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THE EFFECTS OF ANTIPSYCHOTICS IN ASTROCYTIC PLASTICITY AND SOCIAL BEHAVIOR IN AN ANIMAL MODEL OF SCHIZOPHRENIA

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“If my mind can conceive it, and my heart can believe it – then I can achieve it. “
Muhammad Ali

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ABSTRACT

The effects of antipsychotics in astrocytic plasticity and social behavior in an animal model of schizophrenia

Schizophrenia is a debilitating psychiatric disorder that affects approximately 1% of the population and is characterized by psychotic events, as well as cognitive and negative symptoms, namely impairments in social interaction. The discovery of drugs that are able to reduce the psychotic symptoms of the disorder helped to shed some light on the mechanisms involved in the etiology of schizophrenia. However, the high complexity of the disease along with the adverse side effects associated with antipsychotic drugs seem to build a long road to the desired treatment of this severe disorder. Functional and structural studies have reported abnormalities in multiple brain areas, namely the hippocampus, one of the particular regions in which the proliferation of stem cells occurs in the adult brain. Importantly, impairments in adult neurogenesis have been described in the brains of schizophrenic patients. However, the possible role of newly formed glial cells and astrocytic plasticity in the etiology of this disorder and in the effects of antipsychotic drugs remains largely unknown.

In the present study, a neurodevelopmental animal model of schizophrenia based in the prenatal exposure to alkylating agent methylazoxymethanol (MAM) was used to evaluate the impact of different classes of antipsychotic (AP) drugs in gliogenesis, astrocytic remodeling and social behavior in rats. Animals exposed to MAM in the prenatal period (GD17) were treated in adulthood with a first generation AP (haloperidol), two second generation AP´s (clozapine and risperidone) and a third generation AP (aripiprazole). The results revealed significant impairments in social behavior, gliogenesis and astrocytic morphology in animals prenatally exposed to MAM. While chronic treatment with aripiprazole was able to revert the impairments in social behavior and restore the levels of gliogenesis, the first and second generation AP´s haloperidol, clozapine and risperidone revealed a specific effect in restoring the detrimental effects of MAM exposure in astrocytic morphology and complexity. However, no significant differences in the expression of astrocytic-related genes were observed in the hippocampus.

In conclusion, these results suggest that gliogenesis and astrocytic plasticity may play a key role in social behavior in the context of schizophrenia. Furthermore, the effects of different classes of AP drugs in these phenomena may pave a new way in the treatment of the negative symptoms of schizophrenia.

RESUMO

Efeitos de antipsicóticos em plasticidade astrocítica e comportamento social num modelo animal de esquizofrenia

A esquizofrenia é uma doença psiquiátrica debilitante, que afeta aproximadamente 1% da população e que é caracterizada por fenómenos psicóticos, bem como por sintomas cognitivos e negativos, nomeadamente distúrbios em interação social. A descoberta de fármacos que reduzem os sintomas psicóticos da doença contribuiu para o avanço no conhecimento de mecanismos afetados no contexto da esquizofrenia. No entanto, a alta complexidade da doença aliada aos efeitos secundários adversos dos antipsicóticos afastam a possibilidade de um tratamento eficaz para esta grave doença. Estudos funcionais e estruturais mostram que várias áreas estão afetadas nesta doença, nomeadamente o hipocampo, uma das poucas estruturas onde a proliferação de células estaminais ocorre no cérebro adulto. Curiosamente, já foi mostrado que a neurogénese adulta encontra-se afetada nos cérebros de pacientes com esquizofrenia. No entanto, o papel de células gliais recentemente formadas e plasticidade de astrócitos na origem desta doença bem como os efeitos de antipsicóticos a este nível é ainda altamente desconhecido.

Neste estudo, o modelo animal de esquizofrenia de injeção pré-natal do agente alquilante acetato de metilazoximetanol (MAM), foi usado para avaliar o impacto de antipsicóticos (AP) de diferentes classes em gliogénese, remodelação de astrócitos maduros e comportamento social em rato. Animais expostos a MAM durante o período pré-natal (GD17) foram tratados em idade adulta com um AP de primeira geração (haloperidol), dois de AP's de segunda geração (clozapina e risperidona) e um AP de terceira geração (aripiprazole). Os resultados mostram distúrbios significativos ao nível do comportamento social, da gliogénese e da morfologia astrocítica em animais expostos a MAM durante o período pré-natal. Embora o tratamento crónico com aripiprazole tenha permitido reverter os distúrbios em comportamento social e recuperar os níveis gliogénese, os AP's de primeira e segunda geração haloperidol, clozapina e risperidona mostraram um efeito específico relativamente à recuperação dos efeitos prejudiciais da exposição a MAM na morfologia e complexidade astrocítica. No entanto, não foram encontradas diferenças significativas relativamente à expressão de genes relacionados com astrócitos no hipocampo.

