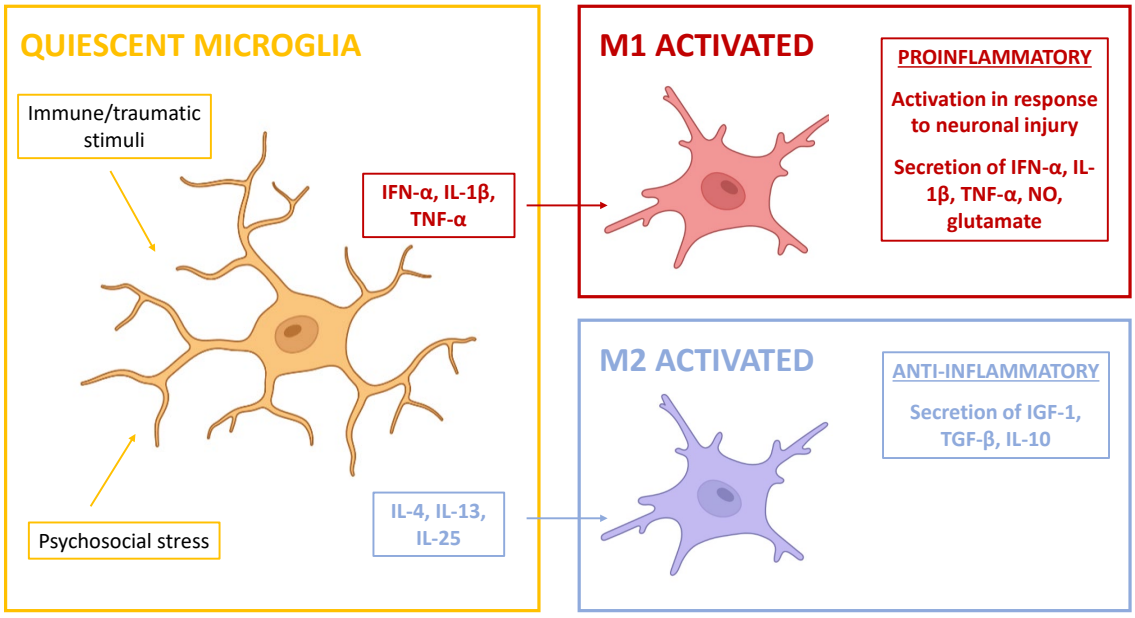


Figure 1.6: Quiescent microglia activation and subsequent effects. Illustration originally created by Natalia Cordero Ruiz for this work, adapted from Howes & McCutcheon, 2017. Abbreviations: IFN-α= Interferon α. IGF-1= Insulin-like Growth Factor 1. IL-1β= Interleukin 1β. IL-4: Interleukin

4. IL-13= Interleukin 13. IL-25= Interleukin 24. NO= Nitric Oxide. TGF-β= Tumor Growing Factor β. TNF-α= Tumor Necrosis Factor α.

However, this balance could be broken in the presence of a genetic susceptibility or immune activation in favor of the expression of proinflammatory genes (Howes & McCutcheon, 2017; Miller & Goldsmith, 2017). Hence, M1 is the proinflammatory stage of microglia, triggered by proinflammatory cytokines such as IFN-γ, IL-1β or TNF-α, whereas M2 is the anti-inflammatory microglia stage, triggered by cytokines such as IL-4, IL-3 or IL-25 (Cherry et al., 2014). The proinflammatory M1 pathway is activated after neuronal injury and leads to the liberation of proinflammatory molecules (such as, NO, IL-1β, TNF-α, IL-6 and glutamate). In homeostatic conditions, M1 is followed by the anti-inflammatory M2 state, and both M1 and M2 pathways are required for an appropriate immune response in the CNS (Cherry et al., 2014). Microglia seem to have an "immunological memory". This implies that an early activation (for example, in the perinatal period), would increase the sensitivity of these cells to low-intensity stimuli in the future, such as low-grade systemic inflammation or chronic stress, then giving rise to a disproportionate inflammatory response mediated by proinflammatory molecules such as IL-6, IL-1β, or TNF-α (Howes & McCutcheon, 2017; Müller, 2018). The increase in microglia activity is also associated with an increase in oxidative stress, through the release of reactive oxygen species and nitrogenous species (Müller, 2018). In addition, there is a feedback between oxidative stress and inflammation, since free radicals also have an inducing effect on the inflammatory response (Steullet et al., 2016). Together, inflammation and oxidative stress, maintained for a long time, could be related to various neurotoxic effects that could even lead to neuronal death (Müller, 2018; Rao et al., 2012; Steullet et al., 2016).

As mentioned above, it is believed that microglia could play an important role in the process of synaptic pruning, which is particularly important in adolescence. This process is considered critical for proper neurodevelopment and maturation of the CNS, and alterations in it are thought to be related to the etiopathogenesis of schizophrenia (Owen, 2016). Some authors suggest that an exaggerated activation of microglia in adolescence or in young adults could lead to excessive synaptic pruning and neuronal death; which would explain the volume losses of the cerebral cortex observed in schizophrenic patients and the consequent appearance of the negative symptoms and cognitive deterioration typical of the disease (Howes & McCutcheon, 2017). The lower figure summarizes the involvement of microglia in the development of the schizophrenia, as described (see **figure 1.7**).

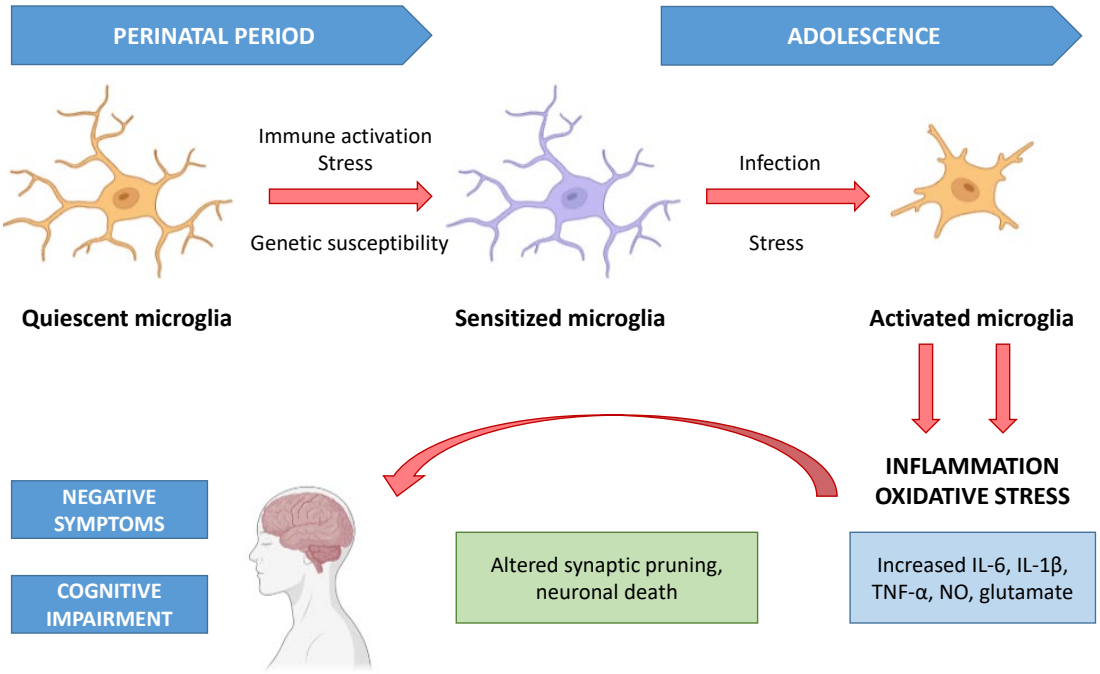


Figure 1.7: Possible mechanisms involving the role of microglia in the pathophysiology of schizophrenia. Illustration originally created by Natalia Cordero Ruiz, adapted from Howes & McCutcheon, 2017. Abbreviations: IL-1β= Interleukin 1β. IL-6: Interleukin 6. NO= Nitric Oxide. TNF-α= Tumor Necrosis Factor α.

1.2.2.2. THE ROLE OF NF-κB IN SCHIZOPHRENIA

The NF-κB family of transcription factors is ubiquitously expressed and it has been mostly studied in the mammalian immune system, where it plays an important role in regulating the expression of genes involved in innate and adaptive immune responses, inflammation, cell survival and proliferation (Vallabhapurapu & Karin, 2009).

As previously pointed, NF-κB pathway has been implicated in schizophrenia (Gandal et al., 2018; Murphy et al., 2020; Volk et al., 2019), making NF-κB an attractive candidate for investigations into the cause of neuroimmune alterations in schizophrenia. NF-κB can exist as homodimers or heterodimers, which remain inactivated in the cytoplasm until they are activated by proinflammatory stimuli and translocated to the cellular nucleus (Murphy et al., 2021). The NF-κB family of inducible transcription factors includes NF-κB1 (p50), NF-κB2 (p52), RelA (also called p65), RelB and c-Rel. NF-κB proteins normally exist as components of inactive cytoplasmic complexes sequestered by members of the inhibitor of κB (IκB) family, which includes the prototypical member IκBα and several structurally related proteins (Sun & Ley, 2008). NF-κB1 (p50) and NF-κB2 (p52) are produced as precursor proteins, p105 and p100, respectively, both

of which contain a carboxy-terminal IκB-homologous region and function as IκB-like NF-κB inhibitors. Proteasome-mediated selective degradation of the C-terminal region of p105 and p100 (termed p100 processing) yields to mature NF-κB1 (p50) and NF-κB2 (p52) (Sun & Ley, 2008). NF-κB dimers need to contain at least one of the Rel subunits to be transcriptionally active, since Rel subunits contain a transactivation domain that allow the NF-κB dimer to initiate transcription on upstream DNA regions of target genes (Hayden & Ghosh, 2008) (**figure 1.8**).

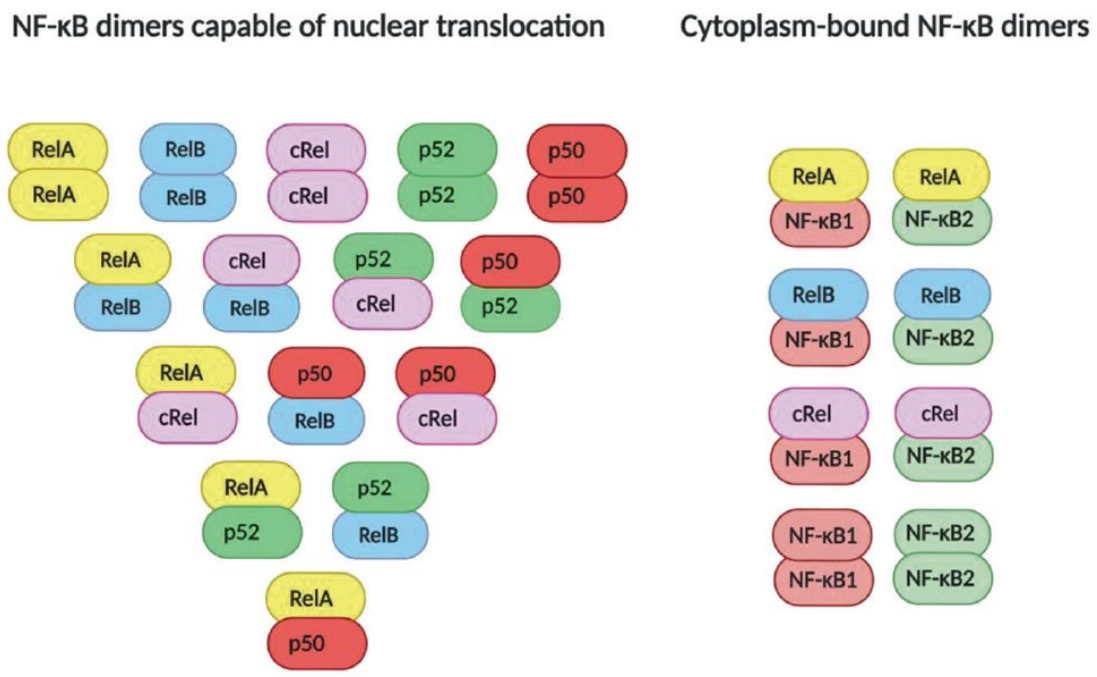


Figure 1.8: NF-κB family. NF-κB homo- or hetero-dimers are composed of any two NF-κB subunits. Murphy et al., 2021. Abbreviations: NF-κB= Nuclear Factor-κB.

Translocation of NF-κB dimers into the nucleus depends entirely on the status of upstream signals, as they cannot move from the cytoplasm without degradation of their bound inhibitors. Upstream signaling cascades trigger breakdown of NF-κB inhibitors. Hence, without changes in NF-κB pathway regulators to facilitate dimer translocation, a higher number of NF-κB dimers stay in the cytoplasm, which may not necessarily mean higher NF-κB mediated gene transcription (Murphy et al., 2021).

NF-κB activation occurs via two major signaling pathways: the canonical and the non-canonical NF-κB signaling pathways. The canonical pathway mediates the activation of NF-κB1 (p50), RelA and c-Rel (also known as canonical NF-κB family members), whereas the non-canonical NF-κB pathway selectively activates p100-sequestered NF-κB members, predominantly NF-κB2 (p52)

and RelB (or non-canonical NF-κB family members) (Sun, 2017). Dimers containing RelA and cRel, most commonly bound to processed NF-κB1 (p50), are induced by the canonical pathway of NF-κB activation, while the dimer formed by RelB and processed NF-κB2 (p52) is induced by the non-canonical pathway of NF-κB activation (Murphy et al., 2021). On the one hand, various immune receptors (pattern recognition receptors PRRs, cytokine receptors, antigen receptors, etc.) trigger the canonical NF-κB pathway, by activating the kinase TGFβ-activated kinase 1 (TAK1). TAK1 triggers an IκB kinase (IKK) complex, formed by catalytic (IKKα and IKKβ) and regulatory (IKKγ) subunits. Upon stimulation, the IKK complex, mainly via IKKβ, phosphorylates members of the inhibitor of κB (IκB) family, such as IκBα and the IκB-like molecule p105, which maintain inactivated NF-κB members in the cytoplasm. IκBα, the principal NF-κB inhibitor, associates with both NF-κB1 (p50) and Rel (RelA or c-Rel), while p105 only associates with NF-κB1 (p50) or Rel (RelA or c-Rel). Upon phosphorylation by IKK, IκBα and p105 are targeted for ubiquitin (Ub)-dependent degradation in the proteasome, ultimately leading to the nuclear translocation of NF-κB, which bind to specific DNA elements (κB enhancers of target genes) (Hayden & Ghosh, 2008; Sun, 2012; Sun, 2017). On the other hand, non-canonical NF-κB pathway is triggered by members of the tumor necrosis factor receptor (TNFR) superfamily, which activate the NF-κB-inducing kinase (NIK). NIK phosphorylates IKKα, which then phosphorylates p100, which leads to the generation of NF-κB2 (p52) and the nuclear translocation of NF-κB2 (p52) and RelB (Hayden & Ghosh, 2008; Sun, 2012; Sun, 2017) (**figure 1.9**).

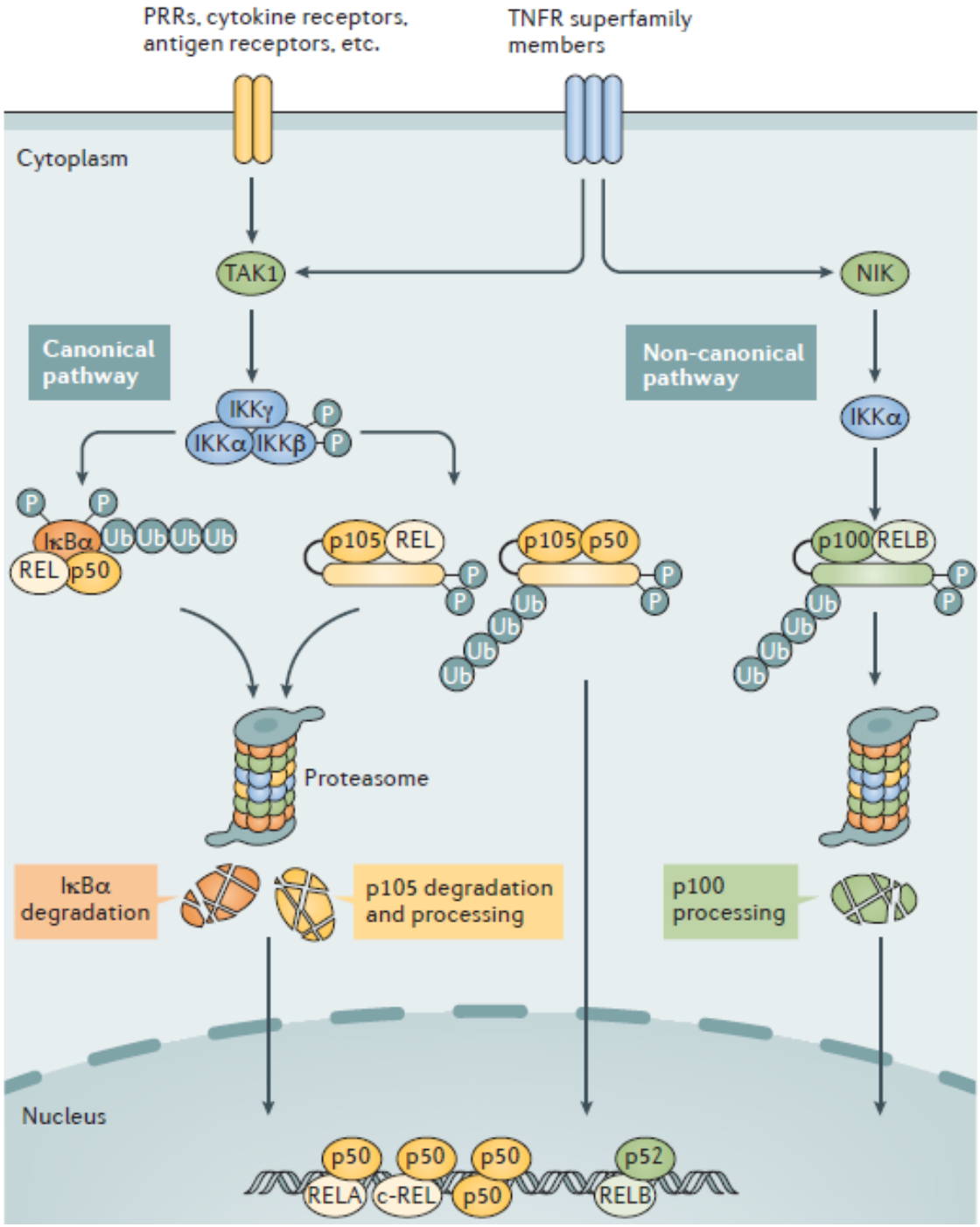


Figure 1.9: NF-κB activation major signaling pathways: the canonical and the non-canonical NF-κB signaling pathways. Sun, 2017. Abbreviations: IκB= Inhibitor of κB. IKK= IκB Kinase. NF-κB= Nuclear Factor-κB. NIK= NF-κB Inducing Kinase. P= Phosphorylation. PRRs= Pattern Recognition Receptors. TAK1= TGFβ-activated kinase 1. TNRF= Tumor Necrosis Factor Receptor. Ub= Ubiquitin.

The proinflammatory pathway triggered by NF-κB is crucial in the counterbalancing mechanisms between proinflammatory and anti-inflammatory pathways (García-Bueno et al., 2014). The NF-κB family has a role in regulating the expression of genes involved in innate and adaptive immune responses, inflammation, cell survival, cell proliferation and synaptic plasticity (Gutierrez & Davies, 2011; Vallabhapurapu & Karin, 2009). As mentioned above, dysregulation of the NF-κB pathway has also been linked to schizophrenia (Gandal et al., 2018; Murphy et al., 2020; Volk et al., 2018). Roussos and collaborators were the first to examine NF-κB alterations in schizophrenia *postmortem* brain samples, finding that the entire NF-κB system was downregulated in several brain regions of schizophrenia subjects, mostly in the temporal cortex (Roussos et al., 2013). However, more recently, overactivity of NF-κB in the PFC specifically has been linked to schizophrenia (Gandal et al., 2018; Volk et al., 2019). In this sense, Gandal et al. (2018) reported an increased gene expression of the NF-κB module pair (gene M5/isoM5), which includes four out of five NF-κB family members [NF-κB1 (p50), NF-κB2 (p52), Rel, RelA] (Gandal et al., 2018). Volk and collaborators reported increased messenger RNA levels for most NF-κB family members (higher mRNA levels of RelA and cRel) and the main NF-κB inhibitor (IκBα) (Volk et al., 2019). In accordance with an overactivity of this pathway in schizophrenia, lower mRNA levels for Schnurri-2, an NF-kB site-binding protein that inhibits NF-kB function, have been reported (Takao et al., 2013). Further, these later findings align with overexpression of immune biomarkers that are under the control of NF-κB—such as IL-6, IL-1β, IL-8 or TNFα—in the brain and blood of schizophrenia patients (for review see Murphy et al, 2021). Conversely, decreased canonical NF-κB transcripts [TLR4, IKKβ, IκBα, IκBβ, IκBε, RelA, c-Rel, and NF-κB1 (p50)] have been reported, in the PFC of subjects with schizophrenia with a neuroinflammation status, characterized by a high brain cytokine profile (Murphy et al., 2020). Finally, genetic association for the *RELA* gene (human gene encoding for the RelA/p65 protein) and *in silico* analysis suggested that the schizophrenia risk genetic variants were associated with downregulation of *RELA* (Hashimoto et al, 2011). In addition, it has been suggested that determination of IκBα, the main inhibitory protein of NF-κB, could be useful in early phases of schizophrenia to assess clinical severity (Cabrera et al., 2016). Moreover, selective inhibition of NF-κB in astrocytes – as these brain cell types appear to have the highest NF-κB expression – has been proposed as an option for treating neuroinflammation in the brain in people with schizophrenia, and has proven to have a neuroprotective effect in many mouse models of neuroinflammation (Murphy et al., 2021).

1.2.3. ANTI-INFLAMMATORY AGENTS IN SCHIZOPHRENIA

Current antipsychotic medication has important therapeutic gaps, as its impact on the cognitive and negative domains of schizophrenia is very limited. There is even the clinical suspicion that atypical antipsychotics could worsen, rather than alleviate, schizophrenia cognitive deficits (Lett et al., 2014), usually contributing to poor functional outcome. Hence, there is an urgent need to identify new molecular targets and to develop novel compounds for more effective and better-tolerated schizophrenia pharmacotherapy that could improve therapeutic effects and safety profile. As previously remarked, inflammatory processes seem to play a pivotal role in the pathogenesis of schizophrenia, and could be related to cognitive and negative deficits of the disease. Based on the inflammatory hypothesis of schizophrenia, the use of drugs with anti-inflammatory properties could be beneficial in the pharmacological approach of the disease (Müller, 2018).

To date, several meta-analyses have investigated treatment effects of drugs with either primary or pleiotropic anti-inflammatory properties as add-on tratments to antipsychotics (Begemann et al., 2012; de Boer et al., 2018; Heringa et al., 2015; Nitta et al., 2013; Nomura et al., 2018; Oya et al., 2014; Shen et al., 2018; Solmi et al., 2017; Sommer et al., 2012; Sommer et al., 2014; Xiang et al., 2017; Zheng et al., 2017; Zheng et al., 2018; Zhu et al., 2018). Various types of drugs, including nonsteroidal anti-inflammatory drugs (NSAIDs) (Nitta et al., 2013; Sommer et al., 2012; Sommer et al., 2014; Zheng et al., 2017), statins (Nomura et al., 2018; Shen et al., 2018), minocycline (Oya et al., 2014; Solmi et al., 2017; Sommer et al., 2014; Xiang et al., 2017), N-acetyl cysteine (NAC) (Sommer et al., 2014; Zheng et al., 2018), estrogens (Begemann et al., 2012; de Boer et al., 2018; Heringa et al., 2015; Sommer et al., 2014; Zhu et al., 2018) and poly-unsaturated fatty acids (PUFAs) (Sommer et al., 2014) have been investigated. A recent meta-analysis including 70 double-blinded, randomized, placebo-controlled clinical trials with more than 4000 subjects (Jeppesen et al., 2020), showed that anti-inflammatory add-on treatment to antipsychotics –including primarily anti-inflammatory compounds such as NSAIDs, minocycline and monoclonal antibodies; or drugs with potential anti-inflammatory properties, such as neurosteroids, NAC, estrogens, PUFAs, statins, and glitazones– showed improvement of psychotic disorders. In this study, drugs with potential anti-inflammatory effect displayed similar effects to primarily anti-inflammatory drugs.

Among all these molecules with anti-inflammatory actions proposed as add-on treatment in schizophrenia, minocycline has gained increased attention, as it has been shown that adjunctive minocycline appears to be efficacious and safe for schizophrenia (Zheng et al., 2019).

Minocycline is a semi-synthetic, second-generation tetracycline, with anti-inflammatory, anti-apoptotic and anti-oxidant properties (Hashimoto & Ishima, 2010; Soczynska et al., 2012), that is able to cross the BBB and has a role in brain protection by inhibiting activated microglia (Levkovitz et al., 2010; Liu et al., 2014). Various clinical trials have studied the use of minocycline as an adjuvant treatment to antipsychotic medication revealing significant improvements in positive (Chaudhry et al., 2012; Khodaie-Ardakani et al., 2014; Liu et al., 2014), negative (Chaudhry et al., 2012; Khodaie-Ardakani et al., 2014; Kelly et al., 2015; Levkovitz et al., 2010; Liu et al., 2014; Zang et al., 2018), and cognitive domains (Levkovitz et al., 2010; Zang et al., 2018) of the disease. Moreover, neuroprotective effects of minocycline have also been reported (Chaves et al., 2015). However, more research would be needed to approve the clinical use of minocycline as an adjuvant medication of antipsychotcs in schizophrenia. The molecular mechanism underlying the beneficial effects of minocycline in schizophrenia symptoms are not fully understood. Further, the targets the molecular and behavioral effects of minocycline should be elucidated. Overall, minocycline holds great promise as a coadjuvant medication for schizophrenia treatment, and the understanding of its molecular mechanisms might contribute to the discovery of new targets that enable the development of new schizophrenia pharmacotherapy.

1.3. EPIGENETICS AND SCHIZOPHRENIA

Epigenetic regulations are defined as the combination of mechanisms causing short- and long-term changes in gene expression without altering the DNA code itself (Tsankova et al., 2007). Growing evidence suggests that epigenetic modifications represent a key mechanism through which environmental factors interact with individual's genetic constitution to affect risk of schizophrenia throughout life (Cariaga-Martinez et al., 2016; Gavin & Akbarian, 2012; Ibi & González-Maeso, 2015; Richetto & Meyer, 2021). As already mentioned, combination of genetic and environmental risk factors is believed to alter normal brain development and maturation, manifesting in a cascade of neurotransmitter and circuit dysfunctions and impaired connectivity in early adulthood, which ultimate leads to schizophrenia onset. While common and rare genetic abnormalities contribute to considerable heritability of schizophrenia, environmental factors are also thought to participate in the etiology of the disorder via epigenetic mechanisms.

1.3.1. BASIC CONCEPTS IN EPIGENETICS

DNA is packed in chromosomes, in a structure called chromatin, which is the complex of DNA, histone and non-histone proteins in the cellular nucleus. The main structural unit of chromatin is the nucleosome, which comprises 147 base pairs of DNA wrapped around a histone octamer that is composed of four pairs of basic histone proteins (H2A, H2B, H3 and H4) (Tsankova et al., 2007) (see **figure 1.10**).

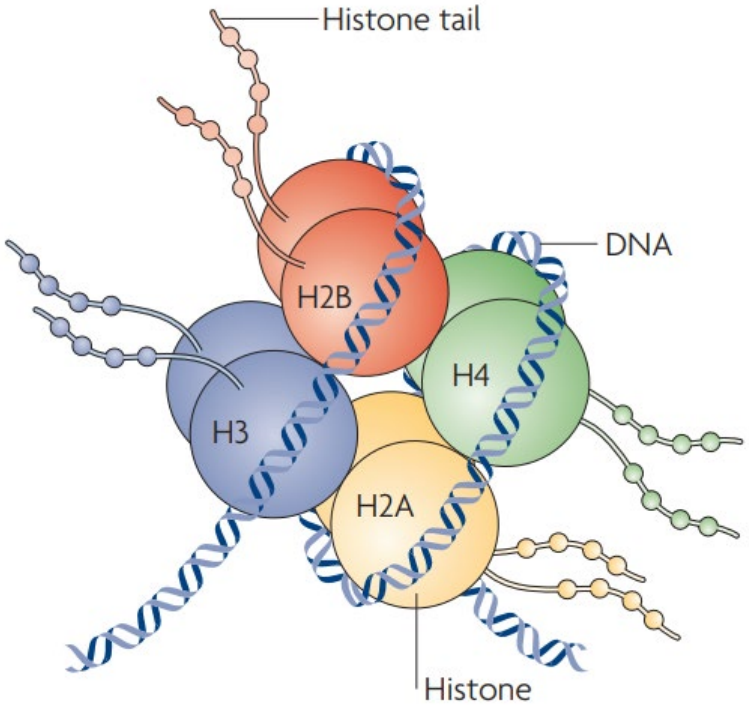


Figure 1.10: Picture of a nucleosome showing a DNA strand wrapped around a histone octamer composed of two copies each of the histones H2A, H2B, H3 and H4. The N-terminal tails of the histones face outward from the nucleosome complex. Tsankova et al., 2007.

Chromatin structure and organization depends on covalent modifications known as epigenetic modifications, including: DNA methylations, expression of noncoding RNAs and histone modifications (Ibi & González-Maeso, 2015; Richetto & Meyer, 2021) (see **figure 1.11**):

DNA methylations

DNA methylation denotes the covalent modification of the DNA at position 50 in the cytosine ring (5mC), which primarily occurs at CpG dinucleotides –DNA regions were a cytosine nucleotide is followed by a guanine nucleotide–. In vertebrates, methylation of CpG dinucleotides within proximal gene promoters is frequently linked to transcriptional repression. 5mC is established and maintained by methyltransferases (DNMTs) and it is oxidized by the ten-eleven translocation (TET) family of dioxygenase proteins to 5-hydroxymethylcytosine (5hmC). TET enzymes further hydroxylate 5hmC to generate 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). 5fC and 5caC are recognized and removed by another enzymes, thymine-DNA glycosylases (TDG), and the created basic site is repaired by the basic excision

repair (BER) pathway, generating an unmodified cytosine. (Ibi & González-Maeso, 2015; Richetto & Meyer, 2021).

Noncoding RNAs

Noncoding RNAs provide an additional level of epigenetic regulation involved in chromatin and nuclear remodeling, gene transcription, translational repression, and degradation of messenger RNAs. Noncoding RNAs can be divided according to their size into long noncoding RNAs (lncRNAs, with >200 nucleotides) and short noncoding RNAs (sncRNAs, with <200 nucleotides). sncRNAs contain small inhibiting RNAs, microRNAs, PIWI(P-element Induced Wimpy)-interacting RNAs, and small nuclear RNAs (Ibi & González-Maeso, 2015; Richetto & Meyer, 2021).

Histone modifications

Histone modifications occur on the N-terminal tails of these proteins. A number of histone modifications exist, including methylation, acetylation, phosphorylation, ubiquitination, and SUMOylation (proteins similar to ubiquitin and considered members of the ubiquitin-like protein family). Histone modifications determine the extent to which chromatin is wrapped around histone proteins. In this sense, loosely coiled chromatin contains transcriptionally accessible DNA regions, whereas tightly coiled chromatin comprises transcriptionally inactive DNA regions. Some histone modifications, such as methylation, are associated with gene activation and repression depending upon the specific position of the histone tail residue. In contrast, other histone modifications, such as acetylation, are commonly associated with transcriptional activation. Hence, histone acetylation is associated with a more flexible and “open” chromatin state that facilitates gene expression and is regulated by the opposite effects of two modifying enzymes: histone acetylases (HAT) and histone deacetylases (HDACs). There are at least 18 classes of HDACs encoded in the human genome, which are commonly divided into four classes. Class I includes HDAC 1, 2, 3 and 8; class II/IIa include HDAC 4, 5, 6, 7, 9 and 10, and HDAC 11 is the only member of class IV. Class I HDACs are mostly localized in the nucleus, whereas class II/IIa are regulated by travelling between nucleus and cytoplasm. HDACs remove acetyl groups from lysine residues in histones, which causes a greater interaction with the DNA, leading to a higher chromatin condensation and decrease in the gene expression (Akbarian, 2014; Ibi & González-Maeso, 2015; Richetto & Meyer, 2021).

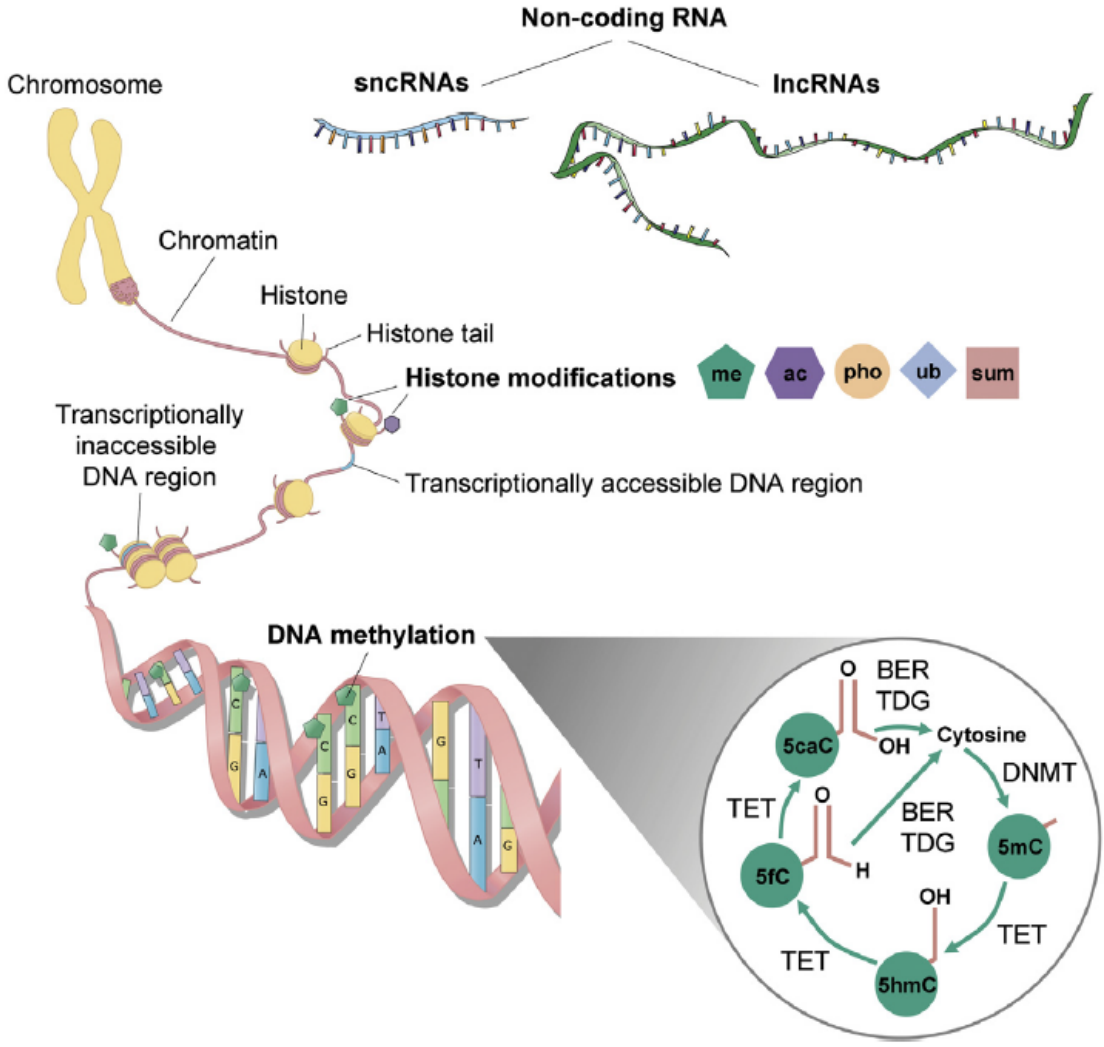


Figure 1.11: Schematic representation of chromatin structure and major epigenetic processes, including: histone modifications, DNA methylation and noncoding RNAs. Richetto & Meyer, 2021. Abbreviations: ac= acetylation. BER= Base Excision Repair. DNMT= DNA Methyltransferase. lncRNAs= long noncoding RNAs. me= methylation. pho= phosphorylation. sncRNAs= short noncoding RNAs. sum= SUMOylation. TDG= Thymine-DNA Glycosylase. TET= ten-eleven translocation. ub= ubiquitination.

1.3.2. EPIGENETIC MODIFICATIONS IN SCHIZOPHRENIA

Epigenetic modifications are thought to play a role in the etiology and pathophysiology of schizophrenia (Peedicayil & Grayson, 2018; Tsankova et al., 2007). Whereas certain epigenetic modifications associated with schizophrenia may have a genetic origin, it is also suggested that epigenetic alterations could be acquired through environmental factors (Richetto & Meyer,

2021). To date, studies have examined the possible involvement of DNA methylations, histone modifications and non-coding RNAs in the etiology and pathophysiology of schizophrenia.

1.3.2.1. DNA METHYLATIONS AND SCHIZOPHRENIA

DNA methylations were among the first epigenetic modifications to be linked with schizophrenia (Richetto & Meyer, 2021). DNA methylations in several schizophrenia candidate risk genes have been studied, including the extracellular matrix protein reelin (*RELN*) (Guidotti et al., 2016), genes involved in GABAergic functions (*GAD1*) (Huang & Akbarian, 2007), dopaminergic functions (*DRD2, DRD3, COMT, IGF2*) (Abdolmaleky et al., 2006; Dempster et al., 2006; Pao et al., 2019; Zhang et al., 2007) or serotonergic functions (*HTR2A*) (Abdolmaleky et al., 2011). More recent approaches have assessed genome-wide DNA methylation profiles in brain tissue of schizophrenia patients, identifying 2104 differentially methylated CpG sites in the PFC of subjects with schizophrenia relative to control subjects (Jaffe et al., 2016). Furthermore, the assay for transposase-accessible chromatin using sequencing (ATAC-seq) is a powerful addition to the genome wide DNA methylation profiling approaches, allowing researchers to assess genome-wide chromatin accessibility and overall changes in epigenetic landscapes and corresponding gene signatures (Buenrostro et al., 2013). ATAC-seq has recently been implemented in schizophrenia research and has already provided preliminary evidence for altered chromatin accessibility in schizophrenia cases compared with control cases (Bryois et al., 2018; Hoffman et al., 2019). Further use of ATAC-seq is expected to provide new knowledge regarding molecular mechanisms of altered epigenetics in schizophrenia.

1.3.2.2. NON-CODING RNAs AND SCHIZOPHRENIA

The majority of studies researching on the putative involvement of non-coding RNAs (ncRNAs) in schizophrenia have focused on microRNAs (miRNAs) (Liu et al., 2018; Ragan et al., 2017; Zhao et al., 2015). This focus on miRNAs arose due to a genetic variation that confers a high risk of schizophrenia, the microdeletion at chromosome 22q11.2, which includes a gene (*DGCR8*) that encodes for a miRNA processing protein (Van et al., 2017). Moreover, a substantial number of studies have reported altered cortical and subcortical expression of various miRNAs in *postmortem* brain samples of patients with schizophrenia, including mir130 (Burmistrova et al., 2007), mir181b (Beveridge et al., 2008), mir497 (Banigan et al., 2013), mir185 (Forstner et al., 2013), mir9 (Topol et al., 2017), mir195, mir301a (Alacam et al., 2016), mir132, mir1307 (Liu et al., 2018) and mir137 (Sakamoto & Crowley, 2018). Notably, miRNAs have also been profiled in

blood samples of subjects with schizophrenia (Du et al., 2019; Lai et al., 2011), but to date, blood miRNA profiling tends to produce equivocal results (Liu et al., 2017; Wu et al., 2016).

1.3.2.3. HISTONE POST-TRANSLATIONAL MODIFICATIONS AND SCHIZOPHRENIA

Numerous studies associate histone post-translational modifications (PTMs) with schizophrenia. Among others, these PTMs include disrupted expression of histone methyltransferases (HMT) in the PFC of schizophrenia patients (Chase et al., 2013; Johnstone et al., 2018). Moreover, chromatin immunoprecipitation followed by sequencing studies (ChIP-Seq) in human *postmortem* brain have found an overrepresentation of open chromatin-associated modifications –histone H3 lysine 4 trimethylation (H3K4me3) and histone H3 lysine 27 acetylation (H3K27ac)– in genes implicated in schizophrenia and specifically in neuronal cells (Girdhar et al., 2018). In this regard, altered histone H3 acetylation and methylation at promoter regions of certain genes in the brain cortex of schizophrenia patients has been reported (Huang et al., 2007; Kurita et al., 2012; Tang et al., 2011). Furthermore, ChIP-seq evaluation of cell type H3K4me3 in PFC of schizophrenia subjects has revealed individual alterations in neurons (Gusev et al., 2019).

Various studies have explored the association between the enzymes that catalyze histone PTMs and schizophrenia. Specifically, HDACs have received special attention. HDACs are implicated in maintenance of neural cell identity, survival, and neuroplasticity, and they are also known to regulate cognitive circuitry (Gilbert et al., 2019). Indeed, HDACs seem to represent a fundamental factor in the processes of epigenetic regulation of genes linked to cognitive processes. While histone acetylation is associated with enhanced cognitive performance, histone deacetylation is related to cognitive impairment. Therefore, dysregulation of HDACs is thought to modulate the establishment and maintenance of aberrant transcriptional programs and behaviors associated with cognitive dysfunctions (Gräff & Tsai, 2013). In this sense, the use of HDAC inhibitors has been proposed in order to restore cognitive functions in disorders coursing with cognitive decline, such as schizophrenia (Fischer, 2014). One of the molecules with HDAC inhibition properties is the anticonvulsant and mood stabilizer valproate. There is the clinical observation that therapeutic doses of valproate act as a HDAC inhibitor, and it has been studied as add-on medication in patients with resistant schizophrenia. In these studies, co-administration of valproate with antipsychotic drugs showed to accelerate the onset of the antipsychotic effects in schizophrenia patients (Gavin et al., 2008; Sharma et al., 2006; Suzuki et al., 2009; Wang et al., 2016). Moreover, several HDAC inhibitors that induce a global up-

regulation of the acetylation of H3, including the benzamide derivative MS-275 (Simonini et al., 2006) and divalproex (Casey et al., 2002; Kelly et al., 2006) have been studied in clinical trials with schizophrenia patients. Focusing on preclinical studies, HDAC inhibitors have proven to have therapeutic potential in rodent models of schizophrenia. For example, suberoylanilide hydroxamic acid (SAHA), a class I and II HDAC inhibitor, has demonstrated to revert molecular and behavioral deficits in animal models of schizophrenia (Kurita et al., 2012). Interestingly, potential mechanisms that HDAC inhibitors act on psychiatric disorders, including schizophrenia, is suggested to be via anti-inflammatory effects, inducing the down-regulation of certain proinflammatory factors such as NF-κB, IL-6 or TNF-α, among others (Qiu et al., 2017).

Alterations in expression levels of several HDACs have been reported, with increased cortical HDAC1 expression (Bahari-Javan et al., 2017; Sharma et al., 2008) and reduced cortical HDAC2 expression (Schroeder et al., 2017) in *postmortem* samples of subjects with schizophrenia. Recently, an *in vivo* positron emission tomography study (PET), using [^{11}C]Martinostat (a radiotracer version of the HDAC inhibitor Martinostat), revealed lower *in vivo* HDAC levels in schizophrenia subjects comparing with marched controls (Gilbert et al., 2019). Authors suggest that these findings provide *in vivo* evidence of HDAC dysregulation in patients with schizophrenia and suggest that altered HDAC expression may affect cognitive function in humans (Gilbert et al., 2019). Besides, HDAC2 has been demonstrated to negatively regulate cognitive functions and synaptic plasticity, as its expression is increased by stress exposition, causing deficiencies in memory formation and synaptic plasticity, and leading to abnormalities in cognitive processes (Gräff & Tsai, 2013; Guan et al., 2009).

Overall, it is increasingly recognized that epigenetic modifications play a critical role in the etiology and pathophysiology of schizophrenia. While certain epigenetic modifications in schizophrenia may have a genetic origin (Ciuculete et al., 2017; Hannon et al., 2016; Montano et al., 2016; Tao et al., 2018), a substantial portion of epigenetic alterations may be acquired via environmental factors and remain stable as “molecular scars”. These “molecular scars” may influence brain functions throughout the entire lifespan and may be transmitted across generations via epigenetic germline inheritance (Hollins & Cairns, 2016; Grayson & Guidotti, 2012; Morishita et al., 2015; Nestler et al., 2015; Rutten & Mill, 2009). In addition, epigenetic modifications are a plausible molecular source of phenotypic heterogeneity and offer a target for therapeutic interventions. Taken together, epigenetic modifications may help to explain missing heritability in schizophrenia (Richetto & Meyer, 2021).

1.4. ANIMAL MODELS OF SCHIZOPHRENIA

Schizophrenia is an exclusive human disorder with subjective symptoms, unknown biomarkers, and genetic and neurobiological alterations that appear in early states of the disease. Consequently, modeling the alterations seen in schizophrenia patients in preclinical models is extremely complicated. Even if there are reasonable correlates in animals (e.g. abnormal social behavior, motivation, working memory, emotion and executive functions), the reproduction may only be approximate, as some of the symptoms (e.g. hallucinations or delusions) cannot be exactly reproduced in animals (Nestler & Hyman, 2010). However, animal models appear as very useful tools to help us understand the pathophysiology of the disease and develop new treatments.

Finding an animal model that replicates the wide symptomatology observed in schizophrenia patients has proven extremely challenging. Ultimately, animal models of schizophrenia should be generated as models of psychosis, cognitive dysfunction and/or negative symptoms, as schizophrenia should no longer be considered as a unitary disorder, but rather as a disorder of component symptom complexes (Ibrahim & Tamminga, 2011).

All useful animal models of schizophrenia should have the appropriate face (symptom homology), construct (replication of theoretical neurobiological rationale and pathology) and predictive (show the expected pharmacological response to treatment by known antipsychotics and potential new adjunct therapies) validity to the clinical disorder being modelled (Jones et al., 2011) (**figure 1.12**).

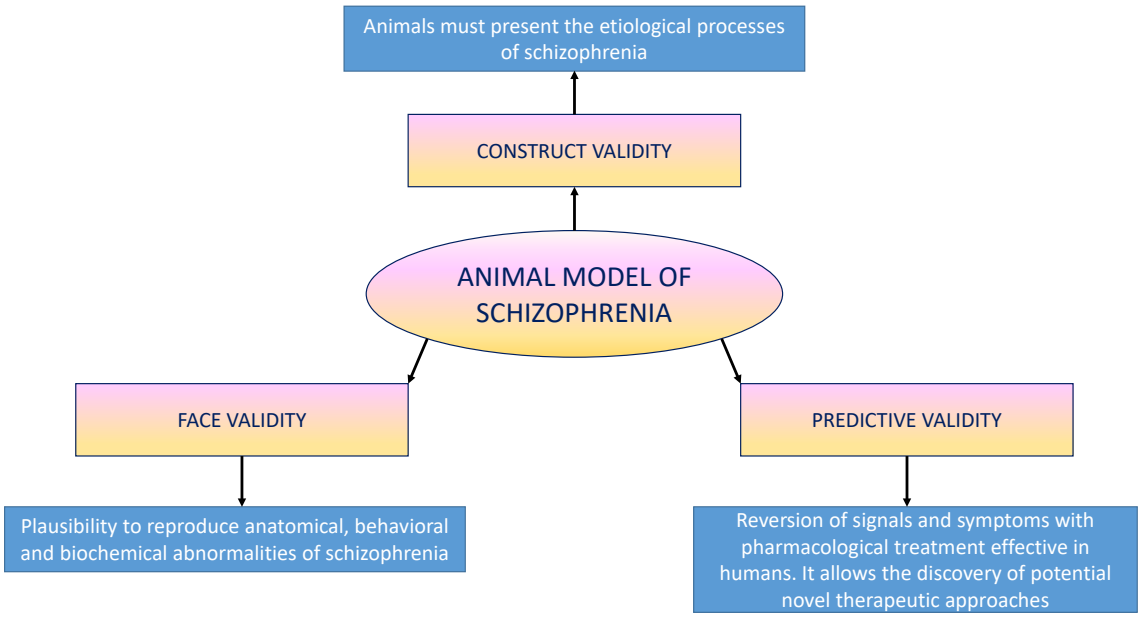


Figure 1.12: Scheme of the three types of validators that animal models of schizophrenia should meet. Illustration originally created by Natalia Cordero Ruiz, modified from Jones et al., 2011.

Many strategies can be used to generate animal models of schizophrenia, including genetic manipulation, drug administration, environmental manipulation or brain lesion, among others. Most representative animal models of schizophrenia are described below.

1.4.1. GENETIC MODELS

As genetic factors are known to play an important role in the etiology of schizophrenia, several animal models based in schizophrenia "candidate genes" have been developed. Classically, genetic animal models of schizophrenia have been generated by downregulating the gene expression of these human "candidate genes". Models based in mutations of susceptible human genes –such as the disrupted-in-schizophrenia 1 gene (*DISC-1*), Nuclear receptor-related 1 gene (*Nurr1*), Neuregulin (*Nrg1*), reelin glycoprotein gene (*RELN*), brain-derived neurotrophic factor (*BDNF*), and others– have demonstrated to resemble certain behavioral, molecular and cellular alterations related to schizophrenia (Guerrin et al., 2021). The disrupted-in-schizophrenia 1 (*DISC-1*) gene is a synaptic protein involved in neurodevelopment, neuro-signaling and synaptic functioning that is implicated in schizophrenia (Johnstone et al., 2011). Nuclear receptor-related 1 (*Nurr1*) protein is an orphan nuclear receptor, critical for development and survival of mesencephalic dopaminergic neurons, known as a potential susceptibility gene in schizophrenia (Buervenich et al., 2000). Neuregulin (*Nrg1*) is involved in the regulation of the expression and activation of neurotransmitter receptors and synaptogenesis, and has also been linked to

increased susceptibility for schizophrenia (Stefansson et al., 2002). Reelin (*RELN*) glycoprotein is involved in synaptic plasticity and brain development that has also been associated with schizophrenia (Ishii et al., 2016). Brain-derived neurotrophic factor (*BDNF*), a neurotrophin involved in brain development, neuroplasticity, neurotransmission and cognition, is also implicated in schizophrenia (Autry & Monteggia, 2012). Finally, animal models based in the downregulation of other genetic mutations associated with schizophrenia have been developed. These genetic mutations include the transporter associated with antigen processing 1 (*TAP1*) (McAllister, 2014), pituitary adenylate cyclase-activating peptide (*PACAP*) (Hashimoto et al., 2007), or synaptosomal-associated protein-15 (*SNAP25*) (Lewis et al., 2003), among others. However, the validity of these animal models has been questioned by some authors, as it is known that multiple genes are implicated in the etiology of the disease, genetic models based in single gene mutations are unlikely to comprise the high complexity of the disorder. Hence, the genetic background of schizophrenia is far more complex than that, and apart from the genetic components, environmental factors play a significant role in developing the illness in human subjects (Białoń & Wąsik, 2022; Nestler & Hyman, 2010).

1.4.2. DRUG-INDUCED ANIMAL MODELS OF SCHIZOPHRENIA

Several drugs acting on brain neurotransmitter systems have been suggested to replicate schizophrenia symptoms in healthy human subjects and rodents. The most common pharmacological approaches include the use of serotonergic hallucinogens (psilocybin, DOI or LSD), dopaminergic psychostimulants (cocaine or amphetamine), non-competitive NMDA receptor antagonists (PCP or ketamine), and the main active compound of *Cannabis sativa*, the Δ^9-tetrahydrocannabinol (THC) (reviewed in Steeds et al., 2015).

It is important to remark that, in contrast to drug models, schizophrenia is a chronic, neurodevelopmental, and episodic disorder, that includes different symptom domains predominating at different stages. Therefore, any purely pharmacological model is likely to be incomplete in the extent to which it can represent the full picture of schizophrenia (Steeds et al., 2015). Nevertheless, while pharmacological models may never be able to accurately mimic all aspects of such a complex disorder as schizophrenia, they offer a valuable insight to the neurobiological mechanisms underlying specific symptomatic domains (Curran et al., 2009). Specially, serotonergic hallucinogens are reported to mimic some aspects of prodromal and first episode psychosis in humans, and low doses in rodents seem to be able to model more symptom domains (Marona-Lewicka et al., 2011).

1.4.2.1. PSYCHEDELIC-INDUCED HEAD-TWITCH RESPONSE

As stated in **section 1.1.3**., the history of research on 5-HT is closely related with modern studies of the neurobiological basis of schizophrenia. The fundamental idea that psychotic states seen in schizophrenia may involve 5-HT dysfunction arose in part from the observed effects exerted by classical serotonergic hallucinogens/psychedelics, such as psilocybin or LSD (Geyer & Vollenweider, 2008).

In humans, psychedelics –LSD, psilocybin (and its active metabolite psilocin), mescaline or DOI– are known to produce profound alterations in perception, mood and cognition. Back in 1956, Keller & Umbreit first administered LSD intravenously to mice and reported "a rapid and violent head shaking" that was easily detectable with a little experience (Keller & Umbreit, 1956). This behavior was designed as the "head-twitch response" (HTR), which is well defined as a side-to-side rotational head movement occurring in mice after psychedelic administration (see **figure 1.13**). HTR is the behavioral signature of psychedelic drugs upon stimulation of the serotonin 5-HT_{2A}R. Other psychoactive drugs such as cocaine, amphetamine or PCP do not produce this characteristic behavior (Halberstadt & Geyer, 2011; De la Fuente Revenga et al., 2019; Nichols, 2016).

HTR is a natural response to activation of cortical serotonin 5-HT_{2A}Rs and mice do not require a previous training in order to perform it. Mice lacking 5-HT_{2A}R gene fail to show a HTR in response to psychedelics, while restoration of cortical 5-HT_{2A}R expression is able to rescue this behavior (González-Maeso et al., 2003; González-Maeso et al, 2007). Thus, HTR has become one of the few behaviors that can reliably distinguish between hallucinogenic and non-hallucinogenic 5-HT_{2A}R agonists, such as lisuride, pergolide or ergotamine, as non-hallucinogenic 5-HT_{2A}R agonists are not able to elicit HTR in rodents (González-Maeso et al., 2003; González-Maeso et al, 2007).

Figure 1.13: Scheme of the side-to-side head movement known as Head-Twitch Response (HTR) elicited by hallucinogenic drugs in mice. Illustration originally created for this work by Natalia Cordero Ruiz.

1.4.2.2. PSYCHOSTIMULANT-INDUCED DRUG MODELS OF SCHIZOPHRENIA

Psychostimulant drugs, such as amphetamine or cocaine, increase synaptic levels of dopamine, and are known to exacerbate psychotic symptoms in schizophrenia patients (Bramness et al., 2012; Farren et al., 2000). Consequently, in rodents, administration of these dopaminergic stimulants is reported to induce repeated or stereotyped behaviors, including locomotion, sniffing and chewing –related to positive psychotic symptoms–, and impaired PPI –marker of sensory gating impairment also seen in schizophrenia patients– (Segal & Mandell, 1974). Moreover, after chronic administration of these psychostimulants, impaired PPI and stereotyped behaviors occur at increasing frequency and duration over time, which is termed "sensitization" (Segal & Mandell, 1974). Particularly, as it occurs in subjects with schizophrenia, amphetamine-sensitized rodents show enhanced dopamine release in response to amphetamine administration when compared to controls (Fletcher et al., 2005; Tenn et al., 2003). Hence, amphetamine-induced sensitization is commonly used as a model for the positive symptoms of schizophrenia, while is not reported to fully resemble the cognitive and negative symptom domains of the disorder (Jones et al., 2011; Wang et al., 2010). Moreover, purely dopaminergic schizophrenia animal models are likely to lead to the development of more dopamine-targeting antipsychotic drugs (first generation of antipsychotics or typical antipsychotics), which is improbable to lead to a great improvement in efficacy or safety (Steeds et al., 2015).

1.4.2.3. NMDA ANTAGONIST-INDUCED DRUG MODELS OF SCHIZOPHRENIA

As explained in **section 1.1.3.**, the NMDA receptor hypofunction hypothesis of schizophrenia has raised as an alternative to the classical dopaminergic hypothesis of the disorder. In this regard, administration of drugs such as the dissociative anesthetics PCP and ketamine, both acting as non-competitive NMDA receptor antagonists, was proposed to generate drug-induced animal models of schizophrenia (Laruelle et al., 2000). In preclinical models, NMDA receptor antagonists induce behavioral, locomotor, cognitive and neurobiological alterations that resemble to those found in schizophrenia patients (reviewed in Steeds et al., 2015). Interestingly, these symptoms were demonstrated to be attenuated (although not completely abolished) with antipsychotic medication (Steinpreis et al., 1994). NMDA antagonist-induced drug models of schizophrenia demonstrate that this receptor could be a key factor in the pathogenesis of the disease, and these models are suggested to have a great value in the developing of new pharmacological therapies targeting the negative and cognitive symptomatology of the disorder (Steeds et al., 2015). Moreover, it should be reminded that NMDA antagonists act on multiple neurotransmitter systems, including the dopaminergic system (Kapur & Seeman, 2002). This action on several overlapping neurotransmitter systems means that non-competitive NMDA antagonists could give more insight into the complex clinical condition of schizophrenia.

Overall, while pharmacological models may never be able to resemble all aspects of such a complex disorder as schizophrenia, they may still be able to provide valuable information into the neurobiological basis that underlie specific symptom domains (Steeds et al., 2015).

1.4.3. ENVIRONMENTAL MODELS OF SCHIZOPHRENIA

Epidemiological studies have provided reliable evidence that the risk of developing schizophrenia is significantly enhanced after prenatal and/or perinatal exposure to various environmental insults (Meyer & Feldon, 2010). Those include maternal exposure to stress (Khashan et al., 2008; Koenig et al., 2002; Koenig, 2006; Malaspina et al., 2008; Selten et al., 1999), infection and/or immune activation (Boksa, 2008; Brown, 2006; Patterson, 2007), nutritional deficiencies (Susser et al., 2008) and obstetric complications (Cannon et al., 2002; McNeil et al., 2000), among others. Moreover, stressful experiences during development (childhood and adolescence), a critical period of the maturation of the neural circuits when the plastic capacity of the brain is maximal (McCormick & Mathews, 2007), have been associated with schizophrenia onset (Kessler et al., 2005). Based on these associations, a great deal of interest has been centered upon the establishment of neurodevelopmental animal models,

which are based on prenatal, perinatal or postnatal exposure to the mentioned environmental stimuli.

1.4.3.1. MIA MODELS

As previously mentioned, maternal infection is an immune-related risk factor associated with schizophrenia, and plethora of basic neuroscience studies have further investigated the role of MIA in schizophrenia. Animal models of MIA are exposed to immunological stimulation at a specific gestational stage. *In-utero* immune activation is mainly modeled by administration of three different substances: First, polyinosinic:polycytidylic acid, Poly (I:C), a synthetic analogue of double stranded RNA, which mimics a viral infection; second, lipopolysaccharide, LPS, a bacterial cell wall endotoxin; and third, human influenza virus (Brown et al., 2004; Meyer, 2014; Wischhof et al., 2015). Besides, other agents have also been used to induce MIA, such as turpentine (Aguilar-Valles et al., 2010; Aguilar-Valles & Luheshi, 2011), a model of clinical trauma that triggers the innate immune response and inflammation (Wusteman et al., 1990); and IL-6, which is reported to be a critical mediator of MIA-induced behavioral and transcriptional changes in the offspring (Smith et al., 2007). MIA models emerge as powerful translational tools to explore the effects of prenatal immune challenge on the developing fetus (Kentner et al., 2019). Overall, rodent and nonhuman primate MIA models have demonstrated a causal relationship between maternal infection and neuropathological and behavioral abnormalities related to a range of neurodevelopmental and psychiatric disorders, including schizophrenia (Patterson, 2009; Brown, 2011; Estes & McAllister, 2016; Choudhury & Lennox, 2021).

MIA animal models present construct, face and predictive validity: they model a known disease-related risk factor, mimic some known biological and/or behavioral pathology and exhibit many disease-related phenotypes, and they respond well to some therapies used to treat schizophrenia (Kentner et al., 2019). However, MIA models use a wide range of protocols that differ on the type, timing, mode of delivery, and dose of the immunogen used (Bilbo & Schwarz, 2009; Fortier et al., 2007; Harvey & Boksa, 2012; Meyer, 2006b), a fact that adds complexity in the replication and interpretation of the outcomes of different preclicical studies with this model.

MIA models appear as useful tools to test the hypothesis that it is the combination of the timing of exposure, the type of immune activation, environmental exposures, co-morbidities (i.e. stress and diet), intensity and duration of MIA, which may determine the nature of brain and behavioral alterations that appear in offspring (Harvey & Boksa, 2012; Ketner et al., 2019). Type

of immunogen used to provoke MIA (viral vs. bacterial and acute vs. chronic) determines the nature of the immune response and downstream phenotypes (Kentner et al., 2019). Timing of exposure is also important, as MIA in early gestation versus MIA in late gestation provoke different fetal immune response and behavioral outcomes in adult offspring (Bilbo and Schwarz, 2009; Fortier et al., 2007; Meyer et al., 2006b). In addition, the dose of the immunogen used to provoke MIA is also crucial, as there might be a threshold of MIA in order to produce schizophrenia-like phenotype in MIA-challenged offspring and no other disease-related phenotypes (Meyer, 2014; Estes & McAllister, 2016). With all these factors in mind, Kentner and collaborators proposed a reporting checklist needed to improve the rigor, reproducibility and transparency of the MIA models. The goal of this report was to standardize the general experiment design (species, immunogenic compounds used, housing, etc.) (Kentner et al., 2019).

As mentioned before, MIA is thought to act as a "disease primer", provoking an altered trajectory of fetal brain development, making the offspring more susceptible to the effect of genetic mutations and environmental exposures, and ultimately leading to the onset of schizophrenia and/or other CNS disorders (Reisinger et al, 2015; Selten, 2010; Estes & McAllister, 2016). Thus, maternal infection-induced inflammation during pregnancy, might act as a primer to make the offspring more susceptible to the effect of "second stressful hits" later in life. In this sense, double-hit models of schizophrenia could represent a more translational alternative in the study of schizophrenia (Guerrin et al., 2021). Particularly, double-hit animal models combining a first adverse event such as genetic predisposition or prenatal insult and a second postnatal insult, may provide valuable insight into understanding disease progression (see **section 1.4.4**).

1.4.3.1.1. POLY (I:C) MIA MODEL

1.4.3.1.1.1. MOLECULAR MECHANISM OF POLY (I:C)

Polyinosinic:polycytidylic acid, Poly (I:C), a synthetic analog of double-stranded RNA (dsRNA), is one of the most widely used substances to provoke MIA in pregnant dams. Upon binding to its receptors, Poly (I:C) is able to selectively activate numerous signaling pathways depending on the conditions. Poly (I:C) is recognized by endosomal Toll-like receptor 3 (TLR3) (Alexopoulou et al., 2001; Matsumoto et al., 2002). Upon recognition, TLR3 activate the transcription factor interferon regulatory factor 3 (IRF3), through the adapter protein Toll-IL-1 receptor (TIR) domain-containing adapter (TRIF, also known as TICAM-1) (Yamamoto et al., 2003). Activation of IRF3 leads to the production of type I IFNs (IFN-α and IFN-β). Moreover, recognition of Poly

(I:C) can lead to the recruitment of TNF receptor-associated factor 6 (TRAF6) or receptor-interacting protein 1 (RIP1), which leads to the activation of the proinflammatory transcription factors NF-κB and AP-1 (Kawai & Akira, 2008). Activation of these pathways triggers the production of inflammatory cytokines and chemokines, such as TNF-α or IL-6, among others (Kawai & Akira, 2008). Furthermore, Poly (I:C) induces the activation of the cytosolic RNA helicases retinoic acid-inducible protein I (RIG-I) and melanoma differentiation-associate gene 5 (MDA-5) (Kato et al., 2006). RIG-I and MDA-5 are critical for host antiviral responses and have essential roles in the recognition of different groups of RNA viruses, as well as in the subsequent production of type-I interferons and proinflammatory cytokines (Kato et al., 2006) (see **figure 1.14**).