Em conclusão, estes resultados indicam que a gliogénese e plasticidade astrocítica parecem estar a ter um papel fundamental ao nível de comportamento social no contexto da esquizofrenia. Além disso, os efeitos de fármacos AP de diferentes classes nestes fenómenos podem ajudar a construir um novo caminho para o tratamento dos sintomas negativos da esquizofrenia.

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ABBREVIATIONS

% - Percentage

µL – Microlitres

5-HT – Serotonin

AB - Antibody

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AP – Antipsychotic

Arip – Aripiprazole

Cloz – Clozapine

Cm - Centimetres

Ctrl – Control

DAPI - 4',6-diamidino-2-phenylindole

dB - Decibel

DISC1 - Disrupted in schizophrenia 1

DSM – Diagnostic and Statistical Manual of Mental Disorders

EPS – Extrapyramidal Symptoms

fMRI – Functional magnetic resonance imaging

GD 17 – Gestational Day 17

GFAP - Glial Fribilary Acidic Protein

Hal – Haloperidol

Hip – Hippocampus

IP - Intraperitoneal

MAM - Methylazoxymethanol acetate

MK-801 – Dizocilpine

MRI – Magnetic resonance imaging

Ms - Milliseconds

NMDA - *N*-methyl-D-aspartate receptor

NRG – Neuregulin

NSC – Neural stem cell

PCP – Phencyclidine

PET - Positron emission tomography

PFA – Paraformaldehyde

PPI – Prepulse Inhibition

PPI - Prepulse inhibition test

Ris – Risperidone

RT-PCR - Real time polymerase chain reaction

Schz – Schizophrenia

SEM - Standard error of the mean

SGZ – Subgranular zone

SPECT - Single-photon emission computed tomography

SVZ – Subventricular zone

TP – Tumble and Play

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Introduction

1. INTRODUCTION

Mental disorders are estimated to contribute for about 13% of global disease, surpassing cancer and cardiovascular diseases (Collins *et al.*, 2011). Therefore, these disorders are not only more frequent than previously thought but also account for a greater burden of disease than previously expected, becoming a major global health challenge of the 21st century (Wittchen *et al.*, 2011).

Schizophrenia is one of the most debilitating and severe mental disorder, that typically starts in late adolescence or early adulthood and is characterized by psychotic episodes, seriously affecting the quality of life of individuals (WHO, 2016).

Even though the discovery of antipsychotic agents contributed to further extend the knowledge about the etiology of schizophrenia, these drugs are still far from being appropriate for the treatment of all symptoms and cause severe side effects which require a delicate balance in the treatment of schizophrenic patients.

1.1. SCHIZOPHRENIA

Psychotic disorders are severe psychiatric conditions characterized by specific psychopathological phenomena in the domains of thought and perception. According to the diagnostic and statistical manual of mental disorders, schizophrenia is the most common psychotic disorder (DSM V, 2013), with a lifetime prevalence of about 1% (McGrath *et al.*, 2008). Furthermore, schizophrenia presents an earlier onset and more severe symptoms in men than in women (Os & Kapur, 2009). Life expectancy of patients with schizophrenia is 2-2.5 shorter than the general population (WHO, 2016), not only due to high suicide rates, but particularly related with premature cardiovascular disease as a result of lifestyle factors, such as smoking, unhealthy diet, poor exercise and obesity (Os & Kapur, 2009) and of treatment with antipsychotic drugs that can increase the risk of diabetes and obesity (Stahl & Muntner, 2013).

1.1.1. RISK FACTORS

By definition, schizophrenia is a disorder that must last for six months or longer, including at least one month of positive symptoms, namely hallucinations (clear and vivid sensorial experiences that occur in the absence of an external stimulus), delusions (fixed beliefs that frequently concern the misinterpretation of experiences and perceptions), disorganized speech, catatonic or grossly disorganized behavior and negative symptoms (DSM, 2013; Stahl & Muntner, 2013). Although the positive symptoms of

schizophrenia are the most dramatic feature of the disorder, negative symptoms such as diminished communication, affective blunting, lack of pleasure from positive stimuli, reduced motivation and social withdrawal have a significant functional impact, determining whether patients have a poor outcome or are successfully reintegrated in their daily life activities (Stahl & Muntner, 2013). Additionally, seven cognitive domains were described as frequently impaired in schizophrenic patients, namely working memory, attention/vigilance, processing speed, reasoning and problem solving, verbal learning and memory, visual learning and memory and social cognition (Kern *et al.*, 2004).

On the pursue of the etiology of schizophrenia multiple developmental, environmental, genetic and epigenetic risk factors have been identified (Millan *et al.*, 2014). Environmental elements, namely the degree of urbanization at birth, family history of psychiatric disorders (Sorensen *et al.*, 2014) and developmental disturbances such as viral infections during pregnancy and obstetric complications are strongly linked to the development of schizophrenia. Other factors such as paternal age and cannabis use have been associated with the onset of the disorder (Matheson *et al.*, 2011). Additionally, genome-wide associations and copy number variant studies have identified several risk loci (Dinan *et al.*, 2014) that contribute to the notion that schizophrenia is a polygenic disorder (Srinivasan *et al.*, 2016); particularly Disrupted-in Schizophrenia 1 (DISC1) and Neuregulin 1 (NRG1) are two major candidate susceptibility genes that might be relevant in the pathogenesis of schizophrenia (He *et al.*, 2016).

1.2. PATHOPHYSIOLOGY OF SCHIZOPHRENIA

1.2.1. Dopamine

For over 40 years, the leading theory regarding the pathogenesis of this disorder has been defined as the dopamine hypothesis of schizophrenia (Howes *et al.*, 2016). This hypothesis rose from clinical studies that demonstrated that dopaminergic agonists and stimulants could worsen psychosis in schizophrenic patients and induce psychosis in healthy individuals (Angrist & Samuel, 1970; Connell, 1957) and also by the finding that antipsychotic drugs exert their effect by affecting the dopamine system (Carlsson & Lindqvist, 1963). This fact was later confirmed by the association of the efficacy of antipsychotics with their degree of affinity for dopamine D2 receptors (Seeman & Lee, 1975). Moreover, post-mortem studies revealed elevated levels of dopamine, its receptors and metabolites in the striatum of schizophrenic patients (Lee & Seeman, 1980; Seeman & Kapur, 2000).

Five main dopamine pathways have been identified in the brain: the mesolimbic dopamine pathway, the mesocortical dopamine pathway, the tuberoinfundibular pathway, the nigrostriatal pathway and also a pathway that involves the thalamus. The mesolimbic dopamine pathway concerns projections of

dopaminergic neurons from the ventral tegmental area of the brainstem to the limbic areas of the brain, particularly the nucleus accumbens in the ventral striatum (Meltzer & Stahl, 1976). This pathway is usually associated to motivation and reward (Salamone & Correa, 2012) has been implicated in amphetamine psychosis (Snyder, 1972) and also in the positive symptoms of schizophrenia (Meltzer & Stahl, 1976). Another key dopamine pathway is the mesocortical pathway that involves dopaminergic neurons that project from the ventral tegmental area to the prefrontal cortex, which is not only associated with executive functions and cognition (Floresco & Magyar, 2006), but is also known to regulate affect and emotions (Laviolette, 2007). Interestingly, an hypofunction of the dopamine system has been observed in this pathway: lesions of dopaminergic neurons in the prefrontal cortex culminated in increased levels of dopamine and D2 receptor density in the striatum (Pycock *et al.*, 1980) whereas the use of dopamine agonists in the prefrontal cortex resulted in reduced levels of dopamine metabolites in the striatum (Scatton *et al.*, 1982). Therefore, it has been hypothesized that the hypofunction of dopamine in this pathway may contribute to the cognitive, negative and affective symptoms of schizophrenia (Laruelle & Abi-Dargham, 2000). The nigrostriatal dopamine pathway comprises the dopaminergic neurons that project from the substantia nigra to the basal ganglia or striatum which control motor function (Engert & Pruessner, 2008). Hyperactivity in this pathway is associated to hyperkinetic movements as dyskinesias and tics (Korchounov *et al.*, 2010), and elevated levels of striatal dopamine have been linked to prodromal symptoms of schizophrenia (Howes *et al.*, 2009). The dopaminergic neurons that project from the hypothalamus to the anterior pituitary constitute the tuberoinfundibular dopamine pathway, which are responsible for the inhibition of prolactin release (Andrews & Grattan, 2002). Finally, a dopamine pathway that innervates the thalamus from several sites as the ventral mesencephalon, the periaqueductal grey matter, the lateral parabrachial nucleus and multiple hypothalamic nuclei has been described in primates (Sánchez-González *et al.*, 2005). Although this pathway remains relatively unexplored, altered levels of dopamine have been reported in the thalamus of patients with schizophrenia (Sánchez-González *et al.*, 2005).