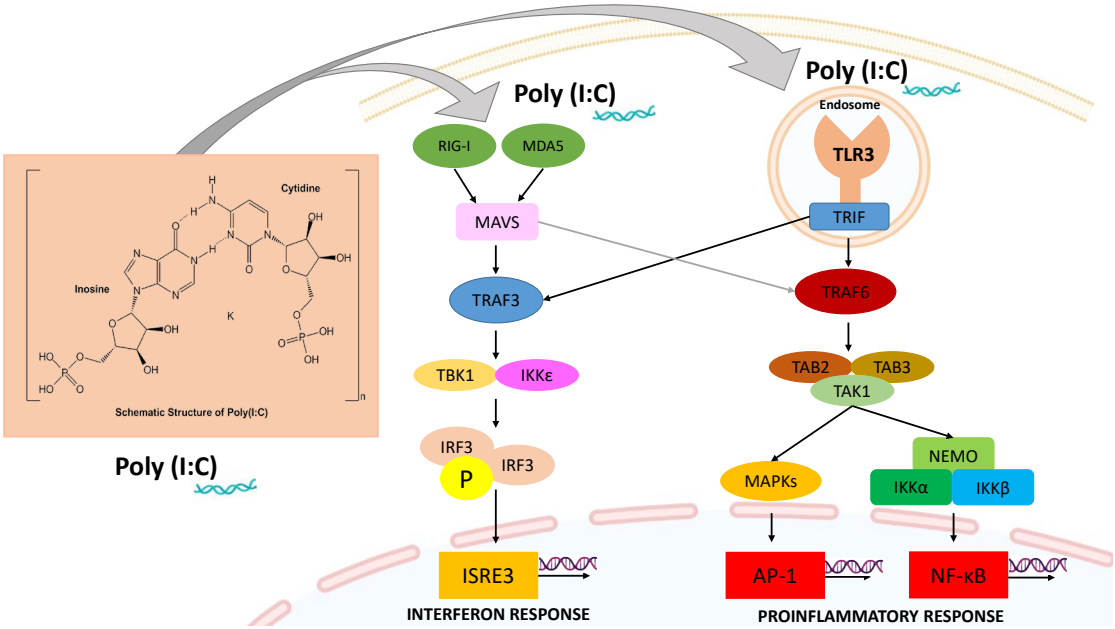


Figure 1.14: Poly (I:C) structure and signaling pathway. Originally developed by Natalia Cordero Ruiz for this work, adapted from Reisinger et al., 2015. Abbreviations: AP-1= Activator Protein 1. IKK= Inhibitors of NF-kB Kinase. IRF3= Interferon Regulatory Factor 3. ISRE3= Interferon-Sensitive Response Element 3. MAVS= Mitochondrial Antiviral-Signaling Protein. MDA5= Melanoma Differentiation-Associate gene 5. MAPKs= Mitogen-Activated Protein Kinase. NEMO= NF-κB Essential Modulator. NF-κB= Nuclear Factor κB. P= Phosphorylation. Poly (I:C)= Polyinosinic:Polycytidylic acid. RIG-I= Retinoic Acid-Inducible Protein I. RIP1= Receptor-Interacting Protein 1. TAB=TAK Binding protein. TAK1= Transforming growth factor beta Activated Kinase 1. TBK1= TANK Binding Kinase 1. TLR3= Toll-Like Receptor 3. TRAF= TNF Receptor-Associated Factor. TRIF= Toll-IL-1 Receptor Domain-Containing Adapter.

1.4.3.1.1.2. POLY (I:C)-INDUCED ALTERATIONS IN MIA MODELS

The Poly (I:C) MIA model was first described by the Weiner laboratory, who reported various behavioral abnormalities associated to schizophrenia in rats exposed to the Poly (I:C) viral mimetic *in* utero (Zuckerman et al., 2003). These alterations included increased sensitivity to drug-induced locomotor activity and disrupted latent inhibition (LI) (Zuckerman et al., 2003). Impaired capacity to ignore irrelevant stimuli is one of the core deficits in schizophrenia (Anscombe, 1987; Nuechterlein & Dawson, 1984), and can be manifested as the loss of latent inhibition (LI), in which repeated inconsequential pre-exposure to a stimulus impairs its subsequent capacity to signal significant consequences.

Poly (I:C) administration is reported to induce an increase in cytokine (including IL-6, IL-1β, TNF-α and IL-10) and interferon type I (IFN-α and IFN-β) production in pregnant dams (Meyer et al., 2006b; Meyer et al., 2009; Song et al., 2011; Reisinger et al., 2015). Consequently, the integrity of the placental barrier becomes compromised, allowing entrance of maternally derived cytokines into the placenta and inducing inflammatory responses in the developing fetus, including in the brain. This leads to structural and developmental disturbances associated with schizophrenia (Reisinger et al., 2015) (see **figure 1.15**). Thus, Poly (I:C)-driven MIA is able to reproduce schizophrenia-related phenotypes in rodents. These abnormalities include deficits in PPI, latent inhibition (LI), social interaction, working memory, novel object exploration, excessive amphetamine-induced locomotion, and anhedonia (Meyer et al., 2005; Ozawa et al., 2006; Smith et al., 2007; Zuckerman et al., 2003; Khan et al, 2014). In further support of the Poly (I:C) model in schizophrenia, some of these abnormalities, including disrupted LI, can be reversed by antipsychotic medication (Ozawa et al., 2006; Zuckerman et al., 2003).

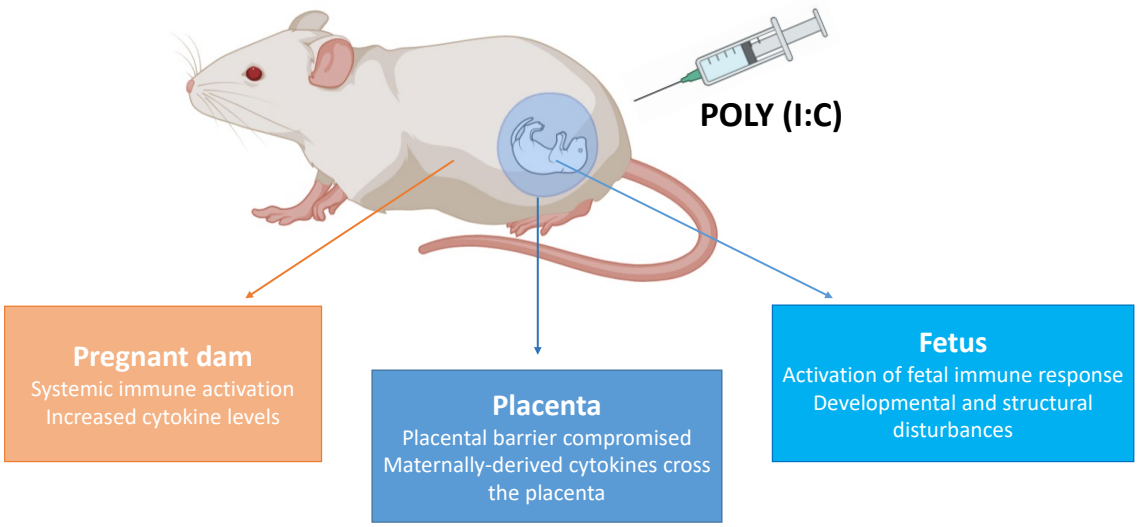


Figure 1.15: The Poly (I:C) rodent model of schizophrenia. Effects of Poly (I:C) administration on the pregnant dam, placental compartment and fetus. Illustration originally developed by Natalia Cordero Ruiz for this work, adapted from Reisinger et al., 2015.

Overall, the Poly (I:C) MIA model has proven to have a high degree of construct validity (in light of the epidemiological evidence of MIA) and face validity (inducing behavioral features related to schizophrenia). Moreover, Poly (I:C) model has also demonstrated to have high predictive validity with some drugs used for the schizophrenia treatment. Indeed, studies show how Poly (I:C)-elicited behavioral deficits can be reverted with antipsychotics as clozapine or haloperidol (Ozawa et al., 2006; Zuckerman et al., 2003), and Poly (I:C)-driven neuroinflammatory alterations can be blocked with chronic paliperidone administration (MacDowell et al., 2017).

1.4.3.1.1.3. RELEVANT FACTORS AND LIMITATIONS OF THE POLY (I:C) MODEL

Despite the high validity showed for the model, many of the behavioral and physiological alterations related to prenatal immune activation with Poly (I:C) are dependent on the prenatal timing (early, mid or late gestation), Poly (I:C) dosing, route of administration (intraperitoneal, sub-cutaneous, intra-venous, intra-muscular, etc.), vendor (Sigma, InvivogGen, etc.), genetic background of mice, and even storage and administration conditions (time of day, room temperature, etc.) (Kentner et al., 2019). Thus, it is important to continually validate Poly (I:C) models to ensure the immune activation elicited by the used product.

Administration of Poly (I:C) is known to activate the immune system, resulting in the release of proinflammatory mediators that induce sickness behavior, indicating an inflammatory response. Consequently, Poly (I:C) administration is known to produce “sickness behaviors” that include

fever, lethargy, reduced food-seeking behavior, reduced sociability, etc. (Dantzer & Kelley, 2007; Bluthé et al., 2000; Kent et al., 1996; Ketner et al., 2019). Hence, one option to validate the potency of the immune response elicited by Poly (I:C) is by the measurement of proinflammatory cytokines (such as IL-6, IL-1β or TNF-α) in immune activated pregnant dams. Immune activation can also be validated by the evaluation of cytokine-induced sickness behaviors in the home cage, including piloerection, lethargy and huddling (Kolmogorova et al., 2017). Finally, one of the most used methods to validate the Poly (I:C)-induced immune response, is to measure changes in the body weight (Hart et al., 1988; Redfern et al., 2017) and temperature (Cunningham et al., 2007; Mueller et al, 2019) after the immune challenge. Poly (I:C)-elicited inflammatory fever response can be verified by measuring body temperature of the immune challenged animals, which can be made by using temperature probes (Kentner et al., 2019). In mice, one of the consequences of Poly (I:C) exposure is a biphasic body temperature response (Cunningham et al., 2007; Mueller et al., 2019). Thus, Poly (I:C) administration generally leads to reduced rectal and surface temperatures. Hence, the predominant thermal response to Poly (I:C) in mice is hypothermia, which is typically preceded by a temporary phase of mild hyperthermia (Cunningham et al., 2007). Thus, there is an association between the elicited inflammatory response and hypothermia in mice (Cunningham et al., 2007; Desbonnet et al., 2022; Mueller et al, 2019). Indeed, the post injection interval of 3 hours after substance administration is reported to correspond to the peak of the hypothermal response to Poly (I:C) administration (Desbonnet et al., 2022; Mueller et al., 2019). Notably, the thermal response to Poly (I:C) in mice is distinct from that in rats, the latter of which typically show a monophasic hyperthermic response to this immunogen (Fortier et al., 2004).

Moreover, different Poly (I:C) batches, even if they are obtained from the same vendor, can induce varying immune responses (Kentner et al., 2019; Mueller et al., 2019). In this sense, Poly (I:C) enriched for high molecular weight dsRNA is able to produce more intense maternal and placental immune responses when comparing to low molecular weight Poly (I:C) (Careaga et al., 2018; Mueller et al., 2019). Another factor to be taken into account is the possible LPS contamination of Poly (I:C) batches (Mueller et al., 2019).

Taking all of this into account, it is highly recommended to have a control of these variables in each pregnant dam, either with one of the proposed measures, or ideally, with all of them.

1.4.3.2. POST-WEANING SOCIAL ISOLATION MODEL

Social isolation is defined as a stressful event that leads to profound changes in both humans and rodent animal models, including: alterations in reactivity to stress, social behavior, function of neurochemical and neuroendocrine system, physiological, anatomical and behavioral changes (Ferdman et al., 2007; Fone & Porkess, 2008; Weiss et al., 2004). Social isolation is considered a potent stressor in both humans and animals (Jones et al., 2011; Liu et al., 2010), and social isolation stress during early stages of life has been proposed to produce signs and symptoms of psychiatric and neurological disorders, including schizophrenia (Mumtaz et al., 2018; Nestler & Hyman, 2010). Moreover, social isolation as a stressor during adolescence has been particularly relevant during the coronavirus disease 2019 (COVID-19) pandemic, which resulted in prolonged social distancing and quarantining, and imposed social isolation on this younger population at a life phase when social experiences are essential to brain development. In addition, as already pointed, maternal immune activation (via prenatal infection) could cause a vulnerability in the offspring that would be further exacerbated as a consequence of stressful insults in sensitive periods of brain development, such as adolescence, contributing to schizophrenia onset (Estes and MacAllister, 2016). Thus, with the risk of COVID-19-caused maternal immune activation, in combination with social isolation stress later in life, is now more important than ever to identify pathophysiological biomarkers of these environmental factors and opportunities for the therapeutic or preventive interventions that can reduce the risk of the disorder onser in exposed individuals (Desbonnet et al., 2022).

In rodents, post-weaning social isolation has profound effects on brain development, leading to a significant behavioral disruption in adulthood reminiscent of schizophrenia (Hill, 2016). Post-weaning social isolation stress has showed to produce a variety of stress-related endocrine changes (Pariante & Lightman, 2008), such as the activation of hypothalamic–pituitary–adrenal (HPA) axis, which ultimately culminates in the release of glucocorticoids, catecholamines, activation of the sympatho-adrenomedullary system and release of oxytocin and vasopressin (Kvetnanský et al., 1995). In several regions of the CNS, social isolation alters the level of various neurotransmitters (dopamine, serotonin, GABA, glutamate etc.), and leads to alterations in receptors sensitivity of NMDA and opioid system (Bledsoe et al., 2011; Fone et al., 1996; Lapiz et al., 2003; Lukkes et al., 2012; Serra et al., 2000; Yorgason et al., 2013). Moreover, social isolation rearing is reported to induce behavioral abnormalities such as locomotor hyperactivity, deficits in sensorimotor gating (PPI), cognitive impairments and alterations in neurotransmitter activity (Jones et al., 1992; Bakshi et al., 1998; McLean et al., 2010).

1.4.4. DOUBLE-HIT MODELS OF SCHIZOPHRENIA

Currently available schizophrenia models, such as the ones based on single gene manipulations and/or pharmacological modulations (e.g. administration of psychedelic drugs or psychostimulants, such as amphetamine) (see **sections 1.4.1** and **1.4.2**, respectively) present deficiencies and discrepancies in terms of construct and face validity. In this sense, double-hit models of schizophrenia could represent a more translational alternative in the study of this psychiatric entity (Guerrin et al., 2021). Double-hit rodent models, using different combinations of prenatal and postnatal risk factors, have been utilized for research on the etiology and pathophysiology of schizophrenia (see **figure 1.16**). Particularly, double-hit animal models combining a first adverse event such as genetic predisposition or prenatal insult and a second postnatal insult, may provide valuable insight into understanding disease progression. Focusing on environmental factors associated with schizophrenia, currently used double-hit models are generated with the combination of two of the following risk factors: maternal infection/immune activation, social/physical stress and administration of different drugs such as NMDA receptor antagonists, corticosterone, cannabis or methamphetamine (for review see Guerrin et al., 2021).

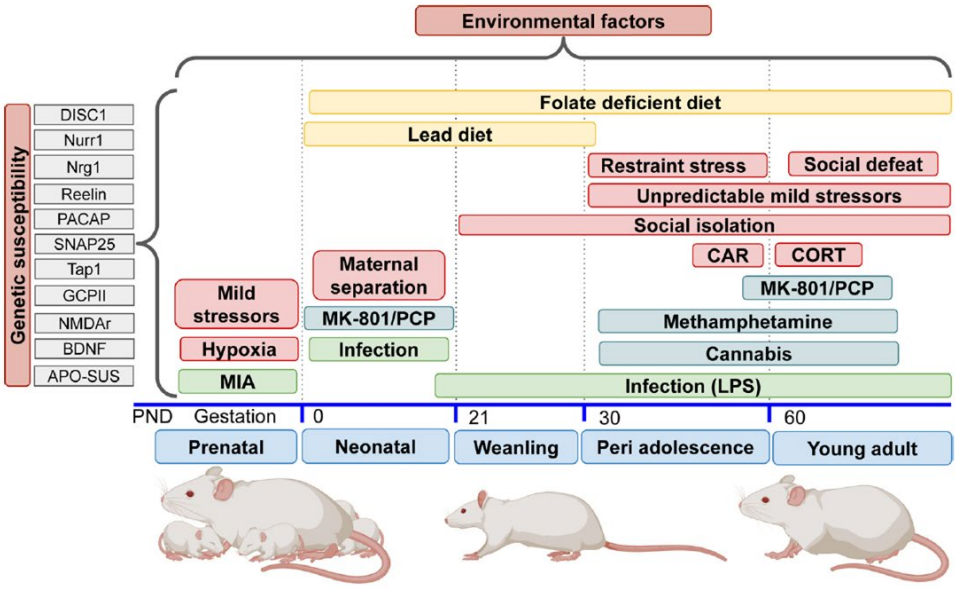


Figure 1.16: Risk factors used to generate double-hit animal models of schizophrenia. Summary of the genetic mutations (DISC-1, Nurr1, Nrg1, etc.) and environmental stressors that have been investigated in double-hit animals. Several environmental risk factors are used in different life periods in order to generate double-hit animal models of schizophrenia. Prenatal risk factors include mild stressors, hypoxia and MIA. Neonatal risk factors include maternal deprivation, infection, administration of NMDA antagonists (PCP, MK801) and nutritional deficits (lead diet, folate deficient diet). Weanling insults include LPS infection, social isolation and nutritional deficits (lead diet, folate deficient diet). Periadolescent risk factors include LPS infection, several stress paradigms (social isolation, restraint stress, unpredictable mild stressors), conditional avoidance training (CAR), drug administration (cannabis, methamphetamine, PCP, MK801) and nutritional deficiencies (folate deficient diet). Finally, early adulthood risk factors include infection with LPS, several stress paradigms (social isolation, restraint stress, unpredictable mild stressors), corticosterone administration (CORT), drug administration (cannabis, methamphetamine, PCP, MK801) and nutritional deficiencies (folate deficient diet). Abbreviations: BDNF= Brain-Derived Neurotrophic Factor. CAR= Conditioning Avoidance training. CORT= Corticosterone. DISC-1= Disrupted-In-Schizophrenia 1. GCPII= Glutamate carboxypeptidase II. LPS= Lipopolysaccharide. MIA= Maternal Immune Activation. NMDAr= N-Methyl-D-Aspartate receptor. Nrg1 = Neuregulin1. Nurr1= Nuclear Receptor-Related 1 protein. PACAP= Pituitary Adenylate Cyclase Activating Peptide. PCP= Phencyclidine. PND= Postnatal Day. SNAP25= Synaptosomal Associated Protein-25. TAP1= Transporter Associated with Antigen Processing 1. Blue box: substance administration; Green box: infection; Red box: stressors; Yellow box: diet factors. Guerrin et al., 2021.

1.4.4.1. DOUBLE-HIT MODELS COMBINING MIA AND A STRESSFUL EVENT IN THE PERIPUBERTY

MIA during pregnancy might act as a primer to make the offspring more susceptible to the effect of stress later in life (Giovanoli et al., 2013). Most MIA animal models are single-factor models, in which the isolated effects of MIA-related exposures are investigated with respect to different phenotypes in the offspring. Thus, these single-factor approaches usually present a lack of analogy, which finally results in a translational gap that reduces the translational value of these models (Brown & Meyer, 2018). In this sense, double-hit animal models represent a more translational alternative, as they combine two environmental factors known to have a significant role in schizophrenia onset (Guerrin et al., 2021). Thus, MIA is believed to have a potential priming effect on the response of the juvenile offspring to a stressor, including social isolation (and other stress paradigms such as unpredictable mild stress or restraint stress) or drug administration (such as methamphetamine, cannabis) (Guerrin et al., 2021).

Specially, studies combining MIA with Poly (I:C) and different stress paradigms in mice (Desbonnet et al., 2022; Deslauriers et al., 2013; Deslauriers et al., 2014; Giovanoli et al., 2013; Giovanoli et al., 2014; Giovanoli et al., 2016) and rats (Monte et al., 2017; Yee et al., 2011; Goh et al., 2020) have gained increasing attention. Regarding behavioral outcomes of these double-hit models, studies show controversial results. While some studies report that combination of Poly (I:C) administration with different stress paradigms in the peripubertal period induces synergistic effects on PPI deficits and hyperlocomotion (Deslauriers et al., 2013; Deslauriers et al., 2014; Giovanoli et al., 2013), other studies do not report these synergistic effects (Deslauriers et al., 2016; Giovanoli et al., 2013; Yee et al., 2011). Indeed, Poly (I:C)-driven MIA is even reported to have a protective effect against the detrimental effects of social isolation rearing on behavioral outcomes such as hyperlocomotion, memory and social behavior (Goh et al., 2020).

Taken together, the majority of the investigations carried out with double-hit models point to the idea that MIA during pregnancy may act as a disease primer in order to make the offspring more susceptible to stress exposure later in life. Nevertheless, given the fact that some authors have claimed that MIA may have protective effects to later adverse events (Goh et al., 2020), or that the combination of MIA and a postnatal stressful event is not enough to potentiate the alterations associated with schizophrenia in the offspring (Deslauriers et al., 2016; Giovanoli et al., 2013; Yee et al., 2011), more research with double-hit rodent models should be conducted in order to elucidate this controversy. Besides, the influence of the sex should be evaluated in these models, as in the majority of the cited investigations this variable has not been taken into account (Deslauriers et al., 2013; Deslauriers et al., 2014; Deslauriers et al., 2016; Giovanoli et

al., 2013; Goh et al., 2020; Yee et al., 2011). Among all the mentioned studies, only the one conducted by Desbonnet and collaborators (2022) has evaluated the influence of the sex on Poly (I:C) and stress induced schizophrenia-related phenotypes (Desbonnet et al., 2022). However, these authors also report no synergistic effects of the combination of Poly (I:C)-driven MIA and stress in the peripubertal period. Overall, further research should be conducted to explore sex-related differences in schizophrenia-related alterations of double-hit models combining MIA and peripubertal stress.

1.4.5. SEX INFLUENCE IN ANIMAL MODELS OF SCHIZOPHRENIA

Sex (a biological construct) and gender (a social construct) differences have repeatedly been reported in various features of schizophrenia, particularly in the age of onset, the course of the disease and response to treatment, reflecting differences in neurodevelopmental processes and cognitive functions (Mendrek & Mancini-Marïe 2016; Riecher-Rössler et al., 2018).

The influence of sex in preclinical models of schizophrenia is poorly studied. To date, most research with preclinical models of schizophrenia, are assessed only in males, or do not even report the sex. In both animals and human beings, males and females differ in their genetic background and hormonally driven behavior and show sex-related differences in brain activity and response to internal and external stimuli (Franceschini & Fattore, 2021).

Despite above-described shortcomings, some studies have evaluated the influence of sex in different animal models of schizophrenia, including the Poly (I:C) model, reporting significant sex differences (Bitanihirwe et al., 2010a; Bitanihirwe et al., 2010b; O'Leary et al., 2014; Ratnayake et al., 2014; Van den Eynde et al., 2014; Zhang et al., 2012). For example, Poly (I:C)-driven MIA is reported to alter behavioral flexibility (reversal learning and set-shifting task) in male, but not in female offspring (Zhang et al., 2012). In other study, prenatal Poly (I:C) exposure induced impaired cognitive flexibility (abnormal latent inhibition) in male but not in female offspring (Bitanihirwe et al., 2010a). While some studies have reported sex-specific alterations in PPI paradigm (O'Leary et al., 2014), other studies including both male and female Poly (I:C) rodent models show PPI deficits in both sexes (Ratnayake et al., 2014; Van den Eynde et al., 2014). Sex-influences in spatial and working memory have also been reported, with discrepant results (O'Leary et al., 2014; Bitanihirwe et al., 2010b). Overall, it appears that sex differences in these schizophrenia-related behavioral paradigms following Poly (I:C) exposure are task-specific and also depend upon the timing of MIA.

The influence of sex on the incidence, onset and course of schizophrenia seem partially linked to neuroprotective action of estrogens in women (Franceschini and Fattore, 2021). Studies using animal models of schizophrenia have consistently confirmed that sex and stress hormones interact to shape the developing brain and modulate behavior (Hill, 2016). Few studies in the literature analyze sex influence in post-weaning social isolation models, with the majority of studies performed using just males or not reporting sex (Hill, 2016). However, some studies with animal models of social isolation report sex-related differences in behavioral outcomes related to schizophrenia. These abnormalities include sex-specific PPI disruption (Powell et al., 2002; Harte et al., 2004; Harte et al., 2007; Swerdlow et al., 2013), sex-specific anxiety related behavior (Powell et al., 2002; Guo et al., 2004; Weiss et al., 2004; Pietropaolo et al., 2008), and sex-related locomotor activity alterations (Abramov et al., 2004; Ko & Liu, 2015). In addition to schizophrenia-related behavioral phenotypes, sex differences on the social isolation induced oxidative stress have been reported (Jiang et al., 2013). Overall, animal models have shown that females are more vulnerable to stress-induced hyper-arousal, but more resilient to stress-induced attention deficits than males (Bangasser et al., 2018).

Hence, studies in animal models of schizophrenia that include a significant number of animals belonging to both sexes provide significant insight into the complexities of these interactions and can direct towards novel therapeutic strategies. Despite some investigations are starting to focus on the sex, animal studies on schizophrenia including both males and females remain scarce and there is little knowledge regarding gender gaps in the alterations observed in different animal models of the disease. Including both sexes in the experimental design of the present PhD project is challenging and adds a big effort; however, working within a framework of translational research is of especial relevance.

2. HYPOTHESIS AND OBJECTIVES

The present work suggests that schizophrenia cognitive and negative deficits may have a neuroinflammatory substrate. The aberrant inflammatory activity shown in schizophrenia may be induced by altered immune responses associated to environmental “hits” in critical periods of brain development. Moreover, activation of inflammatory signaling is proposed to be associated with alterations in histone acetylation in certain genes, which might be underlying cognitive and/or negative symptoms of schizophrenia. Finally, modulation of these aberrant inflammatory pathways may benefit the pharmacological treatment of schizophrenia cognitive and negative deficits.

Thus, to test the premises of previous hypothesis, this Doctoral Thesis addresses the following main objectives:

FIRST: the present work aimed to develop and to validate a double-hit animal model of schizophrenia, based on I) the establishment of a prenatal neurodevelopmental deficit – based on maternal immune activation with Poly (I:C) during the gestation – followed by II) a second stressful event in peripuberty: social isolation stress. This Doctoral Thesis aimed to evaluate in this mouse model the potential schizophrenia-related behavioral alterations, the changes in inflammatory signaling proteins and the expression of HDACs induced by each of the hits and by the combination of both.

For this purpose, the specific objectives of the first aim of the present work are:

i. Evaluation of schizophrenia-related behaviors in the double-hit model, including basal locomotor activity and anxiety-related behaviors (Open Field Test), cognitive deficits (Y-Maze Spontaneous Alternation test, Novel Object Recognition Test), negative-like symptomatology (Social Preference Test) and positive psychotic-like alterations (locomotor response to amphetamine and Head-Twitch Response behavior induced by psilocybin).
ii. Evaluation of the peripheral inflammatory status of the double-hit model, by the assessment of different proinflammatory (IL-6, IFN-γ, TNFα, IL-17A, IL-1β, IL-2) and anti-inflammatory (IL-10) cytokine levels in plasma samples.
iii. Evaluation of the neuroinflammatory status of the double-hit model, by the assessment of the gene and protein expression of the inflammatory signaling protein NF-κβ and its principal inhibitor IκBα in brain cortex samples.

iv. Evaluation of epigenetic alterations related to schizophrenia in the double-hit model, by the measurement of the gene expression of nine different HDACs in brain cortex samples.

SECOND: the present work aims to assess –in the double-hit mouse model– the effect of chronic treatment with the atypical antipsychotic clozapine, the inhibitor of microglia activation minocycline, and co-administration of both drugs on: I) the negative-like and cognitive status and II) the changes in the gene expression of inflammatory signaling proteins, different HDACs, and in the 5-HT_{2A} receptor.

For this purpose, the specific objectives of the second aim of the present work are:

i. Evaluation of the effects of the chronic treatment with clozapine and/or minocycline, on cognitive (Novel Object Recognition Test) and social (Social Preference Test) status of the double-hit model.
ii. Evaluation of the effects of the chronic treatment with clozapine and/or minocycline, on the gene expression of the cytokines IL-6 and IFN-γ in brain cortex samples of the double-hit model.
iii. Evaluation of the effects of the chronic treatment with clozapine and/or minocycline, on the gene expression of NF-κβ and its principal inhibitor IκBα in brain cortex samples of the double-hit model.
iv. Evaluation of the effects of the chronic treatment with clozapine and/or minocycline, on the gene expression of HDAC4 and HDAC8 in brain cortex samples of the double-hit model.
v. Evaluation of the effects of the chronic treatment with clozapine and/or minocycline, on the gene expression of the 5-HT_{2A} receptor in brain cortex samples of the double-hit model.

3. ANIMALS, MATERIALS AND METHODS

3.1. ANIMALS

3.1.1. CD-1® MICE AND C57BL/6 MICE

Wild-type Hsd:ICR (CD-1®) male and C57BL/6 male and female mice were used for all the experiments described in this work. All parental mice were purchased from Envigo RMS Spain S.L. The offspring was handled in the animal facilities of the University of the Basque Country UPV/EHU.

Mice weighting around 25-30 (C57BL/6) and 40-50 grams (CD-1®) were housed at room temperature (22-25 ^{o}C) and 65-70% humidity, on a 12 hour light/dark cycle (lights on between 8 am – 8 pm) and with access to water and food *ad libitum*. Every procedure involving mice in this work was performed in accordance with the European Union Directive (2010/63/UE) for the protection of animals used for experimental and other scientific purposes and approved by the corresponding Ethical Committee of Animal Experimentation (CEEA M20/2017/166) and Biological Agents (CEIAB M30/2018/108) of the University of the Basque Country UPV/EHU.

3.1.2. DOUBLE-HIT ANIMAL MODEL

A double-hit mouse model of schizophrenia was generated by the promotion of a prenatal neurodevelopmental trajectory alteration based on maternal immune activation during gestation, followed by a second stressful event in peripuberty (social isolation stress). First, in order to validate the double-hit animal model of schizophrenia, CD-1® male and female mice were used. For the head-twitch response evaluation after psilocybin administration, C57BL/6 female mice were used. Then, in order to evaluate the effect of a chronic treatment with antipsychotic and/or anti-inflammatory drugs in the double-hit model, wild-type C57BL/6 mice were used.

For the breeding procedures, two females and one male were housed per cage. Every morning before 8 am, females were checked for the presence of the vaginal plug that indicates pregnancy. When a vaginal plug was detected, pregnant females were separated from the males and housed in groups of two or three individuals in different cages. The breeding procedure was the same for both mouse strains used in this work.

3.1.2.1. FIRST HIT: MATERNAL IMMUNE ACTIVATION

First hit or priming event implied maternal immune activation (MIA) by the administration of polyriboinosinic-polyribocytidic acid sodium salt, Poly (I:C), a synthetic analog of dsRNA.

Pregnant mouse dams were randomly assigned to a treatment with a saline solution or Poly (I:C).

Poly (I:C) was dissolved in saline solution (0.9% NaCl) in agitation for a few minutes in a sonic bath. The solution was administered at a dose of 7.5 mg/kg i.p. (volume 5 ml/kg) at GD 9.5 to pregnant dams (**figure 3.1**). Dose of Poly (I:C) was chosen on the basis of preliminary results (MacDowell et al., 2021). Time of administration of Poly (I:C) was chosen based on previous literature data, and corresponds to the first-to-second trimester of gestation in humans (Meyer et al., 2006a; Haddad et al., 2020). Epidemiological studies support a bigger neurodevelopmental risk for maternal infection exposures in the first and second trimester, which has been linked to increased risk of schizophrenia, comparing to other neurodevelopmental disorders (Atladóttir et al., 2010; Brown et al., 2004; Izumoto et al., 1999; Limosin et al., 2003; Mednick et al., 1998).

Poly (I:C) was purchased from Sigma Aldrich®, St. Louis, MO, USA. Batches #095M4049V (for the behavioral and molecular characterization of the double-hit model in CD-1® mice) and #123M4053V (for the chronic pharmacological treatments with antipsychotic and/or anti-inflammatory drugs and HTR experiments to psilocybin administration in C57BL/6 mice) were used.

3.1.2.1.1. VALIDATION OF IMMUNE ACTIVATION AFTER POLY (I:C) ADMINISTRATION

In order to validate the selected protocol to induce MIA, body weight and temperature was recorded in a series of C57BL/6 pregnant dams after Poly (I:C) administration. Body weight (Hart et al., 1988; Redfern et al., 2017) and temperature (Cunningham et al., 2007; Mueller et al, 2019) are known to be affected by immunogens as the Poly (I:C) as part of the inflammatory sickness response. Thus, an accepted validation of the Poly (I:C)-elicited immune response is to measure alterations in the body weight and temperature after this immune challenge (Kentner et al., 2019).

For this purpose, vehicle (saline solution, NaCl 0.9%; volume 5 ml/kg; n= 5) and Poly (I:C) (7.5 mg/kg; volume 5 ml/kg; n= 11) treated C57BL/6 pregnant dams were used. MIA was validated by the measurement of the body weight and temperature. Body weight was measured right before vehicle/Poly (I:C) administration (+ 0 h) (GD 9.5), and 3 h, 6 h, 24 h (GD 10.5) and 48 h (GD 11.5) after vehicle/Poly (I:C) administration. Body temperature was measured right before vehicle/Poly (I:C) administration (+ 0 h) (GD 9.5), and 3 h, 6 h, 24 h (GD 10.5) and 48 h (GD 11.5) after vehicle/Poly (I:C) administration. For this purpose, a thermocouple probe (RET-3 Rectal

Probe for Mice, Bioseb, Montpellier, France) connected to a digital thermometer (BioSeb 8851 K.J.T. Type; Bioseb, Montpellier, France) was inserted at a maximal depth of 9 mm into the rectum and a steady temperature readout was obtained within 10 seconds of the insertion (Mueller et al., 2019).

Further details of how maternal immune activation with Poly (I:C) administration was developed are described below in the **section 3.1.2.1.2**. The information plotted in this section is adapted from the MIA Model Reporting Guidelines Checklist developed by Kentner and colleagues in order to improve the rigor, reproducibility and transparency of the Poly (I:C) MIA model (Kentner et al. 2019).

3.1.2.1.2. CRITICAL INFORMATION FOR THE DEVELOPMENT OF POLY (I:C) ANIMAL MODEL OF MATERNAL IMMUNE ACTIVATION

The information described in this section is adapted from MIA Model Reporting Guidelines Checklist ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) Reporting Guideline & Recommendation. MIA Model Specific Reporting Recommendation (Ketner et al., 2019).

MIA MODEL REPORTING GUIDELINES CHECKLIST

A. STUDY DESIGN.

- **General need for improved reporting in MIA model methods + reporting pilot data:**

- *Details on pilot data***:** 4 experimental groups: (1) Grouped offspring of saline treated mothers (*control*); (2) grouped offspring of Poly (I:C) treated mothers (*MIA*); (3) isolated offspring of saline treated mothers (*SI*); (4) isolated offspring of Poly (I:C) treated mothers (*MIA+SI, double-hit*). Offspring of mothers treated with saline/Poly (I:C) were assigned to the two saline/Poly (I:C) groups, respectively. After weaning, animals were housed individually (social isolation) or in groups or 3-4 individuals. Experimental procedures were analyzed by an experimenter blind to the animals' condition.
    - Ninety-six animals were used for the behavioral evaluation of the double-hit model
        - Within the animals used for the behavioral characterization experiments, two additional pilot batches of animals were used for the Open Field Test and the short-term (2 h) Novel Object Recognition Test, including 45 and 101 animals, respectively. These batches of animals were developed for other projects and were used in the present work for the experiments described.

- A different batch of 64 animals was used for the molecular evaluation of the double-hit model.
- A different batch of 23 female mice was used for the head-twitch response experiments after psilocybin administration.
- A different batch of 139 animals was used for the evaluation of the behavioral effects of the chronic treatment with antipsychotic and/or anti-inflammatory drugs. 64 animals of the total 139 were selected for the corresponding molecular assessment. Notably, for the chronic treatment with antipsychotic and/or anti-inflammatory drugs, only the control and the double-hit groups were used
- Time-line diagrams attached (**figure 3.1** and **figure 3.2**).

B. EXPERIMENTAL PROCEDURES.

➢ **Compounds:**

- *Name of compounds*: Polyriboinosinic-polyribocytidylic acid sodium salt.
- *Catalogue number*: P1530 , Sigma-Aldrich®.
- *Lot number*: #095M4049V (behavioral and molecular characterization of the model). #123M4053V (chronic treatment with antipsychotics and/or anti-inflammatory drugs and head-twitch response after psilocybin administration).
- *Vehicle control used*: saline solution (0.9% NaCl).
- *Route of administration*: Intraperitoneal.
- *Volume administered*: 5 ml/kg.
- *Storage conditions*: Powder at -20^{0}C. Dosing solution freshly prepared each day of administration.
- *Anesthetic*: N/A.

➢ **Housing variables at injection:**

- *Light cycle of animal housing room*: 12h light-dark (light from 08:00 to 20:00, dark from 20:00 to 08:00).
- *Time of the day of injection*: 10:00.
- *Room temperature*: 22 +/- 2 ^{0}C.
- *Did a cage change occur at time of injection*: No.

➢ **Validation of immune activation:**

- *Method used to verify immune activation*: Body weight and temperature monitoring after Poly (I:C) administration.

➢ **Validation of gestational timing:**

- *Method of validating gestational timing*: Gestational timing was validated by vaginal plug detection. The day of vaginal plug detection was considered GD0.5.

C. EXPERIMENTAL ANIMALS.

Provide details of:

- **Species – considerations for appropriate species (mouse, rat, non human primate, other):**
- *Species*: Mouse.
- **Strain – variability in strain can influence model:**
- *Strain*: For the behavioral and molecular characterization of the animal model CD-1® strain was used. A subsample of C57BL/6 mice were used for the head-twitch response experiments to psilocybin administration. For the chronic treatment with antipsychotic and anti-inflammatory drugs C57BL/6 strain was used.
- **Maternal/Offspring Physiological Variables at time of immune challenge – age, body weight:**
- *Maternal age at challenge*: 10 weeks.
- *Maternal body weig*ht: Maternal weight was recorded at GD0.5 (when the vaginal plug was detected) and monitored during pregnancy.
- *Offspring age at challenge*: GD9.5.
- *Offspring sex*: Independent experimental groups of males and females were tested in the behavioral and molecular characterization experiments. For the chronic treatment with antipsychotic and anti-inflammatory drugs, male and female mice were included and analyzed together in each experimental arm.
- *Offspring body weight*: First recorded at weaning.
- **Vendor – even within the same strain, vendor can influence endpoints:**
- *Vendor*: Envigo S.L.
- *Location of Vendor*: Barcelona, Spain.
- *Room/area where animals originated from*: Animal facilities of the University of the Basque Country (room OH 13.2).

D. HOUSING AND HUSBANDRY.

Provide details of:

- **Caging systems:**

- *At breeding:*
  - Material of cage: polycarbonate.
  - Cage dimensions: 15 cm high x 22 cm wide x 45 cm long.
- *After parturition:*
  - Material of cage: polycarbonate.
  - Cage dimensions: 15 cm high x 22 cm wide x 22 cm long.
- *At weaning:*
  - Material of cage: polycarbonate.
  - Cage dimensions: Grouped animals, 15 cm high x 15 cm wide x 22 cm long. Isolated animals, 15 cm high x 12 cm wide x 27 cm long.

- **Animal holding room:**

- *Temperature in room*: 22-25^{0}C.
- *Humidity in room*: 65-70%.
- *Ventilation system*: Yes.
- *Specific pathogen free [SPF]*: No.
- *Are males and females housed in the same or separate room*: Male and female mice were housed in different cages (with the exception of breeding procedures) inside the same holding racks, that were equipped with air filters, ventilation system, and with controlled conditions of light, temperature and humidity.

- **Bedding exchanges/bedding type:**

- *Type of cage bedding used*: Sawdust plus paper nesting material was provided to the grouped animals. Isolated mice were housed without nesting material.
- *Frequency of cage changes per week:*
  - During gestation: Once weekly.
  - During neonatal period: Twice between parturition and weaning (first week after parturition dams and pups were left undisturbed).
  - Following weaning: Once weekly.

- **Breeding – bred on site or timed pregnant, how many different sites (are the same fathers breeding with both experimental and control dams):**

- *Breeding location*: Parental mice used in the breeding procedures and the offspring used in experimental procedures were hosted in the animal facilities of the University of the Basque Country UPV/EHU.

- *Gestational age at shipping*: N/A (breeding procedures were carried out in the animal facilities of the University of the Basque Country UPV/EHU).
- *Biological age of dams*: See above in "C. EXPERIMENTAL ANIMALS".
- *Number of dams bred*:
    - Sixteen dams were successfully mated, and the offspring (96 mice of a total of 196 mice) was used for the behavioral characterization of the double-hit model. The rest of the mice was used in different projects.
    - Nine dams were successfully mated, and their offspring was used for the molecular characterization of the double-hit model (64 mice of a total of 72 mice). The rest of the mice was used in different projects.
    - Twenty-nine dams were successfully mated, and their offspring (a total of 162 mice): (1) 139 mice were used for the behavioral and molecular evaluation after the chronic treatment with antipsychotic and anti-inflammatory drugs. (2) 23 mice were used for the HTR experiments to psilocybin administration.
- *How many times have dams been mated previously*: 0.
- *How many times did the dams mate and not become pregnant*: Dams were mated for 5 days.
- *Are the dams primiparous or multiparous?*: All dams are primiparous.
- *What was the frequency of material handling during the gestational/neonatal period (e.g. cage cleanings, weighing, blood collection manipulations):* Weighting on GD 9.5, cage cleaning once-twice between vaginal plug detection and birth.
- *Biological age of sires:* 10 weeks.
- *Number of sires bred*: Breeding ratio was 1 male : 2 females in every batch generated.
- *How many times have sires been mated previously*: 0.
- *How many times did the sires mate successfully*:
    - CD-1® mice: 16 dams from the initial 20 delivered litters at full term (then the offspring conducted behavioral evaluation or was assigned to other experimental projects); 9 dams from the initial 12 delivered liters at full term (then the offspring were used for the molecular evaluation of the double-hit model) .
    - C57BL/6 mice: 29 dams were successfully mated from the initial 40 (two breeding procedures of 20 females: 10 males each were carried out). A total of 162 pups were born and 139 conducted the chronic treatments with clozapine and/or minocycline, and 23 conducted the head-twitch response experiments.
- *If bred previously, what was the interval between mating times*: N/A.
- *Are sires matched to experimental and control dams*: Sires could mate with a dam treated with saline and then with a dam treated with Poly (I:C), and vice versa.

- *Describe mating design*: 1 male: 2 females.

➢ **Social enrichment – number of cage companions:**

- *Number of cage companions prior to breeding*: Unknown, dams were 10 weeks old when purchased from Envigo SL, and directly mated when arrived to the laboratory facilities.
- *Gestational age when dam separated for parturition*: 10 weeks.
- *Number of cage companions at weaning*: Housed in groups of 3-4 individuals of the same sex or individually.

➢ **Physical enrichment – describe enrichment devices, and when enrichment is in the cage (removed when pups born? Or present throughout study), does the enrichment type change? How frequently?:**

- *Describe what type of enrichment devices (and how many) are included in cage/housing room*: one paper nesting material (fills ¼ of lower cage level). Isolated mice were housed without nesting material.
- *Does enrichment type/access change across study?:* No.

E. SAMPLE SIZE.

Provide details of:

➢ **Maternal N vs offspring N:**

- *What is the total number of dams/litters included in the study:*
    - CD-1® mice: 20 dams/16 litters (behavioral experiments) and 12 dams/9 litters (*in vitro* experiments).
    - C57BL/6 mice: 40 dams/29 litters.
- *What is the total number of offspring per litter included in the study:* All the offspring was included in the study.

➢ **Litter size and sex distribution:**

- *What size was each litter maintained at*: See below in "F. ALLOCATING ANIMALS TO EXPERIMENTAL GROUPS".
- *What age did culling take place at*: See below in "F. ALLOCATING ANIMALS TO EXPERIMENTAL GROUPS".
- *How many males and females were maintained in each litter*: See below in "F. ALLOCATING ANIMALS TO EXPERIMENTAL GROUPS".

➢ **Cross fostering:**

- *Did cross fostering occur*: No.

F. ALLOCATING ANIMALS TO EXPERIMENTAL GROUPS.

- **How many offspring per litter were used in each measure:**
- For the behavioral characterization of the double-hit model in CD-1® mice, 196 pups were born to the 16 dams successfully pregnant, of these only 96 were included in the *in vivo* experiments. The rest of the offspring was used in a different project.
- For the *in vitro* experiments with CD-1® mice, 64 mice were selected of a total of 72 pups born to the 9 dams successfully pregnant, of these 72 animals, 64 were included in the *in vitro* experiments. The rest of the offspring was used in a different project.
- Regarding the chronic treatment with antipsychotic and/or anti-inflammatory drugs in C57BL/6 mice, 162 pups were born to the 29 dams successfully pregnant. 139 of them were included in the chronic treatments and in the in vivo evaluation. Of these 139, 64 were selected for the in vitro experiments. 23 were used for the head-twitch response experiments after psilocybin administration. Litters were maintained at natural size and male:female ratios until weaning.
- **Randomization/matching procedures:**
- *What procedures were used to assign animals to groups:* Animals were divided in groups according to their sex and experimental condition (maternal challenge, isolation). This was made in a way that ensured balanced litter representation across groups.
- **Sex as a biological variable (behavioral and physiological outcomes):**
- *Were both males and females evaluated in each behavioral and physiological outcome*: Yes, both male and female mice were included in all the experimental procedures, with the exception of the females used to evaluate the HTR.

G. EXPERIMENTAL OUTCOMES.

- **Maternal behavior and pup interactions:**
- *If maternal care was evaluated, were there differences following immunogen challenge:* Not evaluated. Mothers and pups were left undisturbed to minimize stress and associated disruption of care.
- **Ages(s) of offspring at behavioral testing/physiological evaluation endpoints**: Behavioral testing was carried out when the offspring were between 11 and 16 weeks old.
- **Order of testing (e.g. behavioral test order):**
- *Where animals evaluated in a counter-balanced order in terms of:*

- Presentation of tests to each animal: No. The order of presentation of the behavioral test was the same for all the animals. Less aversive/stressful test were performed in the first place.
- Order of experimental/control groups run through each test: Yes. Experimental/control animals were counter-balanced in order to avoid differences. Experimental/control animals performed each test once.

➢ **What was the inter-test interval if a single animal underwent a battery of tests**: All behavioral experiments were performed between the weeks 11-16, so, the inter-test interval was 4-5 days. OFT to YMSAT: 5 days. YMSAT to NORT: 5 days; NORT to SPT: 4 days. See section 3.3.1**.**

H. STATISTICAL METHODS.

➢ **Unit of analysis for each data set:**

▪ *Is the unit (n) of each analysis based on number of litters, or number of animals used per group*: the unit of each analysis was based on the number of animals used per group.

3.1.2.2. SECOND HIT: POST-WEANING SOCIAL ISOLATION

After weaning, offspring of saline or Poly (I:C) treated dams were housed in individual cages or in groups of 3-4 animals, until the end of the experimental procedures (**figure 3.1**). Thus, Social Isolation (SI) generated the final four groups: grouped offspring of saline treated mothers (control), grouped offspring of Poly (I:C) treated mothers (MIA), isolated offspring of saline treated mothers (SI), isolated offspring of Poly (I:C) treated mothers (MIA+SI, double-hit).

Socially isolated animals were housed individually in the same facilities than the grouped ones. Isolated animals were able to see, smell and hear the rest of the mice, but they were not able to touch them or socially interact with them. Isolated mice were housed in smaller cages (15 cm high x 12 cm wide x 27 cm long) than the grouped ones (15 cm high x 15 cm wide x 22 cm long) and without nesting material. Social isolation was performed right after the weaning (when mice were 21 days old, PND 21) and had a minimum duration of 8 weeks (PND 77). Behavioral testing started when animals were 11 weeks old (PND 80), and during these experiments isolation was maintained. Isolation conditions were chosen based on preliminary experiments and previous literature data (Fone & Porkess, 2008; Strauss et al., 2014).

3.1.2.3. DOUBLE-HIT MODEL CHARACTERIZATION

Ninety-six CD-1® male and female mice were generated for the behavioral evaluation of the double-hit model. All the 96 mice (48 males and 48 females) were used in the behavioral experiments (12 animals/experimental group/sex). For the acute amphetamine administration, a subsample of 48 mice (24 males and 24 females) was selected (6 animals/experimental group/sex). For the acute psilocybin administration, a different batch of 23 C57BL/6 female mice was used, only belonging to the saline-grouped (control) and MIA+SI (double-hit) experimental groups (11-12 animals/experimental group). Finally, another batch of 64 animals (32 males and 32 females) was generated for the *in vitro* experiments (8 animals/experimental group/sex) (**figure 3.1**).

BEHAVIORAL AND MOLECULAR CHARACTERIZATION OF THE DOUBLE-HIT ANIMAL MODEL

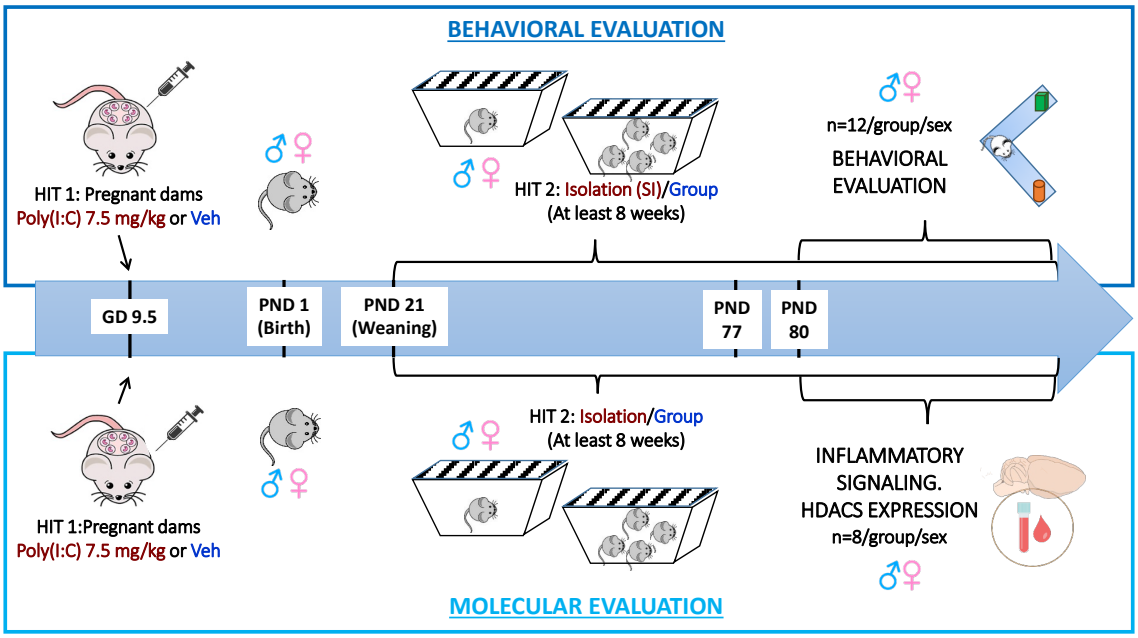


Figure 3.1: Double-hit model characterization. Developmental protocol to generate the schizophrenia double-hit mouse model in male and female mice proposed in this work. Experimental groups generated and validated are shown. Abbreviations: GD= Gestational Day. HDAC= Histone Deacetylase. PND= Postnatal Day. Poly (I:C)= Polyinosinic:polycytidylic acid. Veh= Vehicle.

3.1.2.4. PHARMACOLOGICAL TREATMENTS IN THE DOUBLE-HIT MODEL

For the chronic treatment with antipsychotic and/or anti-inflammatory drugs, another batch of double-hit animals was generated. In this case, C57BL/6 mice were used, and only saline-

grouped (control) and MIA+SI (double-hit) experimental groups were evaluated. A total number of 139 animals were used in these experiments. These animals were divided into four different pharmacological treatments: “Vehicle”, “Clozapine”, “Minocycline”, “Clozapine + Minocycline” (for further details see **section 3.2.3.2**.). 139 mice conducted the behavioral experiments (15-21 animals/experimental group/pharmacological treatment), and for the gene expression experiments, a subsample of 64 animals was selected (8 animals/experimental group/pharmacological treatment). In these experiments, each experimental group was made up of both males and females (**figure 3.2**).

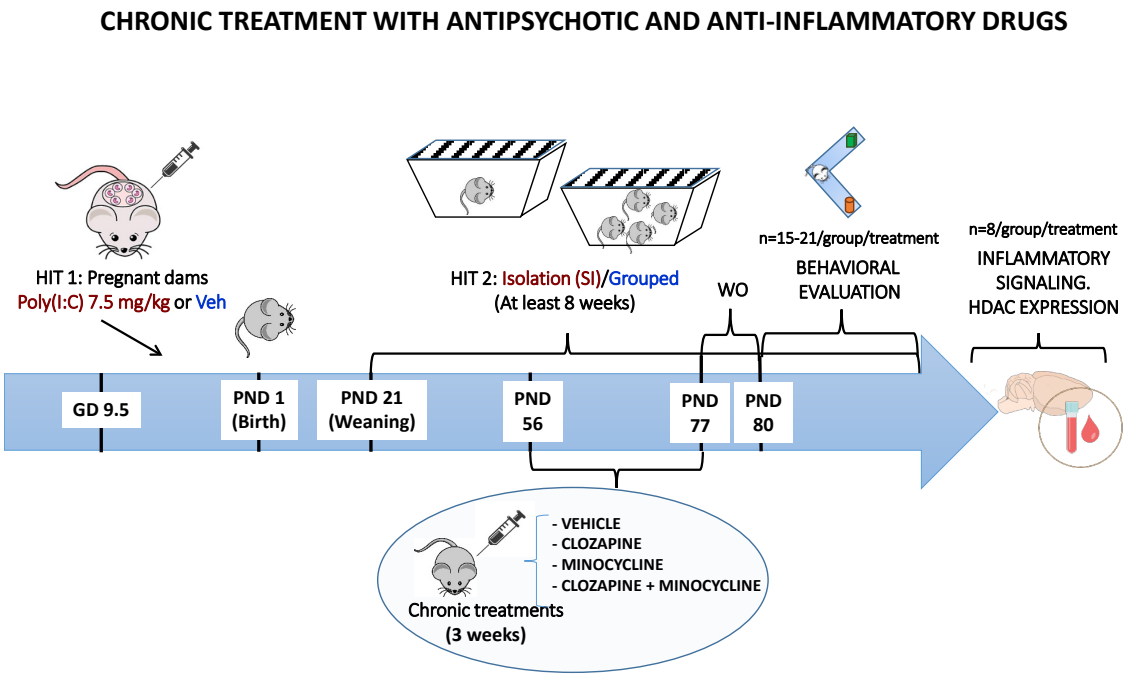


Figure 3.2: Pharmacological treatments in the double-hit model. Developmental protocol to generate the schizophrenia double-hit mouse model proposed in this work. Experimental groups generated along the project and the pharmacological treatments proposed to reverse behavioral and molecular alterations are shown. Abbreviations: GD= Gestational Day. HDAC= Histone Deacetylase. PND= Postnatal Day. Poly (I:C)= Polyinosinic:polycytidylic acid. Veh= Vehicle. WO= Wash-out.

3.2. MATERIALS

3.2.1. PRIMERS AND PROBES

TaqMan® gene expression assays were used (Applied Biosystems™, California, USA) for the quantitative real time polymerase chain reaction (RT-qPCR) gene expression assays with mice brain cortex samples. A detailed description of these gene expression assays is provided in section **3.3.2.2.3**.

3.2.2. ANTIBODIES

For western blot experiments, primary monoclonal antibodies purchased from Santa Cruz Biotechnology, Inc (California, USA) were used. A further description of these primary antibodies is provided in section **3.3.2.3.2**. Regarding the fluorescent secondary antibodies used for western blot assays, the following ones were used: Anti-rabbit Alexa Fluor® 680 conjugated in goat, purchased from Invitrogen (Oregon, USA); and Anti-mouse IRDye™ 800 conjugated in donkey, provided by Rockland Immunochemicals (Pensylvania, USA). For more information regarding secondary antibodies see also section **3.3.2.3.2**.

3.2.3. PHARMACOLOGICAL TREATMENTS

3.2.3.1. ACUTE TREATMENTS

3.2.3.1.1. AMPHETAMINE

Alpha-methyl-pheniletylamine: Catecholamine reuptake blocker and inducer of catecholamine release (Sigma Aldrich®, St. Louis, MO, USA). Amphetamine was dissolved in saline solution (0.9% NaCl) and administered at a dose of 5 mg/kg i.p (volume 5 ml/kg).

3.2.3.1.2. PSILOCYBIN

O-phosphoryl-4-hydroxy-N,N-dimethyltryptamine (and its active dephosphorylated metabolite psilocin, N,N-dimetyltryptamine): Psychoactive alkaloid contained in hallucinogenic mushrooms (THC Pharm, Frankfurt, Germany). Psilocybin was dissolved in saline solution (0.9% NaCl) and administered at a dose of 1 mg/kg i.p. (volume 5 ml/kg).

3.2.3.2. CHRONIC TREATMENT WITH CLOZAPINE, MINOCYCLINE AND COMBINATION OF BOTH DRUGS

C57BL/6 mice were used for the chronic treatment with the atypical antipsychotic clozapine, the inhibitor of the inflammatory activity minocycline and its combination. In these experiments, each experimental group was made up of both males and females. Mice belonging to control (n=18-21) and double-hit experimental groups (n=15-16) were used for each treatment arm.

Clozapine was dissolved in saline solution (NaCl 0.9%) plus acetic acid. As clozapine is a lipophilic compound, it had to be dissolved in an acidic aqueous environment (pH=2), after what the solution was alkalinized with 6N NaOH until a pH of 5 was reached. During this process, sonication, agitation, and heating were used to prevent the formation of drug precipitates. Minocycline was dissolved in saline solution (NaCl 0.9%), and dissolved with heating and agitation to prevent the formation of precipitates.

Control and double-hit mice were injected intraperitoneally (volume 5 ml/kg) with vehicle, clozapine (10 mg/kg/day) and/or minocycline (30/mg/kg/day) during 21 days. Daily dosages were administered every 24 hours. Independently of the treatment group, each animal received 2 injections per day (vehicle + vehicle, vehicle + drug or drug + drug). Of note, the vehicle solutions injected were prepared in the same way as the solutions to dissolve the respective drugs. The selected doses of each drug were chosen from literature (Clozapine: Ibi et al., 2017. Minocycline: Bassett et al. 2021; Wang et al., 2020).

Thus, the generated groups were the following: (1) Control mice treated with vehicle. (2) Control mice treated with clozapine. (3) Control mice treated with minocycline. (4) Control mice treated with clozapine + minocycline. (5) MIA + SI (double-hit) mice treated with vehicle. (6) MIA + SI (double-hit) mice treated with clozapine. (7) MIA + SI (double-hit) mice treated with minocycline. (8) MIA + SI (double-hit) mice treated with clozapine + minocycline. Experimental groups and animals used per group are described in the following table (**table 3.2**):

Table 3.2: Summary of the experimental groups and drug combinations used in the chronic treatment with clozapine and minocycline.

Control			
Vehicle	Clozapine	Minocycline	Clozapine + Minocycline
n= 19	n= 18	n= 18	n= 21
MIA + SI (double-hit)			
Vehicle	Clozapine	Minocycline	Clozapine + Minocycline
n= 16	n= 16	n= 15	n= 16

After a wash-out period of 48h of the last administration of vehicle, clozapine, minocycline or clozapine + minocycline, behavioral evaluation of mice was performed by means of the Novel Object Recognition Test and Social Preference Test. Then, mice were sacrificed and brain cortices were removed and processed for the gene expression experiments. 24-48h lasted since the last behavioral test and the sacrifice (see **figure 3.2**).

3.2.4. OTHER DRUGS AND CHEMICALS

Suppliers and distributors for specific reagents and chemicals will be described throughout the text. Thus, general drugs and chemicals used and providers are described in this section:

- Agilent technologies, Inc (Waldbronn, Germany): Agilent RNA 6000 Nano Kit, RNA 6000 Nano Ladder.
- Applied Biosystems (Foster City, CA, USA): Fast Taqman™ fast universal PCR Master Mix, High Capacity cDNA Reverse Transcription Kit.
- Bio-Rad Laboratories (Hercules, CA, USA): Ammonium persulfate (APS), Bradford Protein Assay, Laemmli buffer, N-N-N-N'-tetramethylenediamine (TEMED) and prestained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) standards.
- Carlo Erba Reagents (Barcelona, Spain): Methanol.
- GE healthcare (Buckinghamsire, UK): Nitrocellulose membranes and Whatman™ cellulose papers 3MM.
- Invitrogen (Barcelona, Spain): DL-Dithiothreitol (DTT), ethylenediamine tetracetic acid (EDTA), Ribopure™ mRNA extraction kit.

- Millipore (Burlintong, MA, USA): MILLIPLEX® MAP Mouse High Sensitivity T Cell Magnetic Bead Panel #MHSTCMAG-70K.
- National diagnostics (Atlanta, GA, USA): Acrylamide 30% - bisacrylamide 0.8%.
- Panreac S.A.U. (Barcelona, Spain): Glacial acetic acid, sucrose and HCl (37%).
- Santa Cruz Biotechnology, Inc (Dallas, Texas, USA): Deacetylation Inhibition Cocktail (ChemCruz®).
- Sigma-Aldrich (Saint Louis, Missouri, USA): 1-Bromo-3-chloropropane (BCP), Bovine serum albumin (BSA), glycine, β-mercaptoethanol, $MgCl_2$, NaCl, NaF, Na_3VO_4, polyoxyethylene (20) sorbitan monolaurate (Tween 20), Protease Inhibitor Cocktail, Tris (2-Amino-2-(hydroxymethyl)-1,3-propanediol) hydrochloride (Tris HCl).
- Water was purified by a Milli-Q Gradient system (Burlintong, Milford, MA, USA).

3.3. METHODS

3.3.1. BEHAVIORAL TESTS

The behavioral tests performed in the present work included: i) the Open Field Test (OFT), ii) the Y-Maze Spontaneous Alternation Test (YMSAT), iii) the Novel Object Recognition Test (NORT), iv)the Social Preference Test (SPT), v) the locomotor response to acute amphetamine administration and vi) the head-twitch response to acute psilocybin administration.

For the double-hit model characterization in CD-1® mice, behavioral evaluation was performed in the following order: first OFT, then YMSAT, then NORT, and finally, SPT. After these behavioral tests, amphetamine was administered to a subsample of mice. Psilocybin administration was performed in an independent batch of C57BL/6 animals. Regarding the behavioral evaluation after the chronic treatment with clozapine and minocycline, only NORT and SPT were performed, in this very order.

For all behavioral assessments, mice were placed into a sound-attenuated room containing the appropriate testing apparatus under the selected illumination conditions for at least 1 h prior to the session. Experimental procedures were conducted between the second and eighth hour of the light phase of the light/dark cycle and were videotaped by a camera set up on the roof of the experimental room in the absence of the researcher, who was blind to the particular experimental condition of the animals before video quantifications.

All the behavioral tests were performed with controlled experimental conditions as it follows:

- Light: The room was equipped with a set of fluorescent ceiling lights with an automatic system that allowed to adjust the lighting conditions to be appropriate for each experiment. To measure the intensity of the light inside the room, a lux-meter was placed in the center of the apparatus were the animals were tested each time.
- Experiment recording: Every trial was recorded by a camera set up on the roof of the experimental room, just above the place were mice were tested. The recording camera was a Sony Color Video Camera SSG-G118, AC 220-240V Hz, Sony Europe Limited, Surrey, UK. Recorded videos were analyzed by means of the SMART Video Tracking software (PanLab/Harvard Apparatus), or by an investigator blind to experimental animal identity by means of the home-made software Behavior Scoring Panel© 2008 by A. DUBREUCQ Version 3.0 beta.

- Cleaning considerations: Male mice were always tested first to avoid them to be distracted by female's corporal smell. After each test session/trial, every apparatus used was washed with 70% ethanol and allowed to dry before placing the next animal.

3.3.1.1. OPEN FIELD TEST (OFT)

OFT is a widely used behavioral test that evaluates locomotor and anxiety-like behaviors in mice (Seibenhener & Wooten, 2015). OFT was assessed in a dark wooden square-shaped maze (45 cm x 45 cm x 40 cm) (**figure 3.3**). Mice performing the OFT required no previous training. Locomotor activity and anxiety-related behaviors were analyzed in 10 minute sessions in the OFT. Total distance traveled (in centimeters) was determined to visualize any differences in locomotor activity between subjects. Time spent (in seconds) in the periphery and in the center of the OFT arena was also determined. Time spent in the periphery or outer zone of the maze, also known as thigmotaxis, is a validated measure of anxiety-related behavior (Simon et al., 1994). Periphery (45 cm x 45 cm) and center (20 cm x 20 cm) zones of the OFT arena were determined by means of the SMART Video Tracking software (PanLab/Harvard Apparatus), that automatically recorded and evaluated mouse movement. The intensity of the light inside the apparatus was settle in 100 lux to ensure anxiogenic conditions.