1.2.2. Glutamate

In the brain, excitatory transmission is mainly glutamatergic, with glutamatergic neurons consuming between 60 and 80 percent of total brain metabolic activity (Rothman *et al.*, 2003). Glutamatergic neurotransmission takes place through ionotropic and metabotropic glutamate receptors, and each are subdivided in 3 groups. Group I metabotropic glutamate receptors (mGluR1 and mGluR5) are mainly postsynaptic, while group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7, mGluR8) are mainly presynaptic and modulate the release of this neurotransmitter (Kew & Kemp, 2005). On the

other hand, ionotropic glutamate receptors are designated accordingly to the agonists initially found to selectively activate them: kainate, a-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) (Dingledine *et al.*, 1999).

The abnormalities in the glutamatergic system were first evidenced by the finding that levels of glutamate were reduced in the CSF of patients with schizophrenia (Kim *et al.*, 1980). However, other research groups were not able to replicate these results (Korpi *et al.*, 1987; Perry, 1982). Nonetheless, the current predominant hypothesis is the dysfunction of the NMDA receptor (Howes *et al.*, 2015). Post-mortem studies revealed that the density of NMDAR1 subunit is reduced in the superior temporal cortex (Humphries *et al.*, 1996) and in the superior frontal cortex (Sokolov, 1998) in patients with schizophrenia. However, overall findings concerning the density of NMDA receptor have been inconsistent (Hammond *et al.*, 2014). Still, current data suggests that alterations in the glutamatergic system may be principally related to aberrant glutamate receptor localization rather than a generalized deficit (Hammond *et al.*, 2014).

The first line of evidence for the involvement of NMDA receptor dysfunction hypothesis of schizophrenia derived from the observation that non-competitive NMDA receptor antagonists, including ketamine, phencyclidine (PCP) and dizocilpine (MK-801), induce immediate psychological conditions that resemble both positive and negative symptoms observed in patients with schizophrenia (Javitt, 2007; Krystal *et al.*, 1994; Morgan & Curran, 2006), which led to the adoption of NMDA receptor antagonists as an approach to model schizophrenia in animals (Howes *et al.*, 2015).

1.2.3. Antipsychotic treatment

Ever since the accidental discovery of the first antipsychotic drugs in the 1950's, the diversity of available treatments for schizophrenia has expanded significantly (Miyamoto *et al.*, 2005; Stahl & Muntner, 2013). The first drug used as an antipsychotic was chlorpromazine, which has antihistaminic properties, even though its antipsychotic effect is not mediated by this feature (Stahl & Muntner, 2013). In fact, the common characteristic to all first generation (or classical) antipsychotics is the high affinity for dopamine D2 receptor (Stahl & Muntner, 2013) and their binding affinity for the D2 receptor is strongly correlated to the therapeutic doses of these drugs (Creese *et al.*, 1976; Miyamoto *et al.*, 2001; Seeman, 1987; Seeman *et al.*, 1976). However, studies have shown that antipsychotic effects are related with a striatal D2 receptor occupancy of 65-70% (Farde *et al.*, 1992; Kapur *et al.*, 1996; Kapur *et al.*, 2000; Nordström *et al.*, 1993) and that dopamine D2 receptor occupancy above 80% dramatically increases the risk of extrapyramidal symptoms (EPS; such as dyskinesia and Parkinsonism) (Farde *et al.*, 1992). Although a low dosage of haloperidol (2-5 mg/day) can induce an occupancy of 60-80% of the D2 receptor

(Kapur *et al.*, 1996; Kapur *et al.*, 1997), the dosage used in the clinical practice must be much higher due to the fact that long-term treatment with classical antipsychotics leads to the upregulation of D2 receptors in humans (Lee *et al.*, 1978; Silvestri *et al.*, 2000). Furthermore, the unspecific blockade of dopamine D2 receptors by classical antipsychotics like haloperidol interferes with the beneficial effects of dopamine. As previously referred, the mesolimbic dopamine system is associated with pleasure and reward, thus the blockade of this system might not only reduce the positive symptoms of the disease, but also block reward functionality, leading to the worsening of negative symptoms and apathy (Stahl & Muntner, 2013). Also, the blockade of dopamine receptors in the tuberoinfundibular pathway interferes with the inhibition of prolactin, resulting in an elevation of plasma concentration of prolactin, a condition designated as hyperprolactinemia (Stahl & Muntner, 2013). This side effect is associated with amenorrhea (i.e., irregular or lack of menstrual periods) and galactorrhea (i.e., breast secretions) thus interfering with fertility, particularly in women, and may also result in a more rapid demineralization of bones (Stahl & Muntner, 2013).