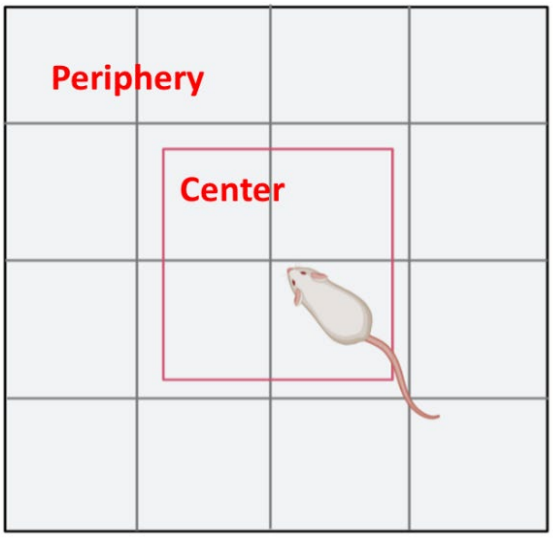


Figure 3.3: Schematic representation of the OFT, that evaluated the locomotor and anxiety-related behaviors. Total distance moved and time spent in the periphery/center of the maze was determined.

3.3.1.2. Y-MAZE SPONTANEOUS ALTERNATION TEST (YMSAT)

Spontaneous alternation behavior comprises the tendency for mice to alternate their (conventionally) non reinforced choices of Y-maze arms on successive opportunities. A validated approach to assess spatial working memory in rodents is to measure the spontaneous alternation behavior in the YMSAT (Hughes, 2004; Ibi et al., 2017). Hence, YMSAT was assessed in a Y-shaped-maze as previously reported (Ibi et al., 2017). Each arm of the maze was identified with a geometrical figure of different shape and color, so that the mice were able to distinguish one arm from another (**figure 3.4**). The arms converged in an equilateral triangular central area. The intensity of the light inside the apparatus was settle in 25 lux.

On the day of the experiment, mice were placed in the maze and the entrance in each arm was scored for 8 min. An entry was considered when the mouse had all four paws in one single arm. A Spontaneous Alternation refers to a triplet of consecutive arm explorations with all three arms being different. Accordingly, the number of correct triplets (exploration of three different consecutive arms) were counted to calculate the percentage of alternation using the following formula: *% Spontaneous Alternation= (Number of Correct Triplets/ Total Number of Triplets) x100*; being the Total Number of Triplets the total number of entries minus two.

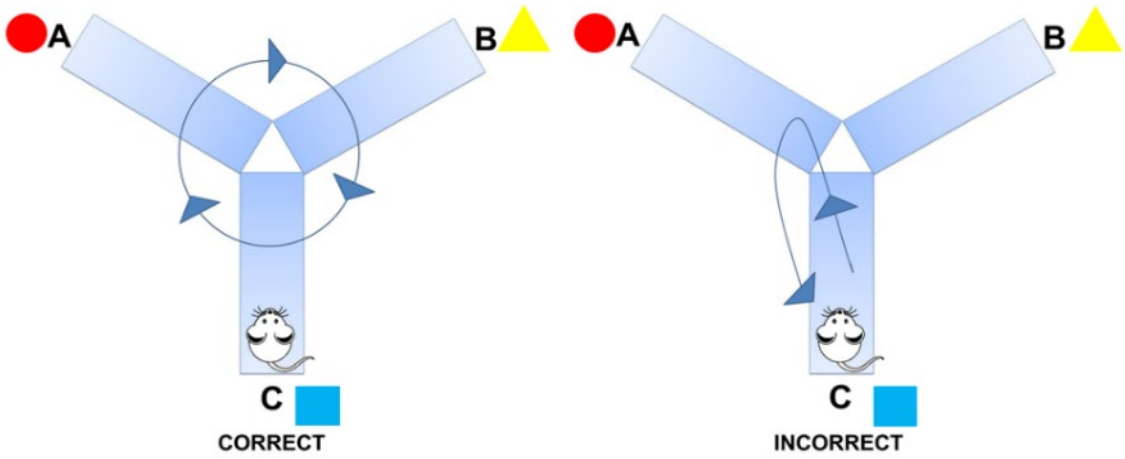


Figure 3.4: Schematic representation of the apparatus where the YMSAT was performed. A correct triplet corresponds to the consecutive exploration of three different arms. Correct and incorrect triplets are shown in the image.

3.3.1.3. NOVEL OBJECT RECOGNITION TEST (NORT)

NORT is a commonly used experimental behavioral task that evaluates the episodic-like declarative memory and it relies on the innate tendency of rodents to explore novelty (Oliveira da Cruz et al., 2020). In this memory task, in the presence of a novel and a previously presented (familiar) object, rodents increase their exploration towards the novel object (Ennaceur & Delacour, 1988). NORT was performed in an L- shaped maze made by two identical perpendicular arms as previously described (Oliveira da Cruz et al., 2020) (**figure 3.5**). Light intensity inside the maze was fixed at 40 lux.

The NORT procedure consisted of the following phases of 9 minutes each:

- In the habituation phase, mice were able to explore the maze in the absence of any objects.
- After 24 hours, in the training phase, mice explored the maze in the presence of two identical objects positioned at the end of each arm. After the training phase, two different timings were chosen to proceed with the next phase, namely “test phase” in order to asses either short-term or long-term episodic-like declarative memory:
    - In an independent pilot group of animals, short-term episodic-like declarative memory was evaluated 2 hours after the training phase. In this test phase (2 hours after the training phase), mice were able to explore two objects: one of them was identical to the one used in the training phase (familiar object) and the other one was new (novel object).
    - In a different set of animals, long-term episodic-like declarative memory was evaluated in the test phase (24 hours after the training phase). In this phase, mice were also able to explore a familiar object (same object than the one used in the training phase) and a novel object (different object than the one used in the training phase).

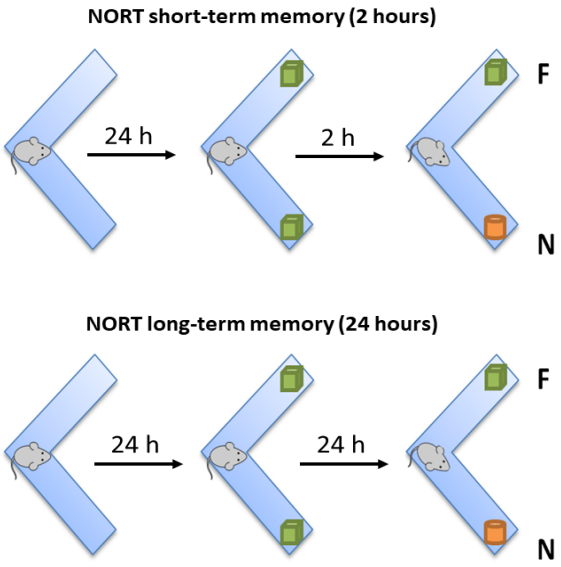


Figure 3.5: Schematic representation of the L-shaped maze used where the NORT experiments were performed. F: Familiar object. N: novel object.

All objects were previously tested to avoid biased preference. Selected objects were different enough from each other (in color tonality, shape and texture) in order to facilitate the discrimination between objects in the test session and include Lego bricks, glass bottles and rubber ducks.

Exploration of an object by a mouse was defined as "directing the nose toward the object at a distance less than or equal to 2 cm" (Leger at al., 2013). Only climbing over or leaning on an object was not considered an explorative behavior. In the test phase, familiar object exploration time (measured in seconds) was defined as the time that the mouse spent exploring the familiar object. Novel exploration time (in seconds) was considered the time that the mouse spent exploring the new object. Total exploration time (in seconds) was calculated as the time that mice spent exploring both novel and familiar objects. Discrimination Index (DI) of the NORT was calculated as the relationship between novel and familiar exploration times as follows: *(Time devoted to the novel object - Time devoted to the familiar object)/ (Time devoted to the novel object + Time devoted to the familiar object)*. DI is the parameter for the evaluation of cognitive impairment (Oliveira da Cruz et al., 2020). Positive DI values (greater than 0) indicate a good discrimination between objects. For example, DI scores greater than 0.2 are considered as high scores, meaning that the novel object was explored 60% of the time and the familiar object 40% of the time.

In the recordings, exploration time during training and test phases (both 2 hours and 24 hours after the training phase) was evaluated manually by means of the home-made software Behavior Scoring Panel-© 2008 by A. DUBREUCQ Version 3.0 beta. Exploration was scored when the animal nose was facing directly within 2 cm of the object and the vibrissae were moving towards. However, exploration was not scored if the animals did not interact with the object or if the animals were standing on the top of the object without facing those (Broadbent et al., 2009, Oliveira da Cruz et al., 2020).

3.3.1.4. SOCIAL PREFERENCE TEST (SPT)

SPT capitalizes on the natural tendency of rodents to interact with each other, considering a lack of interaction as social withdrawal, reflecting the negative symptoms of schizophrenia (Osborne et al., 2017). SPT was assessed in a three-chamber apparatus following a methodology adapted from previous literature (Faure et al., 2019; Wei et al., 2015). The apparatus was a rectangular box made from black Plexiglas, which was divided in three compartments of equal surface by Plexiglas walls. In both lateral compartments, a metallic cylinder was located (**figure 3.6**). Light intensity inside the three-chamber apparatus was set in 25 lux.

The SPT consists into two consecutive phases:

1. First, during the 5-minute habituation phase, the “test mouse” was placed in the center compartment and was free to visit the three empty chambers.
2. During the following 10 min phase, one cylinder was located in each lateral chamber and a “stimulus mouse” of the same age, strain and sex was placed inside one of the cylinders as a social reward. Cylinders had multiple holes to allow breathing, and nose pokes from both mice. The other cylinder was left empty and the “test mouse” was allowed to explore the entire apparatus.

“Stimulus mouse” side placement was counter-balanced between trials. Mice used as “stimulus mouse” were previously trained to get used to the cylinder in order to avoid stress during the trials.

Exploration was considered when the “test mouse” was facing the cylinder with the nose and vibrissae towards it, or when the “test mouse” had the paws on the cylinder. Social exploration time (measured in seconds) was defined as the time that the “test mouse” devoted exploring the cylinder containing the “stimulus mouse”. Non-social exploration time (seconds) was defined as the time the “test mouse” spent exploring the empty cylinder. SPT total exploration

time (seconds) was defined as the time that the "test mouse" spent exploring both the cylinder containing the "stimulus mouse" and the empty one. In order to measure social preference, Social Index was calculated as follows: *(Time devoted to the social exploration - Time devoted to the non-social exploration)/ (Time devoted to the social exploration + Time devoted to the non-social exploration)*.

Once videos were recorded, social, non-social and total exploration times were quantified by means of the Behavior Scoring Panel-© 2008 by A. DUBREUCQ Version 3.0 beta.

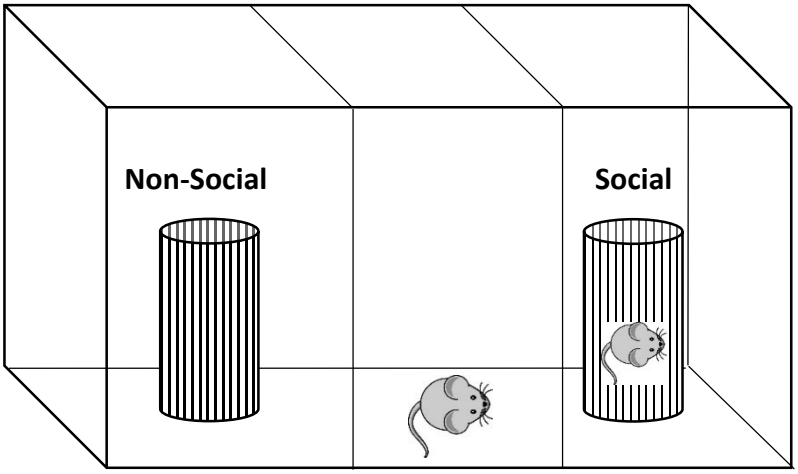


Figure 3.6: Schematic representation of the three-chamber apparatus used in the SPT. Two cylinders were placed in the lateral rooms of the apparatus, one of them was empty (non-social) and the other one contained the "stimulus mouse" (social). "Test mouse" was allowed to explore the three chambers of the SPT apparatus.

3.3.1.5. LOCOMOTOR EVALUATION TO AMPHETAMINE ADMINISTRATION

In animal studies, administration of the psychostimulant drug amphetamine and related compounds stimulates hyperlocomotion and stereotypy (Kokkinidis & Anisman, 1980; Sharp et al., 1987).

Locomotor activity after amphetamine administration was assessed as previously described with minor modifications (Zager et al., 2012). For this purpose, an actimeter (PanLab, SL, Barcelona, Spain) was used, a squared cage with a floor containing sensors that detect and record movements. Mice were individually placed in the apparatus and allowed to habituate for 20 minutes (in order to mitigate novelty-induced effects) before a 100 min test session. Basal locomotor activity was registered during 20 minutes after habituation. Then, amphetamine was intraperitoneally administered (5 mg/kg in 5 ml/kg volume) (Zager et al., 2012) and locomotor

activity was recorded every 20 minutes for 80 minutes after injection. Locomotor activity after amphetamine administration was expressed as percentages of basal locomotion values (% of number of steps).

3.3.1.6. HEAD-TWITCH RESPONSE INDUCED BY PSILOCYBIN ADMINISTRATION

The head-twitch response (HTR) is a rapid side-to-side rotational head movement that occurs in rats and mice after administration of hallucinogenic drugs, which act as serotonergic 5-HT_{2A}R agonists. HTR is widely used as a behavioral assay for 5-HT_{2A}R activation and psychotic response (see **section 1.4.2.1.**, Moreno et al., 2012; Halberstadt & Geyer, 2011).

In order to carry out this assay, mice were individually placed into wooden open field cages in the test room. Twenty minutes after the intraperitoneal administration of 1 mg/kg psilocybin, animals were videotaped for 25 minutes. Afterwards, the recordings were examined for head twitch events quantification.

3.3.2. IN VITRO ASSESSMENTS

3.3.2.1. MOUSE PLASMA AND BRAIN CORTEX OBTAINING

Mice were euthanized by cervical dislocation. On the one hand, for the plasma collection, blood samples were harvested via cardiac puncture right after cervical dislocation and then processed for the ELISA Luminex ® immunoassays as described below (see section **3.3.2.2**). On the other hand, brains were carefully removed from the skulls to separate the brain cortices, which were immediately frozen at -80^{0}C. Cortices were divided into two halves, and right and left cortices were randomly assigned to RT-qPCR and western blot experiments. Cortical samples were processed for gene and protein expression assessment as described below (see **sections 3.3.2.3.** and **3.3.2.4.**, respectively).

3.3.2.2. PERIPHERAL CYTOKINE MEASUREMENT BY MEANS OF ELISA LUMINEX® IMMUNOASSAY

3.3.2.2.1. PLASMA SAMPLE PREPARATION

For the plasma collection, blood samples were harvested via cardiac puncture right after cervical dislocation. 1% dipotassium ethylenediamine tetraacetic acid (EDTA) solution was added as anti-coagulant to the blood samples and those were maintained at 4^{0}C until centrifugation procedure. Within the 2-3 hours after blood extraction, samples were centrifuged for 20 minutes

at 4^{o}C and 2000 x g. Supernatant (plasma) was transferred to another tube and centrifuged at high speed (4^{o}C, 20000 x g for 5 minutes, twice) to separate lipid content. Finally, plasma samples were split into 60 µl aliquots—to avoid multiple freeze/thaw cycles—and stored at -80^{0}C.

3.3.2.2.2. ELISA LUMINEX® IMMUNOASSAY

A high sensitivity multiplex immunoassay purchased from Millipore (MILLIPLEX® MAP Mouse High Sensitivity T Cell Magnetic Bead Panel #MHSTCMAG-70K, Burlintong, MA, USA) was used —following manufacturers' protocol— for the simultaneous quantification of the following cytokines in mouse plasma: IL-6, IFN-γ, TNFα, IL-17A, IL-1β, IL-2 and IL-10. This kit is based on the Luminex ® technology.

The experiment day, plasma samples were thawed completely and mixed well by vortexing and cytokines were measured by Luminex® technology using magnetic bead-based multiple immunoassays. Assays were performed in 96-well plates. Each plate included a calibration curve for each of the cytokines to be measured. Calibration ranges were: IL-6, 0.997-4000 pg/mL; IFN-γ, 0.488-2000 pg/mL; TNFα, 0.488-2000 pg/mL; IL-17A, 0.488-2000 pg/mL; IL-1β, 3.91-16000 pg/mL; IL-2, 0.997-4000 pg/mL; IL-10, 1.34-5500 pg/mL. Two quality controls were also included in all the assays. All samples were run in duplicate. Plates were read on a Luminex 100™ apparatus (Luminex Corporation Austin, TX, USA). Plasma concentration of each cytokine was calculated though a 5-parameter logistic curve-fitting method using the XPONENT® software (Luminex Corporation). For the data analisys, the values of those samples that were below assay range (Out of Range <OOR) —analyte level below the limit of detection— were replaced by the lower detection value for each of the cytokines assessed.

3.3.2.3. REVERSE TRANSCRIPTION QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (RT-qPCR)

3.3.2.3.1. RNA EXTRACTION AND PURIFICATION

Commercial Ambion RiboPure™ kit (Invitrogen), was used for the purification of the RNA extracted from mouse cortex, following the manufacturer specifications.

For tissue disruption, TRIzol® Reagent was used, which is composed of phenol and guanidine thiocyanate in a monophasic solution. Mouse cortex samples (weighting 80 mg approximately)

were homogenized with a grinder in 1 ml of TRIzol® Reagent, then, the homogenate was incubated for 5 min at room temperature (RT). After that, 100 µl of bromochloropropane (BCP) were added. Then, tubes were vortexed, incubated 2-3 min at RT and centrifuged at 12,000 x g for 15 min at 4^0C. After centrifugation, the mixture was separated into a lower, red, organic phase; an interphase; and an upper, colorless, aqueous phase containing the RNA (**figure 3.7**). Subsequently, 400 µl of the aqueous phase were transferred to another tube, mixed with 400 µl of 70% ethanol and vortexed.

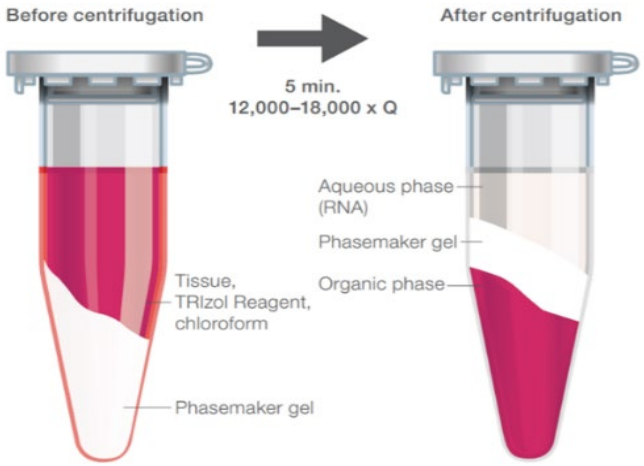


Figure 3.7: Tissue homogenization with TRIzol® Reagent, before and after centrifugation.

After that, 700 µl of the mixture were transferred to a filter cartridge-collection tube assembly and centrifuged at 12,000 x g for 30 seconds at RT, so that RNA was bound to the filter cartridge. The filter was rinsed three times with two different washing solutions and finally the RNA was eluted with 100 µl of RNAse-free water into a Recovery Tube. Finally, mRNA samples were stored at -80^0C. RNA extraction and purification process is schematized in **figure 3.8**.

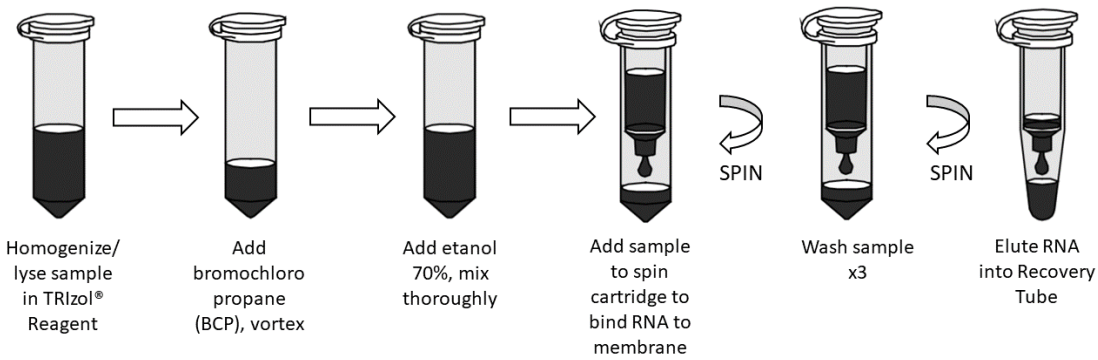


Figure 3.8: RNA extraction and purification process, step by step.

Total mRNA concentration was determined using a Nanodrop® RD-1000 spectrophotometer (Thermo Fisher Scientific, IL, USA). 260/280 and 260/230 ratios of absorbance were also measured by means of the Nanodrop® RD-1000. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA, a value of 2 indicates that the sample is pure. The 260/230 ratio is used as a secondary measure of nucleic acid purity, with an expected value between 2-2.2. Concentrations of the RNA samples included in these experiments were 300 - 500 ng/µl, with 260/280 and 260/230 ratio values of 2-2.2 and 2, respectively. Samples with lower concentrations than 300 ng/µl and lower 260/280 and 260/230 ratios than 2 were excluded.

Moreover, RNA Integrity Number (RIN) was assessed by means of the 2100 Bioanalyzer (Agilent Technologies). The Agilent Expert software allocates a score from zero to 10 to each trace based on the ribosomal RNA peaks and the extent of RNA degradation products. It assigns a number according to how much signal is found between the 5S and 18S band, between the 18S and 28S bands, and after the 28S band. Thus, the RIN number is a measure for the quality of the mRNA extracted. A RIN number of 10 is the maximal score, and only samples with a RIN score ≥ 8 were selected. All RNA samples were assayed for RIN values in the Agilent 2100 Bioanalyzer using Agilent RNA nano chips and Agilent RNA6000 Nano Kit (Applied Biosystems™, Waldbronn, Germany) (Fleige & Ptaffl, 2006; Fleige et al., 2006; Trabzuni et al., 2011).

3.3.2.3.2. REVERSE TRANSCRIPTION

Retrotranscription was performed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems™, California, USA) in 96 well plates, using a StepOne™ System (Applied Biosystems™, California, USA).

For that purpose, 1 µg of RNA sample was set in final volume of 10 µl and then 10 µl of 2X Reverse Transcription Master Mix (containing primers, nucleotides, reverse transcriptase and buffer) were added (**table 3.3**). Moreover, **table 3.4** shows the experimental conditions (temperature, time) of the different steps of the retrotranscription process. A negative control with DEPC-treated water (commercially available nuclease-free water) instead of RNA was included in each plate as negative control, to verify there was not any contamination.

Table 3.3: Experimental conditions for High Capacity cDNA Reverse Transcription Kit.

Component	Volume/Reaction (µl)	Final Concentration
10X RT Buffer	2,0	1X
25X dNTP Mix (100 nM)	0,8	4mM
10X RT Random Primers	2,0	1X
Multiscribe™ Reverse Transcriptase	1,0	50 U
DEPC-treated H_2O	4,2	
Final volume (Mix)	10	
RNA (1 µG)	10	50 ng/µl
Final volume (well)	20	

Table 3.4: Experimental conditions for reverse transcription.

	Enzyme Activation	Retrotranscription	Enzyme Inactivation	Infinite Holding
Temperature	25 ^{0}C	37^{0}C	85^{0}C	4^{0}C
Time	10 min	120 min	5 sec	∞

Two retrotranscription reactions (duplicates) were performed for each RNA sample, in order to minimize potential variability in the retrotranscription process. Total cDNA obtained from the two retrotranscription processes was mixed and diluted to a 10 ng/µl concentration with DEPC-treated water. Samples were stored at -20^{0}C until use.

3.3.2.3.3. qPCR WITH TAQMAN® GENE EXPRESSION ASSAYS

Quantitative real-time Polymerase Chain Reaction (qPCR) experiments were carried out with a StepOne™ System (Applied Biosystems™, California, USA). For that purpose, TaqMan® gene expression assays were used in the following qPCR experiments with the cDNA obtained from mouse cortical samples.

First, mRNA expression of the genes encoding for the neuroinflammatory signaling proteins NF-κB (*Rela*), IκBα (*Nfkbia*) and nine different HDACs (*Hdac1, Hdac2, Hdac3, Hdac4, Hdac5, Hdac6, Hdac7, Hdac8, Hdac9*) was evaluated in the first block of experiments for the double-hit model characterization. Then, changes in the gene expression of the genes encoding for NF-κB (*Rela*),

IκBα (*Nfkbia*), HDAC2 (*Hdac2*), HDAC4 (*Hdac4*), HDAC8 (*Hdac8*), IL-6 (*Il6*), IFN-γ (*Ifng*) and 5-HTR_{2A}R (*Htr2a*) were evaluated in the second block of experiments, after the chronic treatment with clozapine and/or minocycline.

Every sample used in qPCR assays was triplicated in MicroAmp® Fast Optical 96-Well Reaction Plates (Applied Biosystems™, California, USA). Each well in the optical plate contained a final volume (reaction) of 5 µl, including 2 µl of cDNA sample (20 ng) and 3 µl of the reaction mix shown in **table 3.5**.

Table 3.5: Experimental conditions for RT-qPCR in cDNA from mouse brain samples.

Reagent	Volume (µl)	Concentration
2X TaqMan® Fast Universal Master Mix	2,5	1X
TaqMan® Gene Expression Assay	0,25	900 nM
DEPC-treated H2O	0,25	
Final Volume (mix)	3	
cDNA (sample)	2 (20 ng/well)	10 ng/µl
Final Volume (well)	5	

Experimental conditions (temperature, time) of the Fast RT-qPCR protocol carried out in cDNA from mouse brain samples are described in **table 3.6**:

Table 3.6: Protocol for Fast RT-qPCR using TaqMan® gene expression assays.

	Holding stage	Cycling stage: 40 cycles	
	Initial denaturation	**Denaturation**	**Amplification**
Temperature (^{0}C)	95	95	60
Time (sec)	20	1	30

Each TaqMan® gene expression assay included forward and reverse primers (900 nM final concentration) and a FAM™ dye-labeled probe (250 nM final concentration). Only one mRNA was amplified in each PCR reaction (singleplex).

In all the qPCR experiments, relative mRNA levels of each target gene were normalized with the expression of two genes used as endogenous controls (also known as housekeeping genes), as

their gene expression does not variate between samples. Reference genes used in qPCR assays were Glyceraldehyde-3-Phosphate Dehidrogenase (*Gapdh*) and Ribosomal Protein S29 (*Rps29*). See **table 3.7** for predesigned TaqMan® gene expression assays used in each experiment of qPCR. Also, a reference sample (pool) corresponding to a pool of cDNA from a representative portion of the samples used in the experiment, was included in all qPCR experiments to correct the inter-experimental variability in the posterior analyses. With this second correction, it was possible to neutralize inter-plate technical variations. A summary of all the TaqMan® assays used in the gene experiments is plotted in **table 3.7**:

Table 3.7: TaqMan® assays used in the RT-qPCR experiments.

Gene	Gene Name	Interrogated Sequence (NCBI)	Amplicon Length (bp*)	Species	Assay ID
Rela	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	NM_009045.4	67	Mouse	Mm00501346_m1
Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha	NM_010907.2	70	Mouse	Mm00477798_m1
Hdac1	histone deacetylase 1	NM_008228.2	67	Mouse	Mm02391771_g1
Hdac2	histone deacetylase 2	NM_008229.2	90	Mouse	Mm00515106_m1
Hdac3	histone deacetylase 3	NM_010411.2	71	Mouse	Mm00515916_m1
Hdac4	histone deacetylase 4	NM_207225.1	110	Mouse	Mm01299557_m1
Hdac5	histone deacetylase 5	NM_001077696.1	81	Mouse	Mm01246076_m1
Hdac6	histone deacetylase 6	NM_001130416.1	95	Mouse	Mm00515945_m1
Hdac7	histone deacetylase 7	NM_001204275.1	65	Mouse	Mm00469527_m1
Hdac8	histone deacetylase 8	NM_027382.3	85	Mouse	Mm01224980_m1
Hdac9	histone deacetylase 9	NM_001271386.1	84	Mouse	Mm00458456_m1
Il6	interleukin 6	NM_031168.1	78	Mouse	Mm00446190_m1
Ifng	interferon gamma	NM_008337.3	100	Mouse	Mm01168134_m1
Htr2a	5-hydroxytryptamine (serotonin) receptor 2A	NM_172812.2	66	Mouse	Mm00555764_m1
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	NM_001289726.1	107	Mouse	Mm99999915_g1
Rps29	Ribosomal protein S29	NM_009090.2	149	Mouse	Mm02342448_gH

The relative mRNA amount (ΔΔCt) of the target genes for each sample was calculated using the comparative Ct method. ΔΔCt method is based on the comparison of the amount of target mRNA

with that of the housekeeping genes (*Gapdh* and *Rps29*) and relative to a reference sample (pool): $\Delta\Delta Ct = [Ct\ (Target\ gen)_{Sample} - Ct\ (Reference\ gene)_{Sample}] - [Ct\ (Target\ gen)_{Reference\ sample} - Ct\ (Reference\ gene)_{Reference\ sample}]$.

Data analysis was performed by means of StepOne™ Software v2.1 (Applied Biosystems™, California, USA). The relative amount of mRNA (Relative quantity, RQ) was calculated as $2^{-\Delta\Delta Ct} \pm$ S.E.M. The average RQ of reference sample for each target gene was considered as relative value 1.

3.3.2.4. WESTERN BLOT

Western blot experiments were performed in order to evaluate the protein expression of the neuroinflammatory signaling proteins NF-κB (precisely, RelA or p65 member of the NF-κB transcription factor family) and its main repressor IκBα, member of the IκB family of NF-κB inhibitors. These NF-κB family members were chosen based on their relevant role on schizophrenia-related neuroinflammatory alterations, as most of the previous studies have evaluated alterations of the expression of these NF-κB family members in schizophrenia patients and animal models of this disorder.

3.3.2.4.1. PREPARATION OF CYTOSOLIC AND NUCLEAR FRACTIONS

Cytosolic and nuclear fractions were prepared from mouse cortex samples as previously described (Ibi et al., 2017). Approximately 100 mg of tissue samples were homogenized manually in 15 volumes of the homogenization buffer (Tris HCl 5 Mm (pH 7.4) + 0.032 M sucrose + protease inhibitors). Samples were then centrifuged at 1000 x g for 15 min (4^0C). After this centrifugation, supernatant (S1) contained the cytosolic and the membrane fraction, and pellet (P1) contained the nuclear fraction.

Cytosolic fraction (S2): S1 were re-centrifuged at 40000 x g for 15 min (4^0C). Supernatants (S2, cytosolic fractions) were transferred to new Eppendorf tubes and their protein content was determined by Bradford method with BSA as standard in 1:1 and 1:2 sample dilutions.

Nuclear fraction (P1'): P1 fractions were re-suspended in 15 volumes of the homogenization buffer and manually homogenized. Then, samples were centrifuged at 1000 x g for 15 min (4^0C), after which supernatants were discarded and pellets (P1'), containing the nuclear fraction, were re-suspended in 250 µL incubation buffer. Protein content of nuclear fractions (P1') was determined by Bradford method with BSA as standard in 1:5 and 1:10 sample dilutions.

Cytosolic and nuclear fractions were adjusted to 1 mg protein/ml and 1.5 mg protein/ml, respectively, and aliquoted. 6x concentrated Laemmli buffer (16,5%) and β-mercaptoethanol (2,5%) were added to each sample, vortexed and stored at -80^{0}C until western blot experiments.

3.3.2.4.2. WESTERN BLOT

Western blot is a semiquantitative technique used to visualize proteins that have been separated by gel electrophoresis. This gel is placed next to a nitrocellulose membrane and electrical current makes the proteins migrate from the gel to the membrane. Then, the membrane can be probed by primary antibodies specific for the protein of interest and visualized using fluorescent secondary antibodies.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out in order to separate the proteins of the sample according to their molecular weight. Gels of 1.5 mm thick were used, which consisted of an upper phase (Stacking gel, 5% polyacrylamide) and a lower phase (Running gel, 12% polyacrylamide). The composition of these two phases of the gel is explained in **table 3.8**:

Table 3.8: Composition of the running and stacking gels.

	Running Gel (12% polyacrylamide)	**Stacking Gel (5% polyacrylamide)**
30% polyacrylamide	3 ml	640 µl
1.5 M Tris HCl (pH= 8.8)	1.875 ml	-
1 M Tris HCl (pH= 6.8)	-	470 µl
MilliQ H2O	2.5 ml	2.55 ml
10% SDS	75 µl	37.5 µl
TEMED	5.25 µl	3.75 µl
10% APS	52.5 µl	37.5 µl

First, samples were heated at 95^{0}C for 5 min with the purpose of denaturalizing proteins and avoiding protein folding and/or aggregation. Then, equal amounts of protein were loaded into the wells of the SDS-PAGE gel, along with the molecular weight marker (protein amounts loaded are explained in **table 3.10**). The gel was run for 30 min at 60 V and immediately after for 120 min at 120 V of electrical current. The electrophoresis buffer was made of 0.25 M Tris-HCl, 1.92 M glycine and 10% SDS.

Then, proteins were transferred from the gel to a nitrocellulose membrane, which was previously activated for 10 min with milliQ water. Stacks were prepared by tightly packing the gel and membrane with Whatman® filter papers and sponges. Then, stacks were placed into a cassette for the transference. Transference was carried out by applying an electric field of 0.3 A per cassette during 1 h. Transference buffer was made of 0.25 M Tris-HCl, 1.92 M glycine and 20% methanol.

For the antibody staining and immunoreactive signal quantification, membranes were first blocked at RT for 1 h. Then, membranes were incubated with appropriate dilutions of primary antibody overnight at 4^0C in the incubation buffer. The following day, membranes were washed with PBST (PBS + 0.1% Tween 20), and after that, incubated with the recommended dilution of conjugated secondary antibody in incubation buffer at room temperature for 1 h and protected from light. See **table 3.10** and **table 3.11** for the experimental conditions used in the western blot experiments. After washing the membranes with PBST, the immunoreactive signal was detected and quantified by means of the Oddysey Infrared Imaging SYSTEM (LI-COR Biosciences, Nebraska, USA). Immunoreactive signal values of the target proteins were corrected by the loading control (β-actin) and by the reference sample (pool), which was loaded in every gel in order to reduce the inter-experimental variability. Two different pools were generated with a representative portion of nuclear and cytosolic samples used in the western blot experiments, respectively. Four replicates of each sample in at least two independent experiments were made.

Table 3.10: Experimental conditions used in western blot experiments with mice cortex samples.

Target protein	Protein content	Molecular weight	Blocking solution	Incubation solution	Primary antibody dilution	Secondary antibody dilution
NF-κB (RelA/p65)	20 µg	65 kD	Milk 5%, BSA 0.5%	Blocking solution + 0.1% Tween 20	1:500	1:6000
IκBα	20 µg	35-41 kD	Milk 5%, BSA 0.5%	Blocking solution + 0.1% Tween 20	1:500	1:6000
β-actin	20 µg	42 kD	Milk 5%, BSA 0.5%	Blocking solution + 0.1% Tween 20	1:200000	1:10000

Table 3.11: Primary and secondary antibodies used for the detection of the target proteins.

Target protein	Fraction	Primary antibody	Secondary antibody
NF-κB (RelA/p65)	Nuclear	NF-κB p65 Rabbit (Santa Cruz, sc-327) 1:500	Anti-Rabbit Alexa Fluor® 680 in goat (1:6000) Invitrogen (Oregon, USA)
IκBα	Cytosolic	IκBα Rabbit (Santa Cruz, sc-371) 1:500	Anti-Rabbit Alexa Fluor® 680 in goat (1:6000) Invitrogen (Oregon, USA)
β-actin	Both	β-actin Mouse (Abcam®, AB8227) 1:200000	IRDye™ 800 (in donkey) 1:10000 Rockland Immunochemicals (Pensylvania, USA).

3.4. DATA AND STATISTICAL ANALYSIS

Data were analyzed using the Graphpad Prism™ Software version 7 and 9. All the results are expressed as means ± standard error of the mean (SEM). In all statistical analysis, a p-value < 0.05 was considered as statistically significant. Different statistical analysis were performed for the suitable interpretation of the results as explained below.

3.4.1 STATISTICAL ANALYSIS OF THE BEHAVIORAL AND MOLECULAR CHARACTERIZATION OF THE DOUBLE-HIT ANIMAL MODEL

Before the statistical analysis of these experiments, data were inspected for outliers (critical value, Z>1.96) using Grubb's test (GraphPad Software).

3.4.1.1. VALIDATION OF THE IMMUNE ACTIVATION WITH POLY (I:C)

For the statistical analysis of the validation of MIA in pregnant dams, two-way repeated measures analysis of variance (ANOVAs) followed by Bonferroni's multiple comparisons was used. We evaluated differences on the vehicle/Poly (I:C) elicited effects on body weight/temperature within experimental groups or between experimental groups. In this case, factors "time" (expressing the hours lasted from the Poly (I:C) administration) and "maternal immune activation" (indicating MIA, vehicle or Poly (I:C) treatment) were taken into account. F ratios were expressed as F_{TIME}, to express the ability of time to exert an effect; F_{MIA}, to express the ability of MIA to exert an effect; and $F_{INTERACTION}$, to express the interaction between both time and MIA.

3.4.1.2. BEHAVIORAL PROCEDURES: OFT, YMSAT, NORT AND SPT

Three-way ANOVAs followed by Tukey's multiple comparisons test were used to analyze the experiments involving the behavior of the double-hit animal model (OFT, YMSAT, NORT and SPT). Results were analyzed by three-way ANOVAs considering the factors "sex" (male and female mice), "maternal immune activation" (MIA, saline or Poly (I:C) treatment), "social isolation" (SI, presence or absence of social isolation in the puberty period). F ratios were expressed as: F_{MIA}, to express the ability of MIA to exert an effect; F_{SI}, expressing the ability of SI to exert an effect; and F_{SEX}, expressing the ability of Sex to exert an effect. F ratios of the interactions between the evaluated factors were expressed as follows: F_{MIAxSI}, expressing the interaction between both hits; $F_{SEXxMIA}$, expressing the interaction between sex and MIA; F_{SEXxSI},

expressing the interaction between sex and SI; and $F_{SEXxMIAxSI}$, expressing the interaction between sex and both hits.

3.4.1.3. LOCOMOTOR EVALUATION AFTER AMPHETAMINE ADMINISTRATION

Regarding the locomotor evaluation after acute amphetamine treatment, results of males and females were analyzed independently by means of a Three-way repeated measures ANOVA followed by Tukey's multiple comparisons test considering the factors "maternal immune activation", "social isolation" and "time" (effect of time across the particular study). F ratios were expressed as: F_{MIA}, to express the ability of MIA to exert an effect; F_{SI}, expressing the ability of SI to exert an effect; and F_{TIME} expressing differences between the analyzed groups across time. F ratios of the interactions between the evaluated factors were expressed as follows: F_{MIAxSI}, expressing the interaction between both hits; $F_{TIMExMIA}$, expressing the interaction between time and MIA; $F_{TIMExSI}$, expressing the interaction between time and SI; and $F_{TIMExMIAxSI}$, expressing the interaction between sex and both hits.

3.4.1.4. HEAD-TWITCH RESPONSE INDUCED BY PSILOCYBIN ADMINISTRATION

Regarding the statistical comparison between control and double-hit groups in psilocybin induced HTR experiments, an unpaired Student's *t-test* was performed.

3.4.1.5. ELISA LUMINEX, RT-qPCR AND WESTERN BLOT EXPERIMENTS

Three-way ANOVAs followed by Tukey's multiple comparisons test were used to analyze the experiments involving the molecular characterization of the double-hit animal model (Elisa Luminex, RT-qPCR and western blot experiments). Results were analyzed by three-way ANOVA considering the factors "sex" (male and female mice), "maternal immune activation" (MIA, saline or Poly (I:C) treatment), "social isolation" (SI, presence or absence of social isolation in the puberty period). F ratios were expressed as: F_{MIA}, to express the ability of MIA to exert an effect; F_{SI}, expressing the ability of SI to exert an effect; and F_{SEX}, expressing the ability of Sex to exert an effect. F ratios of the interactions between the evaluated factors were expressed as follows: F_{MIAxSI}, expressing the interaction between both hits; $F_{SEXxMIA}$, expressing the interaction between sex and MIA; F_{SEXxSI}, expressing the interaction between sex and SI; and $F_{SEXxMIAxSI}$, expressing the interaction between sex and both hits.

3.4.2. STATISTICAL ANALYSIS OF THE BEHAVIORAL AND MOLECULAR EVALUATION AFTER THE CHRONIC TREATMENT WITH CLOZAPINE AND MINOCYCLINE

In these experiments, data were inspected for outliers by means of the Tukey's outlier test.

An unpaired Student's *t-test* was performed between control and double-hit groups treated with vehicle.

A two-way ANOVA analysis followed by Tukey's multiple comparisons test was performed in the control and double-hit groups, in order to evaluate the effect exerted by clozapine, minocycline and their combination in each of the experimental groups. F ratios were expressed as: $F_{CLOZAPINE}$ (expressing ability of chronic clozapine treatment to exert an effect); $F_{MINOCYCLINE}$ (expressing the ability of chronic minocycline treatment to exert an effect); and $F_{INTERACTION}$ (expressing the ability of the combination of both clozapine and minocycline to exert an effect).

3.4.2.1. INFLUENCE OF SEX ON GENE EXPRESSION EXPERIMENTS AFTER THE CHRONIC PHARMACOLOGICAL TREATMENTS

In order to test the potential influence of sex on the mRNA levels of the different genes after the chronic pharmacological treatments, exploratory two-way or three-way ANOVA analyses were performed including the sex as an additional factor. Due to the low number of animals of each sex per experimental group, we are aware that these analyses might not have enough statistical power to reach firm conclusions.

4. RESULTS

4.1 BEHAVIORAL AND MOLECULAR EVALUATION OF A DOUBLE-HIT MOUSE MODEL OF SCHIZOPHRENIA

4.1.1. VALIDATION OF THE IMMUNE ACTIVATION INDUCED BY THE IMMUNOGEN AGENT POLY (I:C) IN PREGNANT DAMS

According to the "MIA model reporting guidelines checklist" developed by Kentner and collaborators (Kentner et al., 2019) to improve the rigor, reproducibility, and transparency of the Poly (I:C) MIA model, we assessed the maternal immune response induced by Poly (I:C) administration to pregnant dams. For this purpose, body weight and temperature were measured in a subsample of vehicle-treated (saline, NaCl 0.9%; 5 ml/kg, n= 5) and Poly (I:C)-treated (7.5 mg/kg i.p., n= 11) C57BL/6 pregnant dams. Of note, dams that did not suffer an hypothermic response (a drop in the body temperature of ≥ 0.5 ^{0}C) 3 hours after Poly (I:C) administration, were excluded from the study (Mueller at al., 2019). Moreover, a dam showing a vaginal plug at GD 0 and a weight gain of ≥3 g during GD 0.5 and GD 15 was considered as undergoing successful pregnancy (Mueller at al., 2019).

Body weight

Body weight was expressed as the percentage of the weight measured at the moment of vehicle/Poly (I:C) administration (% grams to 0 h, W_0=100%) (**figure 4.1**). Initial body weight was 25.44 ± 1.09 grams in vehicle-treated dams, and 24.52 ± 1.08 grams in Poly (I:C)-treated dams.

Vehicle-treated pregnant dams increased their body weight over time (from 0 to 48h), consistent with normal growth during pregnancy. The % of increase on body weight became significant 48 h after vehicle administration (105.34 ± 1.94 % grams to 0 h; ###p<0.001). In contrast, Poly (I:C) administration induced a decrease on % of body weight compared to W_0, which lasted from 3 h (97.64 ± 0.5 % grams to 0 h; #p<0.05) to 24 h (97.10 ± 0.85 % grams to 0 h; ##p<0.01). 48 h after Poly (I:C) administration a similar % of body weight increase to the observed in vehicle group was measured (103.59 ± 0.85 % grams to 0 h; ###p<0.001). This means that Poly (I:C) administration had a transitory effect on the body weight of pregnant dams that ended 48 h after the Poly (I:C) administration (**figure 4.1**). Thus, the effect of Poly (I:C) administration on body weight is an indirect measure of the sickness behavior induced by this immunogen, and Poly (I:C)-challenged pregnant dams demonstrate to have a transitory systemic response to this substance. Two-way repeated measures ANOVA analysis revealed a significant effect of the time elapsed since vehicle/Poly (I:C) administration [$F_{TIME}(4,56)= 2.81$, $p= 0.03$], and of the Poly (I:C) treatment itself [$F_{Poly\ (I:C)}(1,14)= 5.21$; $p= 0.04$]. Moreover, a significant interaction

between both time and Poly (I:C) was found [$F_{INTERACTION}(4,56)= 2.811$; $p= 0.03$]. Bonferroni´s post-hoc test showed a significant difference in the % of body weight between vehicle-treated and Poly (I:C)-treated animals 24 h after the administration (**p= 0.002, **figure 4.1**).

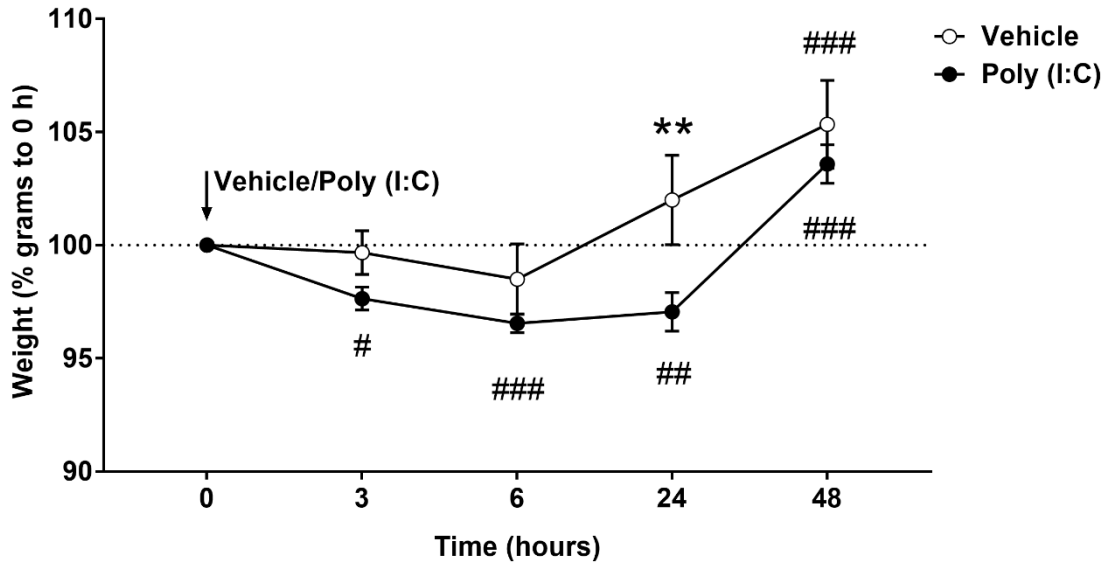


Figure 4.1: Validation of the effect of vehicle and Poly (I:C) administration on the body weight of C57BL/6 pregnant dams. Body weight is expressed as a percentage of the weight measured at the moment of vehicle or Poly (I:C) administration (% grams to 0 h). Vertical arrow indicates the time of administration of vehicle/Poly (I:C). Control (n= 5) and Poly (I:C) (n= 11) mice are plotted. Results were analyzed by means of two-way repeated measures ANOVAs followed by Bonferroni's multiple comparison post-hoc test. Points represent mean ± SEM values. # indicate significant differences detected by Bonferroni's multiple comparison test in the % of body weight over time respect to the initial body weight (W_0 = 100%) in vehicle-treated or Poly (I:C)-treated animals. * indicate a significant difference detected by Bonferroni's multiple comparison test in the % of body weight between vehicle-treated and Poly (I:C)-treated dams at a specific time point (24 h after the administration). P value significance is expressed as **p<0.01; #p<0.05; ##p<0.01; ###p<0.001.

Body temperature

Body temperature was expressed as the percentage of temperature measured at the moment of vehicle/Poly (I:C) administration (% ^{0}C to 0 h, T_0= 100%) (**figure 4.2**). Initial body temperature was 38.24 ± 0.09 ^{0}C in vehicle-treated dams, and 38.11 ± 0.10 ^{0}C in Poly (I:C)-treated dams.

Vehicle administration decreased the body temperature over time, reaching a maximum effect 3 h after the administration (98.38 ± 0.34 % ^{0}C to 0 h; ##p<0.01). Vehicle-treated pregnant dams had a total recovery of their body temperature 24 h after vehicle administration. Administration of Poly (I:C) triggered a more pronounced decrease in the body temperature (96.92 ± 0.37 % ^{0}C to 0 h; ####p<0.001) 3 h after administration compared to that observed in vehicle-treated dams. Body temperature was also recovered 24 h after Poly (I:C) administration. Two-way ANOVA analysis revealed a significant effect of the time elapsed since Poly (I:C) administration and of Poly (I:C) treatment itself [$F_{TIME}(4,56)= 25.51$; $p<0.0001$; $F_{Poly\ (I:C)}(1,14)= 0.97$, $p= 0.34$]. Moreover, a significant interaction between both time and Poly (I:C) was found [$F_{INTERACTION}(4,56)= 2.793$; $p<0.05$] (**figure 4.2**). Bonferroni's multiple comparison test revealed a significant difference between the body temperature of vehicle-treated and Poly (I:C)-treated dams 3 h after the administration (*p<0.05).

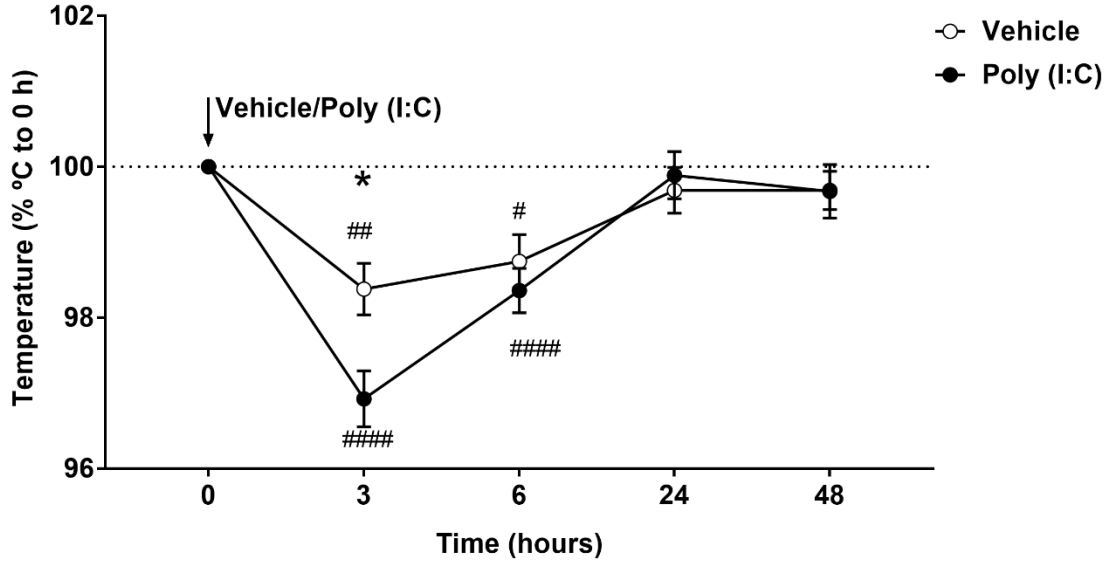


Figure 4.2: Validation of the effect of vehicle and Poly (I:C) administration on body temperature of C57BL/6 pregnant dams. Body Temperature is expressed as a percentage of the temperature measured at the moment of vehicle/ Poly (I:C) administration (% ^{0}C to 0 h, T_0=100%). Vertical arrow indicates the time of administration of vehicle/ Poly (I:C). Control (n= 5) and MIA (n= 11) mice are plotted. Results were analyzed by a two-way repeated measures ANOVA followed by Bonferroni's multiple comparison test. Points are representations of the mean ± SEM values. # indicate significant differences detected by Bonferroni's multiple comparison test in the % of temperature over time respect to the initial temperature (T_0= 100%) in vehicle or Poly (I:C)-treated animals. * indicate a significant difference detected by Bonferroni's multiple comparison test in the % of Temperature over basal between vehicle-treated and Poly (I:C)-treated dams at a specific time point (3 h after the administration). P value significance is expressed as *p<0.05; #p<0.05; ##p<0.01; ####p<0.0001.

4.1.2. EVALUATION OF THE LOCOMOTOR ACTIVITY AND THE ANXIETY-LIKE BEHAVIOR IN THE OPEN FIELD TEST (OFT)

A pilot batch of 45 animals were tested for their basal locomotor activity and the anxiety-like behavior in the Open Field Test (OFT).

Total distance moved in the OFT

Male control mice moved a total distance of 356.17 ± 31.32 cm, while female control mice moved a total distance of 393.09 ± 38.91 cm. See **table 4.1** below for the mean ± SEM values of the distance traveled in the Open Field for all the experimental groups evaluated.

Table 4.1: Total distance moved (cm) for the experimental groups evaluated in the OFT. Total distance moved values are expressed as mean ± SEM.

Total distance moved (cm) in the OFT – Mean ± SEM values		
	Males	**Females**
Control	357.44 ± 24.74	393.09 ± 38.91
MIA	373.56 ± 28.87	405.16 ± 27.53
SI	319.51 ± 26.15	374.20 ± 37.06
MIA + SI	344.54 ± 59.85	327.50 ± 28.12

Total distance moved (expressed in cm) in the OFT was not affected by any of the hits or sex [$F_{MIA}(1,37)= 0.003$, $p= 0.96$; $F_{SI}(1,37)= 1.96$, $p= 0.17$; $F_{SEX}(1,37)= 0.81$, $p= 0.38$] (**figure 4.3**). Moreover, no significant “sex x hit”, “hit x hit” or “sex x hit x hit” interactions were found (see **table 4.2**).

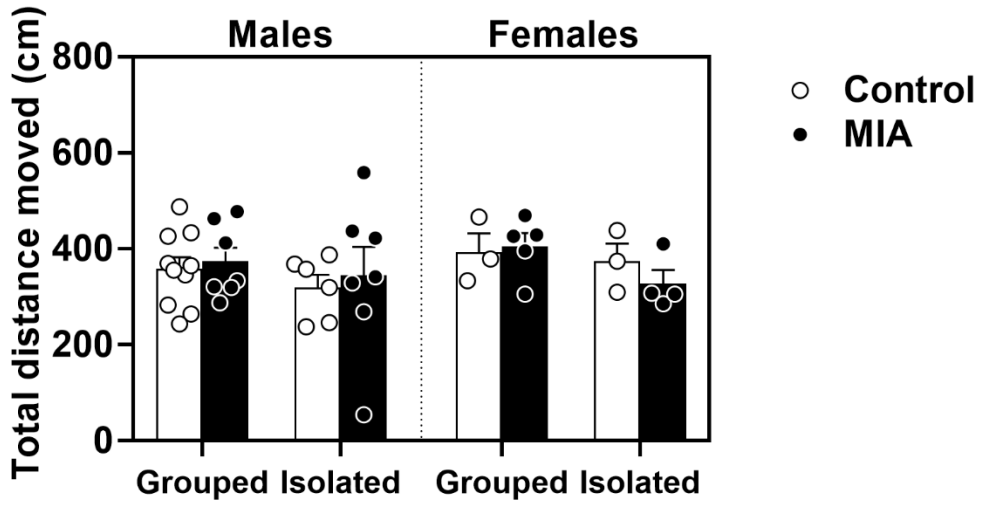


Figure 4.3: Graphical representation of the total distance moved (in cm) in the OFT. Individual distance values (in cm) of male and female mice of the four experimental groups (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Bars are a representation of the mean ± SEM values (n= 3-10 sex/arm). Data were analyzed using three-way ANOVA followed by Tukey's multiple comparisons test.

Table 4.2: Three-way ANOVA analysis conducted in order to evaluate the effect of MIA, SI, sex and their interactions on the total distance moved in the OFT.

Total distance moved (cm) in the OFT – Three-way ANOVA	
MIA	F(1,37)= 0.003, p= 0.96
SI	F(1,37)= 1.96, p= 0.17
Sex	F(1,37)= 0.81, p= 0.38
MIA x SI	F(1,37)= 0.19, p= 0.67
Sex x MIA	F(1,37)= 0.42, p= 0.52
Sex x SI	F(1,37)= 0.06, p= 0.80
Sex x MIA x SI	F(1,37)= 0.33, p= 0.57

Time spent in the periphery/center in the OFT

The mean ± SEM values of time (in seconds, s) that all the experimental groups spent in the periphery and center in the OFT, are shown in tables 4.3 and 4.4 respectively. Male control mice spent a total time of 360.61 ± 25.18 s in the periphery of the Open Field, whereas female control mice spent a total time of 436.83 ± 39.52 s in this area. Thus, female controls spent more time

than males in the periphery of the Open Field. Accordingly, time spent in center of the OF was 236.03 ± 24.94 s for male controls, and 163.17 ± 39.52 s for female controls.

Table 4.3: Mean ± SEM values of the time spent in the periphery of the OFT (s).

Time spent in periphery (s) of the OFT – Mean ± SEM values		
	Males	**Females**
Control	360.61 ± 25.18	436.83 ± 39.52
MIA	344.18 ± 26.66	369.79 ± 27.47
SI	374.28 ± 15.87	490.96 ± 9.26
MIA + SI	395.40 ± 17.37	426.05 ± 31.06

Table 4.4: Mean ± SEM values of the time spent in the center of the OFT (s).

Time spent in center (s) of the OFT – Mean ± SEM values		
	Males	**Females**
Control	236.03 ± 24.94	163.17 ± 39.52
MIA	253.33 ± 26.21	230.21 ± 27.47
SI	225.72 ± 15.87	109.04 ± 9.26
MIA + SI	202.78 ± 16.19	173.95 ± 31.06

Time spent in periphery (s) was significantly increased by the effect of SI [$F_{SI}(1,37)= 4.74$, $p= 0.04$], but not by the effect of MIA [$F_{MIA}(1,37)= 2.50$, $p= 0.12$]. Sex also influenced the time spent in periphery [$F_{SEX}(1,37)= 9.58$, $p= 0.004$], with females spending more time in this area than males (**figure 4.4 a**). No significant "sex x hit", "hit x hit" or "sex x hit x hit" interactions were found in the time that animals spent in periphery (see **table 4.5**).

Consequently, time spent in center (s) was significantly decreased by the effect of SI [$F_{SI}(1,37)= 4.65$, $p= 0.04$], whereas no effect of MIA was found [$F_{MIA}(1,37)= 2.53$, $p= 0.12$]. Time spent in center was also influenced by effect of sex [$F_{SEX}(1,37)= 9.24$, $p= 0.004$], with females spending less time in center than males (**figure 4.4 b**). Overall, this fact seems to point to the presence of a higher degree of anxiety-like behavior in females when compared with male animals. In addition, isolation appears to elicit an anxiogenic effect on mice, by decreasing the time spent in the center of the Open Field, and consequently, promoting a thigmotaxis behavior, which is a

validated measure of anxiety-related behavior in mice. No significant "sex x hit", "hit x hit" or "sex x hit x hit" interactions were found in the time that animals spent in center (see **table 4.5**).

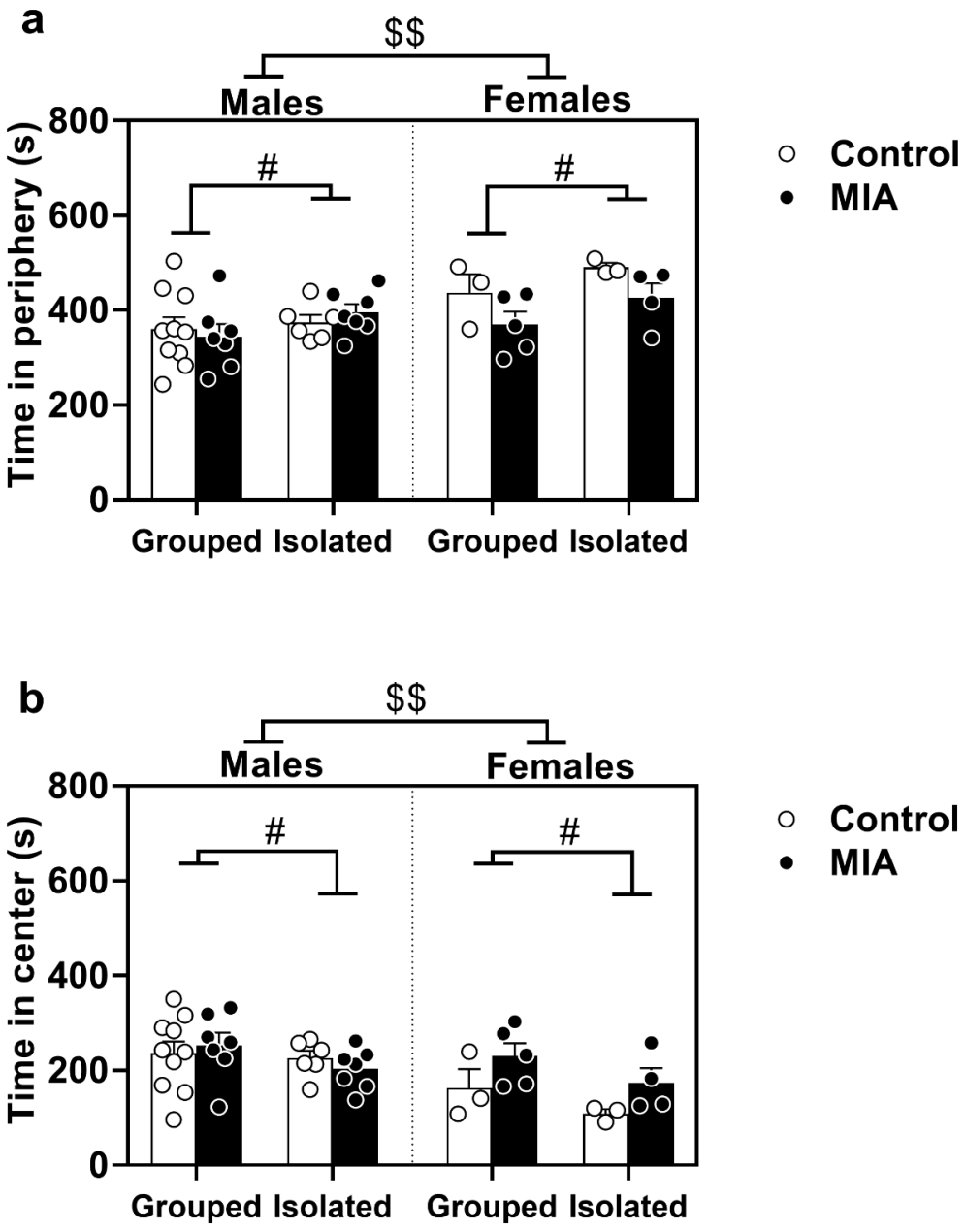


Figure 4.4: Graphical representation of the time spent (s) in periphery (**figure 4.4 a**) and in center (**figure 4.4 b**) in the OFT. Individual time values (s) of male and female mice of the four experimental groups (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Bars are a representation of the mean ± SEM values (n= 3-10 sex/arm). Data were analyzed using three-way ANOVA followed by Tukey's multiple comparisons test. SI significance is expressed as #p<0.05. Sex significance is expressed as $$p<0.01.

Table 4.5: Three-way ANOVA analysis conducted in order to evaluate the effect of MIA, SI, sex and their interactions on the time spent in periphery and center in the OFT.

Time spent in periphery/center of the OFT – Three-way ANOVA		
	Time spent in periphery (s)	**Time spent in center (s)**
MIA	F(1,37)= 2.50, p= 0.12	F(1,37)= 2.53, p= 0.12
SI	**F(1,37)= 4.74, p= 0.04**	**F(1,37)= 4.65, p= 0.04**
Sex	**F(1,37)= 9.58, p= 0.004**	**F(1,37)= 9.24, p= 0.004**
MIA x SI	F(1,37)= 0-24, p= 0.62	F(1,37)= 0.28, p= 0.60
Sex x MIA	F(1,37)= 2.88, p= 0.10	F(1,37)= 2.99, p= 0.09
Sex x SI	F(1,37)= 0.32, p= 0.57	F(1,37)= 0.39, p= 0.54
Sex x MIA x SI	F(1,37)= 0.19, p= 0.66	F(1,37)= 0.23, p= 0.63

4.1.3. EVALUATION OF THE COGNITIVE STATUS IN THE Y-MAZE SPONTANEOUS ALTERNATION TEST (YMSAT) AND NOVEL OBJECT RECOGNITION TEST (NORT)

Animals were tested for their cognitive status using the Y-maze Spontaneous Alternation Test (YMSAT) and the Novel Object Recognition Test (NORT).