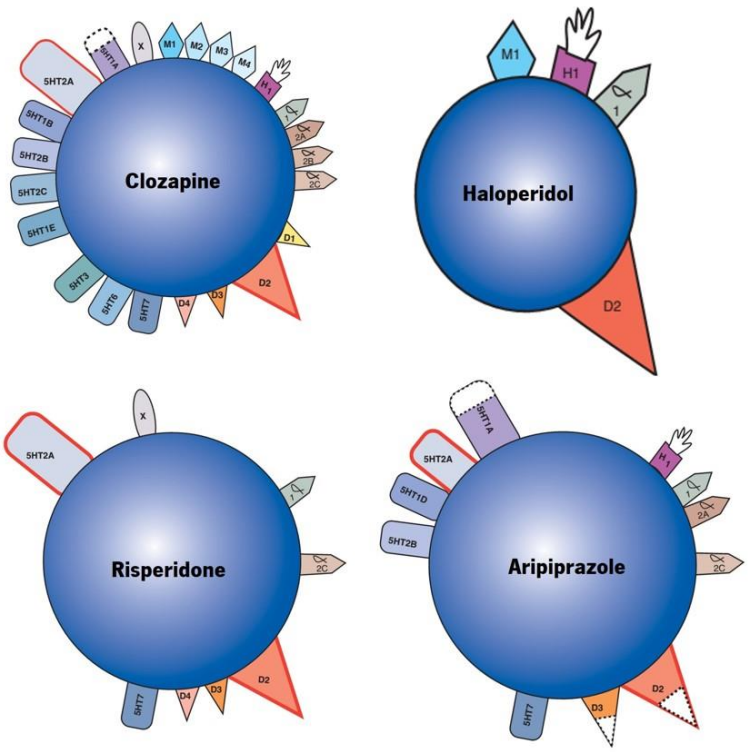
Notwithstanding, atypical or second-generation antipsychotics are drugs that are defined by their serotonin-dopamine antagonism, implicating that 5HT2A antagonism accompanies dopamine D2 receptor antagonism (Miyamoto *et al.*, 2005; Stahl & Muntner, 2013). The stimulation of 5HT2A by serotonin theoretically blocks dopamine downstream release in the striatum, therefore 5HT2A antagonism may interfere with the obstruction of dopamine release by 5HT2A receptors thus reducing EPS and hyperprolactinemia (Stahl & Muntner, 2013) and also contribute to the mood-stabilizing properties of atypical antipsychotics (Brugue & Vieta, 2007).

Clozapine was the first antipsychotic to be described as atypical, considering the reduced EPS and hyperprolactinemia (Stahl & Muntner, 2013). Interestingly, this drug has higher affinity for 5HT2A receptors than it does for D2 receptors and is the only antipsychotic that seems to reduce the risk of suicide in schizophrenic patients (Baldessarini & Frankenburg, 1991; Meltzer *et al.*, 2003), while being the "gold-standard" drug used in patients that present treatment resistance to classical antipsychotics (Chakos *et al.*, 2001). Furthermore, clozapine might even reduce tardive dyskinesia severity in patients suffering from this problem, particularly over long treatment periods (Stahl & Muntner, 2013). However, clozapine is also associated with cardiometabolic complications (such as metabolic syndrome, diabetes mellitus, weight gain and obesity), presenting the highest risk for the development of these side effects compared with other atypical antipsychotic drugs (Zimbron *et al.*, 2016). The administration of clozapine is also associated with agranulocytosis (Hazewinkel *et al.*, 2013), seizures and myocarditis therefore being used only when the alternative treatment is ineffective, rather than as a first-line treatment (Stahl &

Muntner, 2013). Remarkably, another atypical antipsychotic – risperidone – exhibits a very different pharmacological profile, displaying a higher affinity for D2 receptors than other atypical antipsychotics, for example clozapine (Stahl & Muntner, 2013). In fact, risperidone can induce dose-dependent EPS, when administered at higher doses (Chouinard, 1995).

A more recent antipsychotic drug, aripiprazole, acts as a partial agonist of D2 and D3 dopamine receptors, meaning that despite the high occupancy of striatal D2 receptors (Gründer *et al.*, 2009), this drugs binds to the D2 and D3 receptors in a way that allows the balance of dopamine release by not blocking completely the flow of this neurotransmitter while not acting as a stimulant (Stahl & Muntner, 2013). Despite not presenting 5HT2A antagonism at higher affinity than its affinity for dopamine D2 receptors, aripiprazole induces reduced hyperprolactinemia and EPS, unlike other atypical antipsychotic drugs (Stahl & Muntner, 2013).

a)



b)

Receptor	Clozapine	Risperidone	Aripiprazole	Haloperidol
D1	+	+	-	+
D2	+	+++	++++	++++
D3	+	++	++	+++
D4	++	-	+	+++
5-HT1A	-	-	++	-
5-HT1D	-	+	+	-
5-HT2A	+++	++++	+++	+
5-HT2C	++	++	+	-
5-HT6	++	-	+	-
5-HT7	++	+++	++	-
α1	+++	+++	+	+++
α2	+	++	+	-
H1	+++	-	+	-
M1	++++	-	-	-
DA transporter	++		-	
NA transporter	+		-	
5-HT				

Figure 1 – Schematic representation of receptor targets (a) and affinity (b) of different antipsychotics. Each antipsychotic presents different targets and binding affinities. Adapted from Stahl & Muntner (2013) and Miyamoto *et al.* (2005).