4.1.3.1. Y-MAZE SPONTANEOUS ALTERNATION TEST (YMSAT)

Spontaneous alternation (%) was calculated for male and female control (control-grouped) mice performing the Y-maze spontaneous alternation test (YMSAT). As stated in **section 3.3.1.1.**, this parameter refers to a triplet of consecutive arm explorations with all three arms being different, and is calculated with the following formula: *Spontaneous alternation (%) = (number of correct triplets/ total number of triplets) x 100*.

Spontaneous alternation values (%) of all the experimental groups evaluated in the YMSAT are plotted in **table 4.6**. Both male and female control mice performed a high percentage of correct triplets in the YMSAT (% of spontaneous alternation= 60.84 ± 2.39 and 56.13 ± 2.42, respectively) and consequently had the expected spontaneous alternation percentage, which is associated with a correct spatial short-term memory (Hughes, 2004; Ibi et al., 2017).

Table 4.6: Spontaneous alternation values (%) for the experimental groups evaluated in the YMSAT. Spontaneous alternation percentages expressed as mean ± SEM.

Spontaneous alternation (%) in the YMSAT – Mean ± SEM values		
	Males	**Females**
Control	60.84 ± 2.39	56.13 ± 2.42
MIA	58.11 ± 2.10	59.91 ± 2.22
SI	58.76 ± 3.43	52.41 ± 3.39
MIA + SI	58.89 ± 1.45	56.96 ± 1.18

Spontaneous alternation (%) was not affected by any of the hits or sex [$F_{MIA}(1,88)= 0.67$; $p= 0.42$; $F_{SI}(1,88)= 1.28$; $p= 0.26$; $F_{SEX}(1,88)= 2.52$; $p= 0.12$] in the YMSAT (**figure 4.5**). Moreover, no significant "sex x hit", "hit x hit" or "sex x hit x hit" interactions were found (see **table 4.7**).

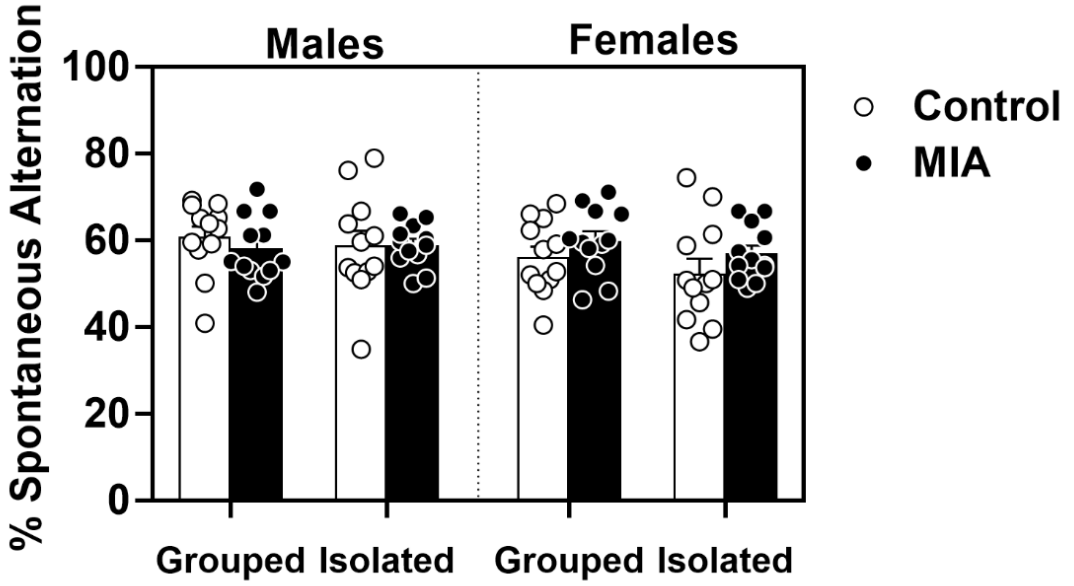


Figure 4.5: Graphic representation of the spontaneous alternation (%) in the YMSAT. Individual spontaneous alternation values (%) of male and female mice of the four experimental groups (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Bars are a representation of the mean ± SEM values (n= 12 sex/arm). Data were analyzed using three-way ANOVA followed by Tukey's multiple comparisons test.

These results show that the spatial working memory, assessed by means of the YMSAT, is not significantly affected by any of the factors analyzed—MIA, SI and sex— or their interactions.

Table 4.7: Three-way ANOVA analysis conducted in order to evaluate the effect of MIA, SI, sex and their interactions on the spontaneous alternation (%) of the YMSAT.

Spontaneous alternation (%) in the YMSAT – Three-way ANOVA	
MIA	F(1,88)= 0.67; p= 0.42
SI	F(1,88)= 1.28; p= 0.26
Sex	F(1,88)= 2.52; p= 0.12
MIA x SI	F(1,88)= 0.26; p= 0.61
Sex x MIA	F(1,88)= 2.41; p= 0.12
Sex x SI	F(1,88)= 0.58; p= 0.45
Sex x MIA x SI	F(1,88)= 0.09; p= 0.77

4.1.3.2. NOVEL OBJECT RECOGNITION TEST (NORT)

The main postulate behind the NORT, is that in the presence of a novel and a previously presented (familiar) object, rodents increase their exploration towards the novel object, which is interpreted as indirect evidence that animals acquired a memory of the familiar object, and thus increase their exploration to the novel one (Oliveira da Cruz et al., 2020). As mentioned in section 3.3.1.2., Discrimination Index (DI), is the main parameter measured for the assessment of cognitive impairment in the NORT, and positive DI values indicate a good discrimination between objects.

Short-term memory evaluation in the NORT

In order to evaluate the impact of MIA, SI, sex and their interactions on short-term episodic-like declarative memory, the test phase of the NORT was performed 2 h after the training session (Oliveira da Cruz et al, 2020) in a pilot batch of animals (n= 101) including the 4 experimental groups for both male and female mice.

Male and female control mice showed a correct discrimination between the familiar and the novel object, with high and positive DI scores (DI males 0.38 ± 0.06; DI females 0.18 ± 0.09). See **table 4.8** for the DI values of all the experimental groups evaluated in the NORT (2h after the training phase).

Table 4.8: DI scores for the experimental groups evaluated in the NORT (2h after the training phase). DI scores are expressed as mean ± SEM.

Discrimination Index (DI) (2h after the training phase) – Mean ± SEM values		
	Males	**Females**
Control	0.39 ± 0.06	0.18 ± 0.09
MIA	0.45 ± 0.11	0.22 ± 0.08
SI	0.36 ± 0.03	0.24 ± 0.08
MIA + SI	0.40 ± 0.12	0.11 ± 0.06

NORT DI (2h) was not influenced by the effect of MIA [$F_{MIA}(1,93)= 0.002$, $p= 0.96$], nor by SI [$F_{SI}(1,93)= 0.28$, $p= 0.59$]. However, sex had a significant effect on NORT DI (2h) [$F_{SEX}(1, 93)= 12.55$, $p= 0.0006$], as females showed lower DI (2h) scores than males (**figure 4.6**). No significant "sex x hit", "hit x hit" or "sex x hit x hit" interactions were found (see **table 4.9**). Interestingly, although not statistically significant, female double-hit group showed worse mean of DI score than their respective control and single-hit groups. Thus, as in the evaluation of the short-term episodic-like declarative memory, double-hit mice showed a modest cognitive impairment, we next evaluated the effect of MIA, SI or combination of both, on long-term memory of male and female animals (NORT performed 24 h after the training phase).

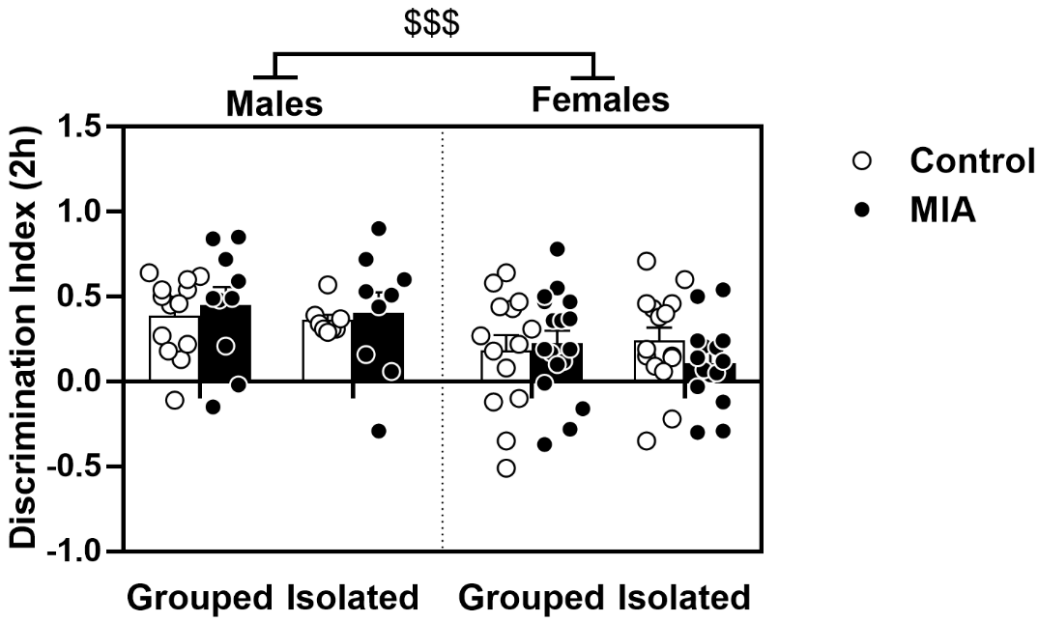


Figure 4.6: Graphic representation of the Discrimination Index (DI) (2 h after the training phase) in the NORT. Individual DI values of male and female mice of the four experimental groups (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Bars represent mean ± SEM values (n= 8-17 sex/arm). Data were analyzed using three-way ANOVA followed by Tukey's multiple comparisons test. Sex significance is expressed as $$$p<0.0001.

Table 4.9: Three-way ANOVA analysis conducted in order to evaluate the effect of MIA, SI, sex and their interactions on the NORT DI 2 h after the training phase.

NORT DI (2h after the training phase) – Three-way ANOVA	
MIA	F(1,93)= 0.002, p= 0.96
SI	F(1,93)= 0.28, p= 0.59
Sex	**F(1,93)= 12.55, p= 0.0006**
MIA x SI	F(1,93)= 0.69, p= 0.41
Sex x MIA	F(1,93)= 0.68, p= 0.41
Sex x SI	F(1,93)= 0.005, p= 0.93
Sex x MIA x SI	F(1,93)= 0.44, p= 0.51

Long-term memory evaluation in the NORT

In order to evaluate the impact of MIA, SI, sex and their interactions on long-term episodic-like declarative memory, the test phase of the NORT was performed 24 h after the training session (Oliveira da Cruz et al, 2020) in all experimental groups.

Thus, 24 h after performing the training session, male and female control mice showed a correct discrimination between the novel and familiar object, as they devoted more time exploring the novel object than the familiar one (see **table 4.10, table 4.11 and table 4.12** for novel, familiar and total exploration values (s) of all the experimental groups evaluated).

Table 4.10: Mean ± SEM values of the novel object exploration times (s) of the NORT.

Novel object exploration time (s) of the NORT – Mean ± SEM values		
	Males	**Females**
Control	35.04 ± 4.45	41.19 ± 8.26
MIA	21.46 ± 4.37	40.67 ± 4.00
SI	30.56 ± 5.51	24.72 ± 3.72
MIA + SI	26.97 ± 6.99	34.85 ± 5.49

Table 4.11: Mean ± SEM values of the familiar object exploration time (s) of the NORT.

Familiar object exploration time (s) of the NORT – Mean ± SEM values		
	Males	**Females**
Control	24.56 ± 2.37	14.50 ± 2.58
MIA	22.57 ± 3.80	14.54 ± 2.28
SI	28.75 ± 2.77	24.59 ± 2.81
MIA + SI	34.97 ± 5.89	28.57 ± 3.84

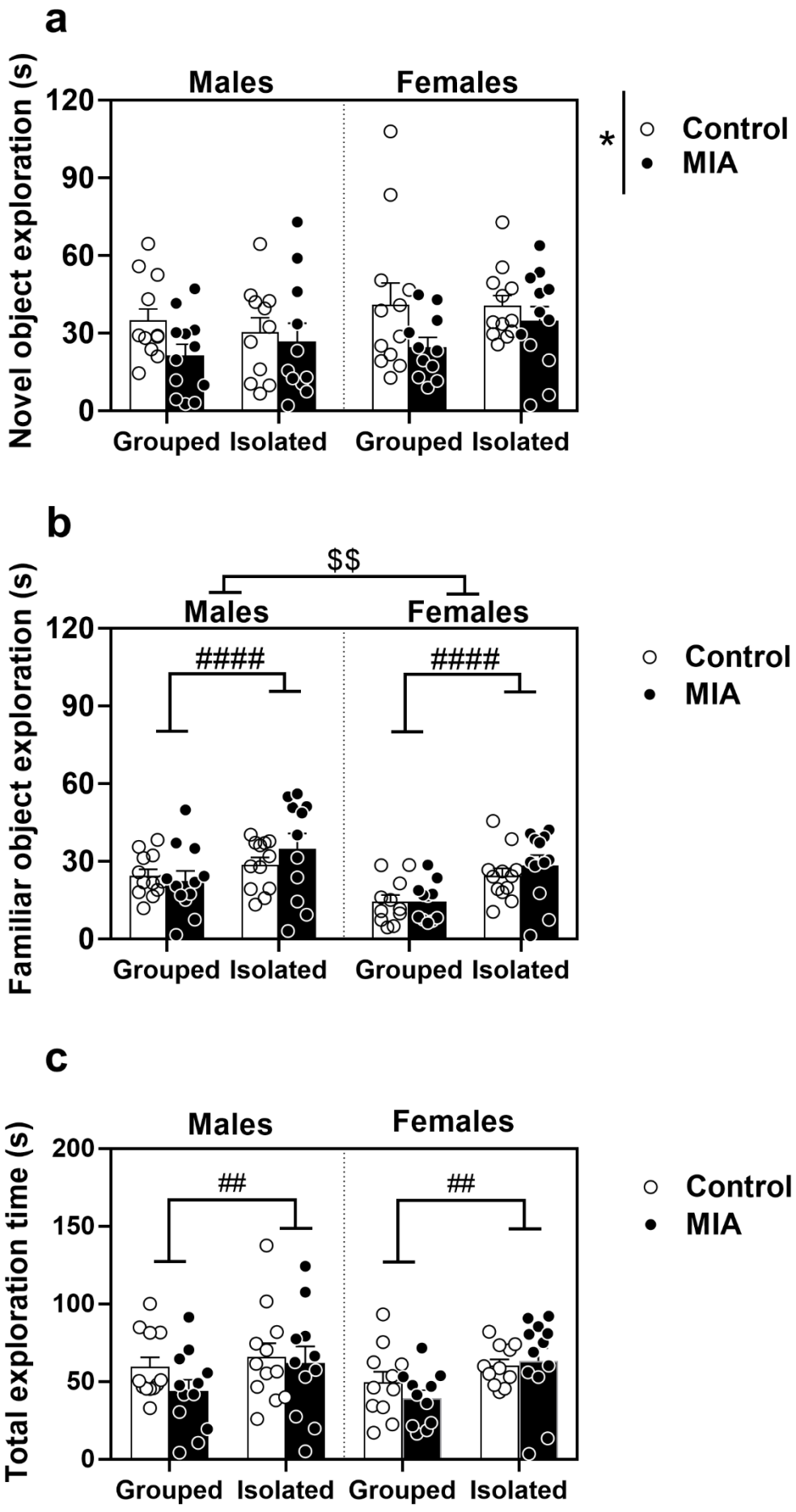
Table 4.12: Mean ± SEM values of the total object exploration time (s) of the NORT.

Total object exploration time (s) of the NORT – Mean ± SEM values		
	Males	**Females**
Control	59.60 ± 6.16	49.61 ± 6.88
MIA	44.03 ± 7.30	39.26 ± 5.31
SI	65.91 ± 8.88	60.40 ± 3.93
MIA + SI	61.94 ± 10.81	63.42 ± 8.30

Novel object exploration time (s) was significantly reduced by the effect of MIA [$F_{MIA}(1,85)= 6.297$; $p= 0.014$] (**figure 4.7 a**). Nevertheless, novel exploration time was not affected neither by the effect of SI [$F_{SI}(1,85)= 0.46$; $p= 0.50$], nor by the effect of sex [$F_{SEX}(1,85)= 3.04$; $p= 0.09$]. No significant "sex x hit" or "sex x hit x hit" interactions were found when analyzing the novel object exploration time by means of a three-way ANOVA (see **table 4.13**).

Familiar object exploration time (s) was significantly increased by the effect of SI [$F_{SI}(1,85)= 17.39$; $p<0.0001$], as isolated mice spent more time exploring the familiar object than grouped mice (**figure 4.7 b**). In addition, familiar object exploration time was significantly influenced by sex [$F_{SEX}(1,85)= 8.622$; $p= 0.004$], with higher familiar exploration times in males than in females. Familiar object exploration time was not affected by MIA [$F_{MIA}(1,85)= 0.71$; $p= 0.40$], and no significant "hit x hit", "sex x hit" or "sex x hit x hit" interactions were found (see **table 4.13**).

Consequently, NORT total exploration time (s), calculated as the time spent exploring both novel and familiar objects, was significantly increased by SI [$F_{SI}(1,84)= 7.76$; $p= 0.007$] (**figure 4.7 c**). However, total exploration time was not influenced by MIA [$F_{MIA}(1,84)= 0.78$; $p= 0.39$], or by sex [$F_{SEX}(1,84)= 1.6$; $p= 0.21$], and no significant "sex x hit", "hit x hit" or "sex x hit x hit" interactions were found (see **table 4.13**). In conclusion, the increase of the total exploration time by effect of SI appears to be a consequence of an increased familiar exploration time by effect of SI.


a
Males
Females
Novel object exploration (s)
120
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60
30
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Grouped
Isolated
Grouped
Isolated
*
Control
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Familiar object exploration (s)
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Control
MIA
c
Males
Females
Total exploration time (s)
200
150
100
50
0
##
##
Control
MIA

Figure 4.7: Graphic representation of the novel (**figure 4.7 a**), familiar (**figure 4.7 b**) and total (**figure 4.7 c**) exploration times (s) in the NORT. Exploration times of male and female mice of the four experimental arms (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Bars represent mean ± SEM values (n=12 sex/arm). Data were analyzed using three-way ANOVA followed by Tukey's multiple comparisons test. MIA significance is expressed as $*p_{MIA}<0.05$. SI significance is expressed as $\#p_{SI}<0.05$. Sex significance is expressed as $\$\$p_{SEX}<0.01$.

Table 4.13: Three-way ANOVA analysis conducted in order to evaluate the effect of MIA, SI, sex and their interactions on the novel, familiar and total exploration times (s) of the NORT.

Exploration times of the NORT – Three-way ANOVA			
	Novel object exploration time (s)	**Familiar object exploration time (s)**	**Total object exploration time (s)**
MIA	**F(1,85)= 6.30; p= 0.014**	F(1,85)= 0.71; p= 0.40	F(1,84)= 0.78; p= 0.39
SI	F(1,85)= 0.46; p= 0.50	**F(1,85)= 17.39; p<0.0001**	**F(1,84)= 7.76; p= 0.007**
Sex	F(1,85)= 3.04; p= 0.09	**F(1,85)= 8.62; p=0.004**	F(1,84)= 1.6; p= 0.21
MIA x SI	F(1,85)= 1.72; p= 1.19	F(1,85)= 1.55; p=0.22	F(1,84)= 1.38; p= 0.24
Sex x MIA	F(1,85)= 0.11; p= 0.75	F(1,85)= 0.0004; p=0.98	F(1,84)= 0.33; p= 0.57
Sex x SI	F(1,85)= 0.30; p= 0.59	F(1,85)= 0.59; p=0.44	F(1,84)= 0.26; p= 0.61
Sex x MIA x SI	F(1,85)= 0.002; p= 0.97	F(1,85)= 0.19; p=0.66	F(1,84)=0.01; p= 0.93

DI scores were calculated from the individual exploration times for all mice evaluated as explained above. Male and female control mice showed a correct discrimination between the novel and the familiar object, with high and positive DI scores when the test phase of the NORT was assessed 24 h after the training session (DI males 0.21 ± 0.03; DI females 0.38 ± 0.07). See **table 4.14** for the NORT DI values (24 h after the training session) of all the experimental groups evaluated.

Table 4.14: DI scores for all the experimental groups evaluated in the NORT (24 h after the training session). DI scores are expressed as mean ± SEM.

NORT DI (24h after the training phase) – Three-way ANOVA		
	Males	**Females**
Control	0.21 ± 0.03	0.38 ± 0.07
MIA	-0.07 ± 0.09	0.23 ± 0.06
SI	0.02 ± 0.12	0.21 ± 0.03
MIA + SI	-0.16 ± 0.10	0.09 ± 0.07

The NORT DI was significantly impaired by both MIA [$F_{MIA}(1,85)= 10.93$; $p= 0.0014$] and SI [$F_{SI}(1,85)= 7.46$; $p= 0.008$]. Moreover, DI scores were significantly influenced by sex [$F_{SEX}(1,85)= 17.27$; $p<0.0001$], being higher in females than in males (**table 4.10** and **figure 4.8**). Although "hit x hit" interaction was not revealed as statistically significant, both male and female double-hit groups showed worse DI scores compared to single-hit groups. This result can be interpreted as a lack of a synergistic effect of the two hits, but it shows an additive effect of their combination in worsening the discrimination index. No other "hit x sex" and "hit x hit x sex" interactions were observed (see **table 4.15**).

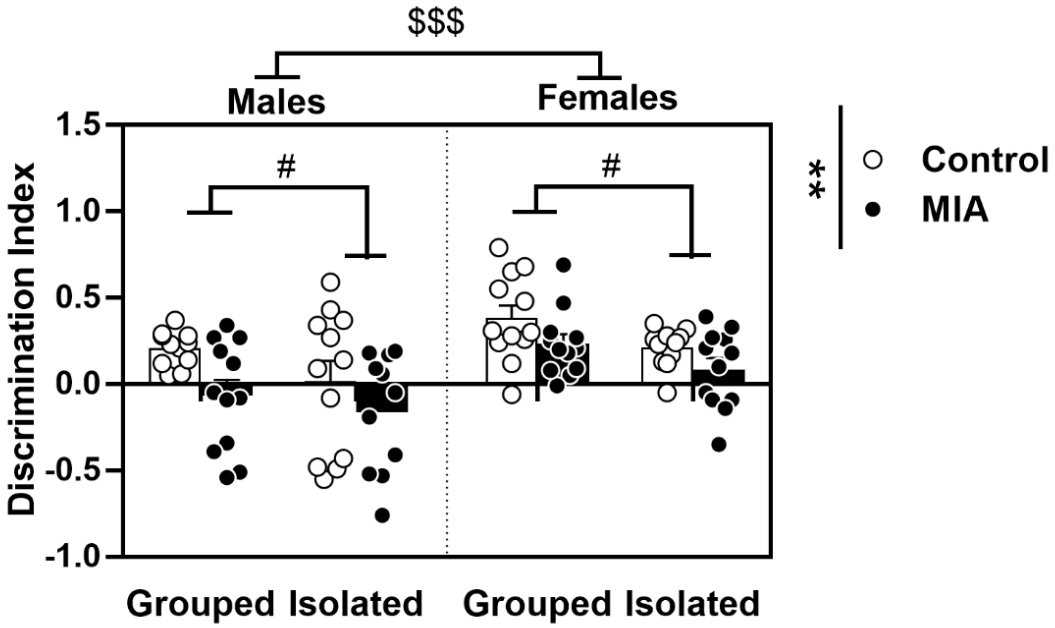


Figure 4.8: Graphic representation of the Discrimination Index (DI) in the NORT. Individual DI values of male and female mice of the four experimental groups (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Bars represent mean ± SEM values (n=12 sex/arm). Data were analyzed using three-way ANOVA followed by Tukey's multiple comparisons test. MIA significance is expressed as **p<0.01. SI significance is expressed as #p<0.05. Sex significance is expressed as $$$p<0.0001.

Table 4.15: Three-way ANOVA analysis conducted in order to evaluate the effect of MIA, SI, sex and their interactions on the NORT DI 24 h after the training phase.

NORT Discrimination Index (DI) – Three-way ANOVA	
MIA	**F(1,85)= 10.93; p= 0.0014**
SI	**F(1,85)= 7.46; p= 0.008**
Sex	**F(1,85)= 17.27; p<0.0001**
MIA x SI	F(1,85)= 0.31; p= 0.58
Sex x MIA	F(1,85)= 0.62; p= 0.43
Sex x SI	F(1,85)= 0.03; p= 0.87
Sex x MIA x SI	F(1,85)= 0.10; p= 0.75

4.1.4. EVALUATION OF THE SOCIAL BEHAVIOR BY MEANS OF THE SOCIAL PREFERENCE TEST (SPT)

Experimental groups were tested for social behavior by means of the Social Preference Test (SPT). Male and female control mice showed a correct sociability, as they devoted more time exploring the social stimulus (a cylinder containing "stimulus mouse" of the same age, strain and sex, as stated in **section 3.3.1.3**) than the non-social stimulus (an empty cylinder). See **table 4.16, table 4.17** and **table 4.18** for social, non-social and total exploration times among the experimental groups evaluated, respectively.

Table 4.16: Mean ± SEM values of the social exploration time (s) of the SPT.

Social exploration (s) of the SPT – Mean ± SEM values		
	Males	**Females**
Control	127.03 ± 11.08	87.70 ± 7.97
MIA	88.30 ± 8.70	98.98 ± 12.76
SI	108.96 ± 10.08	69.31 ± 7.76
MIA + SI	86.04 ± 12.90	53.53 ± 3.96

Table 4.17: Mean ± SEM values of the non-social exploration time (s) of the SPT.

Non-social exploration (s) of the SPT – Mean ± SEM values		
	Males	**Females**
Control	80.52 ± 3.96	60.63 ± 6.92
MIA	80.68 ± 7.67	64.03 ± 6.00
SI	77.67 ± 7.06	57.54 ± 7.51
MIA + SI	74.02 ± 9.16	63.83 ± 4.98

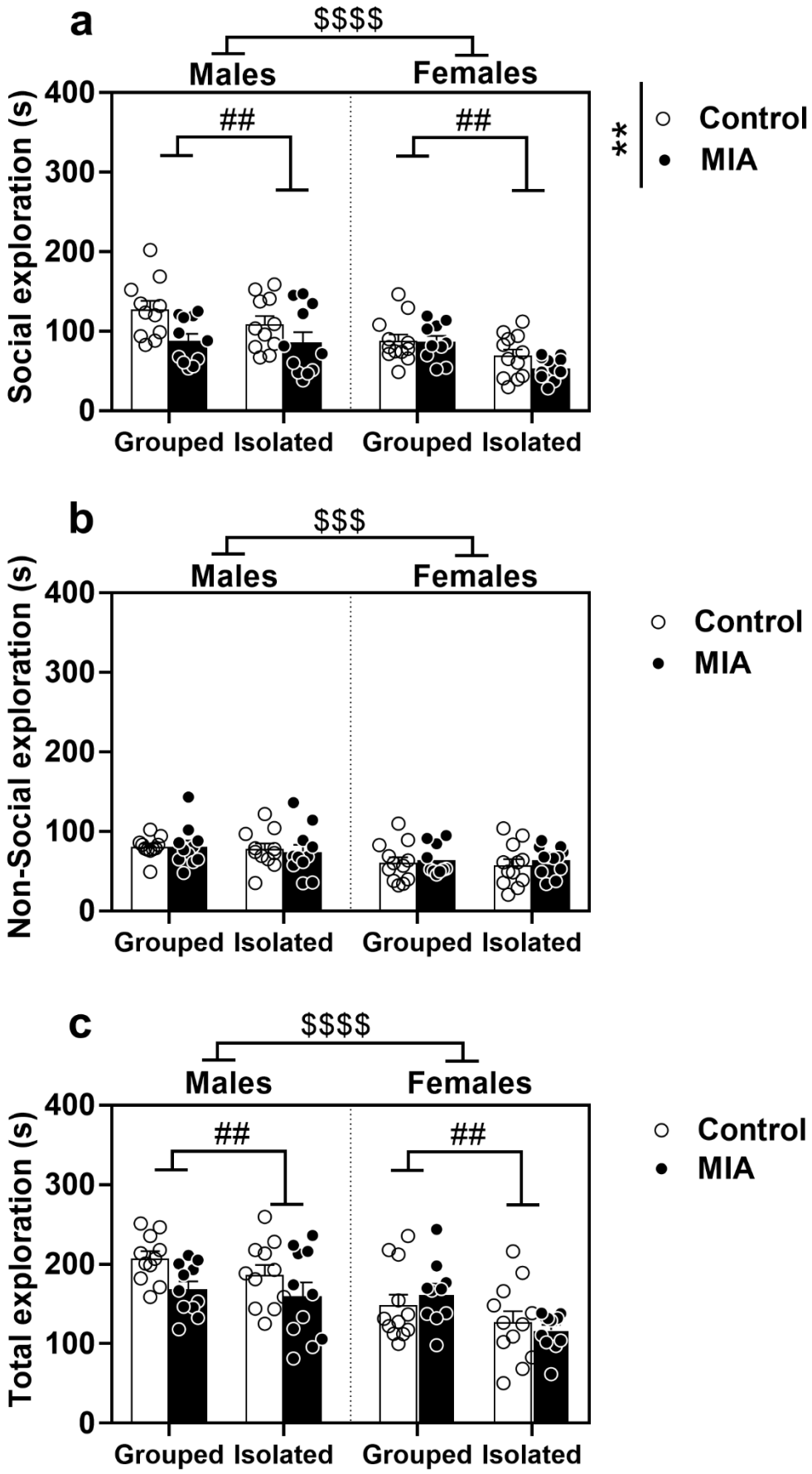
Table 4.18: Mean ± SEM values of the total exploration time (s) of the SPT.

Total exploration (s) of the SPT – Mean ± SEM values		
	Males	**Females**
Control	207.55 ± 8.95	148.33 ± 13.48
MIA	168.98 ± 9.62	163.01 ± 12.64
SI	186.64 ± 12.43	126.85 ± 14.06
MIA + SI	160.06 ± 16.95	117.36 ± 6.62

Social exploration time (s) was significantly reduced by the effect of both MIA [$F_{MIA}(1,82)= 5.96$; $p= 0.02$] and SI [$F_{SI}(1,82)= 9.65$; $p= 0.003$] (**figure 4.9 a**). In addition, a significant effect of sex was found [$F_{SEX}(1,82)= 13.84$; $p= 0.0004$], as males showed higher social exploration times than females. A significant "sex x MIA" interaction was found [$F_{SEXxMIA}(1,82)= 4.45$; $p= 0.04$], as MIA affected male and female social exploration in a different way. Indeed, whereas MIA was associated to a decrease in the social exploration time in all male groups (both grouped and isolated) when compared to control males, MIA only decreased the social exploration compared to controls in female isolated mice, although the interaction MIA x SI x sex did not reach statistical significance ($p= 0.12$). No other significant "sex x SI" or "hit x hit" interactions were found in the social exploration time of the SPT (see **table 4.19**).

Non-social exploration time (s) was not influenced by the effect of MIA [$F_{MIA}(1,82)= 0.10$; $p= 0.75$] or SI [$F_{SI}(1, 82)= 0.44$; $p= 0.51$], but it was significantly affected by sex [$F_{SEX}(1, 82)= 11.89$; $p<0.001$], with females showing lower non-social exploration times than males (**figure 4.9 b**). No significant "sex x hit", "hit x hit" or "sex x hit x hit" interactions were found in the non-social exploration time of the SPT (see **table 4.19)**.

SPT total exploration (s) time was calculated as the time (s) that mice spent exploring both social and non-social stimulus. Total exploration time was significantly decreased by SI [$F_{SI}(1,82)= 7.84$; $p= 0.006$], but it was not significantly affected by MIA [$F_{MIA}(1,82)= 3.00$; $p= 0.09$]. Total exploration time was significantly affected by sex [$F_{SEX}(1,82)= 23.44$; $p<0.0001$], with females showing lower total exploration times than males (**figure 4.9 c**). A significant interaction between sex and MIA was found [$F_{SEXxMIA}(1,82)= 4.12$; $p= 0.04$], meaning that the effect exerted by MIA was different on males and females: while in both grouped and isolated males MIA decreased the total exploration time, MIA increased the total exploration time in grouped females and decreased the total exploration time in isolated females. No other "sex x SI", "hit x hit" or "sex x hit x hit" interactions were found (see **table 4.19**).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Grouped Isolated

Figure 4.9: Graphic representation of the social (**figure 4.9 a**), non-social (**figure 4.9 b**) and total (**figure 4.9 c**) exploration times (s) in the SPT. Exploration times of male and female mice of the four experimental groups (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Bars represent mean ± SEM values (n= 12 sex/arm). Data were analyzed using three-way ANOVA followed by Tukey's multiple comparisons test. MIA significance is expressed as *p<0.05. SI significance is expressed as #p<0.05. Sex significance is expressed as $p<0.05.

Table 4.19: Three-way ANOVA analysis conducted in order to evaluate the effect of MIA, SI, sex and their interactions on the total, social and non-social exploration times of the SPT.

Exploration times of the SPT – Three-way ANOVA			
	Social exploration time (s)	**Non-social exploration time (s)**	**Total exploration time (s)**
MIA	**F(1,82)= 5.96; p= 0.02**	F(1,82)= 0.10; p= 0.75	F(1,82)= 3.00; p= 0.09
SI	**F(1,82)= 9.65; p= 0.003**	F(1,82)= 0.44; p= 0.51	**F(1,82)= 7.84; p= 0.006**
Sex	**F(1,82)= 13.84;p=0.0004**	**F(1,82)= 11.89; p<0.001**	**F(1,82)= 23.44; p<0.0001**
MIA x SI	F(1,82)= 0.17; p= 0.68	F(1,82)= 0.002; p= 0.96	F(1,82)= 0.12; p= 0.73
Sex x MIA	**F(1,82)= 4.45; p= 0.04**	F(1,82)= 0.46; p= 0.50	**F(1,82)= 4.12; p= 0.04**
Sex x SI	F(1,82)= 2.58; p= 0.11	F(1,82)= 0.10; p= 0.75	F(1,82)= 1.16; p= 0.28
Sex x MIA x SI	F(1,82)= 2.50; p= 0.12	F(1,82)= 0.12; p= 0.73	F(1,82)= 1.09; p= 0.30

Social Index scores were calculated from the individual exploration times for all mice evaluated as explained above. Male and female control mice showed a correct sociability, as they had a higher exploration towards the social stimulus than towards the non-social stimulus, consequently showing positive Social Index scores. Moreover, both male and female control mice had higher Social Index scores than single-hit and double-hit groups (see **table 4.20**).

Table 4.20: Social Index scores for the experimental groups evaluated in the SPT. Social Index scores are expressed as mean ± SEM.

SPT Social Index – Mean ± SEM values		
	Males	**Females**
Control	0.20 ± 0.06	0.19 ± 0.05
MIA	0.04 ± 0.07	0.18 ± 0.08
SI	0.16 ± 0.06	0.10 ± 0.05
MIA + SI	0.06 ± 0.08	-0.08 ± 0.05

Social Index was significantly decreased by MIA [$F_{MIA}(1,82)= 6.74$; p= 0.01], and SI [$F_{SI}(1,82)= 4.08$; p= 0.03] (**figure 4.10**). However, Social Index was not affected by the effect of sex [$F_{SEX}(1,82)= 0.11$; p= 0.74]. Moreover, an almost significant "Sex x SI" interaction was found [F(1,82)= 3.81; p= 0.05], since SI seems to have a greater impact in female mice than in male mice. Additionally, female double-hit group showed worse scores in the Social Index compared to single-hit groups (see again **table 4.20** and **figure 4.10**). No significant other "sex x hit", "hit x hit" or "sex x hit x hit" interactions were found in the Social Index of the SPT (see **table 4.21)**.

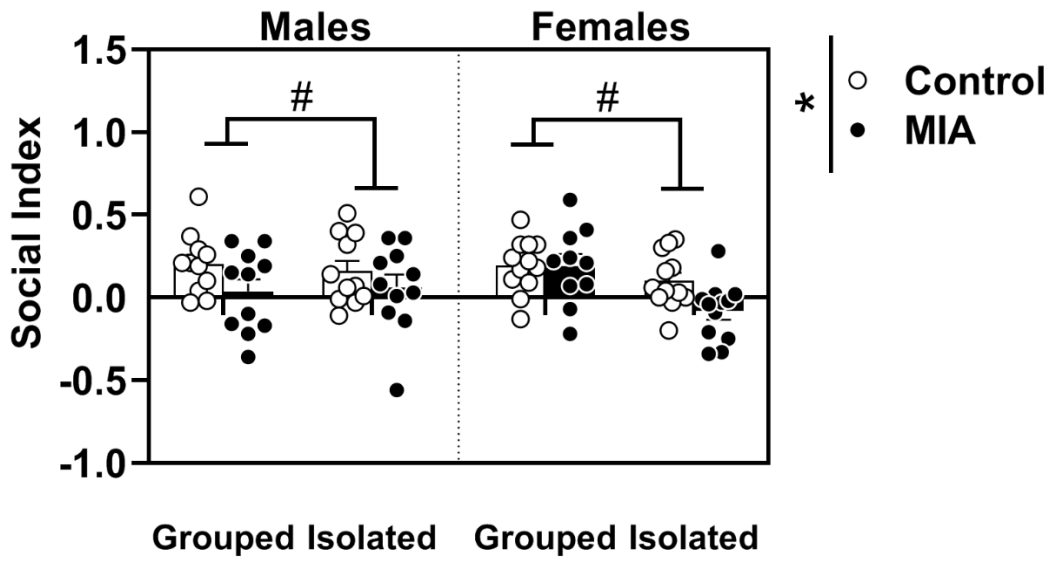


Figure 4.10: Graphic representation of the Social Index of the SPT. Individual Social Index values of male and female mice of the four experimental groups (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Bars represent mean ± SEM values (n=12 sex/arm). Data were analyzed using three-way ANOVA followed by Tukey's multiple comparisons test. MIA significance is expressed as *p<0.05. SI significance is expressed as #p<0.05.

Table 4.21: Three-way ANOVA analysis conducted in order to evaluate the effect of MIA, SI, sex and their interactions on the Social Index of the SPT.

SPT Social Index – Three-way ANOVA	
MIA	**F(1,82)= 6.74; p= 0.01**
SI	**F(1,82)= 4.08; p= 0.03**
Sex	F(1,82)= 0.11; p= 0.74
MIA x SI	F(1,82)= 0.44; p= 0.51
Sex x MIA	F(1,82)= 0.20; p= 0.65
Sex x SI	F(1,82)= 3.81; p= 0.05
Sex x MIA x SI	F(1,82)= 2.02; p= 0.16

4.1.5. EVALUATION OF THE HYPERLOCOMOTION INDUCED BY SYSTEMIC AMPHETAMINE ADMINISTRATION

Locomotor activity after systemic amphetamine administration (5 mg/kg i.p) was assessed for the evaluation of positive schizophrenia-like symptoms. As explained in **section 3.3.1.4.**, locomotor activity was recorded in intervals of 20 minutes. First, animals were placed in the actimeter and allowed to habituate for 20 minutes (time interval 0-20 min). After the habituation, basal locomotor activity was determined (interval 20-40), amphetamine was administered (time 40 min) and locomotor activity was recorded every 20 minutes. Locomotor activity (% of number of steps) after amphetamine administration was expressed as percentages of basal locomotion values.

Absolute basal locomotion values (number of steps in time interval 20-40 min) of male and female mice belonging to the four experimental groups of the study (control, MIA, SI, MIA + SI) are plotted in **table 4.22**. Basal locomotion values were not significantly affected by MIA, SI or sex [$F_{MIA}(1,38)= 2.38$; $p= 0.13$; $F_{SI}(1,38)= 0.46$; $p= 0.50$; $F_{SEX}(1,38)= 2.87$; $p= 0.10$]. However, a significant "MIA x SI" interaction was found [$F_{MIAxSI}(1,38)= 4.52$; $p= 0.04$], meaning that an increase in basal locomotion was found only in grouped mice associated with MIA (see **figure 4.11**). No other "sex x hit" or "sex x hit x hit" significant interactions were found (see **table 4.23**).

Table 4.22: Mean ± SEM values of basal locomotor activity (number of steps in time interval 20-40) for male and female mice belonging to the four experimental groups of the study.

Basal locomotor activity (20 -40 min interval) after amphetamine administration Mean ± SEM values		
	Males	**Females**
Control	1394.67 ± 118.30	1313.83 ± 90.69
MIA	1748.33 ± 65.06	1443.17 ± 115.78
SI	1530.50 ± 178.51	1413.00 ± 80.34
MIA + SI	1504.67 ± 136.91	1343.83 ± 117.74

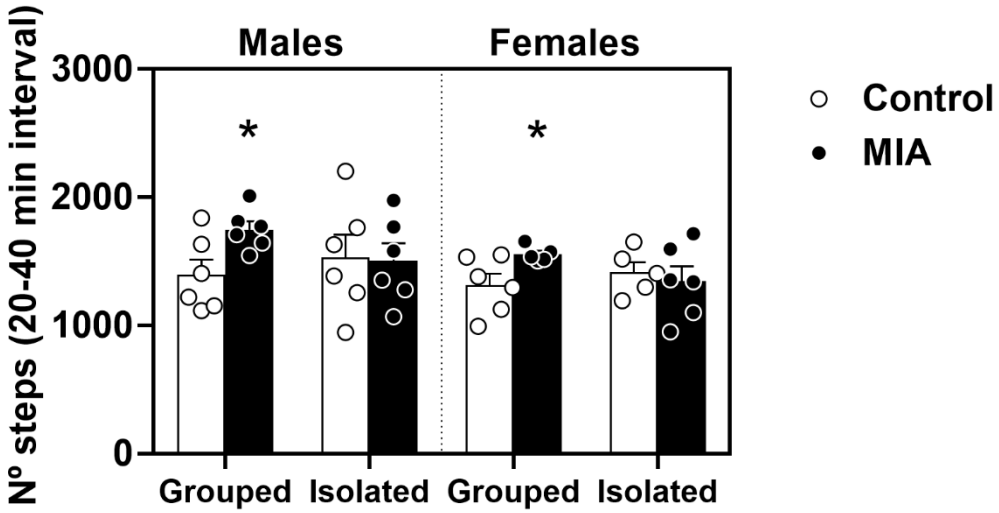


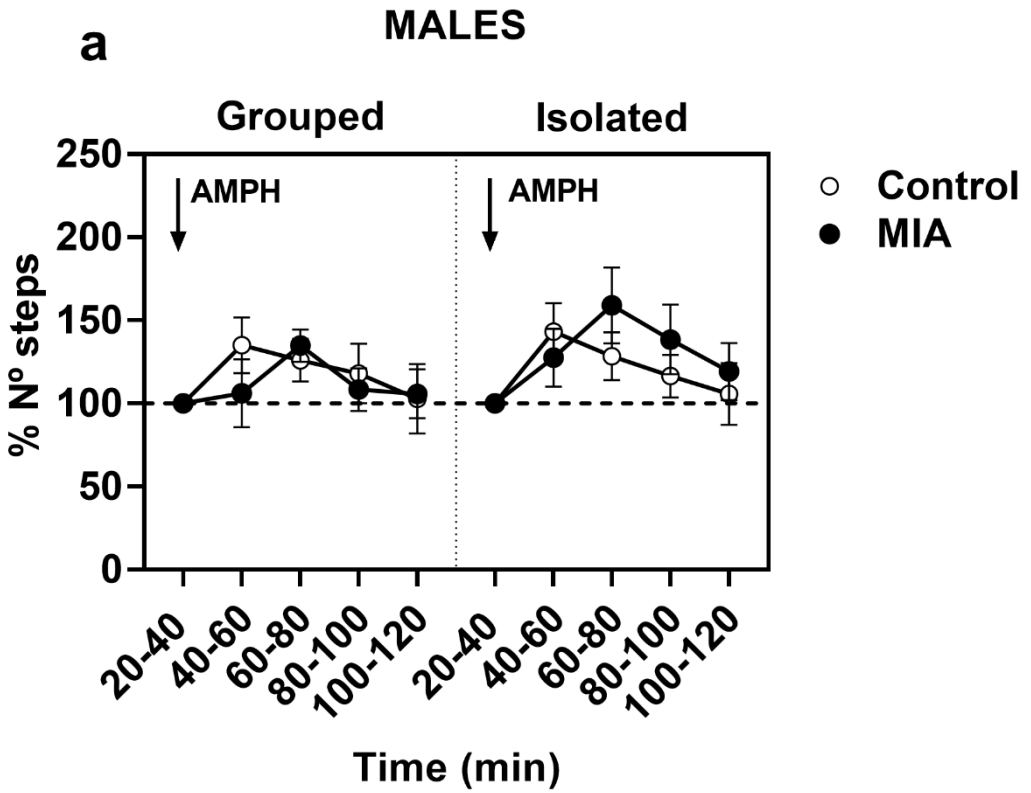
Figure 4.11: Graphic representation of the basal locomotor activity (number of steps in the 20-40 min interval). Individual locomotion values of male and female mice of the four experimental groups (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Bars represent mean ± SEM values (n= 6 sex/arm). Data were analyzed using three-way ANOVA followed by Tukey's multiple comparisons test. MIAxSI significance is expressed as *p<0.05 (inside the graph).

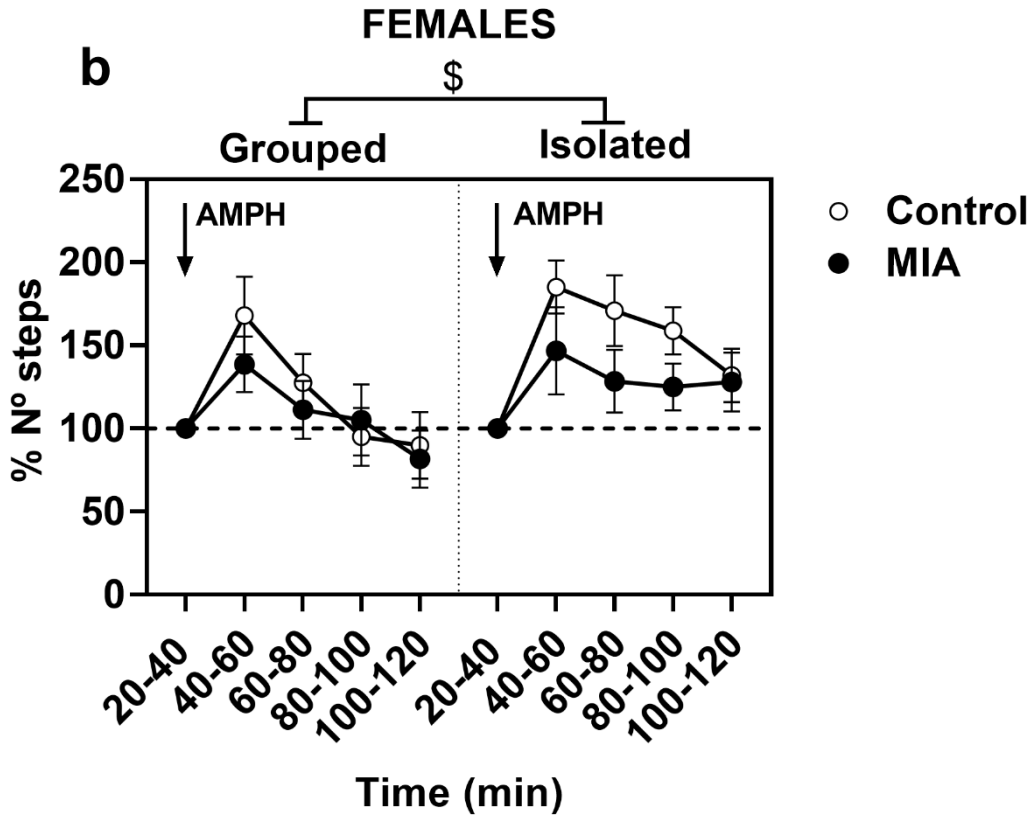
Table 4.23: Three-way ANOVA analysis conducted in order to evaluate the effect of MIA, SI, sex and their interactions on the basal locomotor activity.

Basal locomotor activity (N^0 steps in the 20-40 min interval) – Three-way ANOVA	
MIA	F(1,38)= 2.38; p= 0.13
SI	F(1,38)= 0.46; p= 0.50
Sex	F(1,38)= 2.87; p= 0.10
MIA x SI	**F(1,38)= 4.52; p= 0.04**
Sex x MIA	F(1,38)= 0.22; p= 0.64
Sex x SI	F(1,38)= <0.0001; p= 0.99
Sex x MIA x SI	F(1,38)= 0.04; p= 0.84

In males, systemic amphetamine administration exerted an increase of locomotor activity, reaching a maximal effect of 135.00 ± 16.77% at 40-60 min time interval in control group; 134.83 ± 9.65% at 60-80 min time interval in MIA group; 143.17 ± 17.16% at 40-60 min time interval in isolated group and 159.00 ± 22.82% at 60-80 min time interval in the double-hit group. No statistical differences were observed between compared groups (see **figure 4.12 a** and **table 4.24** for the statistical analysis).

When amphetamine was administered to female mice, similar effects were observed (168.00 ± 23.29% at 40-60 min interval in control group; 138.67 ± 16.73% at 40-60 min interval in MIA group; 185.20 ± 15.85% at 40-60 min interval in isolated group and 146.83 ± 26.27% at 40-60 min interval in the female double hit group). Statistical comparison between groups revealed differences between grouped and isolated animals [F_{SI}(1,19)= 5.36; p= 0.03]. Hence, isolated female mice showed a greater sensitivity to amphetamine-induced hyperlocomotion (see **figure 4.12 b** and **table 4.24** for the statistical analysis).


a
MALES
Grouped
Isolated
AMPH
AMPH
Control
MIA
% Nº steps
250
200
150
100
50
0
20-40
40-60
60-80
80-100
100-120
Time (min)


b
FEMALES
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Grouped
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Control
MIA
% Nº steps
250
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150
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50
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20-40
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60-80
80-100
100-120
Time (min)

Figure 4.12: Graphic representation of the locomotor response induced by systemic amphetamine administration (5 mg/kg i.p.) in male (**figure 4.12 a**) and female (**figure 4.12 b**) mice. Mean locomotion values of male and female mice of the four experimental groups (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Vertical arrow indicates the time of administration of amphetamine (AMPH). Data were analyzed using three-way repeated measures ANOVA followed by Tukey's multiple comparisons test. Data represent the locomotor activity (% of number of steps) after amphetamine administration and are expressed as percentages of basal locomotion values. Points are representations of the mean ± SEM values (n=6/sex/arm). SI significance is expressed as *p<0.05.

Table 4.24: Three-way repeated measures ANOVA analysis conducted in order to evaluate the effect of MIA, SI, time and their interactions on the locomotor activity after systemic amphetamine administration.

	Males	**Females**
MIA	F(1,20)= 0.05; p= 0.83	F(1,19)=2.12; p= 0.16
SI	F(1,20)= 0.82; p= 0.38	**F(1,19)=5.36; p= 0.03**
Time	**F(3.05, 60.92)= 6.49; p= 0.0007**	**F(2.41, 45.82)= 10.88; p<0.001**
MIA x SI	F(1,20)= 0.48; p= 0.50	F(1,19)=0.45; p=0.51
Time x MIA	F(4, 80)= 1.77; p= 0.14	F(4, 76)= 1.06; p= 0.38
Time x SI	F(4, 80)= 0.29; p= 0.89	F(4, 76)= 1.78; p= 0.14
Time x MIA x SI	F(4, 80)= 0.26; p= 0.90	F(4, 76)= 0.49; p= 0.75

4.1.6. EVALUATION OF THE HEAD-TWITCH RESPONSE INDUCED BY SYSTEMIC PSILOCYBIN ADMINISTRATION

Head-Twitch Response (HTR) after systemic psilocybin administration (1 mg/kg i.p.) was evaluated in C57BL/6 female control (vehicle-treated and grouped animals) and double-hit groups (Poly (I:C)-treated and socially-isolated animals).

Student's *t-test* analysis revealed a significant increase in the number of HTR performed by the double-hit group (double-hit: 15.50 ± 1.03 HTR events) compared to the control group (control: 12.00 ± 0.47 HTR events) (t= 3.21; p= 0.005) (**figure 4.13**). Thus, female double-hit mice probe to be more sensitive to the HTR induced by the psychedelic psilocybin than control female mice.

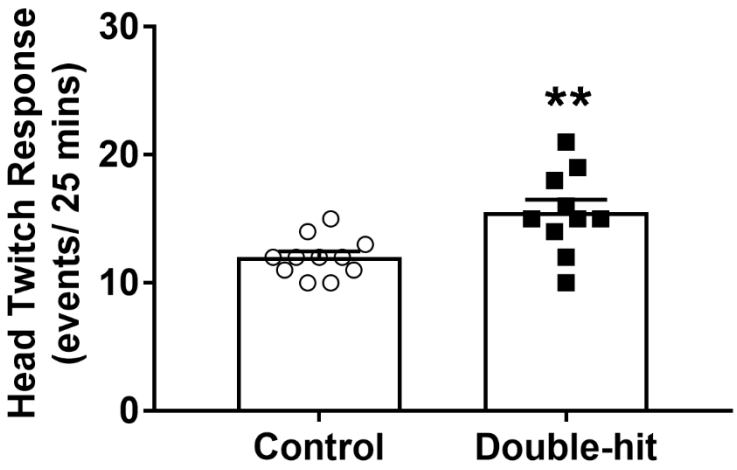


Figure 4.13: Head twitch response induced by psilocybin (1 mg/kg i.p.) in control (n= 12) and double-hit (n= 11) female mice. Number of HTR events performed by the experimental groups were manually assessed during 25 min. Data were analyzed by unpaired Student's *t-test*. Bars are representation of mean values ± SEM. Statistical significance is expressed as **p<0.01).

4.1.7. EVALUATION OF THE PLASMATIC CONCENTRATION OF DIFFERENT CYTOKINES

Proinflammatory (IL-6, IFN-γ, TNFα, IL-17A, IL-1β and IL-2) and anti-inflammatory (IL-10) cytokines were quantified in plasma samples of the four experimental groups by means of the high sensitivity multiplex immunoassay ELISA Luminex®. The male and female mean ± SEM plasma concentration values of the different proinflammatory and anti-inflammatory cytokines for all the experimental groups are shown in **tables 4.25** and **4.26**, respectively.

Plasma concentrations of IL-6 were 2.49 ± 0.65 pg/ml in male control-grouped animals, and 1.50 ± 1.34 pg/ml in female control-grouped animals. MIA induced a significant increase of IL-6 ($F_{MIA}(1,54)= 5.85$, p= 0.02). Plasma concentration of IL-6 was not influenced by the effect of SI [$F_{SI}(1,54)= 0.64$, p= 0.43], nor by sex [$F_{SEX}(1,54)= 0.02$, p= 0.89]. However, a significant interaction between SI and sex was found [$F_{SEXxSI}(1,54)= 5.60$, p= 0.02], as SI induced an increase of IL-6 plasma concentrations in female-isolated mice but not in male-isolated animals (f**igure 4.14 a**). No other "MIA x SI", "Sex x MIA" and "Sex x MIA x SI" interactions were found (see **table 4.27**).

IFN-γ plasmatic concentrations were 0.46 ± 0.11 pg/ml for male control-grouped animals, and of 1.51 ± 0.48 pg/ml for female control-grouped animals. Plasmatic concentration of IFN-γ was not significantly influenced by MIA [$F_{MIA}(1,54)=3.337$; p=0.07], although a non-statistically significant increase of IFN-γ concentration by effect of MIA was detected. In the same way,

plasmatic concentration of IFN-γ was not significantly influenced by SI [$F_{SI}(1,54)= 3.37$, $p= 0.07$], although a non-statistically significant increase of IFN-γ concentration by effect of SI was detected, specifically in female mice [$F_{SEXxSI}(1,54)= 3.50$, $p= 0.07$]. Plasmatic IFN-γ levels were significantly affected by sex [$F_{SEX}(1,54)= 4.67$; $p= 0.04$], with female mice having higher IFN-γ plasmatic levels than male mice (**figure 4.14 b**). No other statistically significant "sex x hit", "hit x hit" or "sex x hit x hit" interactions were found (see **table 4.27**).

Regarding TNF-α, plasmatic levels were 8.35 ± 1.35 pg/ml in male control-grouped animals, and of 10.09 ± 2.38 pg/ml in female control-grouped animals. Plasma concentrations of TNF-α were not influenced by MIA [$F_{MIA}(1,60)= 0.50$, $p= 0.48$]. However, TNF-α levels were significantly reduced by the effect of SI ($F_{SI}(1,60)= 13.06$; $p= 0.0006$). TNF-α plasma levels were not influenced by sex [$F_{SEX}(1,60)= 0.95$, $p= 0.33$] **(figure 4.14 c)**. No other statistically significant "sex x hit", "hit x hit" or "sex x hit x hit" interactions were found (see **table 4.27**).

Plasma concentrations of IL-17A were 10.41 ± 1.48 pg/ml in male control-grouped animals, and of 19.86 ± 4.16 pg/ml in female control-grouped animals. Plasmatic levels of IL-17A were not affected by MIA [$F_{MIA}(1,58)= 0.28$, $p= 0.60$], whereas they were significantly decreased by the effect of SI [$F_{SI}(1,58)= 10.74$, $p= 0.002$], and significantly influenced by sex [$F_{SEX}(1,58)= 19.20$, $p<0.0001$], with higher IL-17A plasmatic concentrations in females than in males. Moreover, a significant MIA x SI interaction was found [$F_{MIAxSI}(1,58)= 6.64$, $p= 0.01$], as MIA-grouped animals had a reduction in IL-17A levels compared to control-grouped animals, while MIA-isolated mice do not present this reduction in IL-17A levels (**figure 4.14 d**) compared to control-isolated mice. Thus, the effect of SI on IL-17A plasma levels is different in control and MIA mice, inducing a reduction in control mice but not in MIA mice. No other "Sex x SI", Sex x MIA" or "Sex x MIA x SI" interactions were found (see **table 4.27**).

IL-1β plasmatic concentrations were 0.70 ± 0.25 pg/ml in male control-grouped animals, and of 1.86 ± 0.16 pg/ml in female control-grouped animals. Plasmatic concentrations of IL-1β were not influenced by the effect of MIA [$F_{MIA}(1,52)= 0.07$, $p= 0.79$] or SI [$F_{SI}(1,52)= 1.05$, $p= 0.31$]. However, a significant effect of sex was found [$F_{SEX}(1,52)= 63.09$, $p<0.0001$], as female mice had higher IL-1β plasma concentrations than male mice. In addition, a significant "Sex x MIA x SI" interaction was found [$F_{SEXxMIAxSI}(1,52)= 4.08$, $p= 0.04$], meaning that SI and MIA had a different effect on male and female IL-1β plasma concentration. Thus, in males, IL-1β concentration was reduced by the effect of both MIA and SI. However, in female-grouped mice, IL-1β plasma concentration was increased in MIA mice compared to control mice; while this increase in IL-1β plasma concentration induced by MIA is not present in female-isolated mice, suggesting that SI

reverts the MIA effect in females (**figure 4.14 e**). No other "Sex x SI", "MIA x SI" and "Sex x MIA" interactions were found (see **table 4.27**).

Regarding the proinflammatory cytokine IL-2, plasma levels were 1.52 ± 0.50 pg/ml in male control-grouped animals, and of 3.07 ± 1.66 pg/ml in female control-grouped animals. Plasmatic levels of IL-2 were not globally influenced by effect of MIA [$F_{MIA}(1,54)= 1.51$, $p= 0.23$] or SI [$F_{SI}(1,54)= 3.21$, $p= 0.08$]. However, a significant effect of sex was found [$F_{SEX}(1,54)= 36.33$, $p<0.0001$], with females having a higher IL-2 plasma concentration. Moreover, a significant "Sex x SI" interaction was found [$F_{SEXxSI}(1,54)= 4.74$, $p= 0.03$], since SI induced a decrease in IL-2 plasma levels in males but a significant increase in females (**figure 4.14 f**). No other "MIA x SI", "Sex x MIA" or "Sex x MIA x SI" were found (see **table 4.27**).

IL-10 plasma concentrations were 4.72 ± 1.00 pg/ml in male control-grouped animals, and of 14.42 ± 4.73 pg/ml in female control-grouped animals. Plasmatic levels of IL-10 were not globbally influenced by effect of MIA [$F_{MIA}(1,56)= 0.31$, $p= 0.58$] or SI [$F_{SI}(1,56)= 0.13$, $p= 0.72$]. However, a significant effect of sex was found [$F_{SEX}(1,56)= 24.79$, $p<0.0001$], with females having higher IL-10 plasma concentrations (see **figure 4.14 g**). Moreover, an almost statistically significant "MIA x SI" interaction [$F_{MIAxSI}(1,56)= 3.45$, $p= 0.07$] was found, since SI induced a decrease of IL-10 plasma concentrations in control mice, whereas in MIA mice SI increased the IL-10 plasma levels. No other "Sex x hit" or "Sex x hit x hit" interactions were found (see **table 4.27**).

Table 4.25: Mean ± SEM values of the plasmatic concentration (pg/ml) of different proinflammatory and anti-inflammatory cytokines obtained in ELISA Luminex® immunoassay experiments conducted in male control, MIA, SI and MIA + SI experimental groups.

Plasmatic levels of different cytokines – Mean ± SEM				
	Males			
	Control	**MIA**	**SI**	**MIA + SI**
IL-6	2.49 ± 0.65	23.24 ± 10.25	1.09 ± 0.45	1.36 ± 0.44
IFN-γ	0.46 ± 0.11	1.26 ± 0.36	0.24 ± 0.00	1.18 ± 0.23
TNF-α	8.35 ± 1.35	7.17 ± 0.46	5.12 ± 0.73	6.10 ± 0.68
IL-17A	10.41 ± 1.48	6.48 ± 1.29	5.25 ± 0.98	5.80 ± 0.90
IL-1β	0.70 ± 0.25	0.10 ± 0.01	0.09 ± 0.00	0.09 ± 0.00
IL-2	1.52 ± 0.50	0.84 ± 0.23	0.62 ± 0.10	0.77 ± 0.19
IL-10	1.33 ± 0.17	1.19 ± 0.15	1.33 ± 0.03	1.28 ± 0.08

Table 4.26: Mean ± SEM values of the plasmatic concentration (pg/ml) of different proinflammatory and anti-inflammatory cytokines obtained in ELISA Luminex® immunoassay experiments conducted in female control, MIA, SI and MIA + SI experimental groups.