1.3. Structural and Functional Morphological Abnormalities in Schizophrenia

Since Eugen Bleuler and Emil Kraepelin (Kraepelin, 1919) first described it over 100 years ago, schizophrenia has been considered a brain disease (Falkai *et al.*, 2011). In 1976, the first computer assisted tomography investigation by Johnstone and colleagues revealed increased volumes of the lateral ventricles in schizophrenia (Johnstone *et al.*, 1976). The technological advances resulted in the development of magnetic resonance imaging (MRI) that pushed the field forward, by allowing better morphological analyses (Falkai *et al.*, 2011). This technique allows the spatial segmentation of brain regions into white and grey matter and cerebrospinal fluid. Studies using MRI have shown deficits in grey matter volume in various brain regions in patients with schizophrenia.

1.3.1. Structural abnormalities in frontotemporal and limbic areas

As previously referred, the prefrontal cortex (PFC) is involved in cognitive executive functions, which are affected in schizophrenia, thus being considered as a key area for the pathophysiology of this disorder (John, 2009; Kinney *et al.*, 1998). Volumetric studies have reported reductions in the PFC (Cannon *et al.*, 2002; Schlaepfer *et al.*, 1994) and functional positron emission tomography (PET) studies reported metabolic hypofrontality (Carter *et al.*, 1998; Schroder *et al.*, 1996). Furthermore, the normal asymmetry verified in the prefrontal cortex (right>left) is reduced in schizophrenia (Bilder *et al.*, 1994), which has been linked to disturbances in the neurodevelopment. Additionally, decreased levels of glucose uptake in the PFC have been associated with negative symptoms in schizophrenic patients (Corcoran & Frith, 1993; Schroder *et al.*, 1996).

Post-mortem and structural MRI studies have reported volume loss in the medial temporal lobe, particularly in the hippocampus, representing one of the most consistent structural abnormalities (Heckers, 2001). Also, post-mortem studies revealed decreased volume of hippocampal subfields, that might be associated to the positive symptoms of schizophrenia (Bogerts, 1997; Bogerts *et al.*, 1990; Bogerts *et al.*, 1993), which was also observed in recent studies using functional magnetic resonance (fMRI) (Haukvik *et al.*, 2015). The hippocampus is involved in emotional regulation and cognition, two affected features in schizophrenia (Heckers & Konradi, 2002). Anatomical and behavioral studies showed that the anterior hippocampus is associated with affect, emotion and stress, while the posterior part essentially involved in cognitive functions (Fanselow & Dong, 2010). Multiple studies have shown a volume reduction in the posterior hippocampus (Becker *et al.*, 1996; Bogerts *et al.*, 1993; Hirayasu *et al.*, 1998; Narr *et al.*, 2001; Rametti *et al.*, 2007; Velakoulis *et al.*, 2001; Yamasue *et al.*, 2004) while other studies shown a decrease

in the volume of the anterior part of the hippocampus in schizophrenic patients (Pegues *et al.*, 2003; Szeszko *et al.*, 2003).

The thalamus transfers peripheral sensory inputs to the cortex, being critical in filtering sensory information, coordinating cognitive input to the cortex and mediating corticocortical connections between regions such as frontal and temporal areas, that are affected in schizophrenia. Structural MRI studies revealed a decrease in the thalamic volumes (Brickman *et al.*, 2004; Gaser *et al.*, 2004). Also, a study using PET and single-photon emission tomography (SPECT) showed decreased levels of metabolic activity in the thalamus associated with cognitive deficits and worsening of negative and positive symptoms (Min *et al.*, 1999).