Plasmatic levels of different cytokines – Mean ± SEM				
Females				
	Control	**MIA**	**SI**	**MIA + SI**
IL-6	1.50 ± 1.34	7.80 ± 1.28	6.29 ± 1.64	14.53 ± 3.73
IFN-γ	1.51 ± 0.48	2.53 ± 0.60	3.62 ± 1.33	30.26 ± 17.27
TNF-α	10.09 ± 2.38	8.84 ± 1.08	6.01 ± 0.96	5.09 ± 1.39
IL-17A	19.86 ± 4.16	14.39 ± 2.41	7.29 ± 1.68	12.94 ± 4.67
IL-1β	1.86 ± 0.16	3.06 ± 0.73	2.39 ± 0.49	2.07 ± 0.50
IL-2	3.07 ± 1.66	8.88 ± 1.57	10.50 ± 5.70	11.33 ± 3.41
IL-10	14.42 ± 4.73	10.46 ± 1.73	9.15 ± 2.41	14.21 ± 4.39

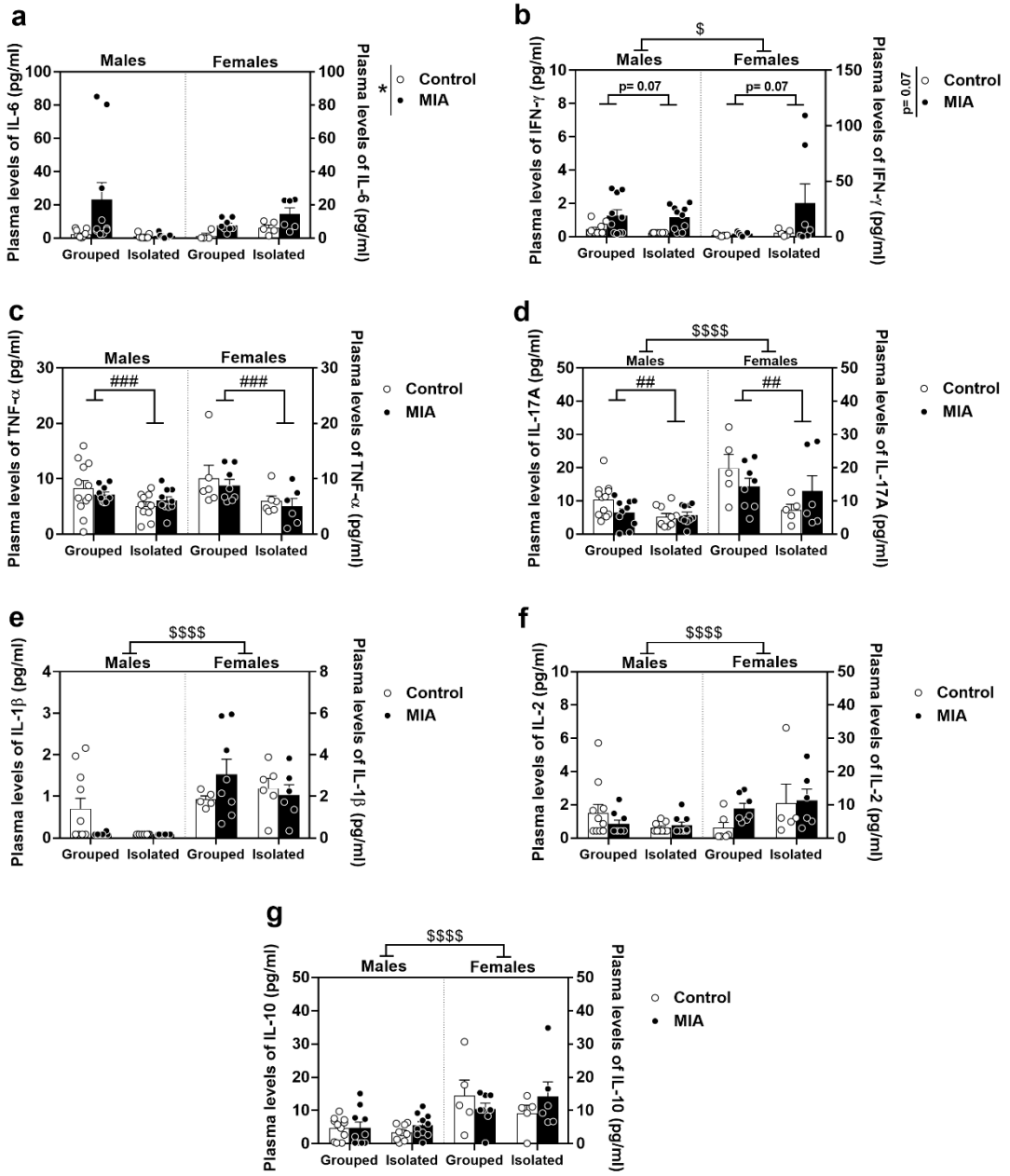


Figure 4.14: Graphic representation of the concentration of the proinflammatory (IL-6, IFN-γ, TNF-α, IL-17A, IL-1β and IL-2) (**figure 4.14 a-f**) and anti-inflammatory (IL-10) (**figure 4.14 g**) cytokines determined in mouse plasma by means of the high sensitivity multiplex immunoassay ELISA Luminex®. Individual concentration values (pg/ml) of male and female mice of the four experimental groups (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Bars represent mean ± SEM values (n= 4-11 sex/arm). Data were analyzed using three-way ANOVA followed by Tukey's multiple comparisons test. MIA significance is expressed as *p<0.05. SI significance is expressed as #p<0.05. Sex significance is expressed as p_SEX$<0.05.

Table 4.27: Three-way ANOVA analysis conducted in order to evaluate the effect of MIA, SI, sex and their interactions on the plasma concentration of different proinflammatory and anti-inflammatory cytokines.

	IL-6	IFN-γ	TNF-α	IL-17A	IL-1β	IL-2	IL-10
MIA	**F(1,54)= 5.85, p= 0.02**	F(1,54)= 3.34, p= 0.07	F(1,60)= 0.50, p= 0.48	F(1,58)= 0.28, p= 0.60	F(1,52)= 0.07, p= 0.79	F(1,54)= 1.51, p= 0.23	F(1,56)= 0.31, p= 0.58
SI	F(1,54)= 0.64, p= 0.43	F(1,54)= 3.37, p= 0.07	**F(1,60)= 13.06,p< 0.001**	**F(1,58)=10.74,p= 0.002**	F(1,52)= 1.05, p= 0.31	F(1,54)= 3.21, p= 0.08	F(1,56)= 0.13, p= 0.72
Sex	F(1,54)= 0.02, p= 0.89	**F(1,54)= 4.67, p= 0.04**	F(1,60)= 0.95, p= 0.33	**F(1,58)=19.20,p<0.0001**	**F(1,52)=63.09,p<0.0001**	**F(1,54)=36.33,p<0.0001**	**F(1,56)= 24.79, p<0.0001**
MIA x SI	F(1,54)= 1.59, p= 0.21	F(1,54)= 2.56, p= 0.12	F(1,60)= 0.55, p= 0.46	**F(1,58)= 6.64, p= 0.01**	F(1,52)= 0.78, p= 0.38	F(1,54)= 0.70, p= 0.41	F(1,56)= 3.45, p= 0.07
Sex x MIA	F(1,54)= 0.19, p= 0.66	F(1,54)= 2.59, p= 0.11	F(1,60)= 0.35, p= 0.56	F(1,58)= 0.35, p= 0.56	F(1,52)= 1.97, p= 0.17	F(1,54)= 2.07, p= 0.16	F(1,56)= 0.04, p= 0.85
Sex x SI	**F(1,54)= 5.60, p= 0.02**	F(1,54)= 3.50, p= 0.07	F(1,60)= 1.10, p= 0.30	F(1,58)= 1.83, p= 0.18	F(1,52)= 0.02, p= 0.88	**F(1,54)= 4.74, p= 0.03**	F(1,56)= 0.02, p= 0.88
Sex x MIA x SI	F(1,54)= 2.32, p= 0.13	F(1,54)= 2.51, p= 0.12	F(1,60)= 0.29, p= 0.59	F(1,58)= 1.20, p= 0.28	**F(1,52)= 4.08, p= 0.04**	F(1,54)= 1.36, p= 0.25	F(1,56)= 1.34, p= 0.25

4.1.8. EVALUATION OF THE mRNA EXPRESSION OF THE GENES ENCODING FOR NF-κB (*Rela*) AND ITS REPRESSOR IκBα (*Nfkbia*)

RT-qPCR experiments were performed in order to evaluate the relative mRNA levels of the genes encoding for NF-κB and IκBα proteins (*Rela* and *Nfkbia*, respectively) in cortical samples of the four experimental groups.

Relative mRNA levels of *Rela* in control animals were 1.20 ± 0.05 in males and 0.91 ± 0.04 in females (see **table 4.28** for mean ± SEM values of *Rela* relative mRNA of all the experimental groups). An increase of *Rela* mRNA levels was associated to MIA [$F_{MIA}(1,56)= 4.31$; $p= 0.04$]. Moreover, *Rela* gene expression was significantly influenced by sex [$F_{SEX}(1,56)= 61.43$; $p<0.0001$], with greater gene expression levels in male than female mice (**figure 4.15 a**). Although the "Sex x MIA" interaction was not significant, the MIA significance is mostly attributable to the increase in *Rela* mRNA levels in male mice. No effect of SI was shown in *Rela* gene expression [$F_{SI}(1,56)= 0.33$; $p= 0.57$]. No other significant "sex x hit", "hit x hit" or "sex x hit x hit" interactions were observed (see **table 4.29**).

Relative mRNA levels of *Nfkbia* in control animals were 1.02 ± 0.04 in males and 1.13 ± 0.08 in females (see **table 4.28** for mean ± SEM values of *Nfkbia* relative mRNA of all the experimental groups). *Nfkbia* gene expression was not modulated by the effect of MIA [$F_{MIA}(1, 56)= 1.23$; $p= 0.27$], SI [$F_{SI}(1, 56)= 2.27$; $p= 0.14$] or sex [$F_{SEX}(1, 56)= 2.14$; $p= 0.15$]. However, only in female mice, MIA was significantly associated to decreased *Nfkbia* mRNA levels [$F_{SEXxMIA}(1, 56)= 7.04$; $p= 0.01$] (**figure 4.15 b**). No other significant "sex x hit", "hit x hit" or "sex x hit x hit" interactions were found (see **table 4.29**).

Table 4.28: Mean ± SEM values of *Rela* and *Nfkbia* relative mRNA in RT-qPCR experiments conducted in male and female control, MIA, SI and MIA + SI experimental groups.

Relative mRNA levels – Mean ± SEM values				
	Rela		***Nfkbia***	
	Males	**Females**	**Males**	**Females**
Control	1.20 ± 0.05	0.91 ± 0.04	1.02 ± 0.04	1.13 ± 0.08
MIA	1.36 ± 0.09	0.89 ± 0.08	1.11 ± 0.10	0.99 ± 0.04
SI	1.16 ± 0.10	0.83 ± 0.05	1.03 ± 0.08	1.35 ± 0.08
MIA + SI	1.34 ± 0.08	0.91 ± 0.06	1.10 ± 0.06	1.09 ± 0.09

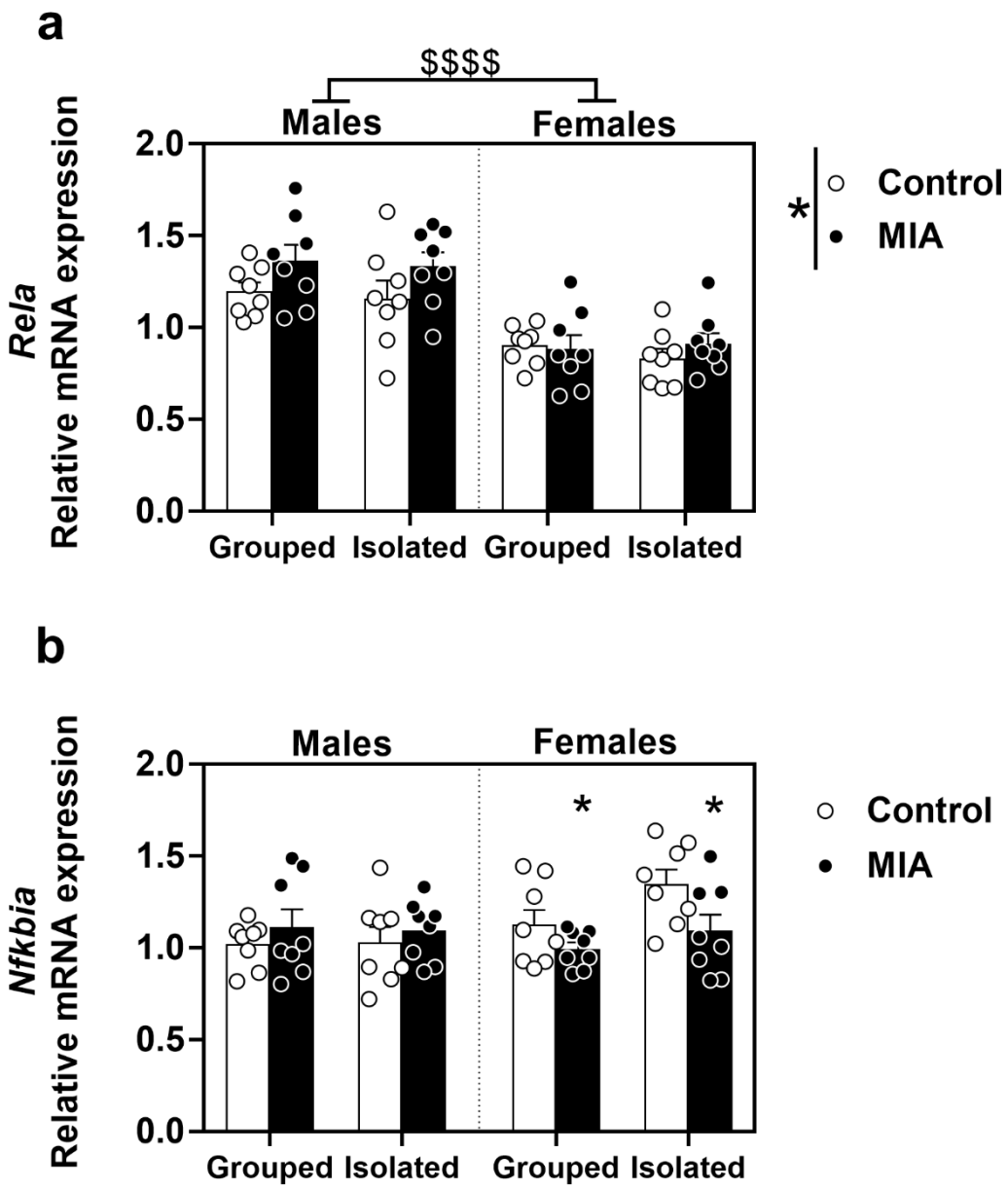


Figure 4.15: Graphic representation of relative mRNA levels of *Rela* (**figure 4.15 a**) and *Nfkbia* (**figure 4.15 b**) obtained by means of RT-qPCR. Individual relative mRNA values of male and female mice of the four experimental groups (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Bars represent mean ± SEM values (n= 8 sex/arm). Data were analyzed using three-way ANOVA followed by Tukey's multiple comparisons test. MIA significance is expressed as *p<0.05. Sex significance is expressed as $$$$p<0.0001. MIAxSex significance is expressed as *p<0.05 (inside the graph).

Table 4.29: Three-way ANOVA analysis conducted in order to evaluate the effect of MIA, SI, sex and their interactions on the mRNA levels of *Rela* and *Nfkbia*.

Relative mRNA levels – Three-way ANOVA		
	***Rela* gene expression**	***Nfkbia* gene expression**
MIA	**F(1,56)= 4.31; p= 0.04**	F(1,56)= 1.23; p= 0.27
SI	F(1,56)= 0.33; p= 0.57	F(1,56)= 2.27; p= 0.14
Sex	**F(1,56)= 61.43; p<0.0001**	F(1,56)= 2.14; p= 0.15
MIA x SI	F(1,56)= 0.32; p= 0.57	F(1,56)= 0.52; p= 0.47
Sex x MIA	F(1,56)= 2.05; p= 0.16	**F(1,56)= 7.04; p= 0.01**
Sex x SI	F(1,56)= 0.01; p= 0.92	F(1,56)= 2.63; p= 0.11
Sex x MIA x SI	F(1,56)= 0.23; p= 0.64	F(1,56)= 0.21; p= 0.65

4.1.9. EVALUATION OF THE PROTEIN EXPRESSION OF NF-κB AND ITS REPRESSOR IκBα

4.1.9.1. CHARACTERIZATION OF THE ANTIBODIES USED FOR THE IMMUNODETECTION OF THE NF-κB AND IκBα PROTEINS IN MOUSE BRAIN CORTEX

4.1.9.1.1. ANTIBODY SELECTION FOR THE IMMUNODETECTION OF NF-κB

The mouse Rela or p65 member of NF-κB family is a 549 amino acids protein, with an estimated molecular weight of 65 kDa.

For the immunodetection of this protein, the sc-327 antibody was used as previously plotted in **table 3.11**. The sc-327 showed a single band around 65 kDa, consistent with the expected molecular weight of Rela/p65. The sc-327 antibody was tested at 1:500 dilution, which was chosen based in previous publications (Ibi et al., 2017).

To determine an appropriate protein load on the electrophoresis gel, the relationship between the amount of protein loaded and the intensity of the immunoreactive signal was evaluated. For that purpose, increasing amounts of protein were loaded (10, 20 and 30 µg) of two different pools made with cytosolic and nuclear fractions, respectively. Finally, a 20 µg load was chosen as a suitable protein amount for the immunodetection of Rela/p65 in mouse brain cortex samples. As Rela/p65 is both expressed in cytosolic and nuclear fractions, membranes were also incubated with the histone 1 (H1) antibody (sc-8030, 1:250 dilution, Santa Cruz) as a marker of nuclear-specificity. H1 is only expressed in the nucleus and has a molecular weight of 32-33 kDa (see **figure 4.16**).

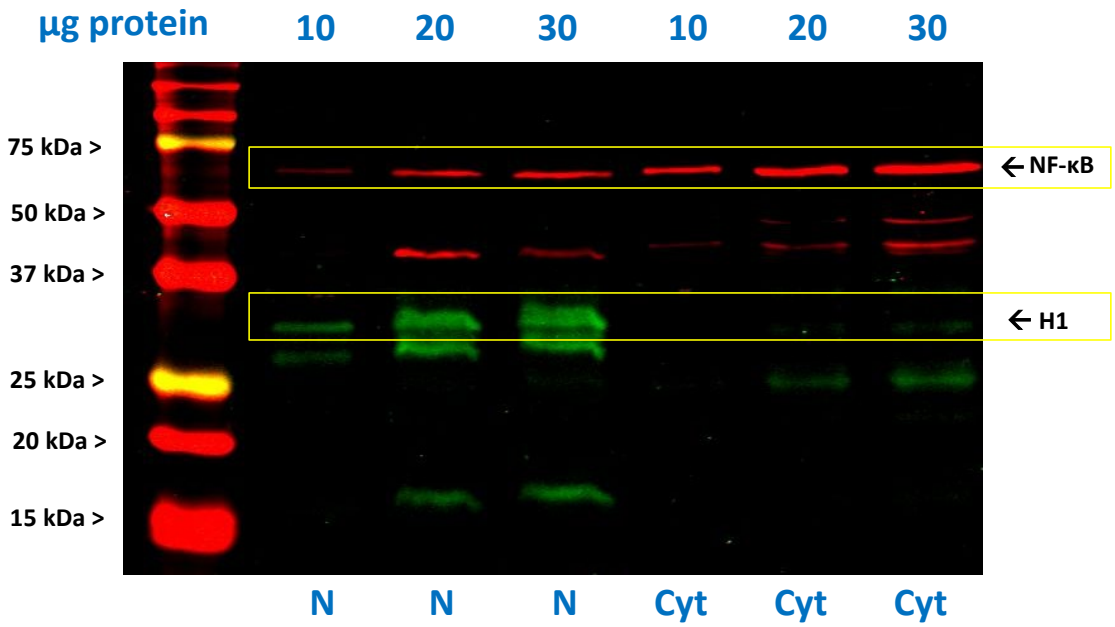


Figure 4.16: Representative image of the immunoreactive signal obtained with the NF-κB (Rela/p65) antibody (sc-327) tested for the western blot experiments. Different protein amounts were loaded (10-30 µg) and antibodies were tested in both nuclear and cytosolic fractions. Membranes were blocked with 5% milk and 0.5% BSA and incubated with 5% milk, 0.5% BSA and 0.1% Tween20. A well-defined band was obtained around 65 kDa corresponding to NF-κB (Rela/p65), in both nuclear and cytosolic fractions (in red). A well-defined band around 32-33 kDa was obtained corresponding to H1 only in nuclear fractions (in green). Abbreviations: Cyt= cytosolic fraction. kDa= kilo Daltons. N= nuclear fraction.

In the western blot experiments in mouse brain cortex, Rela/p65 target protein was measured in nuclear fractions with sc-327 primary antibody, and the immunoreactive signal values were corrected by the loading control protein β-actin.

4.1.9.1.2. ANTIBODY SELECTION FOR THE IMMUNODETECTION OF IκBα

In mouse, IκBα, the principal inhibitor of NF-κB, is a 314 amino acids protein, with an estimated molecular weight of 35-41 kDa.

For the immunodetection of this protein, the sc-371 antibody was used as previously plotted in **table 3.11**. The sc-371 showed a single band around 45 kDa, consistent with the expected molecular weight of IκBα. In this experiments, the sc-371 antibody was tested at 1:500 dilution, based in previous publications (Ibi et al., 2017).

To determine an appropriate protein load on the electrophoresis gel, the relationship between the amount of protein loaded and the intensity of the immunoreactive signal was evaluated. For that purpose, increasing amounts of protein were loaded (15, 20 and 25 µg) of two different pools made with cytosolic and nuclear fractions, respectively. Finally, a 20 µg load was chosen as a suitable protein amount for the immunodetection of IκBα in mouse brain cortex samples. As the IκBα protein is only expressed in cytosol, we incubated the antibodies in both cytosolic and nuclear fractions and we corroborated that IκBα was only expressed in the cytosol (see **figure 4.17**).

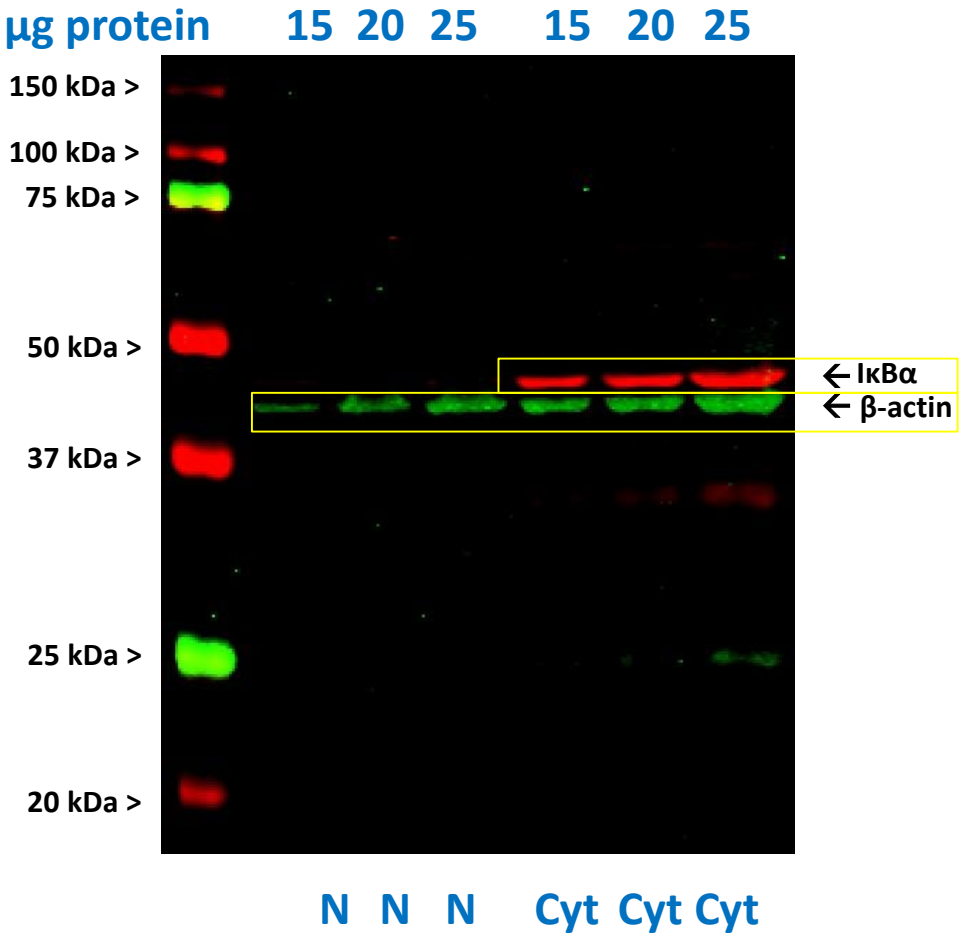


Figure 4.17: Representative image of the immunoreactive signal obtained with the IκBα antibody (sc-371) tested for the western blot experiments. Different protein amounts were loaded (15, 20, 25 µg) and antibodies were tested in both nuclear and cytosolic fractions. Membranes were blocked with 5% milk and 0.5% BSA and incubated with 5% milk, 0.5% BSA and 0.1% Tween20. A well defined band was obtained around 45 kDa corresponding to IκBα only in cytosolic fractions (in red). A well defined band around 42 kDa was obtained corresponding

to β-actin in both cytosolic and nuclear fractions (in green). Abbreviations: Cyt= cytosolic fraction. kDa= kilo Daltons. N= nuclear fraction.

Hence, in the western blot experiments in mouse brain cortex, IκBα target protein was measured in cytosolic fractions with sc-371 primary antibody, and the immunoreactive signal values were corrected by the loading control protein β-actin.

4.1.9.2. WESTERN BLOT EXPERIMENTS FOR THE IMMUNODETECTION OF NF-κB AND IκBα PROTEINS

Western Blot experiments were performed to evaluate protein expression levels of NF-κB (RelA or p65 member of the NF-κB transcription factor family) and IκBα (principal NF-κB repressor belonging to the IκB family) in cortical samples of the four experimental groups.

Protein expression levels of NF-κB in control animals were 0.97 ± 0.05 in males and 1.02 ± 0.07 in females (see **table 4.30** for mean ± SEM values of protein expression of all the experimental groups). NF-κB nuclear expression was not affected by the effect of MIA [$F_{MIA}(1,56)= 0.05$; $p= 0.82$] nor by the effect of SI [$F_{SI}(1,56)= 0.07$; $p= 0.79$]. However, NF-κB protein expression was significantly influenced by sex [$F_{SEX}(1,56)=5.51$ $p= 0.02$], with higher NF-κB expression in females than in males (**figure 4.18 a**). Although non-significant, a "Sex x MIA x SI" interaction was observed [$F_{SEXxMIAxSI}(1,56)= 3.60$; $p= 0.06$], since the effect of SI was different in control and MIA mice and also depending on the sex. Thus, SI induced a decrease in NF-κB protein levels in control male mice, while it induced an increase in control female mice. Moreover, SI increased NF-κB levels in MIA male mice, while it was associated to decreased NF-κB levels in MIA female mice. No other statistically significant "sex x hit" or "hit x hit" interactions were found in these experiments (see **table 4.31**).

Protein expression levels of IκBα in control animals were 0.96 ± 0.04 in males and 1.09 ± 0.04 in females (see **table 4.30** for mean ± SEM values of protein expression of all the experimental groups). IκBα cytoplasmic expression was not affected by MIA [$F_{MIA}(1,56)= 0.21$; $p= 0.65$], nor by SI [$F_{SI}(1,56)=0.14$; $p=0.71$]. However, IκBα protein expression was significantly influenced by sex [$F_{SEX}(1,56)=14.49$ $p<0.001$] with a higher IκBα expression in females than in males (**figure 4.18 b**). No other statistically significant "sex x hit", "hit x hit" or "sex x hit x hit" interactions were found in these experiments (see **table 4.31**).

Table 4.30: Mean ± SEM values of NF-κB and IκBα protein expression in western blot experiments conducted in male and female control, MIA, SI and MIA + SI experimental groups.

Protein expression (immunoreactivity) – Mean ± SEM values				
	NF-κB (RelA or p65)		**IκBα**	
	Males	**Females**	**Males**	**Females**
Control	0.97 ± 0.05	1.02 ± 0.07	0.96 ± 0.04	1.09 ± 0.04
MIA	0.92 ± 0.06	1.09 ± 0.06	0.99 ± 0.03	1.06 ± 0.06
SI	0.90 ± 0.06	1.11 ± 0.08	0.96 ± 0.04	1.08 ± 0.05
MIA + SI	1.02 ± 0.07	1.01 ± 0.04	0.98 ± 0.03	1.12 ± 0.06

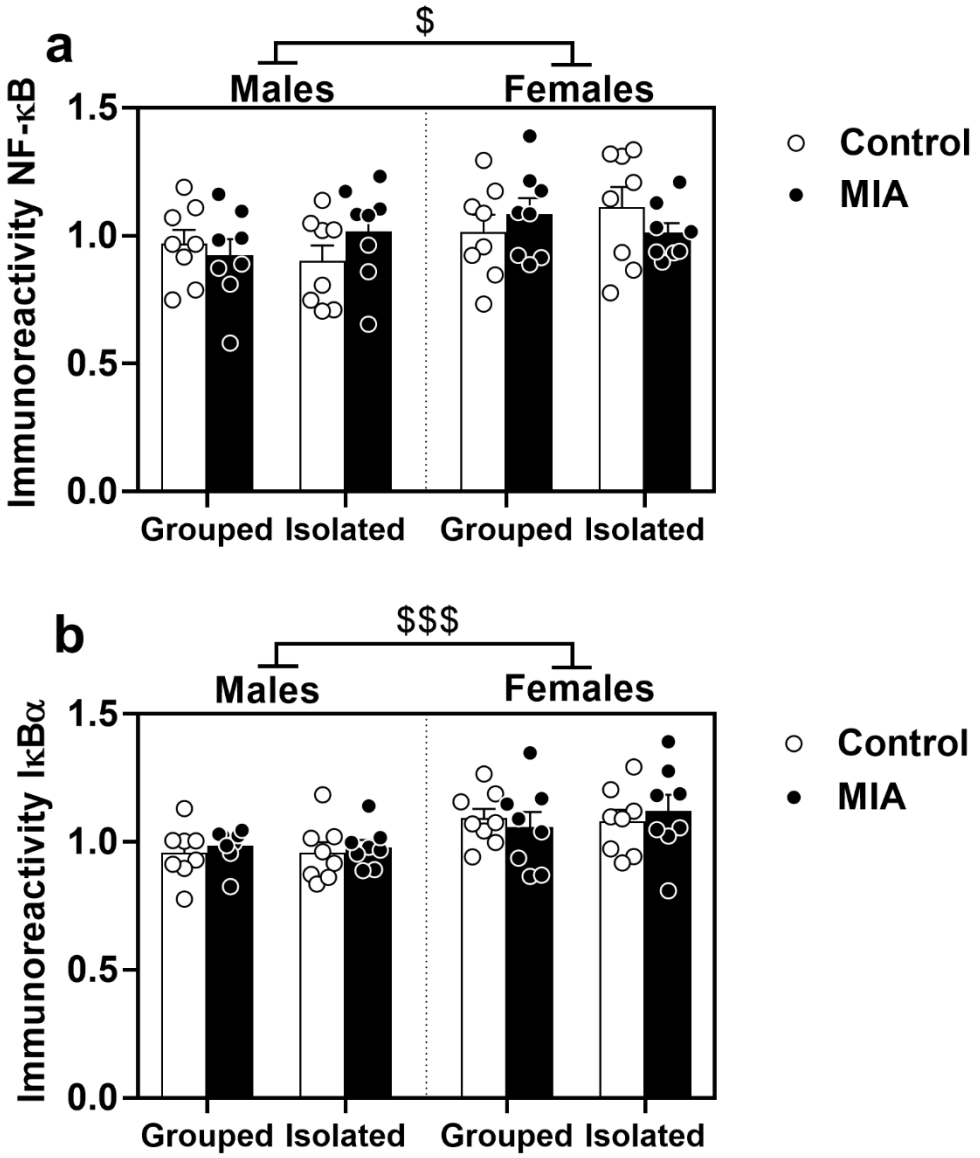


Figure 4.18: Graphic representation of protein expression levels of NF-κB (**figure 4.18 a**) and IκBα (**figure 4.18 b**) obtained by means of RT-qPCR. Individual immunoreactivity values of male and female mice of the four experimental groups (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Bars represent mean ± SEM values (n= 8 sex/arm). Data were analyzed using three-way ANOVA followed by Tukey's multiple comparisons test. Sex significance is expressed as $p<0.05.

Table 4.31: Three-way ANOVA analysis conducted in order to evaluate the effect of MIA, SI, sex and their interactions on the protein expression levels of NF-κB and IκBα.

Protein expression (immunoreactivity) – Three-way ANOVA		
	NF-κB (RelA or p65)	**IκBα**
MIA	F(1,56)= 0.05; p= 0.82	F(1,56)= 0.21; p= 0.65
SI	F(1,56)= 0.07; p= 0.79	F(1,56)= 0.14; p= 0.71
Sex	**F(1,56)=5.51; p= 0.02**	**F(1,56)= 14.49; p= 0.0004**
MIA x SI	F(1,56)= 0.0005; p= 0.98	F(1,56)= 0.31; p= 0.58
Sex x MIA	F(1,56)= 0.35; p= 0.56	F(1,56)= 0.11; p= 0.74
Sex x SI	F(1,56)= 0.0004; p= 0.98	F(1,56)= 0.21; p= 0.65
Sex x MIA x SI	F(1,56)= 3.60; p= 0.06	F(1,56)= 0.45; p= 0.50

4.1.10. EVALUATION OF THE GENE EXPRESSION OF NINE DIFFERENT HDACS

RT-qPCR experiments were performed in order to evaluate the gene expression of nine different HDACs (genes *Hdac1, Hdac2, Hdac3, Hdac4, Hdac5, Hdac6, Hdac7, Hdac8* and *Hdac9*) in the brain cortex of the four experimental groups. See **table 4.32** and **table 4.33** for mean ± SEM values of relative mRNA of the different HDACs of male and female experimental groups, respectively.

Relative mRNA levels of *Hdac1* in control animals were 1.04 ± 0.12 in males and 0.99 ± 0.07 in females. *Hdac1* gene expression was not modulated by the effect of MIA [$F_{MIA}(1,56)= 0.38$, p= 0.54], SI [$F_{SI}(1,56)= 0.61$, p= 0.44] or sex [$F_{SEX}(1,56)= 1.27$, p= 0.26] (**figure 4.19 a**). No other statistically significant "sex x hit", "hit x hit" or "sex x hit x hit" interactions were found in these experiments (see **table 4.34**).

Regarding to *Hdac2*, in control male mice relative mRNA levels were 0.72 ± 0.03 and 0.75 ± 0.02 in control female mice. *Hdac2* gene expression was significantly decreased by MIA [$F_{MIA}(1,56)=5.95$, p= 0.02) (**figure 4.19 b**). No significant effect of SI [$F_{SI}(1,56)= 0.36$, p= 0.55], sex [$F_{SEX}(1,56)= 2.73$, p= 0.10] and no significant "sex x hit", "hit x hit" or "sex x hit x hit" interactions were found (see **table 4.34**).

Relative mRNA levels of *Hdac3* in control animals were 0.98 ± 0.10 in males and 0.90 ± 0.04 in females. *Hdac3* gene expression was not modulated by the effect of MIA [$F_{MIA}(1,56)= 0.32$, p= 0.58], SI [$F_{SI}(1,56)= 0.61$, p= 0.44] or sex [$F_{SEX}(1,56)= 0.40$, p= 0.53] (**figure 4.19 c**). No other

statistically significant “sex x hit”, “hit x hit” or “sex x hit x hit” interactions were found in these experiments (see **table 4.34**).

For *Hdac4,* relative mRNA levels in control animals were 1.01 ± 0.11 in males and 1.10 ± 0.10 in females. *HDAC4* gene expression was decreased by effect of SI [F_{SI}(1,56)= 5.53; p= 0.02] (**figure 4.19 d**). No significant effect of MIA [F_{MIA}(1,56)= 3.16, p= 0.08], sex [F_{SEX}(1,56)= 0.23, p= 0.64] and no significant “sex x hit”, “hit x hit” or “sex x hit x hit” interactions were found (see **table 4.34**).

Relative mRNA levels of *Hdac5* in control animals were 1.04 ± 0.06 in males and 0.85 ± 0.05 in females. *Hdac5* was not modulated by the effect of MIA [F_{MIA}(1,56)= 0.00004, p= 0.98] or SI [F_{SI}(1,56)= 2.90, p= 0.09]. However, *hdac5* gene expression was significantly affected by sex [F_{SEX}(1,56)= 4.49; p= 0.04], with higher mRNA levels in males than in females (**figure 4.19 e**). No other significant “sex x hit”, “hit x hit” or “sex x hit x hit” interactions were found (see **table 4.34**).

In control animals, relative mRNA levels of *Hdac6* were 1.17 ± 0.12 in males and 1.05 ± 0.05 in females. *Hdac6* gene expression was not modulated by the effect of MIA [F_{MIA}(1,56)= 0.007, p= 0.93], SI [F_{SI}(1,56)= 3.08, p= 0.08] or sex [F_{SEX}(1,56)= 0.45, p= 0.50] (**figure 4.19 f**). No other statistically significant “sex x hit”, “hit x hit” or “sex x hit x hit” interactions were found in these experiments (see **table 4.34**).

Regarding *Hdac7,* relative mRNA levels in control animals were 1.33 ± 0.17 in males and 1.12 ± 0.07 in females. *Hdac7* gene expression was not modulated by the effect of MIA [F_{MIA}(1,56)= 0.46, p= 0.50], SI [F_{SI}(1,56)= 1.51, p= 0.22] or sex [F_{SEX}(1,56)= 0.52, p= 0.47] (**figure 4.19 g**). No other statistically significant “sex x hit”, “hit x hit” or “sex x hit x hit” interactions were found in these experiments (see **table 4.34**).

For *Hdac8,* relative mRNA levels in control animals were 1.07 ± 0.10 in males and 1.04 ± 0.08 in females. *Hdac8* gene expression was significantly reduced by effect of SI [F_{SI}(1,56)= 6.81; p= 0.012] (**figure 4.19 h**). No significant effect of MIA [F_{MIA}(1,56)= 0.33, p= 0.57], sex [F_{SEX}(1,56)= 0.25, p= 0.62] and no significant “sex x hit”, “hit x hit” or “sex x hit x hit” interactions were found (see **table 4.34**).

Relative mRNA levels of *Hdac9* in control animals were 1.17 ± 0.14 in males and 0.99 ± 0.04 in females. *Hdac9* gene expression was not modulated by the effect of MIA [F_{MIA}(1,56)= 0.78, p= 0.38], SI [F_{SI}(1,56)= 0.17, p= 0.68] or sex [F_{SEX}(1,56)= 1.90, p= 0.17] (**figure 4.19 i**). No other

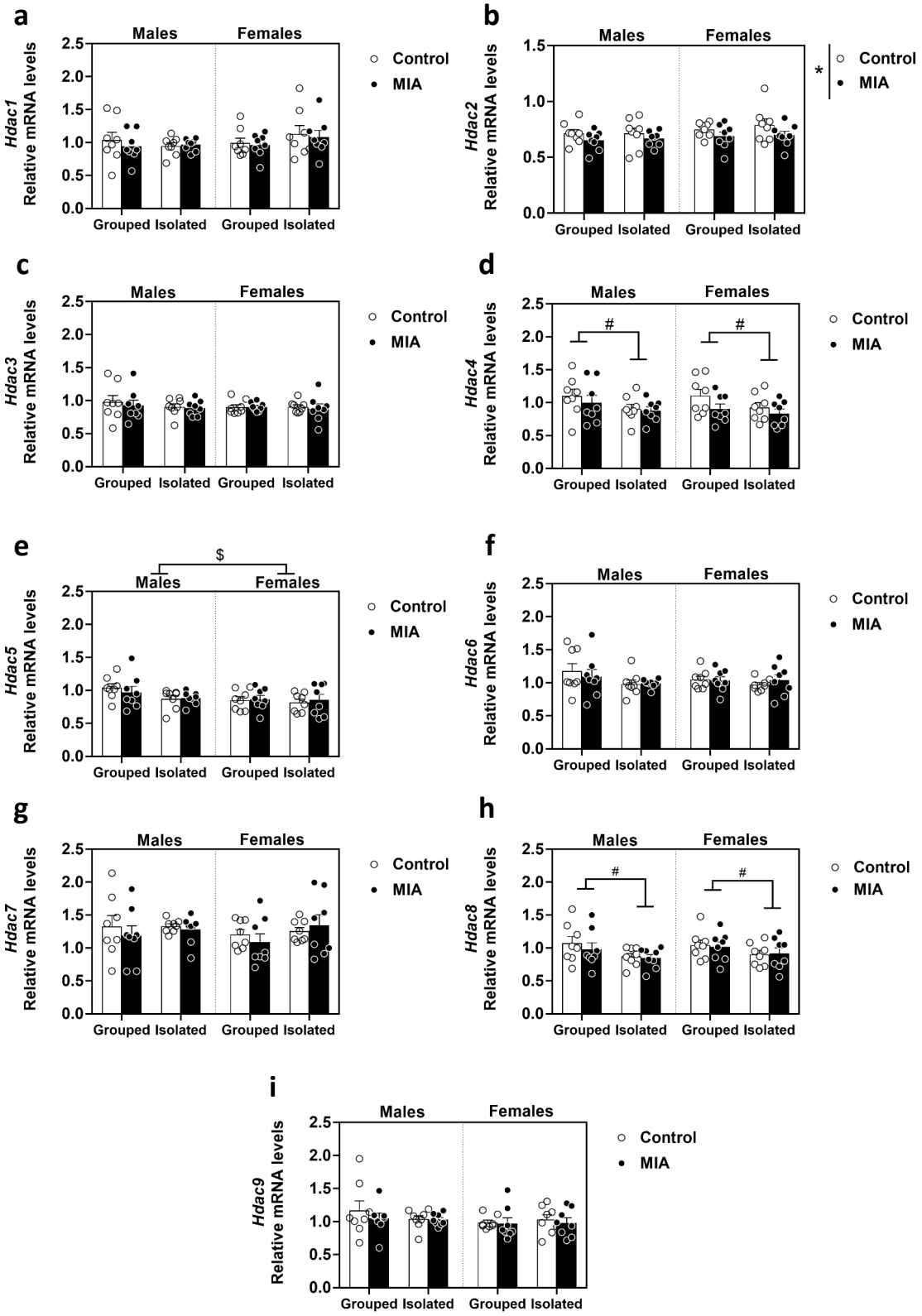
statistically significant “sex x hit”, “hit x hit” or “sex x hit x hit” interactions were found in these experiments (see **table 4.34**).

Table 4.32: Mean ± SEM values of nine different HDACs relative mRNA in RT-qPCR experiments conducted in male control, MIA, SI and MIA + SI experimental groups.

Relative mRNA levels of nine different HDACs – Mean ± SEM				
Males				
	Control	**MIA**	**SI**	**MIA + SI**
Hdac1	1.04 ± 0.12	0.94 ± 0.08	0.95 ± 0.05	0.97 ± 0.03
Hdac2	0.72 ± 0.03	0.65 ± 0.03	0.71 ± 0.05	0.67 ± 0.02
Hdac3	0.98 ± 0.10	0.92 ± 0.08	0.91 ± 0.05	0.89 ± 0.04
Hdac4	1.01 ± 0.11	1.00 ± 0.11	0.91 ± 0.07	0.88 ± 0.06
Hdac5	1.04 ± 0.06	0.97 ± 0.09	0.87 ± 0.05	0.88 ± 0.04
Hdac6	1.17 ± 0.12	1.09 ± 0.11	0.98 ± 0.06	0.99 ± 0.02
Hdac7	1.33 ± 0.17	1.19 ± 0.15	1.33 ± 0.03	1.28 ± 0.08
Hdac8	1.07 ± 0.10	0.98 ± 0.10	0.87 ± 0.05	0.85 ± 0.05
Hdac9	1.17 ± 0.14	1.04 ± 0.08	1.04 ± 0.05	1.03 ± 0.03

Table 4.33: Mean ± SEM values of nine different HDACs relative mRNA in RT-qPCR experiments conducted in female control, MIA, SI and MIA + SI experimental groups.

Relative mRNA levels of nine different HDACs – Mean ± SEM				
Females				
	Control	**MIA**	**SI**	**MIA + SI**
Hdac1	0.99 ± 0.07	0.96 ± 0.06	1.13 ± 0.13	1.08 ± 0.10
Hdac2	0.75 ± 0.02	0.69 ± 0.04	0.79 ± 0.06	0.70 ± 0.04
Hdac3	0.90 ± 0.04	0.90 ± 0.02	0.91 ± 0.03	0.88 ± 0.07
Hdac4	1.10 ± 0.10	0.90 ± 0.07	0.93 ± 0.07	0.83 ± 0.07
Hdac5	0.85 ± 0.05	0.87 ± 0.06	0.82 ± 0.05	0.86 ± 0.08
Hdac6	1.05 ± 0.05	1.04 ± 0.06	0.97 ± 0.32	1.04 ± 0.08
Hdac7	1.12 ± 0.07	1.09 ± 0.13	1.26 ± 0.06	1.34 ± 0.16
Hdac8	1.04 ± 0.08	1.02 ± 0.08	0.90 ± 0.06	0.92 ± 0.08
Hdac9	0.99 ± 0.04	0.97 ± 0.09	1.03 ± 0.07	0.98 ± 0.07


a
Hdac1
Relative mRNA levels
Males
Females
Grouped Isolated Grouped Isolated
Control
MIA
b
Hdac2
Relative mRNA levels
Males
Females
*
Control
MIA
c
Hdac3
Relative mRNA levels
Males
Females
Control
MIA
d
Hdac4
Relative mRNA levels
Males
Females
#
#
Control
MIA
e
Hdac5
Relative mRNA levels
$
Males
Females
Control
MIA
f
Hdac6
Relative mRNA levels
Males
Females
Control
MIA
g
Hdac7
Relative mRNA levels
Males
Females
Control
MIA
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Hdac8
Relative mRNA levels
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Females
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Control
MIA
i
Hdac9
Relative mRNA levels
Males
Females
Control
MIA

Figure 4.19: Graphic representation of relative mRNA levels of nine different HDACs (**figure 4.19 a-i**) obtained by means of RT-qPCR. Individual relative mRNA values of male and female mice of the four experimental groups (control-grouped, control-isolated, MIA-grouped and MIA-isolated) are plotted. Bars represent mean ± SEM values (n= 8 sex/arm). Data were analyzed using three-way ANOVA followed by Tukey's multiple comparisons test. MIA significance is expressed as *p<0.05. SI significance is expressed as #p<0.05. Sex significance is expressed as $p<0.05.

Table 4.34: Three-way ANOVA analysis conducted in order to evaluate the effect of MIA, SI, sex and their interactions on the relative mRNA levels of nine different HDACs.

	MIA	SI	Sex	MIA x SI	Sex x MIA	Sex x SI	Sex x MIA x SI
Hdac1	F(1,56)= 0.38; p=0.54	F(1,56)= 0.61; p= 0.44	F(1,56)= 1.27; p= 0.26	F(1,56)= 0.15; p= 0.70	F(1,56)= <0.001; p>0.99	F(1,56)= 1.77; p= 0.19	F(1,56)= 0.29; p= 0.59
Hdac2	**F(1,56)= 5.95; p= 0.02**	F(1,56)= 0.36; p= 0.55	F(1,56)= 2.73; p= 0.10	F(1,56)= 0.01; p= 0.93	F(1,56)= 0.16; p= 0.69	F(1,56)= 0.11; p= 0.75	F(1,56)= 0.22; p= 0.64
Hdac3	F(1,56)= 0.32; p= 0.58	F(1,56)= 0.61; p= 0.44	F(1,56)= 0.40; p= 0.53	F(1,56)=0.002; p=0.96	F(1,56)= 0.09; p= 0.76	F(1,56)= 0.28; p= 0.60	F(1,56)= 0.17; p= 0.68
Hdac4	F(1,56)= 3.16; p= 0.08	**F(1,56)= 5.53; p= 0.02**	F(1,56)= 0.23; p= 0.64	F(1,56)= 0.56; p= 0.46	F(1,56)= 0.47, p= 0.49	F(1,56)= 0.07; p= 0.79	F(1,56)= 0.02, p= 0.89
Hdac5	F(1,56)=0.00; p=0.98	F(1,56)= 2.90; p= 0.09	**F(1,56)= 4.49; p= 0.04**	F(1,56)= 0.33; p= 0.57	F(1,56)= 0.42; p= 0.52	F(1,56)= 1.46; p= 0.23	F(1,56)= 0.10; p= 0.76
Hdac6	F(1,56)=0.007; p=0.93	F(1,56)= 3.08; p= 0.08	F(1,56)= 0.45; p= 0.50	F(1,56)= 0.70; p= 0.41	F(1,56)= 0.39; p= 0.54	F(1,56)= 1.11; p= 0.30	F(1,56)= 0.003; p= 0.96
Hdac7	F(1,56)= 0.46; p= 0.50	F(1,56)= 1.51; p= 0.22	F(1,56)= 0.52, p= 0.47	F(1,56)= 0.78; p= 0.38	F(1,56)= 0.25; p= 0.62	F(1,56)= 0.41; p= 0.53	F(1,56)= 0.12; p= 0.73
Hdac8	F(1,56)= 0.33; p= 0.57	**F(1,56)= 6.81; p=0.01**	F(1,56)= 0.25; p= 0.62	F(1,56)= 0.27; p= 0.61	F(1,56)= 0.24; p= 0.62	F(1,56)= 0.18; p= 0.67	F(1,56)= 0.01; p= 0.91
Hdac9	F(1,56)= 0.78; p= 0.38	F(1,56)= 0.17; p= 0.68	F(1,56)= 1.90; p= 0.17	F(1,56)= 0.14; p= 0.71	F(1,56)= 0.08; p= 0.77	F(1,56)= 0.82; p= 0.37	F(1,56)= 0.41; p= 0.53

4.2. EVALUATION OF THE BEHAVIORAL AND MOLECULAR EFFECTS OF THE CHRONIC TREATMENT WITH CLOZAPINE, MINOCYCLINE AND THE COMBINATION OF BOTH DRUGS IN THE DOUBLE-HIT MOUSE MODEL

Male and female control and double-hit mice were treated for 21 days with the atypical antipsychotic clozapine, the inhibitor of inflammatory activity minocycline, and the combination of both drugs. After chronic treatment, mice belonging to "vehicle", "clozapine", "minocycline", "clozapine + minocycline" experimental groups performed the NORT and the SPT. After the behavioral evaluation, mice were sacrificed, brains were removed and cortical samples were processed for gene expression assessment. Based on previous results, the expression of genes encoding for proteins NF-κB (*Rela*), IκBα (*Nfkbia*), HDAC2 (*Hdac2*), HDAC4 (*Hdac4*), HDAC8 (*Hdac8*), IL-6 (*Il6*), IFN-γ (*Ifng*) and 5-HT_{2A}R (*Htr2a*) was evaluated.

4.2.1. EFFECT OF THE CHRONIC TREATMENT WITH CLOZAPINE, MINOCYCLINE AND THE COMBINATION OF BOTH DRUGS ON THE COGNITIVE STATUS BY THE NORT

Control and double-hit animals belonging to the four mentioned experimental groups (vehicle, clozapine, minocycline, clozapine + minocycline) were tested for their cognitive status by means of the NORT.

All the animals with a lower DI than -0.1 and/or with a total exploration time lower than 5 seconds (calculated as *Time devoted to the novel object + Time devoted to the familiar object*) were excluded from the study. This criterion is supported by previous researches (Oliveira da Cruz et al., 2020), who claim that animals with very low exploration times (< 5- 10 seconds) should be excluded from the test phase of the NORT. In this batch of experiments, 15-21 animals per experimental group performed the NORT (a total number of 139 animals), however, as it is reflected in the number of data plotted in the following graphs, a high percentage of the mice used in these NORT experiments did not fulfill the mentioned criterion and were excluded from the study.

NORT novel object exploration time

Vehicle-treated control mice spent 23.23 ± 3.56 s exploring the novel object, while vehicle-treated double-hit mice spent 22.27 ± 4.51 s exploring the novel object. Thus, no statistical significances were found in the novel object exploration time between vehicle-treated control and double-hit mice (unpaired Student's *t-test* t= 1.17; p= 0.87) (**figure 4.20**).

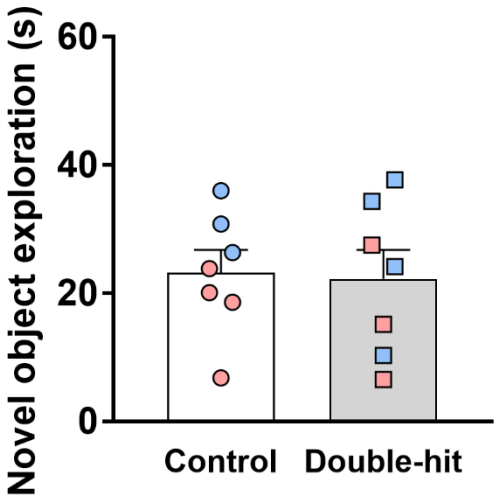

Figure 4.20: Graphic representation of the novel object exploration time (s) of the NORT. Vehicle-treated control (n= 7) and double-hit (n= 7) mice are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using unpaired Student's *t-test*.

In control mice (**figure 4.21 a**), novel object exploration time (s) was not altered by the chronic administration of clozapine, minocycline, or combination of both drugs [$F_{CLOZAPINE}(1,21)= 0.01$, $p= 0.91$; $F_{MINOCYCLINE}(1,21)= 1.57$, $p= 0.22$; $F_{INTERACTION}(1,21)= 0.64$; $p= 0.43$]. Similarly, in the double-hit group (**figure 4.21 b**), novel object exploration time was not altered by any of the pharmacological treatments used in this study [$F_{CLOZAPINE}(1,23)= 0.35$, $p= 0.56$; $F_{MINOCYCLINE}(1,21)= 0.19$, $p= 0.66$; $F_{INTERACTION}(1,21)= 2.01$, $p= 0.17$]. **Table 4.35** summarizes the mean ± SEM values of the NORT novel exploration times (s) of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

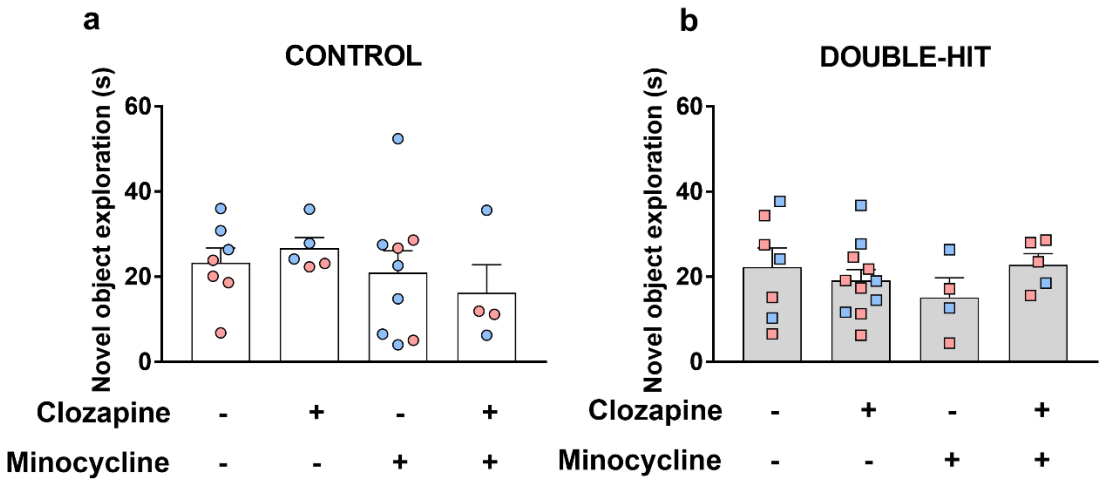

Figure 4.21: Graphic representation of the novel object exploration time (s) of the NORT. Control (**figure 4.21 a**) and double-hit (**figure 4.21 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 4-11/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test.

Table 4.35: Mean ± SEM values for the novel object exploration time (s) of the NORT of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

Novel object exploration time (s) of the NORT – Mean ± SEM				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	23.23 ± 3.56	26.68 ± 2.50	20.91 ± 5.15	16.25 ± 6.57
Double-hit	22.27 ± 4.51	19.11 ± 2.57	15.18 ± 4.59	22.84 ± 2.56

NORT familiar exploration time

Vehicle-treated control mice spent 16.89 ± 3.68 s exploring the familiar object, while vehicle-treated double-hit mice spent 16.44 ± 4.24 s exploring the familiar object. Thus, no statistical significances were found in the familiar object exploration time between vehicle-treated control and double-hit mice (unpaired Student's *t-test* t= 0.08; p= 0.94) (**figure 4.22**).

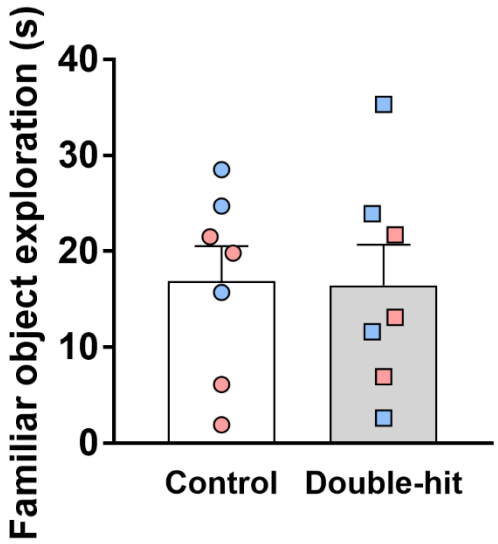

Figure 4.22: Graphic representation of the familiar object exploration time (s) of the NORT. Vehicle-treated control (n= 7) and double-hit (n= 7) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using unpaired Student's *t-test*.

In control mice (**figure 4.23 a**), familiar object exploration was not significantly affected by chronic clozapine administration, minocycline administration, nor by the co-administration of both drugs [$F_{CLOZAPINE}(1,21)= 0.0005$, $p= 0.98$; $F_{MINOCYCLINE}(1,21)= 2.20$, $p= 0.15$; $F_{INTERACTION}(1,21) = 0.03$, $p= 0.87$]. In double-hit mice (**figure 4.23 b**), familiar object exploration was not significantly affected by clozapine or minocycline chronic administration [$F_{CLOZAPINE}(1,21)= 0.004$, $p= 0.95$; $F_{MINOCYCLINE}(1,21)= 2.03$, $p= 0.17$]. However, in both control and double-hit groups, familiar object exploration time appears to be reduced by effect of minocycline, although this reduction does not reach the statistical significance. Moreover, in the double-hit group, clozapine reverted the minocycline-elicited decrease of the familiar object exploration when both drugs were administered together, although this effect was not statistically significant [$F_{INTERACTION}(1,21)= 3.99$, $p= 0.06$]. **Table 4.36** summarizes the mean ± SEM values of the NORT familiar exploration times (s) of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

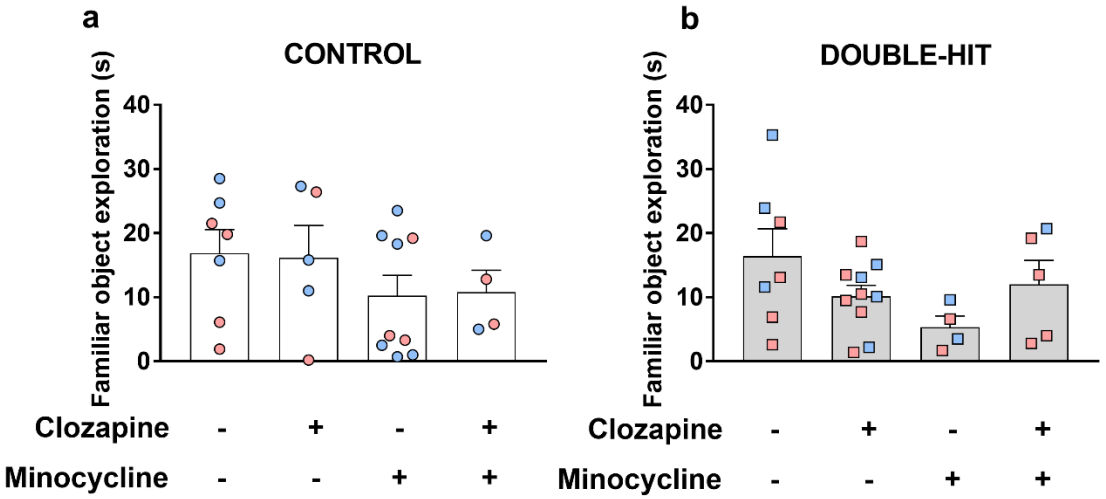

Figure 4.23: Graphic representation of the familiar object exploration time (s) of the NORT. Control (**figure 4.23 a**) and double-hit (**figure 4.23 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 4-10/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test.

Table 4.36: Mean ± SEM values for the familiar object exploration time (s) of the NORT of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

Familiar object exploration time (s) of the NORT – Mean ± SEM				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	16.89 ± 3.68	16.14 ± 5.05	10.23 ± 3.19	10.80 ± 3.41
Double-hit	16.44 ± 4.24	10.18 ± 1.71	5.35 ± 1.74	12.04 ± 3.73

NORT total exploration time

Vehicle-treated control mice had a total object exploration time of 40.11 ± 6.59 s, while vehicle-treated double-hit mice had a total object exploration time of 38.71 ± 7.57 s. Thus, no statistical significances were found in the total exploration time between vehicle-treated control and double-hit mice (unpaired Student's *t-test* t= 1.14; p= 0.89) (**figure 4.24**).

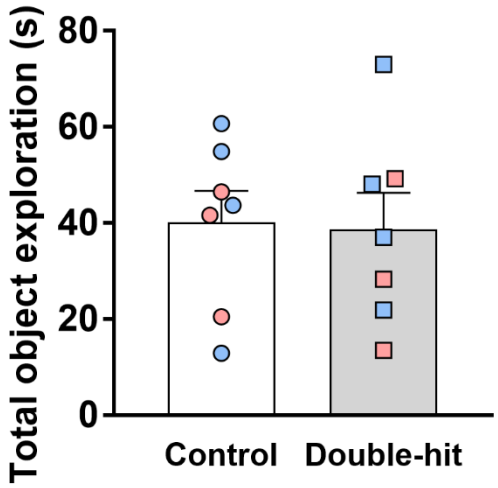

Figure 4.24: Graphic representation of the total object exploration time (s) of the NORT. Vehicle-treated control (n= 7) and double-hit (n= 7) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using unpaired Student's *t-test*.

In control mice (**figure 4.25 a**), NORT total object exploration time was not altered by clozapine administration [$F_{CLOZAPINE}(1,21)= 0.007$; p= 0.93], minocycline administration [$F_{MINOCYCLINE}(1,21)= 2.29$; p= 0.15] or by the co-administration of both clozapine and minocycline [$F_{INTERACTION}(1,21)= 0.17$; p= 0.68]. In double-hit mice (**figure 4.25 b**), NORT total exploration time was not affected by clozapine administration [$F_{CLOZAPINE}(1,22)= 0.08$; p= 0.79], nor by minocycline administration [$F_{MINOCYCLINE}(1,22)= 0.89$; p= 0.36]. However, co-administration of both drugs significantly increased total exploration time, compared to each drug alone [$F_{INTERACTION}(1,22)= 4.96$; p= 0.04]. Thus, whereas administration of either drug alone tended to reduce the total exploration time compared to vehicle-treated double-hit mice, co-administration of both drugs reverted the total exploration times to vehicle values. **Table 4.37** summarizes the mean ± SEM values of the NORT total exploration times (s) of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

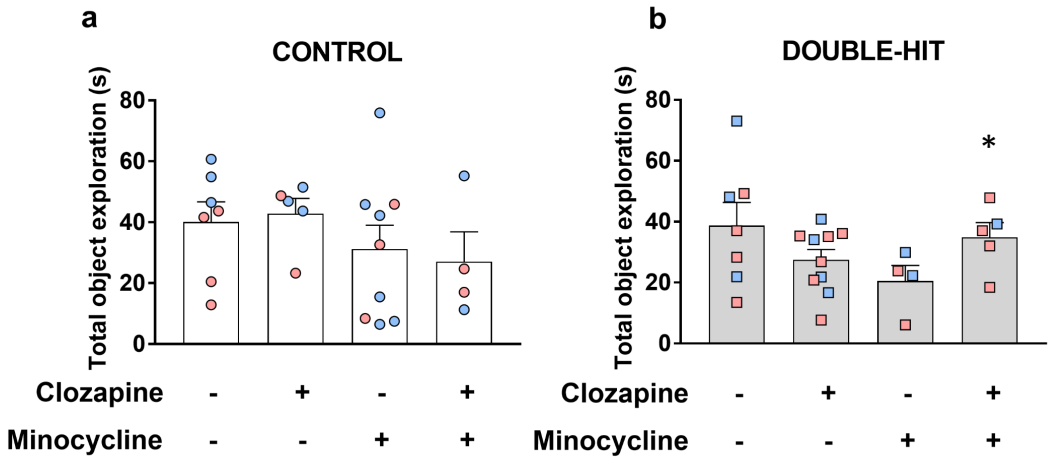

Figure 4.25: Graphic representation of the total object exploration time (s) of the NORT. Control (**figure 4.25 a**) and double-hit (**figure 4.25 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 4-9/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test. Clozapine + minocycline significance in double-hit mice is expressed as * $p<0.05$.

Table 4.37: Mean ± SEM values for the NORT total object exploration time (s) of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

Total object exploration time (s) of the NORT – Mean ± SEM				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	40.11 ± 6.59	42.82 ± 5.04	31.14 ± 7.90	27.05 ± 9.78
Double-hit	38.71 ± 7.57	27.52 ± 3.33	20.53 ± 5.08	34.88 ± 4.85

NORT DI

Vehicle-treated control mice showed NORT DI scores of 0.21 ± 11, while vehicle-treated double-hit mice showed NORT DI scores of 0.02 ± 0.03. Thus, NORT DI was decreased by the effect of MIA+SI, as double-hit mice showed lower DI scores than control mice. However, statistical significance was not reached (unpaired Student's *t-test* t= 1.47; p= 0.17) (**figure 4.26**).

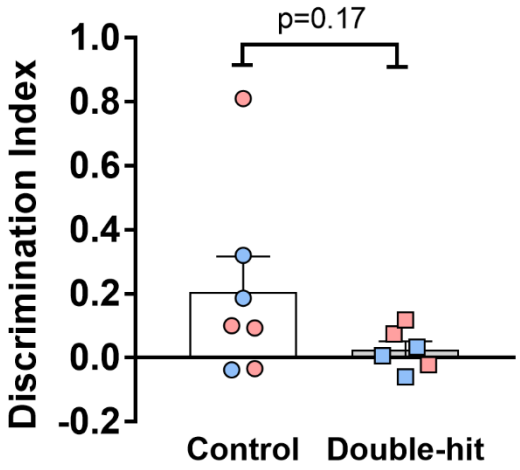

Figure 4.26: Graphic representation of the NORT Discrimination Index (DI). Vehicle-treated control (n= 7) and double-hit (n= 6) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using unpaired Student's *t-test*. Statistical difference between control and double-hit mice is expressed as p= 0.17.

In control mice (**figure 4.27 a**), NORT DI was not altered by any of the pharmacological treatments, when a two-way ANOVA analysis was performed [$F_{CLOZAPINE}(1,21)= 0.16$, p= 0.69; $F_{MINOCYCLINE}(1,21)= 0.03$, p= 0.88; $F_{INTERACTION}(1,21) = 1.76$, p= 0.20]. In double-hit mice (**figure 4.27 b**), both clozapine and minocycline increased the DI scores respect to the DI scores of vehicle-treated animals, although only the minocycline effect probe to be statistically significant [$F_{CLOZAPINE}(1,22)= 0.78$, p= 0.39; $F_{MINOCYCLINE}(1,22)= 6.24$, p= 0.02]. No statistically significant interaction was found between the two treatments [$F_{INTERACTION}(1,23)= 2.38$, p= 0.14]. **Table 4.38** summarizes the mean ± SEM values of the NORT DI of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

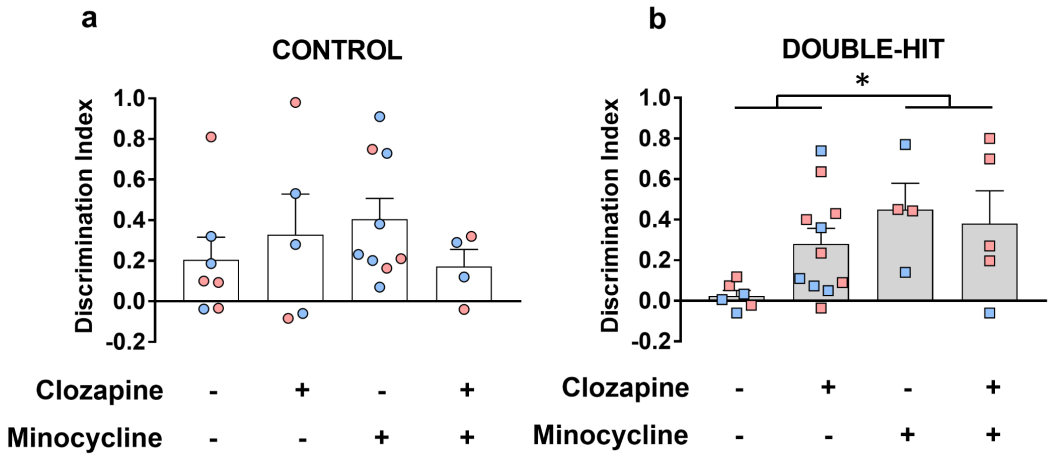

Figure 4.27: Graphic representation of the NORT Discrimination Index (DI). Control (**figure 4.27 a**) and double-hit (**figure 4.27 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 4-9/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test. Minocycline significance in double-hit animals is expressed as * $p<0.05$.

Table 4.38: Mean ± SEM values for the NORT Discrimination Index (DI) of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

NORT DI – Mean ± SEM				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	0.21 ± 0.11	0.33 ± 0.20	0.41 ± 0.10	0.17 ± 0.08
Double-hit	0.03 ± 0.03	0.28 ± 0.08	0.45 ± 0.13	0.38 ± 0.16

4.2.2 EFFECT OF THE CHRONIC TREATMENT WITH CLOZAPINE, MINOCYCLINE AND COMBINATION OF BOTH DRUGS ON THE SOCIAL BEHAVIOR BY THE SPT

48 hours after the NORT, the same control and double-hit animals performed the SPT in order to evaluate their negative-like symptomatology after the chronic treatment with clozapine and/or minocycline.

SPT social exploration time

Vehicle-treated control animals had a social exploration time of 71.60 ± 4.66 s, while vehicle-treated double-hit animals had a social exploration time of 59.52 ± 3.78 s. Thus, a lower social exploration was observed in double-hit mice compared to control mice. This reduction in the social exploration time of the SPT did not reach the statistical significance when an unpaired Student's *t-test* was performed (t= 1.98; p= 0.056) (**figure 4.28**).

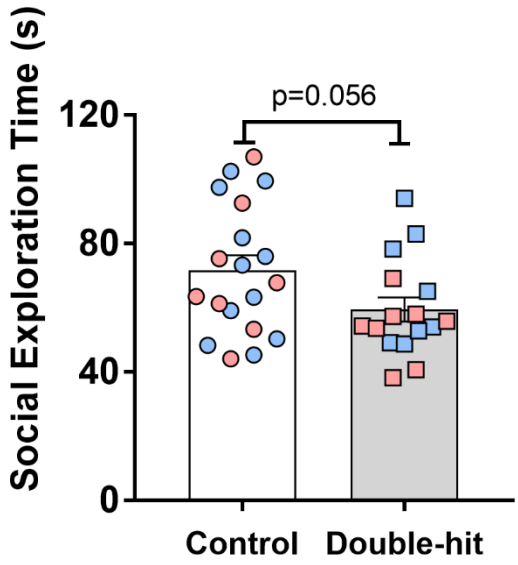

Figure 4.28: Graphic representation of the social exploration time (s) of the SPT. Vehicle-treated control (n= 19) and double-hit (n= 16) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using Student's unpaired *t-test*. Statistical difference between control and double-hit mice is expressed as p= 0.056.

In control mice (**figure 4.29 a**), social exploration time of the SPT was not altered by any of the pharmacological treatments [$F_{CLOZAPINE}(1,72)$= 0.003, p= 0.96; $F_{MINOCYCLINE}(1,72)$= 0.06, p= 0.81;

$F_{INTERACTION}(1,72)= 0.002$, $p= 0.96$], as all the experimental groups spent similar amount of time exploring the social stimulus. Similarly, in double hit mice (**figure 4.29 b**), social exploration time was not affected by any of the pharmacological treatments used in the study [$F_{CLOZAPINE}(1,59)= 1.03$, $p= 0.32$; $F_{MINOCYCLINE}(1,59)= 0.27$, $p= 0.61$; $F_{INTERACTION}(1,59)= 0.26$, $p= 0.27$]. **Table 4.39** summarizes the mean ± SEM values of the SPT social exploration times (s) of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

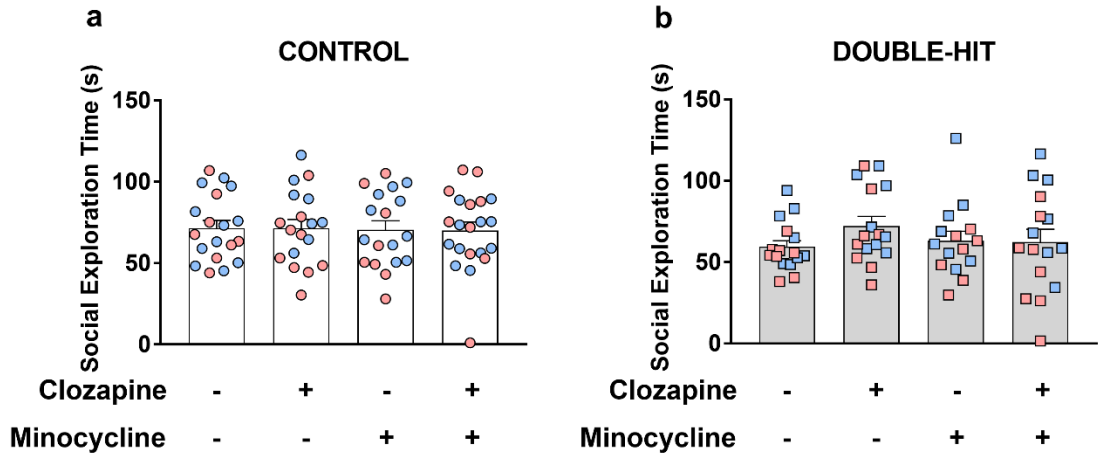

Figure 4.29: Graphic representation of the social exploration time (s) of the SPT. Control (**figure 4.29 a**) and double-hit (**figure 4.29 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 15-21/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test.

Table 4.39: Mean ± SEM values for the social exploration time (s) of the SPT of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

Social exploration time (s) of the SPT – Mean ± SEM				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	71.60 ± 4.66	71.54 ± 5.40	70.61 ± 5.42	70.09 ± 5.24
Double-hit	59.42 ± 3.78	72.24 ± 5.79	63.07 ± 5.88	62.41 ± 7.84

SPT non-social exploration time

Control and double-hit animals spent similar amount of time exploring the non-social stimulus (control: 31.10 ± 1.66 s; double-hit: 31.90 ± 3.35 s). Quantification of the non-social exploration time did not show differences between control and double-hit mice when an unpaired Student's *t-test* was performed (t= 0.12; p= 0.90) (**figure 4.30**).

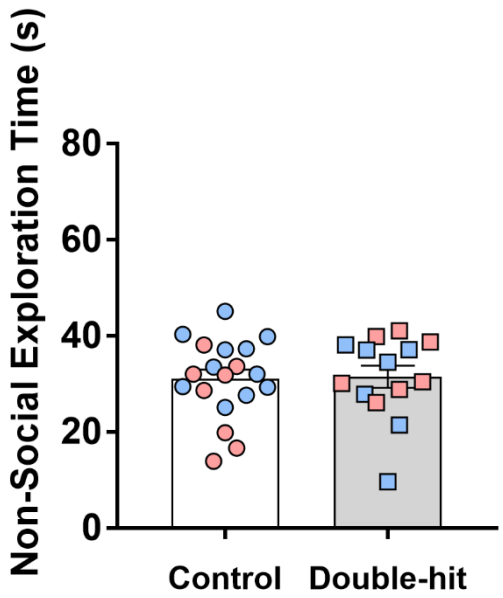

Figure 4.30: Graphic representation of the non-social exploration time (s) of the SPT. Vehicle-treated control (n= 19) and double-hit (n= 14) mice are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using Student's unpaired *t-test*.

In control mice (**figure 4.31 a**), the non-social exploration time was significantly reduced by effect of clozapine [$F_{CLOZAPINE}(1,72)= 7.57$; p= 0.008]. Minocycline treatment elicited a non-significant increase of the non-social exploration time [$F_{MINOCYCLINE}(1,72)= 0.03$, p= 0.87]. A significant interaction was found as co-administration of both drugs reduced the non-social exploration time to a greater extent than with clozapine alone [$F_{INTERACTION}(1,72)= 6.43$, p= 0.013]. Regarding the double-hit experimental group (**figure 4.31 b**), non-social exploration was not affected by the administration of clozapine or minocycline alone [$F_{CLOZAPINE}(1,59)= 0.83$, p= 0.37; $F_{MINOCYCLINE}(1,59)= 0.42$, p= 0.52]. However, co-administration of both drugs exerted a non-significant increase in the non-social exploration time of double-hit animals [$F_{INTERACTION}(1,59)=$

3.59, p=0.06]. **Table 4.40** summarizes the mean ± SEM values of the SPT non-social exploration times (s) of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

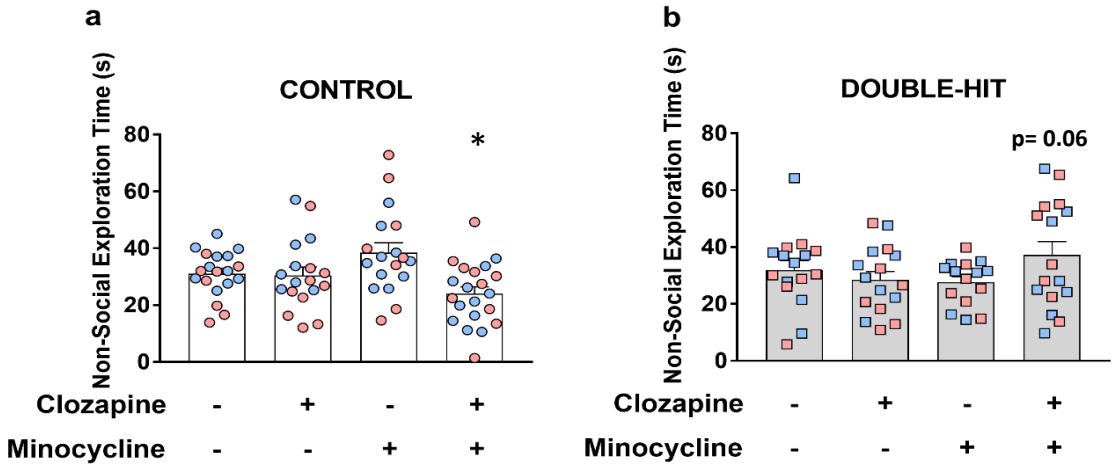

Figure 4.31: Graphic representation of the non-social exploration time (s) of the SPT. Control (**figure 4.31 a**) and double-hit (**figure 4.31 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 15-21/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test. Clozapine + minocycline interaction significance in control animals is expressed as *p<0.05. The non-significant clozapine + minocycline interaction found in double-hit animals is expressed as p= 0.06.

Table 4.40: Mean ± SEM values for the non-social exploration time (s) of the SPT of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

Non-social exploration time (s) of the SPT – Mean ± SEM				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	31.10 ± 1.86	30.52 ± 2.92	38.43 ± 3.53	24.10 ± 2.39
Double-hit	31.90 ± 3.35	28.51 ± 2.95	27.61 ± 2.05	37.27 ± 4.72

SPT total exploration time

Total exploration time (s) of the SPT was calculated for the vehicle-treated control and double-hit animals. Total exploration time was 102.7 ± 5.43 s for vehicle-treated controls. Vehicle-treated double-hit mice showed a lower total exploration time of 91.32 ± 5.45 s. Student´s *t-test* revealed no statistical significance in the total exploration time between vehicle-treated control and double-hit mice (t=1.47; p= 0.15) (**figure 4.32**).

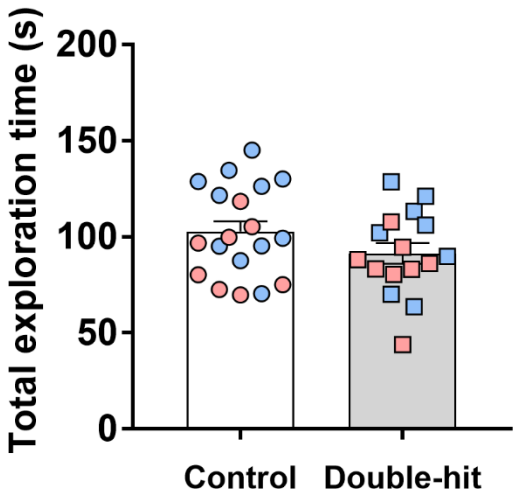

Figure 4.32: Representation of the total exploration time (s) of the SPT. Vehicle-treated control (n= 19) and double-hit (n= 16) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using Student's unpaired t-test.

In controls (**figure 4.33 a**), total exploration time of the SPT was not affected by the effect of clozapine [$F_{CLOZAPINE}(1,72)= 1.48$; p= 0.23], minocycline [$F_{MINOCYCLINE}(1,72)= 0.01$; p= 0.90], nor by the co-administration of both drugs [$F_{INTERACTION}(1,72)= 1.25$; p= 0.27]. In double-hit animals (**figure 4.33 b**), SPT total exploration time was not affected by the any of the pharmacological treatments used in the study [$F_{CLOZAPINE}(1,59)= 1.38$, p= 0.25; $F_{MINOCYCLINE}(1,59)= 0.01$, p= 0.91; $F_{INTERACTION}(1,59)= 0.0008$, p= 0.98]. **Table 4.41** summarizes the mean ± SEM values of the SPT total exploration times (s) of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

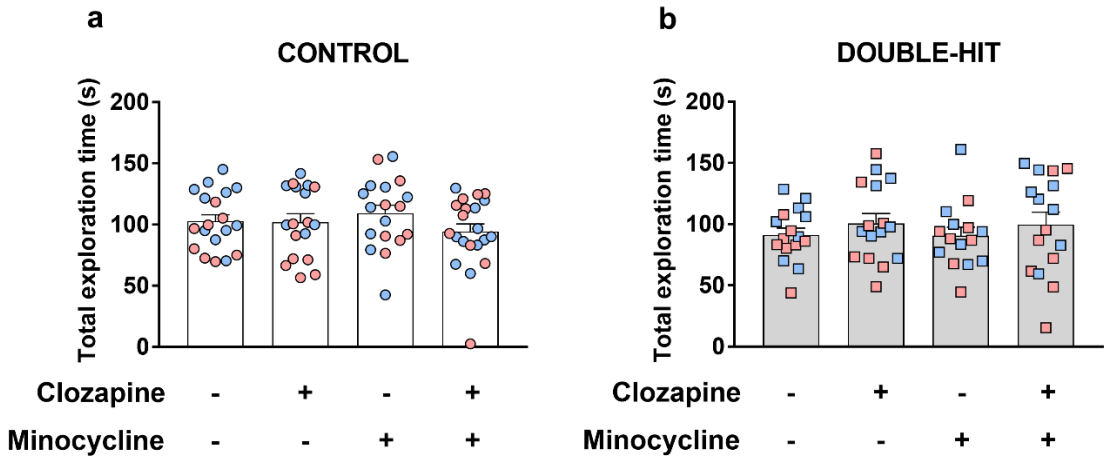

Figure 4.33: Graphic representation of the total exploration time (s) of the SPT. Control (**figure 4.33 a**) and double-hit (**figure 4.33 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 15–21/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test.

Table 4.41: Mean ± SEM values for the total exploration time (s) of the SPT of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

SPT total exploration time (s) – Mean ± SEM				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	102.70 ± 5.43	102.06 ± 6.72	109.04 ± 6.80	94.18 ± 6.41
Double-hit	91.32 ± 5.45	100.76 ± 7.95	90.68 ± 6.97	99.68 ± 10.17

SPT Social Index

SPT Social Index in vehicle-treated control animals was 0.38 ± 0.03, while in vehicle-treated double-hit mice was 0.32 ± 0.06. Unpaired Student's *t-test* showed no significant differences between groups (t= 1.02; p= 0.32) (**figure 4.34**). When the potential influence of sex on the SPT Social Index was analyzed by an exploratory two-way ANOVA including the sex as an additional factor, both "Sex" and "Double-hit" factors were revealed as non-significant [$F_{SEX}(1,31)= 0.34$, p= 0.56; $F_{DH}(1,12)= 1.21$, p= 0.28]. Moreover, no "Sex x Double-hit" interaction was found [$F_{SEXxDH}(1,31)= 1.20$, p= 0.28].

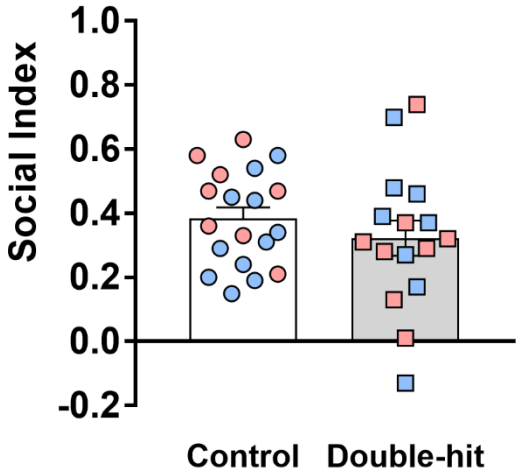

Figure 4.34: Graphic representation of the Social Index of the SPT. Vehicle-treated control (n= 18) and double-hit (n=16) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using Student's unpaired *t-test*.

In control mice (**figure 4.35 a**), Social Index was not altered by any of the pharmacological treatments [$F_{CLOZAPINE}(1,70)= 2.13$, p= 0.15; $F_{MINOCYCLINE}(1,70)= 0.14$, p= 0.71; $F_{INTERACTION}(1,70)= 1.26$, p= 0.26]. Regarding the double-hit group (**figure 4.35 b**), neither clozapine nor minocycline showed a significant effect in the SPT Social Index [$F_{CLOZAPINE}(1,58)= 0.02$, p= 0.89; $F_{MINOCYCLINE}(1,58)= 1.85$, p= 0.18]. However, combination of clozapine and minocycline significantly decreased the SPT Social Index [$F_{INTERACTION}(1,58)= 6.07$, p=0.017], indicating that combination of both clozapine and minocycline may worsen schizophrenia-related negative-like symptomatology. **Table 4.42** summarizes the mean ± SEM values of the Social Index of the SPT of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline. Moreover, the potential influence of sex on the SPT Social Index after the pharmacological treatments was analyzed by a three-way ANOVA including the sex as an additional factor. Sex effect was not significant in control mice [$F_{SEX}(1,66)= 0.03$; p= 0.87]. However, in double-hit mice, sex was revealed as a significant factor [$F_{SEX}(1,66)= 5.00$; p= 0.03] and a significant "Sex x Minocycline" interaction was found [$F_{SEXxMINOCYCLINE}(1,66)= 4.72$; p= 0.03]. This interaction showed that minocycline decreased the Social Index only in female mice. Further, the significant "Clozapine x Minocycline" interaction was also found in these exploratory analyses, regardeless of the sex [$F_{CLOZAPINExMINOCYCLINE}(1,66)= 6.56$; p= 0.01].

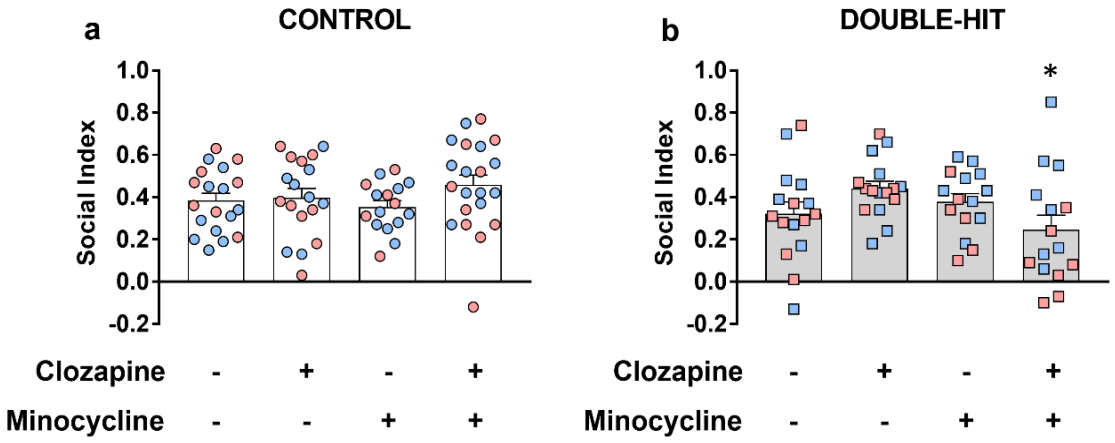

Figure 4.35: Graphic representation of the Social Index of the SPT. Control (**figure 4.35 a**) and double-hit (**figure 4.35 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 15-21/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test. Clozapine + minocycline interaction significance in double-hit mice is expressed as *p<0.05.

Table 4.42: Mean ± SEM values for the Social Index of the SPT of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

SPT Social Index – Mean ± SEM				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	0.38 ± 0.03	0.40 ± 0.04	0.35 ± 0.03	0.46 ± 0.05
Double-hit	0.32 ± 0.06	0.44 ± 0.03	0.38 ± 0.04	0.25 ± 0.07

4.2.3. EFFECT OF THE CHRONIC TREATMENT WITH CLOZAPINE AND MINOCYCLINE ON THE GENE EXPRESSION OF PROINFLAMMATORY CYTOKINES

RT-qPCR experiments were performed to evaluate the effect of chronic administration of clozapine, minocycline and co-administration of both drugs on the relative mRNA levels of the genes encoding for the proinflammatory cytokines IL-6 and IFN-γ (genes *Il6* and *Ifng*, respectively).

***Il6* relative mRNA levels**

Il6 relative mRNA levels in vehicle-treated control mice were 0.63 ± 0.03, while in double-hit mice were 0.73 ± 0.04. Although *Il6* mRNA levels were higher in double-hit mice than in control mice, this increase did not reach the statistical significance when a Student's unpaired t-test was performed (t= 1.91; p= 0.078) (**figure 4.36**). When the potential influence of sex on *Il6* relative mRNA levels was analyzed by an exploratory two-way ANOVA including the sex as an additional factor, sex was revealed as non-significant [$F_{SEX}(1,12)= 1.92$; p= 0.19] and double-hit showed a significant effect on *Il6* mRNA levels [$F_{DH}(1,12)= 5.59$; p= 0.04]. Interestingly, a "Sex x Double-hit" significant interaction was found [$F_{SEXxDH}(1,12)= 7.67$; p= 0.02], meaning that the double-hit had a different impact on *Il6* mRNA levels depending on the sex, as double-hit males had higher *Il6* mRNA levels than double-hit females.

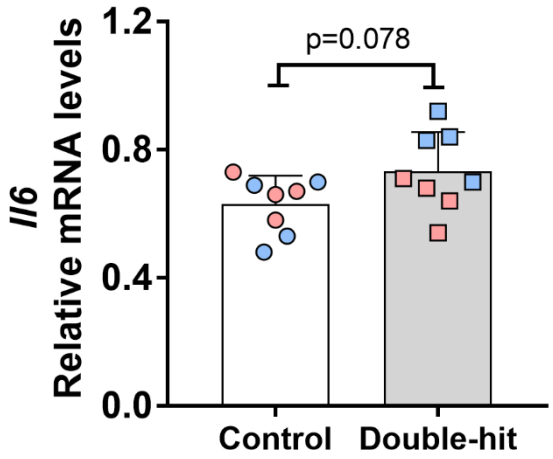

Figure 4.36: Graphic representation of the relative mRNA levels of *Il6*, determined by means of RT-qPCR. Vehicle-treated control (n= 8) and double-hit (n= 8) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using Student's unpaired *t-test*. Statistical difference between control and double-hit mice is expressed as p= 0.078.

In control mice (**figure 4.37 a**), *Il6* relative mRNA levels were not modulated by chronic clozapine, minocycline or by co-administration of both drugs [$F_{CLOZAPINE}(1,28)= 0.62$, p= 0.44; $F_{MINOCYCLINE}(1,28)= 0.39$, p= 0.54; $F_{INTERACTION}(1,28)= 2.27$, p=0.14]. In double-hit mice (**figure 4.37 b**), *Il6* relative mRNA levels were not modulated by any of the pharmacological treatments used

in the study [$F_{CLOZAPINE}(1,28)= 2.15$, $p= 0.15$; $F_{MINOCYCLINE}(1,28)= 0.01$, $p= 0.93$; $F_{INTERACTION}(1,28)= 0.07$, $p= 0.78$]. **Table 4.43** summarizes the mean ± SEM values of the *Il6* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline. Moreover, the potential influence of sex on *Il6* relative mRNA levels after the pharmacological treatments was analyzed by an exploratory three-way ANOVA including the sex as an additional factor. Sex was not significant in control mice [$F_{SEX}(1,24)= 0.53$; $p= 0.47$]. However, in double-hit mice, sex was revealed as a significant factor [$F_{SEX}(1,24)= 3.37$; $p= 0.02$] and a significant "Sex x Clozapine" interaction was found [$F_{SEXxCLOZAPINE}(1,24)= 6.39$; $p= 0.02$], indicating that male double-hit mice had lower *Il6* mRNA levels after clozapine treatment.

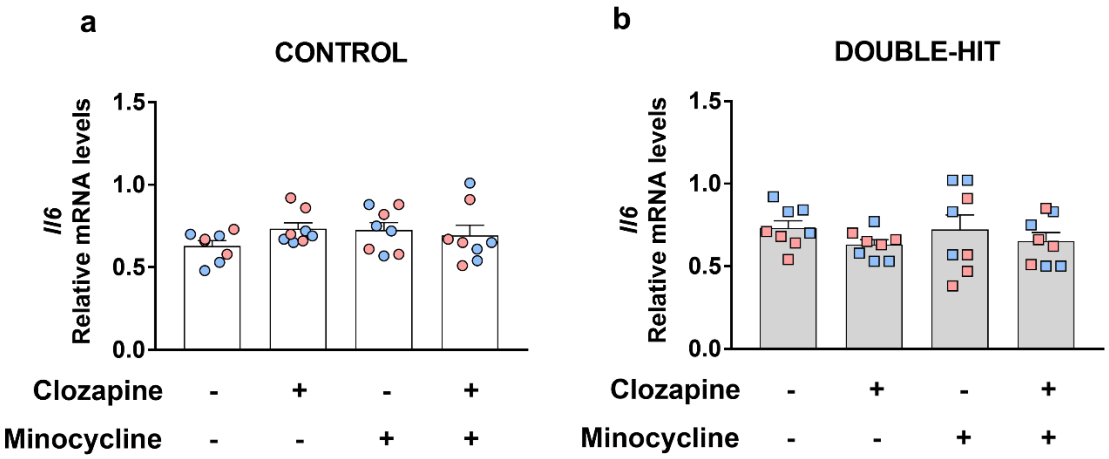

Figure 4.37: Graphic representation of the relative mRNA levels of *Il6*, determined by means of RT-qPCR. Control (**figure 4.37 a**) and double-hit (**figure 4.37 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 8/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test.

Table 4.43: Mean ± SEM values for the *Il6* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

***Il6* relative mRNA levels – Mean ± SEM**				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	0.63 ± 0.03	0.73 ± 0.04	0.73 ± 0.05	0.69 ± 0.06
Double-hit	0.73 ± 0.04	0.63 ± 0.03	0.72 ± 0.09	0.65 ± 0.05

***Ifng* relative mRNA levels**

Ifng relative mRNA levels in vehicle-treated control mice were 1.62 ± 0.35, while in double-hit mice were 1.44 ± 0.33. Student's unpaired *t-test* revealed no differences on *Ifng* relative mRNA levels between vehicle-treated control mice and vehicle-treated double-hit mice (t= 0.37; p= 0.72) (**figure 4.38**). When the potential influence of sex on *Ifng* relative mRNA levels was analyzed by exploratory two-way ANOVA including the sex as an additional factor, sex was revealed as non-significant [$F_{SEX}(1,12) = 2.17$; p= 0.17]. Additionally, no significant effects were found for neither the factor "Double-hit" nor the interaction "Sex x Double-hit" (data not shown).

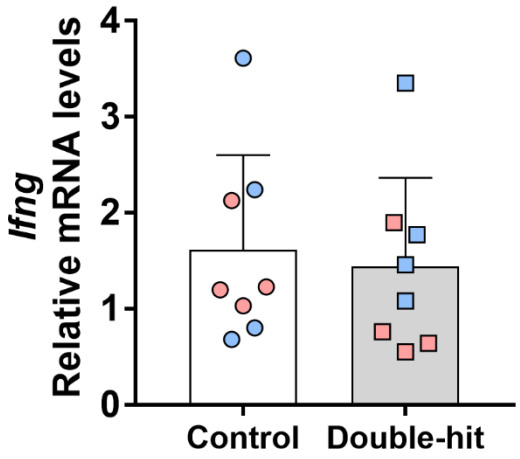

Figure 4.38: Graphic representation of the relative mRNA levels of *Ifng*, determined by means of RT-qPCR. Vehicle-treated control (n= 8) and double-hit (n= 8) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using Student's unpaired *t-test*.

In control mice (**figure 4.39 a**), *Ifng* relative mRNA levels were not modulated by chronic clozapine, minocycline or by co-administration of both drugs [$F_{CLOZAPINE}(1,28)= 0.01$, p= 0.92; $F_{MINOCYCLINE}(1,28)= 0.92$, p= 0.34; $F_{INTERACTION}(1,28)= 1.47$, p= 0.24]. Similarly, in double-hit mice (**figure 4.39 b**), *Ifng* relative mRNA levels were not modulated by any of the pharmacological treatments used in this study [$F_{CLOZAPINE}(1,27)= 0.004$, p= 0.95; $F_{MINOCYCLINE}(1,27) = 0.86$, p= 0.36; $F_{INTERACTION}(1,27)= 0.04$, p= 0.84]. **Table 4.44** summarizes the mean ± SEM values of the *Ifng* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline. Moreover, the potential influence of sex on *Ifng* relative mRNA levels after the pharmacological treatments was analyzed by an exploratory three-way ANOVA analysis including the sex as an additional factor. Sex was not a significant factor, neither in control mice [$F_{SEX}(1,24)= 0.17$; p= 0.68], nor in double-hit mice [$F_{SEX}(1,24)= 1.72$; p= 0.20]. Moreover, no significant "Sex x Clozapine", "Sex x Minocycline" or "Sex x Clozapine x Minocycline" were found in control and double-hit mice (data not shown).

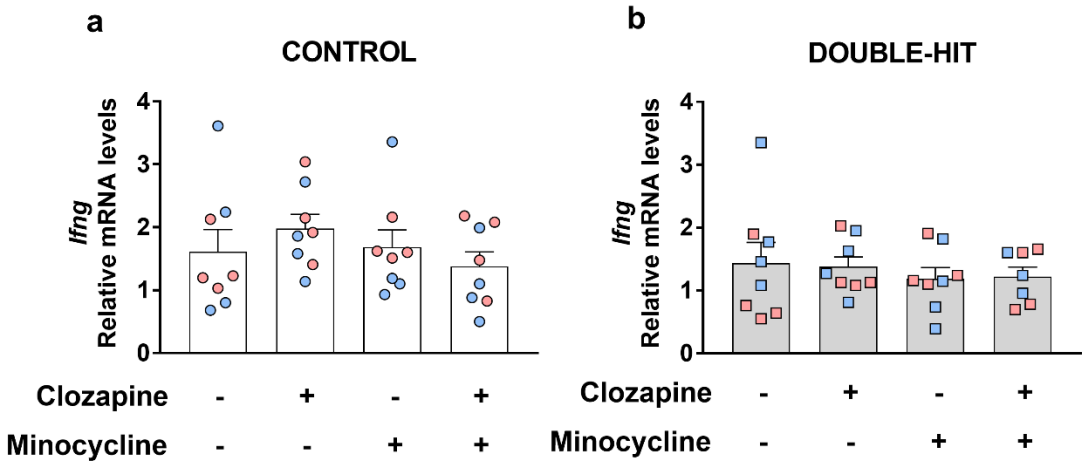

Figure 4.39: Graphic representation of the relative mRNA levels of *Ifng*, determined by means of RT-qPCR. Control (**figure 4.39 a**) and double-hit (**figure 4.39 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 7-8/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test.

Table 4.44: Mean ± SEM values for the *Ifng* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

***Ifng* relative mRNA levels – Mean ± SEM**				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	1.62 ± 0.35	1.98 ± 0.23	1.68 ± 0.28	1.38 ± 0.23
Double-hit	1.44 ± 0.33	1.38 ± 0.16	1.19 ± 0.18	1.22 ± 0.16

4.2.4. EFFECT OF THE CHRONIC TREATMENT WITH CLOZAPINE, MINOCYCLINE AND THE COMBINATION OF BOTH, ON THE GENE EXPRESSION OF NF-κB (*Rela*) AND ITS REPRESSOR IκBα (*Nfkbia*)

RT-qPCR experiments were performed in order to evaluate the effect of the chronic treatment with clozapine and/or minocycline in the expression of the genes encoding for NF-κB (*Rela*) and IκBα (*Nfkbia*) inflammatory signaling proteins in cortical samples of control and double-hit mice.

NF-κB (*Rela*) relative mRNA levels

Rela relative mRNA levels in vehicle-treated control mice were 0.87 ± 0.02; while in double-hit mice were 0.87 ± 0.07. Unpaired Student's *t-test* analysis revealed no significant differences in *Rela* relative mRNA levels between vehicle-treated control and double-hit mice (t= 0.09; p= 0.93) (**figure 4.40**). When the potential influence of sex on *Rela* relative mRNA levels was analyzed by an exploratory two-way ANOVA including the sex as an additional factor, sex was revealed as significant [$F_{SEX}(1,11) = 6.67$; p= 0.03]. Moreover, a significant "Sex and Double-hit" interaction was found [$F_{SEXxDH}(1,11) = 6.67$; p= 0.03], meaning that the double-hit had a different impact on *Rela* mRNA levels depending on the sex, as male double-hit mice had higher *Rela* mRNA levels than female double-hit mice.

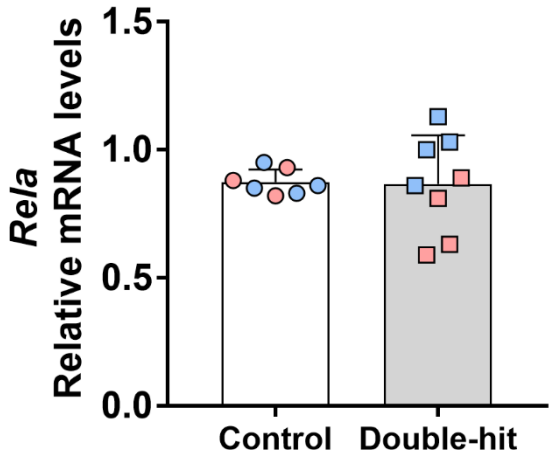

Figure 4.40: Graphic representation of the relative mRNA levels of NF-κB protein (*Rela*), determined by means of RT-qPCR. Vehicle-treated control (n= 7) and double-hit (n= 8) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using Student's unpaired *t-test*.

In control mice (**figure 4.41 a**), the administration of clozapine and minocycline alone induced a non-significant increase in *Rela* relative mRNA levels [$F_{CLOZAPINE}(1,26)= 0.33$, $p= 0.57$; $F_{MINOCYCLINE}(1,26)= <0.001$; $p= 0.99$], which was reverted by co-administration of both drugs [$F_{INTERACTION}(1,26)= 5.7$, $p= 0.02$]. In double-hit mice (**figure 4.41 b**), *Rela* relative mRNA levels were not modulated by any of the pharmacological treatments used in the study [$F_{CLOZAPINE}(1,27)= 0.0002$, $p= 0.99$; $F_{MINOCYCLINE}(1,27)= 0.14$, $p= 0.71$; $F_{INTERACTION}(1,27)= 0.30$, $p= 0.59$]. **Table 4.45** summarizes the mean ± SEM values of the *Rela* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline. Moreover, the potential influence of sex on *Rela* relative mRNA levels after the pharmacological treatments was analyzed by an exploratory three-way ANOVA including the sex as an additional factor. Both in control [$F_{SEX}(1,24)= 7.49$; $p= 0.012$] and in double-hit [$F_{SEX}(1,24)= 6.98$; $p= 0.014$] mice, sex was revealed as significant. In control mice, no significant "Sex x Treatment" interaction was found. However, a significant "Sex x Minocycline" interaction was found in double-hit mice [$F_{SEXxMINOCYCLINE}(1,24)= 8,941$; $p= 0.006$]. Thus, it seems that minocycline treatment is able to restore the altered *Rela* mRNA levels in vehicle-treated double-hit mice to vehicle-treated control valued (see **figures 4.40** and **4.41**).

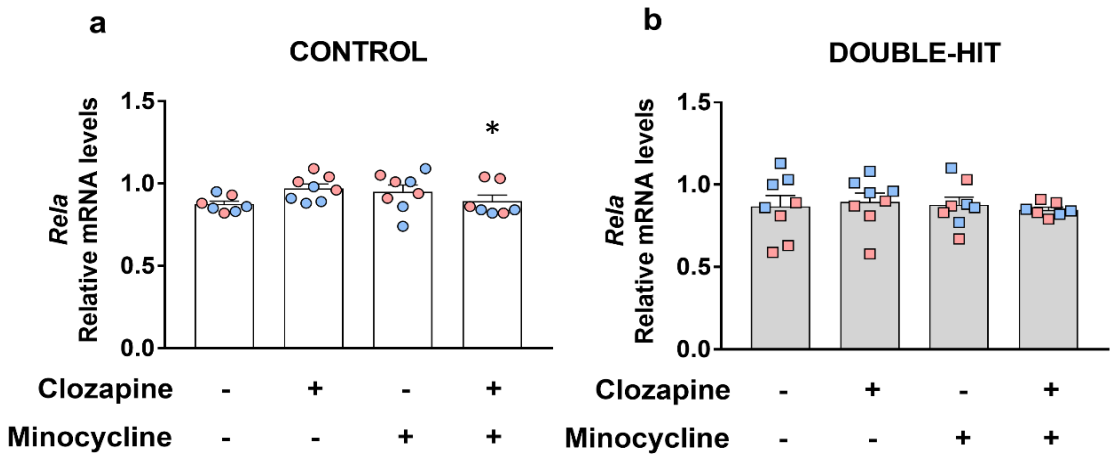

Figure 4.41: Graphic representation of the relative mRNA levels of NF-κB protein (*Rela*), determined by means of RT-qPCR. Control (**figure 4.41 a**) and double-hit (**figure 4.41 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 7-8/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test. Clozapine + minocycline interaction significance in control mice is expressed as *p<0.05.

Table 4.45: Mean ± SEM values for the *Rela* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

***Rela* relative mRNA levels – Mean ± SEM**				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	0.87 ± 0.02	0.97 ± 0.03	0.95 ± 0.04	0.89 ± 0.04
Double-hit	0.87 ± 0.07	0.90 ± 0.05	0.88 ± 0.05	0.85 ± 0.02

IκBα (*Nfkbia*) relative mRNA levels

Nfkbia relative mRNA levels in vehicle-treated control mice were 0.88 ± 0.05, while in double-hit mice were 0.83 ± 0.05. Student's unpaired *t-test* analysis revealed no significant differences in *Nfkbia* relative mRNA levels between vehicle-treated control and double-hit mice (t= 0.71; p= 0.49) (**figure 4.42**). When the potential influence of sex on *Nfkbia* relative mRNA levels was analyzed by an exploratory two-way ANOVA including the sex as an additional factor, sex was

revealed as non significant [$F_{SEX}(1,12) = 0.009$; p= 0.93]. Moreover, "Sex x Double-hit" interaction was not significant [$F_{SEXxDH}(1,12)= 3.396$; p=0.09], although it was close to the statistical significance.

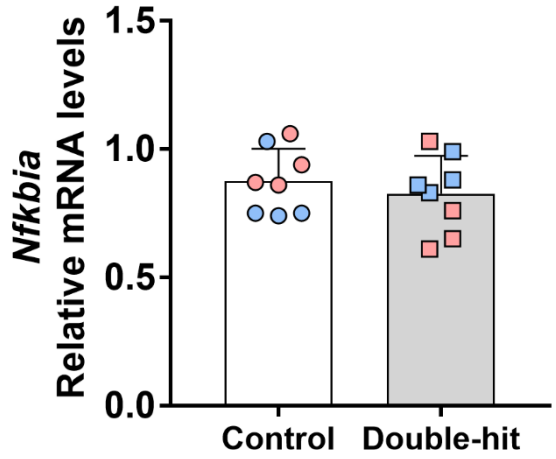

Figure 4.42: Graphic representation of the relative mRNA levels of IκBα protein (*Nfkbia*), determined by means of RT-qPCR. Vehicle-treated control (n= 8) and double-hit (n= 8) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using Student's unpaired *t-test*.

In control mice (**figure 4.43 a**), *Nfkbia* relative mRNA levels were not modulated by the administration of clozapine, minocycline, or by the co-administration of both drugs [$F_{CLOZAPINE}(1,27)= 0.12$, p= 0.73; $F_{MINOCYCLINE}(1,27)= 0.06$, p= 0.81; $F_{INTERACTION}(1,27)= 0.06$, p= 0.81]. Similarly, in double-hit mice (**figure 4.43 b**), *Nfkbia* relative mRNA levels were not modulated by any of the pharmacological treatments used in the study [$F_{CLOZAPINE}(1,28) = 0.48$, p= 0.50; $F_{MINOCYCLINE}(1,28) = 2.25$, p= 0.14; $F_{INTERACTION}(1,28) = 0.31$, p=0.58]. **Table 4.46** summarizes the mean ± SEM values of the *Nfkbia* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline. Moreover, the potential influence of sex on *Nfkbia* relative mRNA levels after the pharmacological treatments was analyzed by an exploratory three-way ANOVA including the sex as an additional factor. Sex was not revealed as significant, neither in control mice [$F_{SEX}(1,24)= 2.60$; p= 0.12], nor in double-hit mice [$F_{SEX}(1,24)= 1.16$; p= 0.29]. No significant "Sex x Clozapine", "Sex x Minocycline" or "Sex x Clozapine x Minocycline" interactions were found in control and double-hit mice (data not shown).

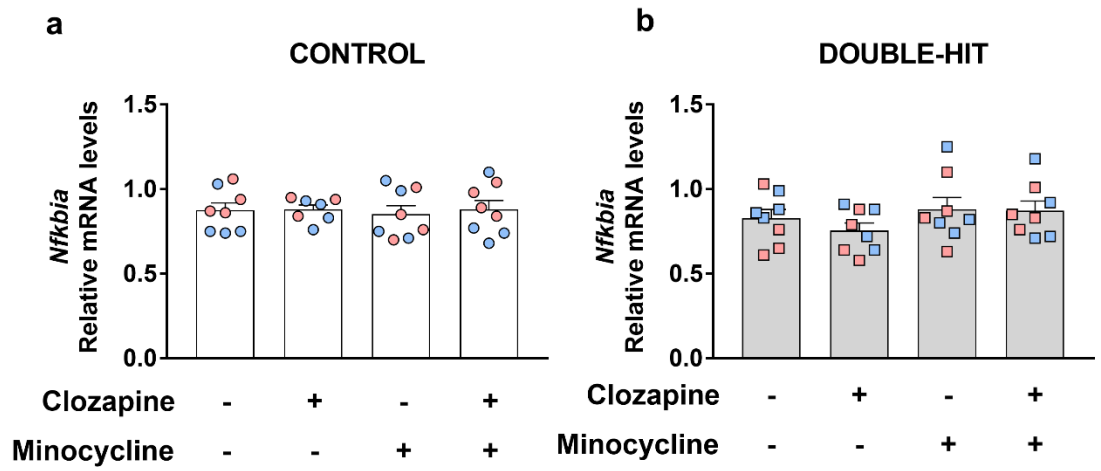

Figure 4.43: Graphic representation of the relative mRNA levels of IκBα protein (*Nfkbia*), determined by means of RT-qPCR. Control (**figure 4.43 a**) and double-hit (**figure 4.43 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 7-8/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test.

Table 4.46: Mean ± SEM values for the *Nfkbia* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

***Nfkbia* relative mRNA levels – Mean ± SEM**				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	0.88 ± 0.05	0.88 ± 0.03	0.85 ± 0.05	0.88 ± 0.05
Double-hit	0.83 ± 0.05	0.76 ± 0.05	0.88 ± 0.07	0.87 ± 0.06

4.2.5. EFFECT OF THE CHRONIC TREATMENT WITH CLOZAPINE, MINOCYCLINE AND THE COMBINATION OF BOTH, ON THE GENE EXPRESSION OF *Hdac2, Hdac4* AND *Hdac8*

RT-qPCR experiments were performed to evaluate the effect of the chronic treatment with clozapine, minocycline and combination of both drugs on the relative mRNA levels of the genes encoding to HDAC2, HDAC4 and HDAC8 proteins (genes *Hdac2*, *Hdac4* and *Hdac8*, respectively).

***Hdac2* relative mRNA levels**

Hdac2 relative mRNA levels in vehicle-treated control mice were 0.84 ± 0.03, while in double-hit mice were 0.83 ± 0.05. Student's unpaired *t-test* analysis revealed no significant differences in *Hdac2* relative mRNA levels between vehicle-treated control and double-hit mice (t= 0.20; p= 0.85) (**figure 4.44**). When the potential influence of sex on *Hdac2* relative mRNA levels was analyzed by an exploratory two-way ANOVA analysis including the sex as an additional factor, sex was revealed as non significant [$F_{SEX}(1,12) = 0.009$; p= 0.93]. However, a significant "Sex x Double-hit" interaction was found [$F_{SEXxDH}(1,12)= 11.97$; p= 0.005], meaning that the effect of Double-hit on *Hdac2* mRNA levels was different between both sexes, as double-hit females had lower *Hdac2* mRNA levels than double-hit males.

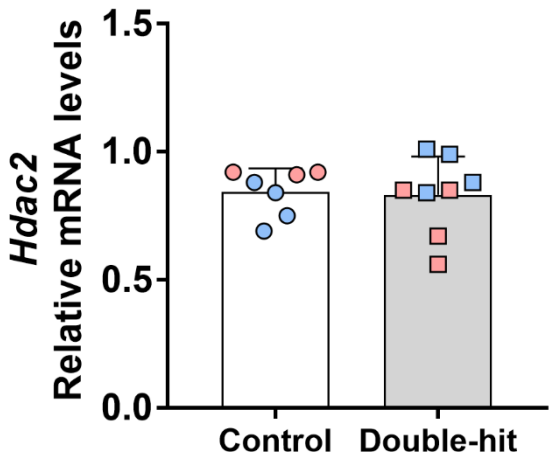

Figure 4.44: Graphic representation of the relative mRNA levels of *Hdac2*, determined by means of RT-qPCR. Vehicle-treated control (n= 7) and double-hit (n= 8) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using Student's unpaired *t-test*.

In control mice (**figure 4.45 a**), *Hdac2* relative mRNA levels were not modulated by the effect of chronic clozapine, minocycline or co-administration of both drugs [$F_{CLOZAPINE}(1,25)= 1.46$, p= 0.24; $F_{MINOCYCLINE}(1,25)= 0.15$, p= 0.71; $F_{INTERACTION}(1,25)= 0.08$, p= 0.78]. Similarly, in double-hit mice (**figure 4.45 b**), *Hdac2* relative mRNA levels were not modulated by any of the pharmacological treatments used [$F_{CLOZAPINE}(1,28)= 0.96$, p= 0.36; $F_{MINOCYCLINE}(1,28)= 0.35$, p= 0.56; $F_{INTERACTION}(1,28)= 0.02$, p=0.88]. **Table 4.47** summarizes the mean ± SEM values of the *Hdac2*

relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline. Moreover, the potential influence of sex on *Hdac2* relative mRNA levels after the pharmacological treatments was analyzed by an exploratory three-way ANOVA analysis including the sex as an additional factor. Sex was not significant neither in control mice [$F_{SEX}(1,24)= 1.51$; $p= 0.23$], nor in double-hit mice [$F_{SEX}(1,24)= 3.74$; $p= 0.07$] –although in the case of double-hit mice, it was close to the statistical significance–. No significant "Sex x Clozapine", "Sex x Minocycline" or "Sex x Clozapine x Minocycline" interactions were found in control and double-hit mice (data not shown).

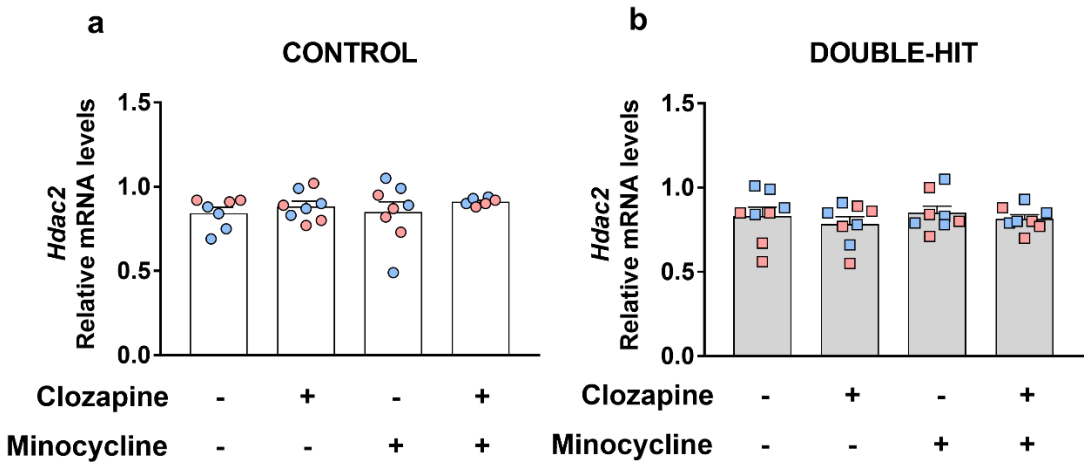

Figure 4.45: Graphic representation of the relative mRNA levels of *Hdac2*, determined by means of RT-qPCR. Control (**figure 4.45 a**) and double-hit (**figure 4.45 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 6-8/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test.

Table 4.47: Mean ± SEM values for the *Hdac2* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

***Hdac2* relative mRNA levels – Mean ± SEM**				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	0.84 ± 0.03	0.88 ± 0.03	0.85 ± 0.06	0.91 ± 0.01
Double-hit	0.83 ± 0.05	0.78 ± 0.04	0.85 ± 0.04	0.82 ± 0.03

***Hdac4* relative mRNA levels**

Hdac4 relative mRNA levels in vehicle-treated control mice were 1.27 ± 0.05; while in double-hit mice were 1.12 ± 0.08. Although double-hit *Hdac4* mRNA levels were lower than control levels, this decrease did not reach the statistical significance when a Student's unpaired *t-test* was performed (t= 1.64; p= 0.12) (**figure 4.46**). When the potential influence of sex on *Hdac4* relative mRNA levels was analyzed by an exploratory two-way ANOVA including the sex as an additional factor, sex was revealed as significant [$F_{SEX}(1,12) = 4.82$; p= 0.04]. In addition, both the "Double-hit" factor [$F_{DH}(1,12)= 6.15$; p= 0.03], and the "Sex x Double-hit" interaction [$F_{SEXxDH}(1,12)= 15.24$; p= 0.002] were found to be significant. Thus, female double-hit mice had lower *Hdac4* mRNA levels than male double-hit mice.

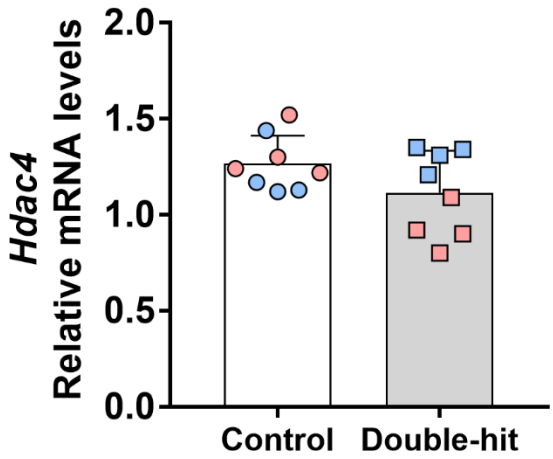

Figure 4.46: Graphic representation of the relative mRNA levels of *Hdac4*, determined by means of RT-qPCR. Vehicle-treated control (n= 8) and double-hit (n= 8) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using Student's unpaired *t-test*.

In control mice (**figure 4.47 a**), *Hdac4* mRNA levels were not modulated by chronic clozapine, minocycline or by co-administration of both drugs [$F_{CLOZAPINE}(1,28)= 0.03$, p= 0.87; $F_{MINOCYCLINE}(1,28)= 1.06$, p= 0.31; $F_{INTERACTION}(1,28)= 0.01$, p= 0.93]. Similarly, in double-hit mice (**figure 4.47 b**), *Hdac4* mRNA levels were not modulated by any of the pharmacological treatments used in the study [$F_{CLOZAPINE}(1,28)= 0.19$, p= 0.67; $F_{MINOCYCLINE}(1,28)= 0.04$, p= 0.85; $F_{INTERACTION}(1,28)= 0.01$, p= 0.92]. **Table 4.48** summarizes the mean ± SEM values of the *Hdac4*

relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline. Moreover, the potential influence of sex on *Hdac4* relative mRNA levels after the pharmacological treatments was analyzed by an exploratory three-way ANOVA including the sex as an additional factor. Sex was not significant –although it was close to the statistical significance– in control mice [$F_{SEX}(1,24)= 3.91$; $p= 0.06$], and no further "Sex x Treatment" interactions were found. However, in double-hit mice [$F_{SEX}(1,24)= 5.03$; $p= 0.03$], sex was revealed as a significant factor. In addition, the "Sex x Clozapine" interaction was close to the statistical significance [$F_{SEXxCLOZAPINE}(1,24) = 3.56$; $p= 0.07$], suggesting that clozapine treatment was able to bring the male and female vehicle values (increased and decreased, respectively) to control values.

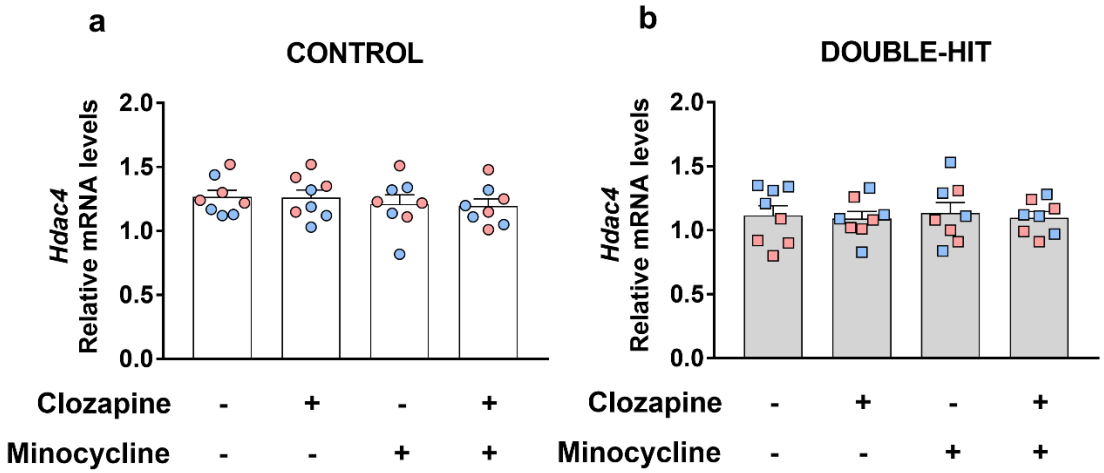

Figure 4.47: Graphic representation of the relative mRNA levels of *Hdac4*, determined by means of RT-qPCR. Control (**figure 4.47 a**) and double-hit (**figure 4.47 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 8/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test.

Table 4.48: Mean ± SEM values for the *Hdac4* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

***Hdac4* relative mRNA levels – Mean ± SEM**				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	1.27 ± 0.05	1.26 ± 0.06	1.21 ± 0.07	1.20 ± 0.05
Double-hit	1.12 ± 0.08	1.09 ± 0.06	1.13 ± 0.08	1.10 ± 0.05

***Hdac8* relative mRNA levels**

Hdac8 relative mRNA levels in vehicle-treated control mice were 1.24 ± 0.05; while in double-hit mice were 1.20 ± 0.08. Control and double-hit mice did not show any differences in *Hdac8* mRNA levels when a Student's unpaired *t-test* was performed (t= 0.43; p= 0.67) (**figure 4.48**). When the potential influence of sex on *Hdac8* relative mRNA levels was analyzed by exploratory two-way ANOVA including the sex as an additional factor, sex was revealed as significant [$F_{SEX}(1,12) = 7.27$; p= 0.02]. Moreover, a significant "Sex x Double-hit" interaction was found [$F_{SEXxDH}(1,12)= 30.67$; p= 0.0001] because the Double-hit factor had a different effect depending on the sex, as it decreased the *Hdac8* mRNA levels in females, and increased *Hdac8* mRNA levels in males.

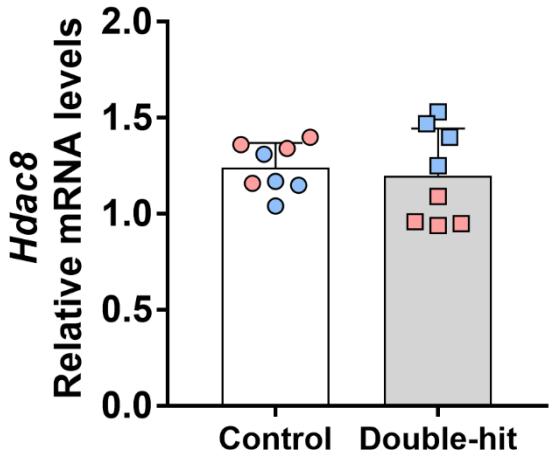

Figure 4.48: Graphic representation of the relative mRNA levels of *Hdac8*, determined by means of RT-qPCR. Vehicle-treated control (n= 8) and double-hit (n= 8) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using Student's unpaired *t-test*.

In control mice (**figure 4.49 a**), *Hdac8* mRNA levels were not modulated by chronic clozapine, minocycline or by co-administration of both drugs [$F_{CLOZAPINE}(1,28)= 1.34$, $p= 0.26$; $F_{MINOCYCLINE}(1,28)= 0.20$, $p= 0.66$; $F_{INTERACTION}(1,28)= 0.34$, $p= 0.56$]. Similarly, in double-hit mice (**figure 4.49 b**), *Hdac8* mRNA levels were not modulated by any of the pharmacological treatments used in the study [$F_{CLOZAPINE}(1,27)= 0.08$, $p= 0.79$; $F_{MINOCYCLINE}(1,27)= 0.06$, $p= 0.81$; $F_{INTERACTION}(1,27)= 0.01$, $p= 0.79$]. **Table 4.49** summarizes the mean ± SEM values of the *Hdac8* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline. Moreover, the potential influence of sex on *Hdac8* relative mRNA levels after the pharmacological treatments was analyzed by exploratory three-way ANOVA including the sex as an additional factor. Sex was not significant –although it was close to the statistical significance– in control mice [$F_{SEX}(1,24)= 3.68$; $p= 0.07$]. Moreover, a significant "Sex x Minocycline" interaction was found in control mice [$F_{SEXxMINOCYCLINE}(1,24)= 5.405$; $p= 0.03$]. In double-hit mice, sex was revealed as a significant factor [$F_{SEX}(1,24)= 5.95$; $p= 0.02$]. No significant "Sex x Double-hit" interaction was found [$F_{SEXxDH}(1,24)= 4.14$; $p= 0.053$].

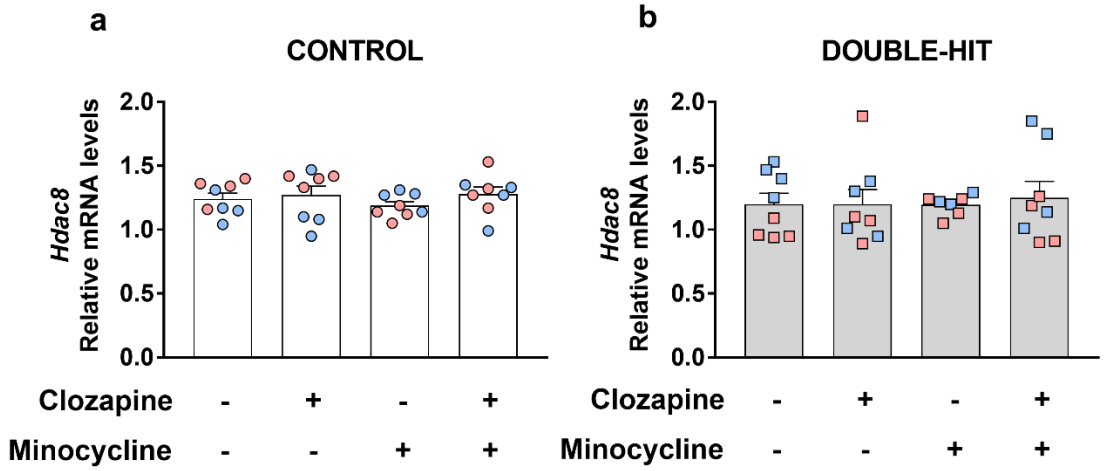

Figure 4.49: Graphic representation of the relative mRNA levels of *Hdac8*, determined by means of RT-qPCR. Control (**figure 4.49 a**) and double-hit (**figure 4.49 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 7-8/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test.

Table 4.49: Mean ± SEM values for the *Hdac8* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

***Hdac8* relative mRNA levels – Mean ± SEM**				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	1.24 ± 0.05	1.27 ± 0.07	1.19 ± 0.03	1.28 ± 0.06
Double-hit	1.20 ± 0.09	1.20 ± 0.12	1.20 ± 0.03	1.25 ± 0.13

4.2.6. EFFECT OF THE CHRONIC TREATMENT WITH CLOZAPINE AND MINOCYCLINE ON THE GENE EXPRESSION OF $5HT_{2A}R$

Gene expression of *Htr2a*, the gene encoding for the 5-HT_{2A}R, was evaluated in cortical samples belonging to control and double-hit mice treated with vehicle, clozapine, minocycline, and combination of both drugs.

Htr2a relative mRNA levels in vehicle-treated control mice were 0.91 ± 0.05, while in double-hit mice were 0.88 ± 0.05. Student's unpaired *t-test* revealed no differences on *Htr2a* relative mRNA levels between vehicle-treated control mice and vehicle-treated double-hit mice (t= 0.53; p= 0.60) (**figure 4.50**). When the potential influence of sex on *Htr2a* relative mRNA levels was analyzed by an exploratory two-way ANOVA analysis including the sex as an additional factor, sex was revealed as non-significant [$F_{SEX}(1,12) = 0.40$; p= 0.54]. Moreover, "Sex x Double-hit" interaction was close to statistical significance[$F_{SEXxDH}(1,12) = 4.58$; p=0.053] , suggesting that the "Double-hit" factor affects the mRNA levels of *Htr2a* in a different manner, thus, eliciting a decrease of the *Htr2a* mRNA levels only in females.