1.3.2. Functional Connectivity Abnormalities

Studies of white matter tracts using diffusion tensor imaging (DTI) of the hippocampus and the fornix body revealed reduced fractional anisotropy, which is a measure of the coherence along white matter tracts, supporting the hypothesis of functional disconnectivity (Kalus *et al.*, 2004; Kuroki *et al.*, 2006; White *et al.*, 2007; Zhou *et al.*, 2008). Furthermore, better performance in cognitive functions such as verbal declarative memory was associated to higher levels of fractional anisotropy of the hippocampus in schizophrenia (Lim *et al.*, 2006). In the entorhinal cortex, a study using DTI and MRI showed a reduction in the volume of this area and a reduction of diffusional anisotropy, suggesting disturbed connectivity to the hippocampus (Kalus *et al.*, 2005) . The neuronal fibers that cross limbic pathways from the posterior hippocampus are connected to the anterior thalamic complex, anterior cingulate cortex, to prefrontal areas and pathways associated with information processing and higher cognition (Fanselow & Dong, 2010; Goldman-Rakic *et al.*, 1984). Therefore, disturbances of connectivity in prefronto-temporal neuronal networks might result in negative and cognitive symptoms (Harrison, 2004; Kuroki *et al.*, 2006; Rajarethinam *et al.*, 2001). Also, in a fMRI study investigating non-articulatory maintenance of phonological information, a subprocess of working memory, reduced connectivity of the prefrontal cortex with the hippocampus and the intraparietal cortex was found in schizophrenic patients (Henseler *et al.*, 2010). A meta-analysis of fMRI studies of executive function has also shown decreased activation of the anterior cingulate cortex, the ventrolateral and dorsolateral prefrontal cortex, and thalamus (Minzenberg *et al.*, 2009). Likewise, disturbances in the prefronto-parietal-thalamic network have been linked to working memory deficits in schizophrenia (Schneider *et al.*, 2007). Therefore, fMRI and DTI studies confirm the hypothesis of dysfunction of the cortico-prefrontal-thalamo-temporo-limbic network in schizophrenia (Weinberg, 1996).

1.4. ANIMAL MODELS OF SCHIZOPHRENIA

Despite the fact that characteristics exclusive to humans such as thoughts cannot be evaluated in animals though representing core features of psychiatric disorders, animal models of complex neuropsychiatric disorders still represent remarkable preclinical tools to study the etiology of this disorder (Powell & Miyakawa, 2006).

Rats are highly sociable animals, that are organized in a structured social system and establish a hierarchy that highly influences their development (Jones *et al.*, 2011). Therefore, social isolation of rat pups at the age of weaning impacts brain development, leading to behavioral impairments in adulthood (Fone & Porkess, 2008; Lapiz *et al.*, 2003), which are not rescued by social re-integration at later stages (Pascual *et al.*, 2006). Post-weaning social isolation rodents causes impairments in sensorimotor gating, augments responses to novelty (neophobia), induces spontaneous locomotor activity, cognitive deficits and increased anxiety, which model some characteristics observable in human patients with schizophrenia (Jones *et al.*, 2011).

In the 1970's, the genetic analysis of a Scottish family with an unusual high prevalence of schizophrenia led to the discovery of a mutation in DISC1 (disrupted in schizophrenia) gene that codes for a scaffolding protein that interacts with other proteins to promote development and growth (Cameron & Glover, 2015). This gene is primarily expressed in the hippocampus of the postnatal brain and its downregulation has been shown to affect cell proliferation in the DG of the adult hippocampus of mice (Mao *et al.*, 2009). DISC1 has also been implicated in the guidance of the migration of new neurons in the DG, and in fact the knockdown of this protein leads to enhanced maturation and aberrant morphology of new neurons in the DG of the adult hippocampus in mice (Cameron & Glover, 2015). Furthermore, knockout mice display reduced brain volume and cortical thickness along with enlarged ventricles (Jaaro-Peled, 2009). Also, subtle impairments in PPI have been reported, which are reverted by treatment with both clozapine and haloperidol (Clapcote *et al.*, 2007; Hikida *et al.*, 2007).

As previously referred, exposure of the neonate to environmental insults during the developmental or perinatal period increases the risk of developing schizophrenia (Lewis & Levitt, 2002). Methylazoxymethanol (MAM) is a natural agent of seeds of cycad plants, that acts as an antimitotic agent that methylates DNA (Matsumot & Higa, 1966) and targets specifically neuroblasts proliferation in the CNS without inducing teratogenic effects in peripheral organs (Cattabeni & DiLuca, 1997). Treatment of pregnant rat dams with MAM selectively impacts neurodevelopment without affecting litter size or pup body weight (Balduini et al., 1991b; Flagstad et al., 2004). In fact, admnistration of pregnant rat dams impacts the brain regions that undergo the fastest development in the fetus, inducing long-lasting

behavioral and anatomical impairments in the offspring (Moore et al., 2006; Lodge and Grace, 2009) which are dependent on the gestational day of admnistration (Talamini et al., 1998; 2000; Fiore et al., 1999). Admnistration of MAM on gestational day (GD) 17, when cortical cell proliferation is considerably diminished leads to a more restricted preferential size reduction in neocortical and limbic areas, including medial prefrontal (PFC), occipital and entorhinal cortices and the hippocampus, and increased neuronal density in the perirhinal cortex (Moore et al., 2006; Matricon et al., 2010). Also, treatment with MAM at GD17 seems to have reasonable face validity for cognitive and positive symptoms, and also construct validity structural impairments and dopaminergic alterations (Jones *et al.*, 2011).