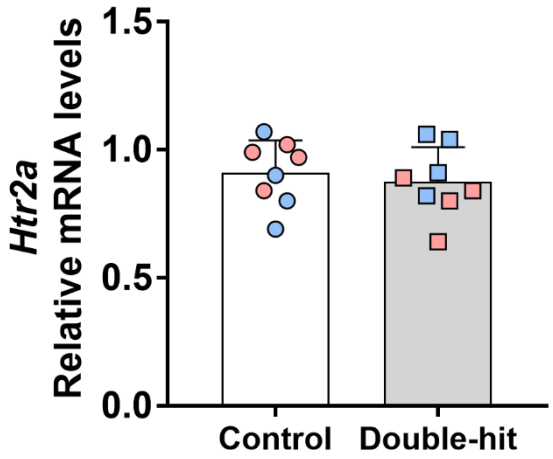

Figure 4.50: Graphic representation of the relative mRNA levels of *Htr2a*, determined by means of RT-qPCR. Vehicle-treated control (n= 8) and double-hit (n= 8) mice are plotted. Male mice are as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values. Data were analyzed using Student's unpaired t-test.

In control mice (**figure 4.51 a**), *Htr2a* relative mRNA levels were not modulated by chronic clozapine, minocycline or by co-administration of both drugs [$F_{CLOZAPINE}(1,26)= 0.45$, $p= 0.51$; $F_{MINOCYCLINE}(1,26)= 0.49$, $p= 0.49$; $F_{INTERACTION}(1,26)= 0.58$, $p= 0.45$]. In double-hit mice (**figure 4.51 b**), *Htr2a* relative mRNA levels were not modulated by any of the pharmacological treatments used in these experiments [$F_{CLOZAPINE}(1,28)= 1.84$, $p= 0.19$; $F_{MINOCYCLINE}(1,28) = 2.07$, $p= 0.16$; $F_{INTERACTION}(1,28)= 0.05$, $p= 0.83$]. **Table 4.50** summarizes the mean ± SEM values of the *Htr2a* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline. Moreover, the potential influence of sex on *Htr2a* relative mRNA levels after the pharmacological treatments was analyzed by an exploratory three-way ANOVA analysis including the sex as an additional factor. In control mice, sex was revealed as a significant factor [$F_{SEX}(1,24)= 5.54$; $p= 0.03$]. In contrast, in double-hit mice, sex was not significant [$F_{SEX}(1,24)= 2.05$; $p= 0.17$]. No other "Sex x Clozapine", "Sex x Minocycline" or "Sex x Clozapine x Minocycline" interactions were found in control and double-hit mice (data not shown).

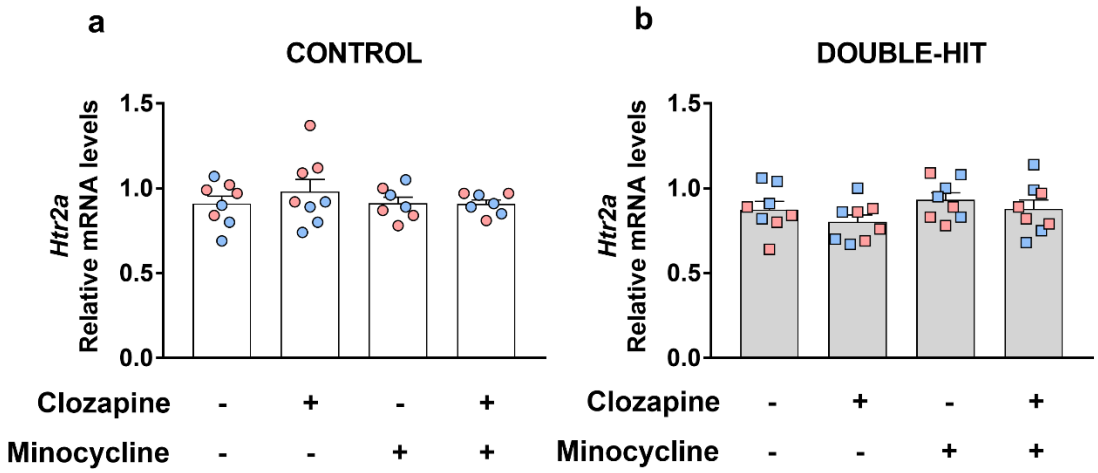

Figure 4.51: Graphic representation of the relative mRNA levels of *Htr2a*, determined by means of RT-qPCR. Control (**figure 4.51 a**) and double-hit (**figure 4.51 b**) mice belonging to "vehicle", "clozapine", "minocycline" and "clozapine + minocycline" experimental groups are plotted. Male mice are represented as blue colored dots/squares and female mice are represented as pink colored dots/squares. Bars are a representation of the mean ± SEM values (n= 7-8/arm). Data were analyzed using two-way ANOVAs followed by Tukey's multiple comparisons test.

Table 4.50: Mean ± SEM values for the *Htr2a* relative mRNA levels of control and double-hit mice treated with vehicle, clozapine, minocycline or clozapine + minocycline.

***Htr2a* relative mRNA levels – Mean ± SEM**				
	Vehicle	**Clozapine**	**Minocycline**	**Clozapine + Minocycline**
Control	0.91 ± 0.05	0.98 ± 0.07	0.91 ± 0.04	0.91 ± 0.02
Double-hit	0.86 ± 0.05	0.80 ± 0.04	0.93 ± 0.04	0.88 ± 0.05

5. DISCUSSION

5.1. DEVELOPMENT AND VALIDATION OF THE DOUBLE-HIT MOUSE MODEL OF SCHIZOPHRENIA

5.1.1. BEHAVIORAL CHARACTERIZATION OF THE DOUBLE-HIT MODEL

5.1.1.1. POLY (I:C)-CHALLENGE ELICITS A SICKNESS BEHAVIOR IN PREGNANT DAMS, CHARACTERIZED BY SIGNIFICANT WEIGHT AND TEMPERATURE LOSS

Poly (I:C) administration mimics the acute phase of a viral infection by activating TLR3 receptors and inducing a "sickness behavior" characterized by changes in body weight and temperature, among other behaviors (such as, lethargy, reduced food-seeking behavior, reduced sociability) (Dantzer & Kelley, 2007; Bluthé et al., 2000; Kent et al .,1996; Kentner et al., 2019). In this regard, several authors have validated the Poly (I:C)-elicited immune response by measuring proinflammatory cytokines (such as IL-6, IL-1β or TNF-α) in immune activated pregnant dams (Ballendine et al., 2015; Meyer et al., 2006b; Song et al., 2011). In addition, Poly (I:C)-driven immune activation has been validated by the evaluation of cytokine-induced sickness behaviors in the home cage (piloerection, lethargy and huddling) (Kolmogorova et al., 2017). Finally, one of the most used methods for the immune response validation is to measure changes in the body weight (Hart et al., 1988; Redfern et al., 2017) and temperature (Cunningham et al., 2007; Mueller et al, 2019) after the immune challenge. Measuring the effects of Poly (I:C) on body weight and temperature is a well-established way of demonstrating the effects of maternal immune activation (Kentner et al., 2019), that can be assessed by using laboratory scales and temperature probes, respectively. Thus, in this work, maternal immune response elicited by Poly (I:C) administration was validated by the measurement of the body weight and temperature of vehicle-treated and Poly (I:C)-treated pregnant dams.

In the present work, body weight of Poly (I:C)-challenged pregnant dams was significantly reduced until 24 hours after Poly (I:C) administration. This transitory loss of maternal body weight elicited by Poly (I:C) was recovered 48 hours after Poly (I:C) administration, when Poly (I:C)-treated pregnant dams started gaining weight again (as a normal process during pregnancy) (see **figure 4.1**). The loss of maternal body weight as a part of the sickness behavior induced by Poly (I:C) is well described and validated (Cunningham et al., 2007). Interestingly, in our study vehicle-injected controls also showed a slight decrease in body weight 6 hours after vehicle administration (98.59 ± 1.56 % grams to 0 h). This body weight loss was minor and transitory, and it was fully recovered 24 hours after the insult. In contrast, Poly (I:C)-challenged animals had a more robust and long-lasting body weight reduction that was evident between 3 hours (97.64 ± 0.5 % grams to 0 h) and 24 hours (97.10 ± 0.85 % grams to 0 h) after Poly (I:C) administration. Vehicle-elicited body weight loss is also reported in the literature (Cunningham et al., 2007) and could be associated to a stress response after the injection.

Furthermore, core body temperature was significantly reduced 3 hours after the Poly (I:C) challenge (96.92 ± 0.37 % ^{0}C to 0 h) and this decrease was not recovered until 24 hours after the administration (see **figure 4.2**). Measurement of core body temperature is also a well-established method of Poly (I:C)-elicited MIA validation (Kentner et al., 2019), as Poly (I:C) challenge is associated with a flu-like acute phase response, including hypothermia in small species such as mice (Cunningham et al., 2007; Traynor et al., 2006). Consistent with previous studies in mice (Cunningham et al., 2007; Desbonnet et al., 2022; Mueller et al., 2019), in our study, administration of Poly (I:C) led to reduced rectal temperatures. Hence, the predominant thermal response to Poly (I:C) in mice is hypothermia, which in other studies has been shown to be preceded by a temporary phase of mild hyperthermia (Cunningham et al., 2007). Notably, the thermal response to Poly (I:C) in mice is distinct from that in rats, the latter of which typically shows a monophasic hyperthermic response to this immunogen (Fortier et al., 2004). These changes in the temperature are proven to be correlated with increased plasma concentrations of key inflammatory markers, such as, IL-6, IL-1β, TNF-α and IFN-β (Cunningham et al., 2007; Desbonnet et al., 2022; Mueller et al., 2019). Notably, a slight decrease in body temperature in vehicle-treated control animals was also observed (98.38 ± 0.34 % ^{0}C to 0 h, 3h after vehicle administration), as reported in previous studies (Cunningham et al., 2007). However, vehicle-treated control animals had a significantly milder temperature loss than Poly (I:C)-challenged pregnant dams, that was recovered 24 hours after the administration. Once again, we suggest that this temperature loss in controls could occur due to the stress response to the injection.

Hence, taking into account the significant effects on body weight and temperature elicited by Poly (I:C) administration to pregnant dams, we can assume that this immunogen substance provoked a sickness response in our mice that mimicked the innate immune response to a viral infection.

5.1.1.2. SOCIAL ISOLATION INDUCES ANXIOGENIC-LIKE BEHAVIOR IN THE OFT

The Open Field Test (OFT) is one of the most commonly used platforms to evaluate the general ambulatory activity and anxiety-related emotional behaviors in animal models (Seinbenhener & Wooten, 2015). The spontaneous activity in the open field represents a behavioral paradigm that mimics the natural conflict in mice between their tendency to explore a novel environment and aversion to open spaces (Ennaceur et al., 2006). If total distance moved is similar between experimental groups, further analysis of emotional behaviors are simplified because locomotor activity is effectively removed from the equation. Thus, prior to test anxiety-like behavior is important to evaluate the general ambulatory activity of the rodents, since if the locomotor activity is altered, other behaviors that rely on animals' ambulatory activity (such as the time spent in periphery/center) can be also affected, leading to a misinterpretation of the data (Seinbenhener & Wooten, 2015).

In our study, MIA, SI, or combination of both hits did not induce any alteration in the spontaneous locomotor activity of the different groups of animals. Hence, as total distance moved in the four evaluated groups was similar, we were able to analyze the thigmotaxis behavior. Rodents show natural aversion to large, open and unknown environments and OFT elicits a feeling of openness in the center of the maze. Thus, thigmotaxis or wall-hugging behavior is observed in most rodent species and is linked to anxiety-related behaviors (Crawley, 1999). The degree of thigmotaxis has been validated as a measure of anxiogenic behavior in mice, and thigmotaxis increases as anxiety levels rise (Simon et al., 1994). In our study, we did not see any significant effect exerted by MIA on the time spent in the center/periphery of the OFT. Our results disagree with the general believe claiming an anxiogenic-like behavior with increased time spent in periphery (and decreased time spent in center) of the OFT in the offspring of Poly (I:C)-challenged dams (Hao et al., 2019; Li et al., 2021; Su et al., 2022). Discrepancies with our results may be explained because these studies were performed in rats and vary in the dose and prenatal timing of Poly (I:C) administration. Nonetheless, in accordance with our results, these studies also report unaltered ambulatory activity (total distance moved) associated with MIA in the OFT. In contrast, in our study, time spent in periphery (thigmotaxis behavior) was significantly increased in socially isolated mice compared to grouped ones, and consequently, time spent in center was significantly reduced in these groups of animals (see **figure 4.4**). In concordance with our results, other studies report increased anxiety-like behavior in the OFT following similar stress exposure (Amiri et al., 2016; Fone & Porkess, 2008; Huang et al., 2017; Koike et al., 2009; Lander et al., 2017; Lukkes et al., 2009). Therefore, we can conclude that social isolation increased the anxiety-like behavior. In this regard, the combined effects of prenatal immune activation with Poly (I:C) (5 mg/kg i.v. in GD12.5) and post-weaning social isolation (PND21 – PND80) in a double-hit mouse model have been investigated in a recent study (Desbonnet et al., 2022). These authors reported increased anxiety-like behavior in the OFT –with decreased time spent in center and increased time spent in periphery– associated to SI in both male and female mice. They further demonstrated that the anxiogenic-like effect of SI was more pronounced in female mice. These findings demonstrate the significant sex-dependent effect on adult schizophrenia-related phenotypes as demonstrated by the greater impact of SI stress on anxiety-like behaviors in females relative to males. In contrast, our findings suggest that the anxiogenic-like response exerted by SI affected both sexes in a similar way. However, female mice appear to have a higher basal sensitivity to anxiogenic-like behaviors in the OFT, as females spend more time in the periphery, and less time in the center of the OFT than males. These facts make interesting to evaluate the effects induced by different hits in the anxiety-related behaviors in an independent manner for each sex.

Thus, in the present study we analyzed the combined effects of a prenatal immune challenge and post-weaning social isolation rearing on the ambulatory activity and anxiety-related behaviors in the OFT.

In this case, combination of MIA and SI does not potentiate the anxiety-like behaviors in our mice. This is consistent with other authors reporting no synergistic effects in anxiety-related behaviors in a mouse double-hit model of Poly (I:C)-induced MIA and post-weaning social isolation stress (Desbonnet et al., 2022). In our study, we did not show a different effect of SI in males and females in the time spent in the periphery/center of the OFT, since both sexes presented an anxiety-like effect induced by SI. Few studies analyze sex-influence in socially isolated rodents displayed anxiety-related behaviors. In these studies, sex-specific responses in various anxiety-related behavioral paradigms (including OFT or elevated plus maze) have been reported. These studies show controversial conclusions, but in general terms, it is believed that post-weaning isolation has a sex-specific effect on emotional behaviors such as anxiety, with females having a more susceptible response than males (Desbonnet et al., 2022; Hill, 2016; Huang et al., 2017). Although we did not report a greater impact of the SI-induced anxiogenic-like behavior in female mice, we did notice that females presented a higher basal susceptibility to anxiety-related behaviors.

5.1.1.3. NON-ALTERED SPATIAL SHORT-TERM MEMORY IN THE YMSAT AND IMPAIRED LONG-TERM EPISODIC-LIKE DECLARATIVE MEMORY IN THE NORT ASSOCIATED TO MIA AND SI

Neuropsychiatric disorders such as schizophrenia are associated with cognitive impairment, including learning, memory and attention deficits. In rodents, a validated method to asses spatial short-term memory is to measure the spontaneous alternation behavior in the Y-maze test (YMSAT), which comprises the tendency for mice to alternate their (conventionally) non-reinforced choices of Y-maze arms on successive opportunities (Hughes, 2004; Ibi et al., 2017). In our study, spontaneous alternation behavior in the YMSAT was not altered in any of the experimental groups evaluated (see **figure 4.5**).

There is some evidence reporting altered spatial short-term memory in Poly (I:C) MIA models (Hao et al., 2019; Li et al., 2021; Richetto et al., 2013; Schroeder et al., 2019; Viola et al., 2019). However, these studies differ on the species used (mice, rats) and MIA protocol used (different Poly (I:C) dose, gestational timing of Poly (I:C) administration, etc.). Spatial working memory has also proved to be altered in Poly (I:C) offspring in other behavioral tests evaluating this cognitive domain, such as in the touchscreen trial-unique nonmatching-to-location task (Gogos et al., 2020). Moreover, alterations in this task were sex-specific, as male, but not female Poly (I:C) offspring displayed a deficit in spatial working memory. MacDowell and colleagues (2017) also evaluated the spatial working memory deficits in male and female offspring (analyzed together) of pregnant dams treated withPoly (I:C) (MacDowell et al., 2017). In this case, this cognitive domain was evaluated by means of the alternation task in the T-Maze, that showed to be impaired in the Poly (I:C) offspring.

In addition, altered spatial working memory by spontaneous alternation performance in the YMSAT has been reported in a double-hit mouse model of schizophrenia, combining postnatal immune activation with Poly (I:C) as a first hit or priming event, and peripubertal unpredictable stress as the second hit (da Costa et al., 2021). However, in this investigation the experimental design of the double-hit model was different from our study. Poly (I:C) was administered perinatally in a lower dose (2 mg/kg i.p.) and during three consecutive days (from PND5 to PND7), followed by a peripubertal unpredictable stress paradigm (from PND35 to PND43). Impaired spatial working memory in the YMSAT has been shown in other double-hit model of schizophrenia, in this case combining a social isolation period and ketamine administration in adult rats (Estaphan et al., 2021). In this study, impaired cognitive performance in the YMSAT was associated to SI. However, we must take into account the important methodological difference of this study compared to ours, as the isolation period induced by Estaphan and collaborators was conducted during the adulthood, and not during adolescence. This important construct difference could explain the discrepancy with our results.

In spite to the fact that we did not find an impairment in the short-term spatial working memory in the YMSAT, we decided to explore further alterations in different cognitive domains associated with schizophrenia in our double-hit model. In this regard, one of the most extended behavioral paradigm that evaluates cognitive impairment of schizophrenia rodent models is the NORT, a widely used behavioral test that assesses the short-term and long-term episodic-like declarative memory (Oliveira da Cruz et al., 2020).

NORT short-term memory (assessed 2 hours after the training session) was not affected in any of the experimental groups evaluated (control, MIA, SI, MIA + SI) (see **figure 4.6**). Male and female control groups showed a correct discrimination between the novel and the familiar object, showing positive and high DI scores (0.39 ± 0.06 in control males; 0.18 ± 0.09 in control females). Interestingly, despite both sexes showed a correct discrimination, DI scores in female control mice were lower than in control male mice. Of note, we noticed that female double-hit mice showed lower DI scores than the rest of the experimental groups of the study (0.40 ± 0.12 in male double-hit group; 0.11 ± 0.06 in female double-hit group), indicating a poorer cognitive performance in this female experimental group. Overall, these findings suggest that there could be a sex-related effect in the short-term memory in the NORT.

Several studies report deficits in the NORT short-term recognition memory in the offspring of Poly (I:C)-challenged dams (Gray et al., 2019; Li et al., 2014; Osborne et al., 2017; Osborne et al., 2019). Gray and colleagues demonstrated that the offspring of Poly (I:C)-treated pregnant Wistar rats showed reduced novel object preference relative to controls (Gray et al., 2019). In this case, pregnant rats were

challenged with 5 mg/kg i.v. Poly (I:C) on GD14, and NORT was assessed in an open field box, 15 minutes or 4 hours after the training phase, in a test phase of 5 minutes long (Gray et al., 2019). Li and colleagues showed impaired NORT short-term memory of the offspring of C57BL/6J pregnant mice challenged with a single intraperitoneal injection of 20 mg/kg of Poly (I:C) in GD9.5. For that purpose, NORT short-term episodic-like memory was assessed in an open field arena, where animals were tested 10 minutes after the training phase (Li et al., 2014). Osborne and collaborators reported, in two different studies (Osborne et al., 2017; Osborne et al., 2019), impaired novel object discrimination with lower DIs in offspring from Poly (I:C)-challenged pregnant Sprague-Dawley rats compared to the corresponding controls, and reduced total exploration time. Osborne and colleagues administered Poly (I:C) on GD15 in a dose of 4 mg/kg i.v., and NORT was performed in an open field box, 1 hour after the training phase, in a 5 minute test phase.

Overall, even if all these studies showed an impaired short-term memory and cognitive performance in the NORT associated with prenatal Poly (I:C) challenge, it is important to remark that these investigations present several methodological differences among them. They differ in the species (mice, rats) and strains used; in the timing of prenatal Poly (I:C) administration, dose, route of administration and duration of the insult; and finally, in the NORT protocol itself. Importantly, the behavioral paradigm used by all these authors (Gray et al., 2019; Li et al., 2014; Osborne et al., 2017; Osborne et al., 2019) to evaluate the short-term episodic-like memory differs from the one used in our study, as the time elicited between the training phase and the test phase is very heterogeneous. Moreover, all these authors performed the NORT in a squared-shaped open field arena, whereas in our experimental conditions, an L-shaped maze was used. The L-shaped maze increases the mouse exploration time and reduces variability compared to other arenas used to assess NORT, and both long- and short-term NORT memory can be easily and accurately quantified using this paradigm (Oliveira da Cruz et al., 2020).

Besides, we evaluated the long-term episodic-like memory by performing the NORT 24 hours after the training session. When assessing the NORT long-term memory, we also confirmed that both male and female control mice showed a correct discrimination between the presented objects, as they increased their exploration towards the novel object (0.21 ± 0.03 in control males; 0.38 ± 0.07 in control females). In contrast, MIA-exposed, SI-exposed and double-hit mice showed a cognitive impairment in the NORT long-term memory, with decreased DI scores compared to the controls' DI scores. In addition, the object discrimination was influenced by sex, as females had higher DI scores than males (contrary to what we observed with the short-term memory evaluation in the NORT). All these findings indicate that each single-hit exposure is able to produce, in both males and females, a cognitive impairment that diminish the animal's ability to distinguish between novel and familiar objects. Indeed, male and female MIA+SI mice, the double-hit groups, showed the lowest DI scores compared to the other groups

of same sex (-0.16 ± 0.10 in double-hit males; 0.09 ± 0.07 in double-hit females). Altogether, these results suggest that, despite combination of both hits is not able to produce a synergistic impairment in cognitive performance (no "hit x hit" significant interaction was found), the expected additive effects of both hits are shown in both sexes (see **figure 4.8**).

Our findings are consistent with previous reports demonstrating impairments in the NORT long-term recognition memory in the offspring of Poly (I:C)-challenged pregnant dams (Ozawa et al., 2006; Prades et al., 2017; Wolff et al., 2011). Overall, regardless of differences in the animal model construct and in the NORT experimental protocol, it appears that MIA-induced cognitive impairments related to long-term recognition memory are common and consistent in preclinical studies. Ozawa and collaborators (2006) showed that the adult offspring of Poly (I:C)-treated pregnant dams showed impaired NORT DI, which was reversed by sub-chronic atypical antipsychotic clozapine administration (Ozawa et al., 2006). In this case, pregnant Balb/c mice were administered intraperitoneally with 5 mg/kg Poly (I:C) in every six consecutive days from GD12 to GD17, and NORT was assessed in an open field box, 1 day after the training session, in a 5 min test phase. In the investigation conducted by Prades and collaborators (2017), the offspring of Poly (I:C) treated pregnant C57BL/6J mice showed long-term memory deficits in the NORT (Prades et al., 2017). In this case, pregnant mice were treated with 5 mg/kg i.p. Poly (I:C) on GD9.5, and NORT was assessed in an open field arena, in three phases (habituation, training and testing) during three consecutive days. Thus, 24 hours lasted between the habituation and the test phases. In another study performed with Sprague-Dawley rats, the offspring of Poly (I:C)-challenged dams displayed impaired long-term object recognition memory in the NORT, as MIA mice spent a reduced proportion of their total exploration time exploring the novel object of the NORT (Wolff et al., 2011). These authors administered Poly (I:C) on GD15 in a dose of 4 mg/kg i.v., and NORT was displayed in an open field arena, with 24 hours lasting from the training phase to the test phase, which lasted 5 minutes.

Findings on the consequences of post-weaning social isolation on novel object recognition are conflicting and particularly scarce in mice (Fone & Porkess, 2008). According to our findings in SI-reared mice, some studies show impaired recognition memory after post-weaning SI (Bianchi et al., 2006; Koike et al., 2009; Voikar et al., 2005). Impairments in memory storage, retrieval and learning have already been shown in socially isolated animals in a number of other tasks (Bianchi et al., 2006). Conversely, other authors report enhanced cognitive performance induced by SI with increased preference for novel objects in the NORT (short-term recognition memory), which is suggested to reflect enhanced sensitivity to novelty (Lander et al., 2017).

Social isolation is considered an amplifying component of genetic and neurodevelopmental models of schizophrenia, or in other words, as a secondary or complementary component of a double-hit model

(Guerrin et al., 2021). Hence, some studies using double-hit rodent models of schizophrenia with Poly (I:C) exposure and SI in the peripubertal period have been conducted (Chang et al., 2020; Desbonnet et al., 2022; Lukasz et al., 2013). Chang and collaborators (2020) developed a double-hit model on C57BL/6J mice, based on the combination of Poly (I:C) administration at GD9 and SI, which was conducted from PND 28 to PND56, until the beginning of the experimental procedures. Of note, isolation was maintained during all the behavioral procedures. These authors reported that either of the single-hit manipulations caused deficits on novel object recognition with impaired DI scores, but the combination of the two hits did not further exacerbate the disabilities. In this study, short-term object recognition memory was assessed in the NORT, with a time delay of 10 minutes between the training and the test phases of the experiment (Chang et al., 2020). Desbonnet and colleagues (2022) recently developed a double-hit mouse model of schizophrenia in C57BL/6J mice. In this study, pregnant dams were challenged with 5 mg/kg i.p. Poly (I:C) on GD12.5, and the offspring was submitted to a social isolation rearing from PND21-25, to PND34-36 or PND55-80, to evaluate schizophrenia-related adolescent or adult behaviors, respectively. NORT short-term memory was evaluated in a 5-minute session, beginning 1 hour after the training session in an open field arena. NORT short-term memory was not affected in adolescent nor in adult offspring, as there were no significant effects of MIA or SI on NORT short-term memory evaluation. Moreover, in concordance with our results, the capacity to discriminate between the novel and familiar object was significantly different between adult males and females, with males having a greater performance than females (in the short-term memory evaluation, see **figure 4.6**). Interestingly, we observed the opposite when evaluating the long-term memory, where females showed a better discrimination than males (see **figure 4.8**) (Desbonnet et al., 2022). Finally, Lukasz et al. (2013) developed a double-hit model of schizophrenia in Wistar rats by combing postnatal Poly (I:C) exposure and social isolation from PND25, until the end of the behavioral procedures (PND80). NORT long-term recognition memory function (evaluated 24 hours after the training session) was disrupted in both Poly (I:C)-treated and isolated animals. However, once again combining the Poly (I:C) challenge with SI, did not exacerbate the behavioral deficits seen with isolation rearing alone. However, it is important to remark that these authors conducted a postnatal Poly (I:C) insult, which entails a big difference in terms of the construct of the model compared to our study (Lukasz et al., 2013).

Altogether, our results showed impaired long-term memory performance in the NORT in single-hit and double-hit groups, but combining the Poly (I:C) challenge with social isolation failed to exacerbate the cognitive deficit or to produce a synergistic effect between both hits (although NORT DI scores in male and female double-hit groups were the lowest). Overall, we demonstrate that MIA-exposed and SI-exposed animals have a cognitive deficit when performing the NORT, although combination of both MIA+SI is not able to further potentiate this deficit. However, combination of both prenatal and

postnatal insults is able to amplify schizophrenia-related cognitive deficits, making our double-hit model a valid tool to study this particular domain of the disease.

Notably, the majority of the discussed studies in this section are performed exclusively in males, with the exception of the studies conducted by Desbonet et al. (2022) and Gray et al. (2019), who evaluated the sex influence in the NORT cognitive performance. On the one hand, Desbonet and collaborators (2022) reported that the NORT short-term memory was significantly different between adult males and female mice, with males having a greater performance than females (Desbonet et al., 2022). On the other hand, Gray and colleagues (2019) reported that the MIA-related reduction in novel object preference appeared to be more prominent in males compared to female rats (Gray et al., 2019). In this regard, our work provides novel information on the sex influence on cognitive assessment in the NORT, as we observe that sex-influence has opposite directions on the short-term and long-term memory in males and females on this task. Whereas females appear to have a poorer cognitive performance (lower DI scores) than males in the short-term memory evaluation, they show greater cognitive performance than males when the NORT long-term memory is evaluated.

5.1.1.4. IMPAIRED SOCIAL BEHAVIOR IN THE SPT ASSOCIATED TO MIA AND SI

Impaired social behavior is one of the hallmark negative symptoms of schizophrenia (Foussias & Remington, 2010). As mice are highly social animals, social interaction can be efficiently studied under experimental conditions (Crawley, 2007), so we tested the sociability of our double-hit animal model by means of the SPT. We first demonstrated that male and female control mice showed a correct sociability as they had a higher exploration towards the social stimulus (a "stimulus mouse" of the same strain, sex and age) than towards the non-social stimulus (Social Index was 0.20 ± 0.06 in control males and 0.19 ± 0.05 in control females). Single-hit MIA and SI mice displayed a lower social exploration in the SPT compared to control mice. However, combination of both hits failed to have a synergistic effect on SPT social exploration time (see **figure 4.9 a**). As expected, the decrease showed in the SPT total exploration time is attributable to the decreased SPT social exploration times (see **figure 4.9 c**). Moreover, SPT social, non-social and total explorations were significantly affected by sex, with female mice showing lower exploration times than males. SPT Social Index was significantly decreased by the effect of both MIA and SI (see **figure 4.10**). Notably, female double-hit mice showed lower Social Index scores than the rest of the male and female experimental groups. Moreover, SI appeared to have a greater impact in females than in males impairing the Social Index. Overall, our results showed impaired sociability associated to both MIA and SI, with decreased social preference in the SPT. In this case, combination of both hits did not show a synergistic interaction, but it is important to remark that both MIA and SI contribute negatively to the social behavior of the model. Thus,

although combining the Poly (I:C) prenatal challenge with SI in the peripuberty failed to exacerbate the social deficit seen with either hits alone, we can affirm that our model is a useful tool to study schizophrenia-related social deficits. Moreover, we demonstrated that social behavior in the SPT is sex-dependent, as male and female mice displayed different social, non-social and total exploration patterns in this task.

Our findings are consistent with a body of evidence showing deficits in social behavior in Poly (I:C)-challenged rodents (Aavani et al., 2015; Amodeo et al., 2019; Bitanihirwe et al., 2010; Osborne et al., 2017; Osborne et al., 2019; Su et al., 2022). In this regard, Aavani and colleagues (2015) reported impaired social interaction in adolescence in a C57BL/6 mouse MIA model of schizophrenia. These authors administered daily intraperitoneal Poly (I:C) injections between GD13 and GD15 at a dose of 20 mg/kg (Aavani et al., 2015). In the same line, Amodeo et al. (2019) showed decreased social approach behavior on the offspring of pregnant C57BL/6J mice treated with 20 mg/kg i.p. Poly (I:C) on GD12 (Amodeo et al., 2019). Consistent with our results, Bitanihirwe et al. (2010) described impaired social interaction on C57BL/6J mice born after prenatal exposure to 5 mg/kg i.v. Poly (I:C) on GD17 (Bitanihirwe et al. 2010). In two different studies, Osborne et al. reported that Sprague-Dawley rats born to Poly (I:C) treated dams (4 mg/kg i.v. on GD15) had deficits on social interaction (Osborne et al., 2017; Osborne et al., 2019). Finally, Su an collaborators (2022) described a social interaction deficit in a Sprague-Dawley rat model of MIA of Poly (I:C) administration on GD15 at a dose of 5 mg/kg i.p. (Su et al., 2022). Once again, these studies differ on the animal species, strain, Poly (I:C) dose, timing, route of administration and duration of the insult. Despite all this heterogeneity on the construct of the MIA model, gestational Poly (I:C) challenge appears as a consistent tool for the study of the schizophrenia negative-like symptomatology.

In accordance with our results, social isolation rearing is also known to induce impaired social behavior (Ferdman et al., 2007; Koike et al., 2009). Notably, these studies differ on the species and isolation-rearing protocols used. On the one hand, Ferdman et al. (2007) performed an isolation protocol in rats that lasted from postnatal week three or four, until postnatal week seven or eight, respectively (Ferdman et al., 2007). On the other hand, Koike and colleagues (2009) isolated three-week old mice for a period of four weeks (Koike et al., 2009).

In the same line of our results, combination of Poly (I:C) challenge and post-weaning social isolation in double-hit models of schizophrenia does not have a synergistic effect on social behavior deficits (Desbonnet et al., 2022; Lukasz et al., 2013). In the study conducted by Desbonnet and collaborators (2022) with a double-hit C57BL/6J mouse model of schizophrenia, MIA did not result in any social deficits in males or females. In contrast, and only in females, SI resulted in impaired sociability as demonstrated by a lack of preference for the social interaction (Desbonet et al., 2022). In addition,

Lukasz and colleagues (2013) developed a double-hit model by combining postnatal Poly (I:C) exposure and social isolation from PND25 to 80. There were no significant differences following either isolation rearing or postnatal Poly (I:C) challenge in preference for the social interaction. It is important to remark that these authors conducted a postnatal Poly (I:C) insult, which entails a big difference compared to our model in terms of the construct basis (Lukasz et al., 2013).

Hence, we demonstrate that the combination of a prenatal immune challenge with Poly (I:C) and post weaning social isolation is able to induce long-term behavioral abnormalities relevant to the negative symptomatology of schizophrenia. Although it does not seem to exacerbate the effects of MIA on social behavior, exposure to post-weaning social isolation stress also has an impact on the negative-like symptomatology that is particularly more evident in female mice. In this case, SI may act as a "complementary" hit that is not able to potentiate the effects of MIA on the model's social behavior. Combination of both hits appears to have an impact on the same neuronal circuits implicated in social behavior. Thus, we suggest that our double-hit model comprises the schizophrenia negative-like symptomatology (as well as other disease domains). Ideally, our final goal is to develop a translational animal model of schizophrenia that is able to resemble various symptom domains of such a complex disorder as schizophrenia.

5.1.1.5. INCREASED LOCOMOTOR RESPONSE TO AMPHETAMINE ASSOCIATED TO SI IN FEMALE MICE

Amphetamine has classically been reported to induce schizophrenia-like positive psychotic symptoms in healthy subjects. Regarding preclinical studies, descendants born to Poly (I:C)-treated mothers have shown to display signs of hyperdopaminergia, manifested by an increased sensitivity to the locomotor-stimulating effects of amphetamine (Meyer et al., 2005; Meyer et al., 2008; Weber-Stadlbauer et al., 2021; Zuckerman et al., 2003). This finding is reminiscent of a hyperdopaminergic state, which in turn is a pathological hallmark of psychosis in patients with schizophrenia (Abi-Dargham et al., 2004; McCutcheon et al., 2019). In contrast with these reports, in our study, MIA mice did not reveal an enhanced sensibility after amphetamine administration, which is supported by other studies reporting unaltered susceptibility in the hyperlocomotion to amphetamine in MIA offspring (Mehan et al., 2017). The discrepancy with the studies that report enhanced sensitivity to amphetamine treatment could possibly be explained because the experimental conditions in the above-mentioned investigations differ from ours. We used a higher amphetamine dose of 5 mg/kg i.p. (Zager et al., 2012), whereas these studies used a lower dose of 2.5 mg/kg i.p. in mice (Meyer et al., 2008; Weber-Stadlbauer et al., 2021), and 1 mg/kg i.p. in rats (Zuckerman et al., 2003). We decided to administer a higher dose based on previous experiments performed in the same mouse strain, the CD-1®-Swiss mice (Zager et al., 2012). This discrepancy between studies on the amphetamine dose chosen might be attributable to

the different mouse strains used in the studies. Whereas the high amphetamine dose of 5 mg/kg was used in CD-1®-Swiss mice (Zager et al., 2012), the lower 2.5 mg/kg doses were used in C57BL/6 mice (Meyer et al., 2008; Weber-Stadlbauer et al., 2021). In low doses (~1 mg/kg for rats, and ~2 mg/kg for mice), systemic amphetamine administration readily induces an increase of locomotor activity in rodents (Robbins & Iversen, 1973; Robinson and Becker, 1986), and the effects are mediated via dopaminergic transmission targeted at the nucleus accumbens (Creese & Iversen, 1975; Pijnenburg et al., 1976; Essman et al., 1993). It has been classically suggested that low dose amphetamine administration elicits these effects via enhanced impulse-dependent dopaminergic transmission in the nucleus accumbens (Weiner et al., 1996; Heidbreder & Feldon, 1998; Murphy et al., 2000). Hyperlocomotion to low doses of amphetamine is interpreted as a sign for functional imbalance in mesolimbic dopaminergic transmission underlying the symptom genesis of schizophrenia (Laruelle, 2000). Thus, the dopaminergic imbalance hypothesis of schizophrenia claims that enhanced activity in the mesolimbic dopaminergic system together with hypoactive mesocortical dopaminergic projections to the PFC contribute to the pathophysiology of this disorder (Abi-Dargham & Moore, 2003; Winterer & Weinberger, 2004). In this regard, there is a correlation between the positive psychotic symptoms of schizophrenia and the increase of dopamine release in the striatum (Laruelle et al., 1999), while negative or cognitive dysfunctions of schizophrenia patients seems to be related to dopaminergic deficits in PFC (Davis et al., 1991; Slifstein et al., 2015).

In the present study, an increased basal locomotor activity was found only in MIA grouped mice (see **figure 4.11**). Further, socially isolated female mice showed an enhanced amphetamine-induced hyperlocomotion (see **figure 4.12 b**) compared to grouped females, independently of the MIA condition. According to our results, some other authors (Lampert et al., 2017; Pietropaolo et al., 2008) have reported sex differences in the effect of acute amphetamine administration in socially isolated rodents. In concordance with our results showing greater impact of amphetamine on SI females, Lampert and collaborators (2017) reported a potentiated sensibility to amphetamine (10 mg/kg i.p.) in socially isolated female Wistar rats (but not in males) (Lampert et al., 2017). However, the specie used (rat), the amphetamine dose chosen (10 mg/kg i.p.) and the isolation protocol conducted (from PND21 to PND65) differ from our study. Interestingly, Pietropaolo and colleagues (2008) reported increased locomotor reaction to amphetamine (2.5 mg/kg i.p.) only in socially isolated C57BL/6J male mice (Pietropaolo et al., 2008). In this case, both our study and the one conducted by Pietropaolo et al. are conducted in mice, however, the strain used is different (C57BL/6J), the amphetamine dose administered is lower (2.5 mg/kg compared to our 5 mg/kg) and the isolation protocol is shorter (4-week isolation period compared to our 8-week isolation period). Lander and collaborators (2017) reported enhanced locomotor response to amphetamine treatment (2 mg/kg i.p.) in C57BL/6 male mice subjected to an isolation period of 3 weeks during mid-adolescence (PND38-PND61) (Lander et

al., 2017). In this case, both the mouse strain and the amphetamine dose used are different from our study. All this variability in the studies' design could explain the different results between investigations.

As already discussed, the effects of amphetamine administration on locomotor activity are commonly associated with increased dopamine release in the mesolimbic pathway, and a study showed that a 6-week period of post-weaning SI enhances the effects of amphetamine on phasic dopamine release and DA-transporter activity in the rat nucleus accumbens and dorsomedial striatum (Yorgason et al., 2016). Thus, the increased sensitivity after systemic amphetamine administration that we observed in isolated females might be interpreted as consequence of an enhanced dopaminergic release in the mesolimbic pathway.

Hence, in our double-hit model, MIA does not appear to reflect this behavioral alteration related to this particular positive domain of schizophrenia, whereas SI does it, at least in females. Once again, combination of MIA+SI did not induce a synergistic effect on the hypersensitivity to amphetamine administration. As mentioned above, in our study, MIA-driven behavioral alterations appear to be related to the cognitive and negative domains of schizophrenia, whereas SI-elicited behavioral outcomes appear to be associated also to the positive-like symptomatology of the model. This fact supports, especially in females, our double-hit animal as a complete translational model that is able to mimic positive, negative, and cognitive schizophrenia domains.

5.1.1.6. INCREASED HEAD-TWITCH RESPONSE TO PSILOCYBIN IN DOUBLE-HIT MICE

Psychedelic drugs, including psilocybin (and its active metabolite psilocin), produce psychotic symptoms and cognitive deficits in humans without known mental pathology similar to those observed in schizophrenic patients, and are able to exacerbate psychotic symptomatology in subjects with schizophrenia (Nichols, 2016; Vollenweider et al., 1998). Even if animal models cannot capture the alterations of perception, cognition, and mood produced by psychedelics in humans, rodents exhibit behavioral proxies of human hallucinogenic effects (González-Maeso et al., 2007). As explained in **section 1.4.2.1.**, systemic administration of psychedelics (such as psilocybin, LSD or mescaline) in rodents elicits head twitch response (HTR). This side-to-side movement of the head stands a high degree of specificity, as it is not seen with other psychoactive drugs such as cocaine, phencyclidine, or amphetamine (De La Fuente Revenga et al., 2020). Psychedelic-induced HTR is reliably and robustly elicited 5-HT_{2A}R agonists and is absent in 5-HT_{2A} knockout mice, thus, demonstrating that is a cortical 5-HT_{2A}R-dependent behavior in mice (González-Maeso et al., 2007). Psilocybin and its active metabolite psilocin are substances with predominant agonist activity on serotonin $5HT_{2A/C}$ and $5HT_{1A}$ receptors, although they also bind to other serotonin and dopamine receptors (Passie et al., 2006; Tylš

et al., 2014). However, 5-HT_{2A}R agonism is considered necessary for psilocybin hallucinogenic effects (Nichols, 2004; Erkizia-Santamaría et al., 2022) and psilocybin has demonstrated to elicit a significant HTR *Htr2a*$^{+/+}$ mice, but not in *Htr2a*$^{-/-}$ mice (González-Maeso et al., 2007).

In the present study, HTR induced by the hallucinogenic 5-HT_{2A}R agonist psilocybin was significantly increased in MIA + SI female mice compared to controls (see **figure 4.13**). Consistent with these findings, abnormalities in expression and behavioral function of the 5-HT_{2A}R have been shown in offspring born to Poly (I:C)-challenged mothers (Holloway et al., 2013). In this sense, the hallucinogenic 5-HT_{2A}R agonist DOI has been reported to exert an increased HTR in Poly (I:C) MIA model (Holloway et al., 2013). Poly (I:C) protocol used in this research differs from our experimental conditions, as it was administered on GD9.5 in a intraperitoneal injection of 5 mg/kg, a lower dose than ours (7.5 mg/kg). In addition, Holloway and collaborators (2013) also report increased [^{3}H]ketanserin binding in frontal cortex of mice born to Poly (I:C)-challenged mothers, indicating altered density of the 5-HT_{2A}R (Holloway et al., 2013). However, the effects of hallucinogenic drugs targeting the 5-HT_{2A}R on double-hit models combining Poly (I:C)-driven MIA and post-weaning SI remain unexplored.

Studies examining the effects 5-HT_{2A}R hallucinogenic agonists in social isolation models are scarce. However, we must underline the research conducted by Sakaue and collaborators (2002), who reported an enhanced DOI-induced HTR in isolated mice compared to grouped mice (Sakaue et al., 2002). This study was performed with male ddY mice that were subjected to a social isolation period of at least 6 weeks. Conversely, previous authors reported a reduced HTR to 5-methoxy-N, N-dimethyltryptamine (5-MeO-DMT) administration in male Swiss NMRI mice isolated for a brief period of 7 days (Courdereau et al., 1995). 5-MeO-DMT is a naturally occurring tryptamine considered as classical psychedelic, which primarily acts as an agonist at the 5-HT_{1A} and 5-HT_{2A} receptors (reviewed in Reckweg et al., 2022).

To the best of our knowledge, our work is the first one to study the effects of the psychedelic psilocybin on a double-hit animal model of schizophrenia. The enhanced HTR to psilocybin is a behavior that resembles cortical 5-HT_{2A}R-mediated positive-like symptomatology of schizophrenia in our double-hit model. Additionally, it would be interesting to assess the $5HT_{2A}$ receptor density and functionality in the model. Of note, our study presents some limitations, as we only performed the HTR experiments in female double-hit mice. Thus, further experiments addressing the HTR in both sexes and in the single-hit experimental groups (MIA and SI, separately) would be also necessary.

Notably, while all the behavioral and molecular characterization experiments were performed with CD-1® mice, HTR experiments after psilocybin administration were conducted with C57BL/6 mice. In this sense, the mouse strain used is known to contribute in the hallucinogen-elicited HTR response (Canal & Morgan, 2012). In this regard, Canal & Morgan (2012) reviewed the HTR induced by the

psychedelic DOI across mouse strains, reporting that C57BL/6 mice had a significantly higher number of events (average 48-68 HTRs at a dose of 1 or 1.5 mg/kg DOI in 10 min) than CD-1® mice (28-29 in the same conditions). Thus, based on this previous literature data and in our pilot results, we decided to switch the strain used and perform the HTR experiments with C57BL/6 mice.

5.1.2. MOLECULAR CHARACTERIZATION OF THE DOUBLE-HIT MODEL

5.1.2.1. ALTERED PERIPHERAL PROINFLAMMATORY PROFILE ASSOCIATED TO MIA AND SI

Numerous investigations have reported alterations in inflammatory/immune signaling and its regulatory mechanisms in the pathophysiology of schizophrenia when evaluated in peripheral blood cells and in *postmortem* brain studies (Khandaker et al., 2015; Leza et al., 2015). An imbalance between pro- and anti-inflammatory signaling has been observed in the plasma of subjects with schizophrenia, with a down-regulation of endogenous antioxidant and anti-inflammatory mechanisms (Martínez-Gras et al., 2011). Besides, different meta-analyses indicate increased levels of pro-inflammatory cytokines in schizophrenia patients (including increased serum concentrations of proinflammatory IL-6) (Dickerson et al., 2013; Fernandes et al., 2016; Goldsmith et al. 2016; Khandaker et al., 2014; Miller et al., 2011; Perry et al., 2021; Potvin et al., 2008; Upthegrove et al. 2014).

Regarding preclinical studies, various investigations have examined peripheral inflammatory changes in rodent and non-human primate models of MIA. Studies in rodents show that Poly (I:C)-driven MIA is able to increase peripheral innate proinflammatory cytokine levels in offspring, including IL-1β, IL-6, TNF-α and IFN-γ (Alexopoulou et al., 2001; Garay et al., 2013; Gilmore et al., 2005; Mueller et al. 2021). In a recent systematic review and meta-analysis including 45 papers studying the effects of maternal Poly (I:C) administration on immune mediators in the offspring, gestational Poly (I:C) challenge proved to increase IL-6 both in brain and serum concentrations (Hameete et al., 2021). Moreover, in a study conducted in non-human primates long-term peripheral immune alterations were observed in the MIA offspring. In this investigation, pregnant rhesus macaques were treated with Poly (I:C) and the offspring were followed until 4 years of age, with blood collected at the end of their first and fourth years of life to assess dynamic cellular immune function by multiplex immunoassays. At one year of age, MIA offspring displayed elevated plasma production of innate inflammatory cytokines IL-1β, IL-6, IL-12p40, and TNF-α. At four years of age, the MIA exposed offspring continued to display elevated plasmatic IL-1β, and there was also a pattern of an increased production of T-cell helper type (TH)-2 cytokines, IL-4 and IL13 (Rose et al., 2017).

Of note, post-weaning social isolation rearing in rodents has also been reported to alter the cytokine milieu. In this regard, a recent report evaluated the peripheral blood concentration of proinflammatory

(IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines in male Wistar rats subjected to a prolonged social isolation period (since weaning and during 10 weeks) (Corsi-Zuelli et al., 2019). These authors reported a decreased peripheral blood concentration of the anti-inflammatory cytokine IL-10, suggesting that prolonged social isolation after the weaning may contribute to the proinflammatory phenotype associated with schizophrenia. In another study, proinflammatory (IL-6, TNF-α and IFN-γ) and anti-inflammatory (IL-4) cytokines were measured in plasma samples of Wistar rats subjected to an 8-week isolation period that started after the weaning (PND21) (Möller et al., 2013). These authors reported a significant increase in TNF-α and IFN-γ and a decrease in IL-6 and IL-4 plasma concentrations associated with SI, and they concluded that SI induced a proinflammatory state. Finally, Ko and Liu examined the effects of social isolation rearing in male and female Sprague-Dawley rats that were isolated for 4 weeks (from PND21-23 to PND49-51) (Ko & Liu, 2015). Socially isolated male and female rats showed increased concentrations of plasmatic IL-6, IL-1β, TNF-α and IFN-γ. Altogether, these studies suggest that post-weaning social isolation in rodents could induce a peripheral proinflammatory state by increasing the blood concentration of proinflammatory cytokines and decreasing anti-inflammatory cytokines.

In agreement with all these previous investigations, our study revealed an increase of the plasmatic concentration of the proinflammatory cytokine IL-6 in response to prenatal Poly (I:C) treatment. In addition, SI exerted a sex-dependent increase in IL-6 plasma concentration, only in female mice (see **figure 4.14 a**). Changes in cytokine concentrations are a driving factor behind the effects of MIA on neurodevelopment (Estes & McAllister, 2016), and for example, IL-6 administration on pregnant dams provokes symptoms similar to those of MIA caused by Poly (I:C) (Smith et al., 2007). Thus, IL-6 is believed to play a central role in the effects of MIA. As already stated, social isolation rearing in rodents is also known to increase plasmatic IL-6 concentrations (Ko & Liu, 2015). Moreover, in our study, SI exerted a significant decrease on TNF-α (see **figure 4.14 c**) and IL-17A (see **figure 4.14 d**) plasma concentration, suggesting that SI-driven alterations could not be related with the model's peripheral proinflammatory status, but might contribute to an impaired homeostasis of cytokine milieu. Combination of MIA and SI failed to provoke a synergistic effect. In addition, sex-related differences were found in the plasmatic concentration of various cytokines (IFN-γ, IL-17A, IL-1β, IL-2, and IL-10), with overall higher levels in females compared to males. "Sex x SI" interactions were found in IL-6, IFN-γ and IL-2 levels, with females presenting increased plasmatic concentrations of these proinflammatory cytokines. This could suggest that SI may have a greater impact in females, as we saw in amphetamine experiments (go to **section 4.1.5.**). In this regard, studies evaluating the sex influence on the proinflammatory cytokine profile of Poly (I:C) and social isolation models are scarce. For example, in the previously mentioned systematic review and meta-analysis of poly(I:C)-induced maternal immune activation model including 45 studies, the sex of the offspring studied was either

mixed (28 studies), or male (11 studies), or even no reported (7 studies) (Hameete et al., 2021). Regarding the mentioned studies performed with models of SI, only the one performed by Ko & Liu (2015) evaluated the sex-influence on social isolation induced alterations in the cytokine milieu (Ko & Liu, 2015). Thus, further experiments assessing the peripheral immune profile on Poly (I:C)-challenged and socially isolated animals would be needed in order to elucidate the influence of the sex.

Overall, we can conclude that our MIA and SI mice show an imbalance in the proinflammatory cytokine profile. However, the alterations induced by either hit alone are not potentiated by combination both, as we do not report synergistic effects of MIA and SI on the proinflammatory cytokine levels. Finally, these alterations are sex-specific, with overall females having higher plasmatic concentrations of these proinflammatory markers.

5.1.2.2. ALTERED CORTICAL NF-κB AND IκBα GENE EXPRESSION ASSOCIATED TO MIA, AND UNALTERED NF-κB AND IκBα PROTEIN EXPRESSION

A major proinflammatory pathway is the one triggered by the activation of the transcription factor NF-κB. Its principal repressor, IκBα, maintains NF-κB dimers in the cytoplasm of unstimulated cells, blocking its translocation to the nucleus (Hayden & Ghosh, 2008; Murphy, 2021; Sun, 2012; Sun, 2017). Thus, degradation of IκBα leads to increased NF-κB translocation to the nucleus (Hayden & Ghosh, 2008; Murphy, 2021; Sun, 2012; Sun, 2017). As discussed before, immune-related abnormalities are commonly reported in schizophrenia patients. Precisely, dysregulation of the NF-κB pathway has been associated with this disorder, with several studies reporting overactivity of the NF-κB pathway in the PFC of subjects with schizophrenia (Gandal et al., 2018; Murphy et al., 2020; Volk et al., 2015; Volk et al., 2018). Besides, IκBα in cytosolic extracts is decreased in schizophrenia patients compared with those in healthy subjects, suggesting the presence of a chronic proinflammatory status (García-Bueno et al., 2014).

Among all the members of the NF-κB family, we decided to evaluate the gene and protein expression of *Rela*, the gene encoding for Rela/p65 protein, and *Nfkbia*, the gene encoding for IκBα protein, based on their importance inside the family. Hence, *Rela* is the gene encoding for the domain-containing proteins RelA/p65 of the NF-κB complex, whereas *Nfkbia* gene provides instructions for making one piece (the alpha subunit) of the IKK protein complex, a group of related proteins that regulates the activity of NF-κB (see **section 1.2.2**.).

When we evaluated the relative mRNA levels of *Rela* and *Nfkbia* in brain cortex of the double-hit model, we observed that *Rela* mRNA levels were significantly increased by effect of MIA, while *Nfkbia* relative mRNA expression was significantly reduced by effect of MIA. Of note, MIA-modulation of *Rela*

and *Nfkbia* gene expression was sex-specific, as the *Rela* increase was much more evident in male MIA mice, whereas *Nfkbia* was only decreased in MIA female mice. Thus, our model presented a sex-dependent proinflammatory alteration related to the expression of these genes associated to MIA (see **figure 4.15**). Hence, these findings in our mouse model go in concordance with the findings on human schizophrenia subjects. However, MIA driven changes in the gene expression of NF-κB and IκBα in mice brain cortex did not appear to be related to differences in nuclear and cytosolic protein levels, respectively, as western blot experiments revealed no differences between experimental groups. However, we did see that females had higher protein levels of both NF-κB and IκBα compared to males, regardless of the MIA or SI condition (see **figure 4.18**).

Previous reports have studied the effects of prenatal and postnatal Poly (I:C) administration on the gene and protein expression of different members of the NF-κB family, and its relation with schizophrenia-related symptomatology (MacDowell et al., 2017; Song et al., 2011; Volk et al., 2015). These reports, however, show very diverse findings. MacDowell and collaborators (2017) described an increased translocation of NF-κB to the nucleus due to the degradation of cytoplasmic IκBα in frontal cortex of mice exposed to a prenatal Poly (I:C) treatment (5 mg/kg i.p. on GD9.5) (MacDowell et al., 2017). Precisely, they reported increased protein expression of Rela/p65 in nuclear extracts, and decreased IκBα protein levels in cytosolic extracts of brain cortices of the offspring of Poly (I:C)-challenged dams. These authors propose that Poly (I:C)-driven activation of TLR3 pathway induces degradation of the inhibitory subunit IκBα by IKK, facilitating NF-κB translocation to nucleus where it activates transcription of inflammatory cytokines and enzymes that lead to reactive oxygen and nitrogen species accumulation. In the same line of these findings, inhibition of NF-κB is reported to suppress the immune response induced by Poly (I:C), as the administration of the NF-κB inhibitor pyrrolidine dithiocarbamate, has demonstrated to prevent Poly (I:C)-related alterations in both pregnant rats and their offspring (Song et al., 2011). Finally, Volk and colleagues (2015) demonstrated that adult mice receiving 20 mg/kg i.p. Poly (I:C) injections during three consecutive days (but not the offspring with prenatal Poly (I:C) exposure), had increased frontal cortex mRNA levels of NF-κB1 and NF-κB2 members of the NF-κB family (Volk et al., 2015).

To the best of our knowledge, our study is the first one to evaluate changes in the NF-κB proinflammatory pathway in an environmental double-hit model of schizophrenia based on prenatal Poly (I:C) administration followed by SI. Indeed, we did not find previous studies that investigated the effects of post-weaning social isolation on NF-κB signaling. Our findings demonstrate that Poly (I:C) is able to cause long-lasting changes in NF-κB and IκBα mRNA levels in the brain cortex associated to MIA, that might be related with a neuroinflammatory status. Yet, these alterations were not modulated by the effect of SI. Thus, inflammation-associated alterations of our model appear to be provoked by prenatal Poly (I:C) exposure.

The NF-κB transcriptional complex is composed of NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel (Volk et al., 2018). We only evaluated the protein expression of the NF-κB family member RelA/p65 in mouse brain cortex, which is one of the canonical members of the of NF-κB family (Sun, 2017). We chose to measure RelA/p65 based on previous findings claiming that this particular NF-κB family member was altered in the postmortem brain samples of schizophrenia subjects (Gandal et al., 2018; Murphy et al., 2020; Volk et al., 2019), as well as in the Poly (I:C) model (MacDowell et al., 2017). However, taking into account the complexity of the NF-κB family, it could be possible that even if RelA protein expression is not altered, other members of the NF-kB family were disrupted in the double-hit model. Furthermore, activation of NF-κB mainly occurs via phosphorylation of IkB kinase (IKK) inhibitory molecules, including IκBα (Viatour et al., 2005). Phosphorylation of the NF-κB subunits has a profound effect on the function of NF-κB. Site-specific phosphorylation of NF-κB subunits controls interactions with other factors, and the stability, degradation and transcription activity of NF-κB dimers (Christian et al., 2016). Thus, it would also be interesting to study the protein expression of the phosphorylated forms of NF-κB subunits in our double-hit model. Hence, although we did see changes in the mRNA levels of *Rela* and *Nfkbia*, these are not correlated to alterations in their encoding protein levels. Notably, it is important to remark that the relationship between mRNA and protein is complex and mRNA-protein levels are often poorly correlated (Greenbaum et al., 2003; Koussounadis et al., 2015). Moreover, whereas gene expression experiments were performed processing all the brain cortex tissue, in the western blot experiments Rela/p65 and IκBα protein levels were only measured in prepared nuclear or cytosolic fractions of brain cortex respectively. IκBα is only expressed in the cytosolic fraction, hence, we can confirm that the levels of IκBα measured in our western blot experiments correspond to the total expression of this protein. In contrast, as Rela/p65 is known to be expressed both in the cell cytoplasm and in nucleus, it could be possible that the cytosolic expression was altered. Further experiments evaluating Rela/p65 protein levels in cytosolic fraction or in total tissue homogenates (including both nuclear and cytosolic fractions) would be needed to better explore changes in the expression of Rela/p65 associated with MIA and SI. Besides, experiments exploring other NF-kB family members and/or phosphorylated forms of NF-kB family subunits could be performed in order to elucidate the role of the these neuroinflammatory signaling proteins in our mouse model of schizophrenia. For example, we suggest that measuring the phosphorylated form of IκBα in the cytoplasmic fraction of these mouse brain cortex samples would be an interesting approach, as translocation of NF-κB from the cytoplasm to the nucleus depends entirely on the phosphorylation of its main inhibitor IκBα in the cytoplasm. Other possible experiments that could be performed in the future would be ChIP-seq or ChIP-PCR assays with Rela, in order to evaluate if MIA, SI or combination of both affect to which gene promoter regions and to wich extent is Rela binding acting as a transcription factor.

5.1.2.3. ABERRANT HDAC GENE EXPRESSION IN THE BRAIN CORTEX ASSOCIATED TO MIA AND SI

The absence of knowledge about the precise mechanisms that lead to schizophrenia onset, together with the fact that its etiology cannot be explained only by genetic heritage, raised the idea that this disorder may appear as a consequence of combination of both prenatal (genetic and/or environmental) and postnatal environmental factors. In this regard, several studies have addressed the potential epigenetic alterations in schizophrenia. Among them, alterations related to chromatin condensation have been the most thoroughly studied in schizophrenia. Recently, researchers have focused on the role of the enzymes responsible for these processes. Specifically, impaired expression of HDACs has been observed in subjects with schizophrenia (Benes et al., 2007; Gilbert et al., 2019; Schroeder et al., 2017; Sharma et al., 2008), a fact that has been related to an altered chromatin condensation that would eventually lead to an unbalanced expression of many genes.

In this sense, *in vivo* neuroimaging studies report histone deacetylase dysregulation in schizophrenia patients suggesting that altered HDAC expression –in either direction– may impact cognitive deficits in humans with schizophrenia (Gilbert et al., 2019). In PET neuroimaging studies, schizophrenia patients also show lower relative HDAC expression in several brain areas including the dorsolateral prefrontal cortex (DLPFC), dorsomedial prefrontal cortex and orbitofrontal gyrus, and higher relative HDAC expression in the cerebral white matter, pons, and cerebellum compared with healthy controls (Gilbert et al., 2019). In this study, the relative [^{11}C]Martinostat (the radiotracer version of the HDAC inhibitor Martinostat) brain uptake was compared between subjects with schizophrenia and schizoaffective disorder and matched healthy controls, using magnetic resonance-PET (MR-PET) (Gilbert et al., 2019). In another study with *postmortem* DLPFC samples of subjects diagnosed with schizophrenia, decreased *HDAC2* mRNA levels were reported, while *HDAC1* mRNA levels remained unaltered (Schroeder et al., 2017). Conversely, previous studies reported higher *HDAC1* mRNA levels in the PFC and hippocampus of subjects with schizophrenia, compared to controls, and no changes on *HDAC2* and *HDAC4* gene expression (Benes et al., 2007; Sharma et al., 2008). Sharma et al. suggested that the higher *HDAC1* mRNA levels could be linked to schizophrenia (Sharma et al., 2008). However, whereas in the two previously cited studies (Gilbert et al., 2019; Schroeder et al. 2017) the effect of the pharmacological treatment of the patients was addressed, Sharma et al. did not evaluate the effect of the medication (antipsychotics, mood stabilizers, antidepressants, stimulants, sedatives) of the subjects evaluated (diagnosed with schizophrenia and bipolar disorder) (Sharma et al., 2008). Similarly, Benes et al. included patients with both schizophrenia and schizoaffective disorder diagnosis in their study, and did not evaluate the effect of antipsychotic drugs or mood stabilizers (Benes et al., 2007). Thus, changes in the *HDAC1* mRNA levels may be associated to the effect of pharmacological medication in these patients. Besides, clinical studies suggest that drugs such as valproate (drug acting

as a nonspecific HDAC inhibitor that is approved for the treatment of epilepsy and bipolar disorder), are efficacious when given chronically in combination with atypical antipsychotic drugs, including clozapine, olanzapine and risperidone (Suzuki et al., 2009). However, the mechanism of action of valproate related to this improvements in patients with schizophrenia remains unknown. Besides the nonspecific inhibition of HDACs, it is thought to act on different targets modulating GABAergic neurotransmission and to block voltage-gated ion channels. Moreover, HDACs are shown to regulate gene transcription of cytokines (Lu et al., 2005; Villagra et al., 2009), thus, altered epigenetic regulation of histones could be related to the aberrant inflammatory signaling reported in schizophrenia patients. Overall, studies in patients with schizophrenia (both *in vivo* and with *postmortem* brain tissue), report dysregulated HDAC expression (in both directions), that is suggested to be linked with aberrant inflammatory signaling.

Moreover, various studies have revealed the importance of other histone PTMs in the pathophysiology of schizophrenia. First, increased expression of another type of enzymes, the histone methyltransferases (HMTs), has been linked with schizophrenia (Chase et al., 2013; Johnstone et al., 2018). Studies of chromatin immunoprecipitation followed by sequencing (ChIP-Seq) in *postmortem* human brain samples, have shown an over representation of open chromatin-associated modifications in schizophrenia-related genes, specifically in neuronal cells. These modifications include histone H3 lysine 4 trimethylation (H3K4me3) and histone H3 lysine 27 acetylation (H3K27ac) (Girdhar et al., 2018). In addition, studies in *postmortem* brain samples of subjects with schizophrenia reveal altered H3 acetylation and methylation at promoter regions of various genes (Huang et al., 2007; Kurita et al., 2012; Tang et al., 2011). Recently, ChIP-Seq evaluation of cell-type H3K4me3 in PFC samples of subjects with schizophrenia showed individual alterations in neurons (Gusev et al., 2019). Altogether, all these evidence suggests that epigenetic alterations of histones might play an important role on the pathophysiology of schizophrenia. Recently, the González-Maeso Lab conducted an epigenomic characterization in postmortem prefrontal cortex samples of subjects with schizophrenia, discovering cell-type specific epigenetic differences at regions with susceptibility genetic loci (such as *NRG1*, *RGS4* or *HTR2A*), revealing the relevance of epigenetic alterations in the prefrontal cortex of schizophrenia subjects (Zhu et al., 2021).

Regarding schizophrenia preclinical models, studies that research on the effects of MIA on epigenetic regulation of histones are scarce. In this sense, global hypoacetylation of histone H3 (at lysine residues 9 and 14) and histone H4 (at lysine residue 8) in the brain cortex from juvenile offspring prenatally exposed to Poly (I:C) has been described, demonstrating that significant epigenetic changes occur in response to Poly (I:C) exposure *in utero* (Tang et al., 2013). Another study reports an increased global HDAC activity in female offspring exposed to prenatal maternal Poly (I:C), suggesting sex-related differences on HDAC expression (Pujol López et al., 2016). Moreover, another study evaluated the

protein expression of different HDACs, showing reduced HDAC1 expression by MIA in female mice, increased HDAC6 expression by MIA regardless of the sex, and no changes in HDAC2, HDAC3 and HDAC4 (Pujol López et al., 2016). In contrast with the latter finding, we reported decreased HDAC2 expression associated to MIA and decreased HDAC4 and HDAC8 associated to SI. However, these authors measured the protein expression of different HDACs whereas we evaluated the mRNA levels, but as it is well reported, mRNA and protein levels could be not correlated. Further, discrepancies on the results could be also explained because MIA protocol used by Pujol López and colleagues (2016) differs from ours, as these authors administered 5 mg/kg Poly (I:C) i.v. on GD17. To the best of our knowledge, the mRNA levels of different HDACs in brain cortex samples of a double-hit mouse model of schizophrenia combining prenatal Poly (I:C) exposure and post-weaning SI has not been addressed. Given the scarce number of studies in the literature, further investigations would be necessary to understand the role of epigenetic mechanisms of histones in rodent neurodevelopmental models of schizophrenia. In addition, a body of evidence suggests that HDAC inhibition may be beneficial in disorders coursing with cognitive impairment, by restoring neuronal function and promoting synaptic plasticity. In this sense, HDAC inhibitors are reported to confer cognitive enhancement in mice (Kurita et al., 2012; Schroeder et al., 2013). However, to date, the effects of HDAC inhibitors have not been assessed in neurodevelopmental models of schizophrenia such as the Poly (I:C) MIA model.

Our mouse model shows altered HDAC gene expression in the brain cortex, with decreased *Hdac2* mRNA levels associated to MIA (see **figure 4.19 b**), and decreased *Hdac4* (see **figure 4.19 d**) and *Hdac8* (see **figure 4.19 h**) mRNA levels associated to SI. Besides, reduced HDAC expression pattern associated to the respective hits was the same in both male and female mice, thus we did not observe any "sex x hit" interactions. Our findings agree with the decreased *HDAC2* mRNA levels found in postmortem DLPFC samples of subjects with schizophrenia (Schroeder et al., 2017), suggesting that the present animal model has a high degree of translational value. Conversely, this MIA-induced downregulation of HDAC2 gene expression, disagrees with previous reports showing increased HDAC2 expression associated to Poly (I:C) (Pujol López et al., 2016). To our knowledge, no other authors have studied the effects of SI on alterations in HDAC expression. However, some studies report altered epigenetic modifications in social isolation models, such as global histone acetylation, histone methylations, DNA methylations or microRNA expression (Chang et al., 2020; Chen et al., 2020; Li et al., 2016; Loureiro et al., 2022). Of note, only *Hdac5* (see **figure 4.19 e**) relative mRNA levels are sex-dependent, as male mice show higher *Hdac5* mRNA levels than females, regardless of experimental group they belong to. Moreover, we hypothesize that this reduced gene expression of various HDACs associated to either MIA or SI could be responsible —maybe through a mechanism that alters inflammatory signaling— of the poor cognitive performance seen when evaluating the cognitive status in the NORT. Thus, as aberrant HDAC expression is suggested to be related to alterations in the inflammatory signaling of

schizophrenia patients (Lu et al., 2005; Villagra et al., 2009), it would be possible that this MIA/SI-driven alterations on HDACs expression could be related to MIA/SI-driven inflammatory alterations in our model.

To sum up, in this first part of this Doctoral Thesis, we aimed to characterize a translational double-hit mouse model of schizophrenia. We demonstrated that our model presented positive, negative and cognitive deficits associated with schizophrenia. Regarding the behavioural assessment, positive-like behavioral alterations in the model appeared as a hypersensitivity to amphetamine administration associated with SI and increased head-twitch response to psilocybin administration associated with both MIA and SI. Interestingly, the enhanced sensitivity to amphetamine was a sex-specific behavior, as only appeared in female mice. In contrast, we only tested the HTR to psilocybin in female mice, so it would be interesting to test the effects of this psychedelic drug also in male mice. Negative-like deficits in the model appeared as impaired social behavior in the SPT, which were provoked both by effect of MIA and by SI. Further, our model presented a long-term memory impairment in the NORT cognitive task, also associated both to MIA and SI. We did not find synergistic effect between both hits in none of these schizophrenia-related alterations. However, even if combination of MIA and SI does not potentiate these alterations, detrimental effects provoked by either hit are not reverted or counterbalance by the other hit and for some alterations are even additive. Altogether, the behavioral evaluation suggest that our double-hit model appears to have schizophrenia-related cortical alterations that impair cognition and sociability and exacerbate cortical 5-HT_{2A} receptor-dependent head-twitch response (at least in females). Moreover, alterations related to the dopaminergic mesolimbic pathway might be also present in the model in females, as showed with the enhanced locomotor response to amphetamine.

Moreover, we explored the immune and epigenetic-related alterations that might underly the schizophrenia-related behaviors in the model. The double-hit model presented an imbalance in the peripheral cytokine milieu, with elevated proinflammatory cytokines by effect of MIA (IL-6 and IFN-γ), and also reduced proinflammatory cytokines by effect of SI (IL-17A and TNF-α). Furthermore, the gene expression in the frontal cortex of the proinflammatory signaling proteins NF-κB and IκBα was altered by effect of MIA towards a proinflammatory status. In this case, alterations were sex-specific, as MIA elicited an increase of NF-κB gene expression mainly in male mice, whereas the IκBα decrease was only present in female mice. We hypothesize that these cortical inflammatory alterations could be responsible of the cognitive symptoms seen in the model. Finally, our model presented an aberrant HDAC expression in the frontal cortex, with decreased HDAC2 gene expression by effect of MIA, and decreased HDAC4 and HDAC8 expression by effect of SI, regardless of the sex. A gene expression imbalance of these cortical epigenetic enzymes could also be responsible to the cognitive deficits of the model.

Overall, our double-hit model appears as a translational model of schizophrenia, presenting a high construct and face validity, and showing positive, negative and cognitive domains of the disorder, as well as immune and epigenetic alterations, in both sexes. Thus, the final step in order to completely validate the model was to assess the predictive validity of the double-hit model by testing the effects of approved antipsychotic medications on the behavioral and molecular alterations of the model, as discussed in the next section of the present work.

5.2. EVALUATION OF THE EFFECT OF CHRONIC TREATMENT WITH CLOZAPINE, MINOCYCLINE AND THE COMBINATION OF BOTH DRUGS ON BEHAVIOR AND GENE EXPRESSION IN THE DOUBLE-HIT ANIMAL MODEL OF SCHIZOPHRENIA

As a result of the large volume of animals needed to perform this second part of the study involving the pharmacological treatments with clozapine and/or minocycline, we were not able to include the four experimental groups (control, MIA, SI, MIA + SI) used in the behavioral and molecular validation of the model. Thus, only animals belonging to control and double-hit groups were included in the study. Furthermore, given that the characterization experiments support that the double-hit model always presents at least the same alterations as each hit individually (and sometimes additive alterations of both hits), the exclusive use of control and double-hit groups in this second part of the study is well justified. Of note, and because the large amount of animals needed for the study, each experimental group included both male and female mice. Moreover, while double-hit model characterization experiments were performed with Hsd:ICR (CD-1®) mouse strain, behavioral and molecular assessments after the pharmacological treatments were performed with C57BL/6 mouse strain. The fact that a vast proportion of scientific studies performed with mice use the C57BL/6 mouse strain encouraged us to choose this mouse strain for the second part of the study. Altogether, we are aware of the limitations of our study, and we hypothesize that all these differences (experimental groups, sex variable, mouse strain) could influence in the lack of reproducibility of several outcomes between the characterization experiments and the experiments after the pharmacological treatments.

5.2.1. MINOCYCLINE TREATMENT REVERTS THE NORT COGNITIVE IMPAIRMENT IN THE DOUBLE-HIT MODEL

When we performed the NORT after the three-week chronic treatment with clozapine, minocycline and combination of both drugs, we realized that a high proportion of the mice belonging to all the experimental groups (about a 60% of the total number of animals) had very low total exploration times. Thus, we applied a criterion in order to exclude the animals that were not performing the test correctly. This criterion is proposed by Oliveira da Cruz and collaborators (2020), who state that if the NORT total exploration time is very low (< 5-10 s) in the training and/or test sessions, the results of these mice must not be considered (Oliveira Da Cruz et al., 2020). These authors suggest that this phenomenon could be caused due to two possibilities. First, the objects in the maze could not be adequate for the task (i.e., they may

induce stress or anxiety to the animals). Alternatively, they propose that the object could be improperly cleaned and it could contain odors of previous animals. They suggest that the objects should be re-evaluated in order to understand whether there is an intrinsic preference or aversion. In our study, we already used the same objects when characterizing the cognitive performance of the double-hit model. In this first batch of characterization experiments, mice belonging to all the experimental groups displayed high exploration times and performed the test correctly. Thus, we can affirm that the objects used in our NORT experiments were adequate for the task. Moreover, we properly cleaned all the objects with 70% ethanol between trials, and furthermore, we first tested male mice in order to avoid distractions caused by females' odors. The reason why this second batch of animals performed the NORT incorrectly (but not the SPT) remains unclear. Possibly, it could be attributable to the change on the mouse strain, as the characterization experiments were performed with CD-1® mice, and the behavioral experiments after the chronic pharmacological treatments were performed with C57BL/6 mice. There is a vast evidence in the literature supporting the C57BL/6 mice as suitable strain for performing behavioral paradigms such as the NORT, so the reason why these animals failed to perform the NORT adequately is still a non-answered question. Another possible explanation could be what Leger et al. (2013) propose, that the failed NORT performance can be attributable to high levels of stress in the tested animal (Leger et al., 2013). Although all experimental conditions for the NORT assessment in C57BL/6 mice were the same as in the first set of characterization experiments with CD-1® mice, it might be possible that the level of stress of this second batch of animals during the NORT assessment was higher, so that they did not performed the test properly. Even if we tried to control all the possible stressors (silence in the experimental room, adequate light intensity, habituation of the animals to the experimental room), we are aware that there could be other stressors that we may have not detected. Moreover, the proportion of animals that did not fulfill the proposed criteria was constant in all the experimental groups regardless of the pharmacological treatment, thus, we can affirm that the poor NORT performance was not caused because of the induction of the double-hit or by any drug used in the study. In the present NORT experiments, all the animals included in the analysis, regardless of the experimental group they belonged to, fulfilled the following criteria: they had higher total exploration times than 5 seconds and higher DI scores than -0.1.

Once the animals were selected based on the proposed criteria, we observed lower DI scores in vehicle-treated double-hit mice compared to vehicle-treated controls (see **figure 4.26**), confirming that double-hit mice had a cognitive deficit when performing the NORT in absence of a pharmacological treatment. Moreover, chronic minocycline administration was able to

revert the long-term memory impairment that double-hit mice showed in the NORT (see **figure 4.27 b**). We hypothesized that this effect was reached due to the anti-inflammatory properties of minocycline (see below for detailed discussion). Of note, clozapine also increased the NORT DI, although this increase did not reach the statistical significance. Further, co-administration of both clozapine and minocycline did not increase the DI scores to a higher extent than minocycline alone. Interestingly, pharmacological treatments assessed in this study did not affect the NORT cognitive performance of the control group. This proves that reversal of the cognitive performance due to the pharmacological treatment can only occur when a previous deficit exists, hence, in the double-hit model.

Previous investigations support that clozapine is able to alleviate the cognitive impairment (in the NORT and other behavioral tasks) induced by prenatal Poly (I:C) exposure in rodents (Meyer et al., 2010; Ozawa et al., 2006). In this regard, Ozawa and colleagues (2006) reported that sub-chronic administration of clozapine (5 mg/kg i.p., daily during 2 weeks) was able to improve the NORT cognitive deficit after *in utero* Poly (I:C) administration (5 mg/kg i.p. from GD12 to GD17) in Balb/c mice (Ozawa et al., 2006). Moreover, chronic administration of clozapine to C57BL/6J mice (5 mg/kg/day i.p. during 3 weeks), significantly improved the prenatal Poly (I:C) (5 mg/kg i.v on GD17) induced working memory deficits in the Morris water maze (Meyer et al., 2010). Consistent with these reports, our double-hit model showed enhanced cognitive performance in the NORT after clozapine treatment, although this increase on the recognition memory failed to be statistically significant. We administered clozapine in a dose of 10 mg/kg/day, intraperitoneally, during 3 weeks, to Poly (I:C)-challenged mice (in a dose of 7.5 mg/kg i.p. on GD9.5). Of note, we must take into account that these authors used different clozapine dosages and administration routes, mice strains or Poly (I:C) protocols (different doses, administration routes and timing). Further, other authors have assessed the effects of other antipsychotic drugs on the cognitive performance of rodent models born to Poly (I:C) treated dams (Lian et al., 2022; MacDowell et al., 2017). In this regard, Lian and collaborators (2022) recently showed an enhanced cognitive performance in the NORT of the offspring of Poly (I:C)-challenged dams after the chronic treatments with three different atypical antipsychotics: aripiprazole, olanzapine and risperidone (Lian et al., 2022). In this study, pregnant Sprague-Dawley rats were challenged with 5 mg/kg i.p. Poly (I:C) on GD15, and the offspring were treated for 4 weeks (from PND22 to PND50) with the mentioned atypical antipsychotic drugs. MacDowell et al. (2017) examined the effects of chronic paliperidone administration on working memory deficits measured by the alternation task T-maze in a MIA model (MacDowell et al., 2017). In this investigation, authors administered 5 mg/kg i.p. Poly (I:C) to C57BL/6J pregnant mice, and the adult offspring were

treated with the atypical antipsychotic paliperidone for 21 consecutive days, which reverted the MIA-induced cognitive deficits.

Moreover, antipsychotic medication has also proven to revert schizophrenia-related cognitive deficits in animal models of social isolation (Li et al., 2019; Möller at al., 2013). In this sense, Li and collaborators (2019) reported that cognitive deficits in a model of SI were reverted by clozapine treatment (Li et al., 2019). In this study, Sprague-Dawley rats were subjected to a SI period of 5 weeks (from PND21 to PND56) and treated with 1 mg/kg i.p. clozapine for 10 days. Moreover, in a Sprague-Dawley rat model of post-weaning social isolation (from PND21 to PND77, 8 weeks), sub-chronic clozapine administration (5 mg/kg/day, during 14 days) proved to revert the SI-induced cognitive deficits in the NORT (Möller et al., 2013).

Interestingly, it has been described that clozapine treatment enhanced cognitive deficits in a double-hit model of neonatal PCP administration followed by post-weaning social isolation (Hamieh et al., 2021). Hamieh and collaborators (2021) induced a SI period to PCP-treated Wistar rats, which started after the weaning and ended at the end of the behavioral experiments, in postnatal week (PNW) 19. They administered clozapine (3 mg/kg i.p.) starting from a week before behavioral tests (PNW10) until the end of the experiments (PNW19) and reported an attenuation of cognitive flexibility deficits in the model in the operant reversal learning task (Hamieh et al., 2021).

As antipsychotics are known to have a very limited efficacy for the treatment of cognitive deficits in schizophrenia patients (reviewed in Hagan & Jones, 2005; Lett et al., 2014; Spark et al., 2022), efforts have been made to find a more effective pharmacological treatment for this “orphan symptoms” of the disorder. Thus, if clozapine is not able to revert cognitive disfunction in patients with schizophrenia, it could be possible that the schizophrenia animal models exposed above that undergo an improvement on schizophrenia-related cognitive impairments, are not translational models of the disorder. In this sense, anti-inflammatories, and more precisely, minocycline, appear as a promising pharmacological alternative. Several clinical trials have been developed in order to study the beneficial effects of minocycline as an add-on medication to antipsychotics (Chaudry et al., 2012; Jhamnani et al., 2013; Khodaie-Ardakani et al., 2014; Kelly et al., 2011; Kelly et al., 2015; Levkovitz et al., 2010; Liu et al., 2014; Qurashi et al., 2014; Zhang et al., 2019a; Wehring et al., 2018; Zhang et al., 2018). Some of these studies report enhancements in cognitive performance of schizophrenia patients in treatment with co-adjuvant minocycline to their antipsychotic medication. Given that current antipsychotics are not able to address cognitive deficits and that inflammation is known to have an important

etiological and pathophysiological role in schizophrenia, combination of antipsychotics with anti-inflammatory drugs could represent a promising pharmacological approach in the treatment of schizophrenia cognitive dysfunction. In this regard, Levkovitz and collaborators (2010) reported that the co-administration of antipsychotic medication (clozapine, risperidone, olanzapine or chlorpromazine) and minocycline reduced cognitive dysfunction (mainly executive functions: working memory, cognitive shifting, cognitive planning) in patients with early-phase schizophrenia diagnosis (Levkovitz et al., 2010). In another double-blind placebo-controlled clinical trial, clozapine and minocycline co-administration showed to improve working memory deficits in a schizophrenia population with persistent symptoms (Kelly et al., 2015). Later, Zhang and co-authors (2019) described that adjunctive minocycline treatment to antipsychotic medication (in this case, risperidone) was effective in improving cognitive deficits of schizophrenia patients and proposed that this beneficial effect of minocycline could be related to reducing pro-inflammatory cytokines through microglia inhibition (Zhang et al., 2019a). Conversely, other authors do not report this enhanced cognitive performance after co-adjuvant minocycline treatment in schizophrenia patients (Weiser et al., 2019). Anyway, despite these discrepancies, recent meta-analyses support the use of minocycline as a co-add medication in the pharmacological treatment of schizophrenia. Thus, these meta-analyses point to the superiority of minocycline versus placebo on reducing the general psychopathology of schizophrenia, and especially on improving the negative deficits, while not on improving cognitive performance (Jeppesen et al., 2020; Zheng et al., 2019).

Moreover, several investigations have reported a reversion of cognitive deficits in different schizophrenia animal models after minocycline treatment (Liaury et al., 2014; Levkovitz et al., 2007; Fujita et al., 2008; Mattei et al., 2017; Monte et al., 2013; Zhang et al., 2019b; Zhu et al., 2014a). However, all these papers are very diverse (as they use different models of schizophrenia: environmental, pharmacological etc.) and there is a lack of methodological consistency throughout the studies. On the one hand, some authors have investigated the effects of minocycline treatment on the reversion of cognitive deficits in environmental models of schizophrenia, including MIA models (Mattei et al., 2017; Zhu et al., 2014a). Focusing on the Poly (I:C) MIA model, and in agreement with our results, it has been reported that chronic minocycline administration (3 mg/kg oral, starting on PND70-80, and carried out for 5 weeks) is able to revert the NORT cognitive impairment produced by prenatal Poly (I:C) challenge (5 mg/kg i.p. on GD15) in C57BL/6 mice (Mattei et al., 2017). Besides, minocycline treatment to Sprague-Dawley rats (intragastric administration of 40 mg/kg from PND42 to PND56), alone and in combination with the atypical antipsychotic risperidone, was able to rescue the NORT cognitive

deficits in the LPS model (Zhu et al., 2014a). Regarding other environmental models beyond MIA, minocycline administration (20 and 40 mg/kg i.p. from PND28 to PND37) has proved to prevent the impaired cognitive deficit in the attentional set-shifting test (ASST, a behavioral paradigm that evaluates cognitive flexibility) in a C57BL/6J mouse model of adolescent social stress (Zhang et al., 2019b). Nevertheless, the majority of authors researching on the effects of minocycline treatment on schizophrenia animal models use pharmacological models of glutamate NMDA receptor antagonist administration (MK801, PCP, ketamine) (Levkovitz et al., 2007; Fujita et al., 2008; Monte et al., 2013), or other drugs such as the psychostimulant metamphetamine (Mizoguchi et al., 2008). In this sense, chronic minocycline administration (35 mg/kg i.p. three consecutive days before the behavioral testing) has demonstrated to be able to revert cognitive deficits in the Morris water maze (measuring visual-spatial memory) in a MK801 model of schizophrenia developed in Sprague-Dawley rats. Interestingly, minocycline reverted these cognitive effects of MK801 and this effect was similar to that of haloperidol (Levkovitz et al., 2007). In other study, a 14-day minocycline treatment (40 mg/kg i.p. from PND15 to PND28) attenuated the NORT cognitive impairment in a PCP mouse of schizophrenia developed in ICR mice, suggesting that minocycline treatment could be a potential therapeutic drug for schizophrenia cognitive deficits (Fujita et al., 2008). Moreover, minocycline (25 or 50 mg/kg/day i.p. during 14 days) prevented and reverted schizophrenia-related cognitive deficits in the Y-maze (spatial short-term memory) in a schizophrenia Swiss mouse model of ketamine administration (Monte et al., 2013). Additionally, enhanced cognitive performance in the NORT is described in a ICR mouse model of schizophrenia based on methamphetamine administration after minocycline treatment (20-40 mg/kg i.p. for 7 days, one day prior to the NORT) (Mizoguchi et al., 2008). Despite all these studies report an enhancement of schizophrenia-related cognitive deficits, it is important to remark that drug-induced schizophrenia models are not able to accurately mimic all the spectrum of the disorder, as they present discrepancies in terms of construct and face validity. Hence, studying the effects of minocycline treatment in double-hit models represent a more translational alternative. In this sense, Giovanoli et al. (2016), explored the effects of preventive chronic minocycline administration on a double-hit C57BL/6J mouse model of schizophrenia, combining prenatal immune activation with Poly (I:C) (1 mg/kg i.v. on GD9.5) and sub-chronic unpredictable stress. In this case, minocycline (30 mg/kg/day, dissolved in drinking water) was administered during the stress exposure (between PND30 and PND40) in order to prevent subsequent behavioral alterations, including PPI deficits and enhanced sensitivity to psychotomimetic drugs (Giovanoli et al., 2016). However, in the present study we did not explore the effects of preventive minocycline treatment on cognitive alterations, but the reversal of this pharmacological approach on the schizophrenia-related behaviors in the double-

hit model. Thus, the beneficial effects described by Giovanoli and collaborators (2016) were a consequence of a preventive minocycline treatment, while in our study, minocycline reverts an already settled schizophrenia-related cognitive impairment. In this sense, our study provides a further step and a valuable information on the effects of minocycline treatment on cognitive dysfunction of a double-hit animal model of schizophrenia.

To our knowledge, current scientific literature lacks preclinical studies that investigate the effects of combination of minocycline and antipsychotics in schizophrenia-like cognitive deficits. Thus, the present results have an important translational value, as chronic clozapine and minocycline administration or combination of both drugs appear to be able to enhance the cognitive performance of the double-hit model in the NORT and specially, significant beneficial effects on cognition were linked to minocycline administration. Overall, a body of clinical and preclinical evidence support the minocycline treatment as a promising therapeutic alternative for the improvement of schizophrenia-related cognitive deficits. Our findings come to an agreement with these previous reports showing reversal of cognitive deficits after minocycline administration. Moreover, the present project is the first one to show enhancement of cognitive impairment after chronic minocycline administration in a double-hit animal model of schizophrenia of MIA with Poly (I:C) and post-weaning social isolation. This project may encourage attempts to explore the use of the tetracycline minocycline treatment in neuropsychiatric diseases coursing with cognitive deficiencies. In addition, the identification of schizophrenia patients with underlying inflammatory status, as well as the detection of inflammatory biomarkers that enable the diagnosis and prognosis of the disorder, could help to detect those patients who could benefit more from co-adjuvant minocycline treatment.

5.2.2. CO-ADMINISTRATION OF CLOZAPINE AND MINOCYCLINE IMPAIRED THE SOCIAL BEHAVIOR IN THE SPT

In this batch of experiments, we were not able to replicate the social deficit seen in the double-hit model characterization experiments when evaluating the schizophrenia negative-like symptomatology in the SPT (see **section 4.1.4.**). This loss of significance could be attributable to the change of mouse strain used. While in the characterization experiments, we used CD-1® mice, behavioral experiments after the chronic clozapine and/or minocycline treatments were performed with C57BL/6 mice. Thus, in this second batch of experiments, SPT social exploration was lower –although this decrease did not reach the statistical significance– in vehicle-treated double-hit animals when compared to vehicle-treated controls (see **figure 4.28**). However, this

decreased SPT social exploration in vehicle-treated double-hit mice, did not affect the Social Index, which was not statistically different between vehicle-treated controls and double-hit animals (see **figure 4.34**). Moreover, co-administration of clozapine and minocycline elicited a significant decrease in the SPT Social Index in the double-hit group (see **figure 4.35 b**). While clozapine treatment alone elicited a non-significant increase of the Social Index, this increase was reverted when both clozapine and minocycline were administered together. Interestingly, this detrimental effect on sociability appeared to be more pronounced in double-hit female mice treated with the combination of both drugs. Hence, our data suggests that the atypical antipsychotic clozapine in combination with minocycline treatment may worsen schizophrenia negative-like symptomatology, especially in females.

As mentioned before, antipsychotics, including clozapine, have proven to have a very limited efficacy on alleviating negative symptoms in schizophrenia patients (Erhart et al., 2006; Mizuno et al., 2020). However, clozapine has shown to revert negative-like deficits in various schizophrenia animal models (Amiri et al., 2021; Hamieh et al., 2021; Möller et al., 2011; Qiao et al., 2000; Vasconcelos et al., 2015). Thus, if antipsychotics such as clozapine are not able to revert negative deficits in schizophrenia patients, whereas schizophrenia animal models show improved negative-like deficiencies after clozapine treatment, this would mean that these animal models of the disorder would not have the adequate face validity. Hence, more translational animal models would be needed. In this regard, our double-hit model of schizophrenia could represent a more translational tool that would enable the evaluation of the current available pharmacotherapies as well as potential new ones. Focusing on drug-induced models, clozapine (both clozapine alone in a dose of 2.5 mg/kg i.p. and clozapine in combination with an antioxidant, α-lipoic acid) was reported to increase social deficits induced by ketamine administration in mice (Vasconcelos et al., 2015). Similarly, clozapine treatment (10 mg/kg/day s.c. during 7 days) attenuated deficits in social behavior in a PCP-induced animal model of schizophrenia (Qiao et al., 2000). Clozapine has also shown to improve social deficits in environmental animal models of schizophrenia, including the ones based on social isolation stress. Amiri and collaborators (2021) developed an animal model of schizophrenia by inducing a post-weaning social isolation during 8 weeks. Chronic clozapine administration (oral 2.5 mg/kg/day during 28 days) attenuated the effects of social isolation on social interaction test (Amiri et al., 2021). In the same line, in another study, sub-chronic clozapine treatment (5 mg/kg/day i.p. during 11 days) reversed social interaction deficits induced by 8-week social isolation rearing in rats (Möller et al., 2011). Interestingly, the effects of chronic clozapine administration have also been examined in double-hit animal models of schizophrenia. In a

study using a rat double-hit model based on neonatal PCP administration and peripubertal social isolation, chronic clozapine treatment (3 mg/kg i.p. since PNW9, until the end of the behavioral procedures) proved to revert social deficits (Hamieh et al., 2021). Nagai and colleagues developed a mouse double-hit model of schizophrenia based on gene-environment interactions. Thus, neonatal DN-DISC1 (dominant-negative form of disrupted-in-schizophrenia 1) transgenic mice were repeatedly injected with Poly (I:C) from PND2 to PND6 in a dose of 5 mg/kg s.c., and then treated with clozapine (3 mg/kg, orally, one week before the experiments). Clozapine did not ameliorate the impaired social behavior in Poly (I:C)-treated DN-DISC1 mice (Nagai et al., 2011). Overall, in the literature, clozapine treatment appears to improve social deficits in various schizophrenia animal models (Amiri et al., 2021; Hamieh et al., 2021; Möller et al., 2010; Qiao et al., 2000; Vasconcelos et al., 2015), whereas some authors report no improvements on social behavior after clozapine treatment (Nagai et al., 2011). To the best of our knowledge, the effects of chronic clozapine on the negative-like social performance of a double-hit model of schizophrenia combining prenatal Poly (I:C) challenge and peripubertal social isolation have not been assessed. Consistent with the investigation developed by Nagai and collaborators (Nagai et al., 2011), we did not observe an enhancement of the social behavior after chronic clozapine treatment, but an impairment of the sociability of our double-hit model after combining clozapine and minocycline.

In clinical studies, minocycline is presented as a valuable add-on medication to antipsychotics for the treatment of negative symptoms of schizophrenia. The majority of the clinical trials conducted with minocycline in combination with antipsychotics report a significant improvement in schizophrenia negative deficits (Chaudry et al., 2012; Khodaie-Ardakani et al., 2014; Levkovitz et al., 2010; Liu et al., 2014; Zhang et al., 2018). Conversely, some other clinical studies also report no beneficial effects on the negative symptomatology after adjunctive minocycline administration (Deakin et al., 2018; Weiser et al., 2019). In this regard, a recent meta-analysis found that minocycline was superior to placebo in improving general symptomatology, and especially negative symptoms of schizophrenia (Zheng et al., 2019). This is supported by another meta-analysis that also indicates the superiority of minocycline versus placebo, highlighting a more significant effect in reducing negative symptoms compared to other anti-inflammatories evaluated in the treatment of schizophrenia (Jeppesen et al., 2020).

Moreover, some preclinical studies support minocycline as a promising pharmacological alternative for the treatment of schizophrenia-like negative deficits in different animal models of schizophrenia (Mattei et al., 2014; Monte et al., 2013; Zhu et al., 2014b; Zhu et al., 2014c). Regarding drug-induced schizophrenia models, Monte and collaborators (2013) explored the

effects of sub-chronic minocycline administration (25 or 50 mg/kg/day i.p. during 14 days) on a ketamine-induced schizophrenia-like negative deficits, showing that minocycline treatment was able to revert social deficits (Monte et al., 2013). Minocycline treatment has also shown to alleviate schizophrenia-like social deficits on environmental models of the disorder. In this regard, minocycline pretreatment (40 mg/kg/day i.p. from PND7 to PND9) prevented deficits on social interaction in a schizophrenia model of neonatal LPS injection (Zhu et al., 2014c). Focusing on schizophrenia MIA models, sub-chronic minocycline treatment (40 mg/kg/day i.p. during 14 days, from PND42 to PND56) attenuated social interaction deficits in the adult offspring of pregnant mice exposed to Poly (I:C) (20 mg/kg i.p. on GD9.5) (Zhu et al., 2014b). Mattei and colleagues (2017) developed a MIA model by injecting Poly (I:C) (5 mg/kg i.p., GD15) to pregnant mice, and demonstrated that minocycline treatment (3 mg/kg oral, starting from PND70-80, and lasting 5 weeks) on adult MIA offspring reverted completely the Poly (I:C)-related social deficits in the SPT (Mattei et al., 2017). Overall, studies in the literature support minocycline treatment as a promising pharmacological approach to the treatment of schizophrenia-like negative deficits in various animal models this disorder. In contrast with these previous reports, we did not find an enhancement of the social behavior after minocycline treatment in our double-hit model, but an impairment of the social behavior in the SPT when combining minocycline and clozapine.

In contrast with all the mentioned reports showing beneficial effects of both clozapine and minocycline on social behavior, our study showed that combination of these drugs might worsen schizophrenia-related negative symptomatology, mainly in female mice. However, the mentioned studies evaluate the effects on negative-like symptomatology of clozapine and minocycline alone, and not in combination. Thus, the present work is the first one to examine the effects of chronic co-administration of clozapine and minocycline on negative-like phenotypes of a double-hit animal model of schizophrenia. Interestingly, whereas minocycline showed to have beneficial effects on improving cognitive deficits in our double-hit model, we did not observe minocycline-elicited positive effects on the social behavior, contrary to what the clinical studies report. Further, when combining both clozapine and minocycline, no synergistic effects were found on potentiating the cognitive performance of the model, and we saw a worsening of negative-like symptoms. Therefore, it would be key to elucidate the molecular mechanisms that induced this worsening in sociability after the co-administration of clozapine and minocycline.

5.2.3. CHRONIC CLOZAPINE AND/OR MINOCYCLINE ADMINISTRATION DOES NOT MODULATE PROINFLAMMATORY CYTOKINE GENE EXPRESSION IN THE BRAIN CORTEX OF THE DOUBLE-HIT MODEL

We analyzed the gene expression in mouse frontal cortex of IL-6 and IFN-γ in this set of chronic-treatment experiments. The election to assess these two proinflammatory cytokines was based on the previous results in the model characterization experiments where the plasma levels of IL-6 and IFN-γ showed to be upregulated by i) the effect of MIA regardless of the sex and ii) the effect of SI specifically in females. Of note, these previous experiments were performed in plasma samples, whereas the current ones were performed in brain cortex. Therefore, we were aware about the possibility that peripheral cytokine levels might not correlate with central cytokine mRNA expression levels.

On the one hand, gene expression levels of the proinflammatory cytokine IL-6 were higher in the brain of double-hit mice compared to controls (see **figure 4.36**), and especially in double-hit male mice, who showed higher *Il6* mRNA levels than double-hit females. Chronic administration of clozapine and/or minocycline did not modulate the gene expression of this interleukin in the frontal cortex of control or double-hit mice (see **figure 4.37**). Notably, we suggest that it might be a sex effect in the cortical mRNA levels of *Il6*, as we detected increased *Il6* mRNA levels in vehicle-treated double-hit males compared to vehicle-treated control males. Moreover, exploratory analysis evaluating the effect of the sex variable reported a significant "Sex x Clozapine" interaction in double-hit animals, suggesting that the increased levels of *Il6* could be reverted by clozapine, only in males.

On the other hand, the gene expression of the proinflammatory cytokine IFN-γ was not different between vehicle-treated control mice and double-hit mice (see **figure 4.38**), and it was not modulated by chronic administration of clozapine and/or minocycline (see **figure 4.39**).

A body of evidence points to an anti-inflammatory effect of antipsychotics as one of the possible beneficial effects that these drugs exert in schizophrenia patients. In this sense, stimulation of anti-inflammatory cytokines, such as IL-10, is reported to be a mechanism elicited by various antipsychotic drugs in schizophrenia patients, and is thought to regulate the deleterious inflammation in the disorder (reviewed in Leza et al., 2015).

Studies evaluating the effects of antipsychotic medication on brain cytokines in schizophrenia MIA models are scarce, as the majority of the studies assess the effects of these drugs on peripheral levels of pro- and anti-inflammatory cytokines. However, some authors have demonstrated that antipsychotic medication is able to modulate the central proinflammatory

cytokine profile in MIA models of schizophrenia. In the study conducted by MacDowell and collaborators (2017), chronic administration of the atypical antipsychotic paliperidone (0.05 mg/kg i.p. during 21 days) proved to revert the accumulated proinflammatory mediators in a Poly (I:C) mouse model of schizophrenia (5 mg/kg i.p. Poly (I:C) on GD9.5). Thus, chronic paliperidone treatment reverted the Poly (I:C)-elicited increase of IL-6 and IFN-α mRNA levels in mouse frontal cortex (MacDowell et al., 2017).

Moreover, some studies have investigated the central pro- and anti-inflammatory cytokine profile in animal models of social isolation stress. In this regard, several authors have reported significant reductions on IL-6, IL-1β and IL-10 in the hippocampus of SI rats (Dunphy-Doherty et al., 2018; Lopizzo et al., 2021). However, studies that evaluate the effect of antipsychotic treatment on the central cytokine milieu of animal models of social isolation stress are scarce. In this sense, some authors have reported that antipsychotic medication is able to attenuate the central proinflammatory cytokine profile induced by social isolation stress. Thus, in a post-weaning SI rat model (8-week SI), chronic clozapine treatment (oral 2.5 mg/kg/day during 28 days) reverted SI-elicited increase on gene expression of IL-6 and IL-1β in rat PFC (Amiri et al., 2021). In another study, clozapine administration (20 mg/kg/day i.p. for 21 days) proved to attenuate the increased levels of IL-1β and TNF-α in the frontal cortex of rats subjected to a social isolation period of three weeks (Todorović & Filipović, 2017).

Minocycline treatment is found to be neuroprotective and highly effective in inhibiting microglia activation and associated neuroinflammation in the MIA by Poly (I:C) model of schizophrenia (Mattei et al., 2014). Mattei and colleagues administered minocycline chronically (3 mg/kg dissolved in drinking water during approximately 70 days, from PND60 to PND128) to the offspring of Poly (I:C)-challenged pregnant rats (4 mg/kg i.v., GD15). They reported increased relative mRNA levels of IL-1β and TNF-α expression in the hippocampus of the offspring born from Poly (I:C)-treated rats, that was attenuated by minocycline treatment (Mattei et al., 2014). Later, these same authors demonstrated that minocycline administration (3 mg/kg oral, from PND70-80 and during 5 weeks) was able to revert increased IL-6 hippocampal relative mRNA levels associated with prenatal Poly (I:C) administration (5 mg/kg i.p. on GD15) (Mattei et al., 2017). Moreover, Wang and collaborators (2017) demonstrated that minocycline treatment (50 mg/kg i.p. during 7 days) was able to attenuate the increased brain IL-6, IL-1β and TNF-α relative mRNA levels in a rat model of post-weaning social isolation (from PND21 to PND63, six weeks) (Wang et al., 2017). Besides, chronic minocycline treatment is known to block the proinflammatory cytokine IL-1β expression (measured by using particle-based flow cytometry) in the hippocampus and prefrontal cortex of a double-hit model of MIA by means of Poly (I:C)

and stress in puberty (Giovanoli et al., 2016). Of note, in this study, minocycline was administered during the stress exposure, while in our project minocycline was administered after the stress exposure. Thus, in the study conducted by Giovanoli et al. minocycline was reported to have preventive effects on the central proinflammatory cytokine production. However, these authors did not assess the effects of preventive minocycline treatment on IL-6 or IFN-γ cortical levels of their double-hit model of schizophrenia.

Our study is the first one to report the effects of chronic clozapine and/or minocycline administration on the cortical proinflammatory cytokine mRNA expression profile in an environmental double-hit mouse model of schizophrenia. We first reported increased peripheral plasma levels of IL-6 associated to MIA (see **figure 4.14 a**). When we evaluated the brain expression of these cytokines, we detected a sex-dependent modulation of *Il6* mRNA levels in the frontal cortex of double-hit mice, with double-hit males having higher levels than controls ($p= 0.078$). The increased *Il6* levels in male mice were then reverted by clozapine treatment. All this evidence suggest that there might be an increased neuroinflammatory status in our double-hit model that could be reverted by antipsychotic medication. Of note, the statistical analyses evaluating the sex effect are exploratory and a larger number of animals would be needed to reach the required statistical power.

5.2.4. CHRONIC CLOZAPINE AND/OR MINOCYCLINE ADMINISTRATION DOES NOT MODULATE NF-κB AND IκBα GENE EXPRESSION IN THE BRAIN CORTEX OF THE DOUBLE-HIT MODEL

Relative mRNA levels of *Rela*, the gene encoding for NF-κB protein, were not different between vehicle-treated controls and vehicle-treated double-hit animals (see **figure 4.40**). We suggest that this lack of replicability with the double-hit model characterization experiments (go to section **4.1.8.**) could be explained because differences in the mRNA expression were sex-specific. Thus, in the characterization experiments, *Rela* levels were significantly increased by MIA mainly in male mice. Because of the large volume of animals needed to perform this second part of the study involving the pharmacological treatments with clozapine and/or minocycline, we were not able to include independent experimental groups of each sex. According to this argument, when we tested the potential influence of sex on *Rela* mRNA levels, "Sex" was revealed as a significant variant, and, further, we found a "Sex x Double-hit" interaction. This suggests that the double-hit factor has a different impact on *Rela* mRNA levels depending on the sex, and consequently, males and females should be analyzed separately. Indeed, male double-hit mice had higher *Rela* mRNA levels compared to females, as we observed in the

characterization experiments. Thus, we probably were unable to see the significant increase on *Rela* levels because we analyzed both sexes together. However, due to the low number of animals of each sex per experimental group (four males and four females included in each experimental arm), we are aware that these analyses might not have enough statistical power to reach firm conclusions. Moreover, when we evaluated the effect of chronic clozapine and/or minocycline administration in control and double-hit animals, we saw that *Rela* mRNA levels were not modulated by any of the pharmacological treatments (see **figure 4.41**). Yet again, when we studied the potential influence of sex on *Rela* relative mRNA levels after the pharmacological treatments, "Sex" variable was significant in control and in double-hit mice. Furthermore, a significant "Sex x Minocycline" interaction was found only in double-hit mice. These findings again suggest that males and females should be analyzed separately to see drug-induced modulations in *Rela* mRNA levels in double-hit mice. Moreover, minocycline treatment appears to increase *Rela* mRNA levels in female double-hit mice, whereas it decreases *Rela* mRNA levels in double-hit male mice, reaching control values. In conclusion, further experiments with a larger number of animals and including both sexes should be performed in order to clarify the sex-dependent effects of chronic clozapine and/or minocycline treatments on *Rela* mRNA levels.

Similarly, relative mRNA levels of *Nfkbia*, the gene encoding for IκBα protein, were not different between vehicle-treated controls and vehicle-treated double-hit animals (see **figure 4.42**). Once again, this discrepancy with the double-hit model characterization experiments (go to section **4.1.8**) could be explained because changes on *Nfkbia* were sex-dependent (in this case affecting only to female mice) and only associated to MIA and not to the combination of MIA + SI studied in these experiments. However, in these latter experiments we did not observe an effect of the "Sex" variable, nor any "Sex x treatment" interactions. Alike, *Nfkbia* mRNA levels were not modulated by clozapine, minocycline or combination of both drugs, neither in controls, nor in double-hit mice (see **figure 4.43**). Hence, gene expression of inflammatory signaling protein IκBα seems not to be related to treatment-dependent behavioral effects in the double-hit model.

Studies in patients and animal models demonstrate that schizophrenia-associated alterations in the protein, gene expression and activity of the NF-κB signaling pathway are modulated by antipsychotic treatment. In this sense, Murphy and colleagues (2020) studied the effects of antipsychotic treatment on the mRNA expression of various members of the NF-κB family in *postmortem* frontal cortex samples of schizophrenia patients (Murphy et al., 2020). These authors demonstrated that the mRNA levels of some of the NF-κB family members, including NF-κB2 and IκBα, were positively correlated with lifetime antipsychotic exposure. Moreover, García-Bueno et al. (2016) reported that NF-κB (Rela/p65) protein expression measured in

nuclear extracts was higher in the PFC of patients with schizophrenia compared to controls, while no changes in the protein levels of IκBα in cytosolic extracts were found (García-Bueno et al., 2016). Further, these authors reported that antipsychotic-free patients had higher Rela/p65 protein expression than controls, suggesting that antipsychotic treatment could reduce the protein expression of this proinflammatory signaling protein. In contrast, Ibi and collaborators (2017), reported unaltered Rela/p65 immunoreactivity, but decreased IκBα immunoreactivy in western blot experiments in *postmortem* brain tissue of subjects with schizophrenia treated with antipsychotic medication compared to controls (Ibi et al., 2017). In addition, these authors evaluated the effects of chronic clozapine administration on the mRNA levels of *Rela*, its inhibitor *Nfkbia* and other proteins of the NF-κB family in mouse brain cortex. Chronic clozapine administration decreased *Nfkbia* mRNA levels in mouse brain cortex, whereas this treatment did not affect *Rela* mRNA levels. Moreover, these authors also studied the effects of clozapine treatment on Rela/p65 and IκBα immunoreactivity in mouse brain cortex samples, finding that Rela/p65 protein expression was unaltered, whereas IκBα protein levels were decreased in mice chronically treated with clozapine. Overall, Ibi et al (2017) suggest an IκBα-mediated increase of the nuclear translocation of the cytoplasmic NF-κB associated with antipsychotic treatment. Of note, these experiments were performed in control mouse brain samples and not in an animal model of schizophrenia. Now continuing with preclinical investigations, another atypical antipsychotic drug, paliperidone, showed to block the increase of nuclear content of NF-κB and the decrease of cytoplasmic protein levels of IκBα in mice exposed to Poly (I:C) (MacDowell et al., 2017). The effects of the atypical antipsychotic risperidone have also been examined in MIA-induced changes in the NF-κB signaling pathway (Casquero-Veiga et al., 2019). In this study, risperidone administration to the offspring of rats treated with Poly (I:C) elicited a reduction of NF-κB activity (measured with a commercially ELISA system) in nuclear extracts of the PFC, hippocampus and amygdala. Moreover, these authors reported a MIA-induced increase of IκBα immunoreactivity in cytosolic extracts of the PFC, hippocampus and amygdala, which was further increased by the effect of risperidone. Casquero-Veiga et al. (2019) suggest that the trend towards increased NF-κB activity in the in the MIA model may explain the increased expression of its inhibitory subunit IκBα as a compensatory mechanism to restore inflammatory balance.

As already stated, minocycline has been suggested to be an inhibitor of microglial activation (Soczynska et al., 2012), however, the exact mechanism behind minocycline activity on microglia remains unclear (Nettis, 2021). One of the suggested mechanisms is the inhibition of the NF-κB pathway, in fact, in murine microglia-derived cell lines (BV-2) minocycline prevented the

degradation of the inhibitory subunit of IκBα, thus reducing NF-κB translocation to nucleus and its activation, which resulted in decreased transcription of proinflammatory mediators (Nikodemova et al., 2006). A few studies have examined the effects of minocycline administration on the expression of proinflammatory markers –including the NF-κB signaling pathway– in different animal models of schizophrenia, including the MIA model and the SI model. For example, Romero-Miguel and collaborators (2021) reported that minocycline treatment during adolescence prevented proinflammatory alterations (reduced iNOS protein expression in the PFC and caudade-putamen brain areas) in a MIA rat model of prenatal Poly (I:C) administration (Romero-Miguel et al., 2021). However, these authors did not study the effects of preventive minocycline administration on the expression of NF-κB family members in the MIA model. Focusing on animal models of adolescence stress, we found evidence demonstrating that in a rat model of post-traumatic stress disorder induced by inescapable foot shock, significant activation of the NF-κB pathway in PFC and hippocampus of stressed animals was reversed by minocycline treatment (Wang et al., 2018). However, the effects of minocycline treatment in SI-induced NF-κB pathway alterations remain unstudied. In our investigation, minocycline treatment failed to modulate the relative mRNA levels of NF-κB or its repressor IκBα.

Thus, the present study is the first one to evaluate the effects of chronic clozapine and/or minocycline administration on the relative mRNA levels of the genes encoding for NF-κB and IκBα proteins on a double-hit animal model of schizophrenia. Of note, these experiments were performed only in control and double-hit animals (and not in single-hit groups) and without evaluating the influence of the sex variable with independent male and female experimental groups. Further experiments would be needed to evaluate the effects of chronic clozapine and minocycline administration on the relative mRNA levels of NF-κB and its repressor IκBα in both male and female double-hit mice and in all single-hit and double-hit experimental groups.

5.2.5. CHRONIC CLOZAPINE AND/OR MINOCYCLINE ADMINISTRATION DOES NOT MODULATE HDAC GENE EXPRESSION IN THE BRAIN CORTEX OF THE DOUBLE-HIT MODEL

We decided to study whether chronic administration of the antipsychotic clozapine and the inhibitor of inflammatory activity minocycline affected the relative mRNA levels of *Hdac2*, *Hdac4* and *Hdac8*. These particular HDACs were selected based on the previous double-hit characterization experiments, as *Hdac2* mRNA levels were downregulated by effect of MIA and *Hdac4* and *Hdac8* resulted to be downregulated by effect of SI. However, in the conditions of

the present experiments we were not able to replicate the downregulation in the gene expression of the mentioned HDACs. Moreover, pharmacological treatments did not modulate the gene expression of HDAC2, HDAC4 and HDAC8, neither in controls, nor in the double-hit model. It is important to remark, that in the gene experiments performed with CD-1® mice, modulation of *Hdac2*, *Hdac4* and *Hdac8* was not sex-specific, hence, both sexes presented the same gene expression pattern. Nevertheless, in this batch of experiments performed with C57BL/6 mice, changes in the gene expression of these HDACs appear to be sex-dependent. Based in the exploratory analyses that we performed to evaluate the sex influence in the gene expression of these HDACs, female double-hit mice of the C57BL/6 strain appear to be more sensitive, with decreased mRNA levels of *Hdac2*, *Hdac4* and *Hdac8*. Thus, in this case, it could be possible that we did not reproduce our previous results because of the different mouse strain used, as female C57BL/6 mice appear to be more sensitive to the effects of MIA+SI than males.

Hence, in the present experiments with experimental groups comprised by both sexes, we were not able to replicate the decrease on *Hdac2* mRNA expression associated to MIA detected in the double-hit model characterization experiments (see **section 4.1.10.**). Thus, *Hdac2* relative mRNA levels were not different between vehicle-treated control and double-hit mice (see **figure 4.44**). However, a significant "Sex x Double-hit" interaction was found, showing that double-hit females appear to have lower *Hdac2* mRNA levels than double-hit male mice. Thus, it seems that we replicated the finding of the characterization experimnets of deacreased HDAC2 mRNA levels only in double-hit females when using C57BL/6 strain. Interestingly, regarding pharmacological treatments and contrary to what González-Maeso and collaborators report, we did not see an increase in the *Hdac2* mRNA in clozapine-treated control mice (see **figure 4.45 a**) (Kurita et al., 2012). Similarly, HDAC2 gene expression was not affected neither in double-hit mice after the pharmacological treatments studied (see **figure 4.45 b**). To our knowledge, there are no other studies assessing the effects of minocycline administrations (or other anti-inflammatory drugs) on the expression of HDAC2 in mouse models of schizophrenia.

Hdac4 mRNA levels were non-significantly reduced in vehicle-treated double-hit mice respect to vehicle-treated controls (see **figure 4.46**). Again, even if we were able to see a tendency, we were not able to reproduce the significant *Hdac4* mRNA decrease showed in the characterization experiments (plotted in **section 4.1.10**). In this case, we did find a significant influence of the "Sex" variable and a significant "Sex x Double-hit" interaction on *Hdac4* mRNA levels. Thus, we suggest that gene expression of HDAC4 is, again, sex-specific. Indeed, female double-hit mice (pink squares) appear to have a lower *Hdac4* mRNA levels than male double-hit mice (blue squares), suggesting that the "double-hit" had a greater impact on *Hdac4* mRNA levels in

C57BL/6 females than in males. Thus, further experiments analyzing males and females separately should be performed in order to corroborate in this mouse strain the results seen in the characterization experiments. Moreover, consistent with Kurita et al. (Kurita et al., 2012), clozapine treatment did not modulate *Hdac4* mRNA levels in control mice (see **figure 4.47 a**). In addition, *Hdac4* mRNA levels were not modulated after clozapine and/or minocycline administration in double-hit mice (**figure 4.47 b**). However, in double-hit mice, an interaction "sex x clozapine" was close to statistical significance after performing an exploratory three-way ANOVA analysis, which again indicates that males and females should be analyzed separately, as differences on *Hdac4* mRNA levels after the pharmacological treatment in double-hit mice appear to be sex-specific. Further experiments should be performed in independent groups of male and female double-hit mice. Besides, to our knowledge, there are no other studies assessing the effects of minocycline administrations (or other anti-inflammatory drugs) on the expression of HDAC4 in mouse models of schizophrenia.

Again, we did not replicate the SI-driven decrease in *Hdac8* mRNA levels showed in **section 4.1.10.**, as *Hdac8* relative mRNA levels were similar in vehicle-treated controls and vehicle-treated double-hit mice (see **figure 4.48**). However, exploratory two-way ANOVA including the sex as an additional factor revealed that sex had a significant influence on *Hdac8* mRNA levels of vehicle-treated controls and double-hit mice. Moreover, we found a significant "Sex x Double-hit" interaction, suggesting that the double-hit factor affected the *Hdac8* mRNA levels in a different way depending on the sex. Indeed, we observed a reduction of *Hdac8* mRNA levels in vehicle-treated female double-hit mice compared to vehicle-treated controls but not in male double-hit mice. This could explain the reason why we were not able to replicate the decrease on *Hdac8* mRNA levels when both males and females were analized together. Consistent with González-Maeso and colleagues, *Hdac8* mRNA levels were not modulated after clozapine treatment in control mice (see **figure 4.49 a**) (Ibi et al., 2017; Kurita et al., 2012). In double-hit mice, *Hdac8* mRNA expression was not modulated by any pharmacological treatment used in the study (see **figure 4.49 b**). To our knowledge, there are no other studies assessing the effects of minocycline administrations (or other anti-inflammatory drugs) on the expression of HDAC8 in mouse models of schizophrenia.

González-Maeso and collaborators reported that the HDAC2 expression was elevated in *postmortem* frontal cortex of human subjects with schizophrenia treated with atypical antipsychotic drugs (Kurita et al., 2012). As untreated schizophrenia subjects did not show an alteration on HDAC expression, these authors concluded that dysregulation of HDAC2 expression was a consequence of antipsychotic drug medication, and nota biochemical marker

of schizophrenia in *postmortem* human brain (Kurita et al., 2012). Moreover, in agreement with the effects of the antipsychotic treatment on schizophrenia patients, these authors assessed the gene expression of nine different HDACs (*Hdac1*, *Hdac2*, *Hdac3*, *Hdac4*, *Hdac5*, *Hdac6*, *Hdac7*, *Hdac8*, and *Hdac9*) in mouse frontal cortex after chronic clozapine treatment, discovering that *Hdac2* relative mRNA levels were increased in clozapine-treated animals compared to vehicle-treated animals (Kurita et al., 2012). Later, these same authors demonstrated that augmentation of HDAC2 expression upon chronic clozapine treatment negatively affected cortical synaptic remodeling and cognitive processes through a signaling mechanism that involves serotonin 5-HT_{2A}R-dependent upregulation of NF-κB activity (provoked by down-regulation of its repressor IκBα). In this study, they also reported unaffected *Hdac1* and *Hdac8* mRNA levels after chronic clozapine administration in mouse frontal cortex (Ibi et al., 2017).

There is some evidence regarding the effects of antipsychotic and anti-inflammatory treatments in epigenetic alterations in animal models of socially isolation. In this sense, Chen and colleagues (2020), administered the atypical antipsychotic quetiapine to adolescent mice undergoing a social isolation period and observed that quetiapine increased levels of tri-methylation of histone H3 at lysine 9 (H3K9me3, which is linked to active transcription) in mature oligodendroglial cells in the PFC of SI mice (Chen et al., 2020). These authors suggested that quetiapine could modulate epigenetic status toward the beneficial direction for oligodendroglial maturation in socially isolated mice. Furthermore, Wang and collaborators (2017) used the social isolation stress model in rats to characterize the levels of histone methylation (Wang et al., 2017). They found that SI increased the levels of neuronal di-methylation of histone H3 at lysine 9 (H3K9me2, a repressive epigenetic mark), in the hippocampus. Interestingly, minocycline treatment triggered downregulation of H3K9me2 expression, suggesting that the detrimental effects of SI on epigenetic marks could be alleviated by minocycline treatment.

To our knowledge, the present work is the first one to evaluate the effects of chronic clozapine and/or minocycline administration on the gene expression of HDAC2, HDAC4 and HDAC8 in a translational double-hit mouse model of schizophrenia. We did not find modulations on the expression of these genes after the pharmacological treatments in any of the experimental groups. Moreover, the alterations on the gene expression seen when validating the model were not replicated in this batch of experiments. However, we hypothesize that this lack of replicability could be explained due to the differences on the experimental groups respect to the characterization part, mainly due to the mouse strain and the sex influence. Further experiments should be conducted with a larger sample of male and female mice in order to

evaluate the impact of clozapine and/or minocycline treatments on the gene expression of these HDACs in each sex of the double-hit model.

5.2.6. CHRONIC CLOZAPINE AND/OR MINOCYCLINE ADMINISTRATION DOES NOT MODULATE 5-HT_{2A} RECEPTOR GENE EXPRESSION IN THE BRAIN CORTEX OF THE DOUBLE-HIT MODEL

Given that 5-HT_{2A}R receptors have been implicated in the pathophysiology of schizophrenia, several *postmortem* studies have evaluated the influence of antipsychotic medication on the expression of 5-HT_{2A}R in patients with this disorder. García-Bea and colleagues (2019) reported a reduction of *HTR2A* mRNA in antipsychotic-treated schizophrenia patients that was not present in antipsychotic-free schizophrenia subjects (García-Bea et al., 2019). In this sense, previous *postmortem* studies report decreased *HTR2A* mRNA levels in subjects with schizophrenia under antipsychotic medication (Hernandez & Sokolov, 2000; López-Figueroa et al., 2004).

In agreement with these studies in *postmortem* brain tissue, when comparing *Htr2a* mRNA levels between vehicle-treated controls and vehicle-treated double-hit animals, we did not report any significant differences (see **figure 4.50**). In this regard, other studies using MIA models have evaluated the gene expression of 5-HT_{2A}R –without assessing the influence of clozapine treatment– showing controversial results. Moreno et al. (2011) evaluated the relative *Htr2a* mRNA levels in the frontal cortex of mouse born to influenza virus-infected mothers, a validated MIA model, reporting significantly higher *Htr2a* mRNA levels in the influenza offspring than in controls (Moreno et al., 2011). Further, increased 5-HT_{2A} mRNA and protein expression has been reported in Poly (I:C)-treated mice (MacDowell et al., 2021; Malkova et al., 2014) and LPS-challenged rats (Wischhof et al., 2015). Conversely, a recent study shows that *Htr2a* mRNA expression in the PFC of Poly (I:C) rats is significantly downregulated compared to controls (Su et al., 2022). Although we did not report an altered mRNA expression of the gene coding for 5-HT_{2A}R, our female double-hit mice displayed a supersensitive head-twitch response, a proxy of hallucinogenic behavior to the administration of 5-HT_{2A}R agonist, psilocybin. Thus, even if we did not find increased mRNA levels of *Htr2a*, we can affirm that there is a supersensitivity of the 5-HT_{2A}R in our model. Of note, as these experiments were performed only in female mice, further studies in male double-hit mice should also be performed to corroborate this phenotype.

Furthermore, in the present study, gene expression of 5-HT_{2A}R was not modulated by any of the pharmacological treatments in any of the experimental groups (control or double-hit) (see **figure 4.51**). This finding was surprising, as clozapine has been reported to modulate the gene

expression of 5-HT_{2A}R in animal studies. Hence, to date, various investigations have evaluated the effects of chronic antipsychotic medication –including clozapine– on the gene expression of 5-HT_{2A}R in wild type rodents. In this sense, *Htr2a* mRNA levels in brain cortical samples of rats chronically treated with haloperidol and clozapine have been assessed, revealing that only clozapine treatment significantly reduced *Htr2a* brain cortical relative mRNA levels (García-Bea et al., 2019). Moreover, in wild type mice, chronic clozapine has proven to downregulate *Htr2a* mRNA levels in mouse frontal cortex in RT-qPCR assays (Kurita et al., 2012). Our study shows that *Htr2a* mRNA levels are not modulated by chronic clozapine administration – nor by the combination of clozapine and minocycline–. Interestingly, in another study, a 21-day chronic treatment with clozapine also reduced *Htr2a* mRNA levels in mouse somatosensory cortex, but this reduction was only seen when mice were sacrificed one day after the last clozapine administration, and not when mice were sacrificed 7, 14 and 21 days after the last clozapine administration (Moreno et al., 2013). In this sense, we performed the *Htr2a* mRNA levels assessment two weeks after the end of the chronic treatments and we did not find modulation of the expression of this gene by any of them. Thus, in view of these results, it seems that the potential clozapine-induce modulation of cortical *Htr2a* mRNA levels is not long-lasting but exert a short-term transient decrease of *Htr2a* mRNA levels. Studies on antipsychotic-induced modulation of 5-HT_{2A}R gene or protein expression in animal models of schizophrenia are scarce. In this regard, MacDowell and colleagues reported unaltered 5-HT_{2A}R protein expression after chronic paliperidone treatment in Poly (I:C) model, as this antipsychotic was unable to revert the increase in 5-HT_{2A}R expression associated with Poly (I:C) (MacDowell et al., 2021). Thus, our results might go in concordance with this latter investigation. Overall, further experiments in our double-hit model evaluating the protein expression of the 5-HT_{2A}R as well as its functionality in both male and female mice would be needed to further investigate the role of this receptor on the pathophysiology of schizophrenia and its modulation by antipsychotic treatment.

To our knowledge, no previous investigations have evaluated the effect of chronic minocycline treatment on *Htr2a* relative mRNA levels. Indeed, this is the very first study evaluating the *Htr2a* mRNA levels after chronic clozapine and/or minocycline treatments in a mouse double-hit model of schizophrenia. Given the fact that we already detected an increased head-twitch behavior in our double-hit model, which is directly related with an altered 5-HT_{2A}R function, it would be very interesting to address the protein expression and functionality of the 5-HT_{2A}R after the proposed pharmacological treatment in the double-hit model.

To sum up, in this second part of the present Doctoral Thesis, we aimed to study the predictive validity of the double-hit animal model, by assessing the effects of clozapine and minocycline administration on several schizophrenia-related behavioral and molecular alterations presented by the model. We chose to study the effects of chronic administration of clozapine, an approved atipycal antipsychotic that is currently used in refractory schizophrenia cases, and micnocyline, a tretracycline with anti-inflammatory properties that has been proposed as an add-on medication to antipsychotics in clinical trials. Chronic minocycline treatment reverted the NORT cognitive deficits in double-hit mice, suggesting that this drug could be effective for alleviating the schizophrenia-related cognitive impairment. In contrast, chronic co-administration of clozapine and minocycline impaired the negative-like symptomatology in double-hit mice, suggesting that combination of both drugs should be evaluated carefully in patients with predominantly negative symptomatology. In addition, we did not observe a modulation exerted by clozapine and/or minocycline treatments on the cortical gene expression of the proinflammatory cytokines IL-6 and IFN-γ, the inflammatory signaling proteins NF-κB and IκBα, histone deacetylases or 5-HT_{2A}R, in none of the experimental control and double-hit groups comprised by both males and females. However, this could be attributable to the change of the mouse strain used and/or the sex-dependent impact of the hits and pharmacological treatments on the expression of several of these genes. Overall, the double-hit model shows predictive validity and we suggest that it could be a valuable tool to study the effects of novel pharmacological approaches.

6. CONCLUSIONS

From the results obtained in the present work, it can be concluded that:

1. The double-hit animal model presented schizophrenia-related cognitive, negative and positive symptoms, provoked either by the individual or combined effects of prenatal immune activation with Poly (I:C) and post-weaning social isolation. The detrimental behavioral effects induced by each hit were not reverted or counterbalanced by the other, and for some alterations were even additive. However, the combination of maternal immune activation and social isolation failed to have a synergistic effect. Cognitive and negative-like symptomatology observed in the double-hit model was evident both in male and female mice. However, some of the hit-induced behavioral alterations were sex-specific. Thus, the model showed positive-like psychotic symptoms in female mice.

2. The double-hit model presented peripheral and cortical inflammatory alterations, as well as epigenetic dysregulations. Maternal immune activation elicited inflammatory-related alterations in the offspring of Poly (I:C)-challenged dams. Maternal immune activation increased the peripheral blood levels of the proinflammatory cytokine IL-6 in both sexes. Moreover, maternal immune activation increased the brain cortex gene expression of the inflammatory signaling protein NF-κB and decreased the gene expression of its inhibitor IκBα in a sex-dependent manner. Finally, maternal immune activation elicited a significant decrease on the brain cortex gene expression of HDAC2 in both sexes. In addition, social isolation was able to decrease the cortical gene expression of HDAC4 and HDAC8, regardless of the sex, which suggested an imbalance of epigenetic regulatory mechanisms in the model. Overall, we suggest that double-hit-driven cortical proinflammatory alterations, as well as the altered cortical pattern of HDAC expression, could be responsible of schizophrenia-related symtomatology of the model.

3. In the present double-hit model, chronic administration of the atypical antipsychotic clozapine alone was not able to enhance neither cognitive nor social schizophrenia-related behaviors. Chronic treatment with the inhibitor of the inflammation minocycline, alone or in combination with clozapine, was able to revert the cognitive deficits observed in the double-hit model. Whereas minocycline treatment had no effect on social behavior, chronic co-administration of clozapine with minocycline impaired

social behavior of double-hit mice. Thus, combination of both drugs could be beneficial to treat schizophrenia cognitive deficits, but it shoud be evaluated with caution in patients with predominantly negative symptomatology.

4. Chronic treatment with clozapine and/or minocycline did not modulate the cortical gene expression of the proinflammatory cytokines IL-6 and IFN-γ, the inflammatory signaling proteins NF-κB and IκBα, histone deacetylases or 5-HT_{2A}R, in none of the experimental control and double-hit groups comprised by both male and female C57BL/6 mice. In these experimental conditions, we were not able to reproduce the alterations found in the gene expression in the double-hit model characterization experiments in CD-1® mice. We hypothesize that this lack of reproducibility could be be attributable to the change of the mouse strain used for the chronic treatments and/or the sex-dependent impact of the hits and pharmacological treatments on the expression of these genes.

5. Overall, our model presents a high construct, face and predictive validity and appears as a useful translational tool for the study of the pathophysiology of schizophrenia in both sexes. While combination of maternal immune activation and social isolation fails to produce synergistic effects on schizophrenia-related behavioral and molecular alterations, these effects are often additive and provide valuable information of the alterations underlying the disorder. The results obtained with the double-hit model suggest that the combination of atypical antypsichotic and anti-inflammatory drugs might be a beneficial approach in the treatment of cognitive deficits of discrete schizophrenia patients with proinflammatory profile.

7. REFERENCES

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