1.5. Neural Stem Cells

Neural stem cells (NSCs) can be described as cells that present the capability of generating all the cell types in the brain, contrasting with neural progenitors (NPs) that present more restricted potential (Homem *et al.*, 2015). Neural stem and progenitor cells (NSPCs) expand through symmetric self-renewing divisions in the beginning of development. Later, these cells divide asymmetrically, meaning that one of the daughter cells undergoes differentiation after one or several rounds of transit amplifying cells, while the other daughter cell continues a proliferating progenitor (Homem *et al.*, 2015).

1.5.1. Neurogenesis and Gliogenesis

Both neurogenesis (the formation of new neuronal cells) and gliogenesis (generation of new glial cells) occur primarily in the subgranular zone of the hippocampus (SGZ) and in the subventricular zone, since the particular ontogeny of these brain regions allows the preserved capability of generating proliferating cells in the adult brain (Rusznak *et al.*, 2016). These brain regions are capable of both neurogenesis and gliogenesis in the adult, particularly after stimulation or injury (Rusznak *et al.*, 2016) .

In the SVZ, a particular type of NSCs – B cells –resemble mature astrocytes and express astrocytic molecular markers, including glial fibrillary acidic protein (GFAP) (Apple *et al.*, 2016). These cells generate transit-amplifying progenitors – C cell – which in turn give rise to neuroblasts – A cells – that migrate along the rostral migratory stream until the olfactory bulb (OB) (Apple *et al.*, 2016; Kronenberg *et al.*, 2007). In rodents, neuronal progenitor cells generated in the SVZ travel through the rostral migratory stream, reaching the olfactory bulbs, where they differentiate into periglomerular cells, interneurons or granule cells (Alvarez-Buylla & Garcia-Verdugo, 2002; Gheusi *et al.*, 2013; Lazarini *et al.*, 2014; Sakamoto *et al.*, 2014). Adult olfactory neurogenesis is required for specific forms of olfactory behavior (Wang *et al.*, 2015) and other studies have shown that gliogenesis also occurs in the rodent olfactory bulb as well as in the SVZ of

patients with multiple sclerosis (Aguirre & Gallo, 2004; Nait-Oumesmar *et al.*, 2008). Even though olfactory neurogenesis in humans is not the same as in rodents as a result of the variable relevance of olfactory stimuli in the survival of the species (Maresh *et al.*, 2008), under some conditions human olfactory neurogenesis can be a prominent feature, for example in Parkinson's disease and depression (Huisman *et al.*, 2004; Maheu *et al.*, 2015). Nevertheless, the number of new neurons generated in the human olfactory bulb is lower than the level of neurogenesis observed in the rodent olfactory bulb (Bergmann *et al.*, 2012). However, the adult human SVZ retains active neurogenesis (Ernst & Frisén, 2015; Knoth *et al.*, 2010; Sanai *et al.*, 2011) which possibly reflects that if the cells generated in the SVZ are not migrating to the olfactory bulbs, then possibly these cells are migrating to the neighboring striatum, which might explain the pronounced striatal neurogenesis observed in the human brain (Ernst *et al.*, 2014).

The main role of the SGZ is to generate new granule cells in the dentate gyrus of the hippocampus (Lepousez *et al.*, 2015). Studies in rodents have shown that hippocampal-dependent behaviors activate more newly generated cells than older granule cells (Ramirez-Amaya *et al.*, 2006; Snyder *et al.*, 2009a; Snyder *et al.*, 2009b). A study revealed three characteristics of adult hippocampal neurogenesis in humans: 1) a larger proportion of neurons in the hippocampus are replaced in the adult human brain; 2) the age-dependent decline of neurogenesis in the hippocampus is less marked in human brains; and 3) in rodents, hippocampal neurogenesis leads to a net increase in the number of neurons in the dentate gyrus, whereas the continuous neurogenesis in the hippocampus contributes to a pool of neurons with specific functional properties in humans (Spalding *et al.*, 2013). Therefore, these results indicate that hippocampal neurogenesis in humans is essential in maintaining hippocampal functions (Cameron & Glover, 2015; Lepousez *et al.*, 2015). Likewise, the generation of astrocytic cells also occurs in the hippocampus of the adult human brain (Eriksson *et al.*, 1998).

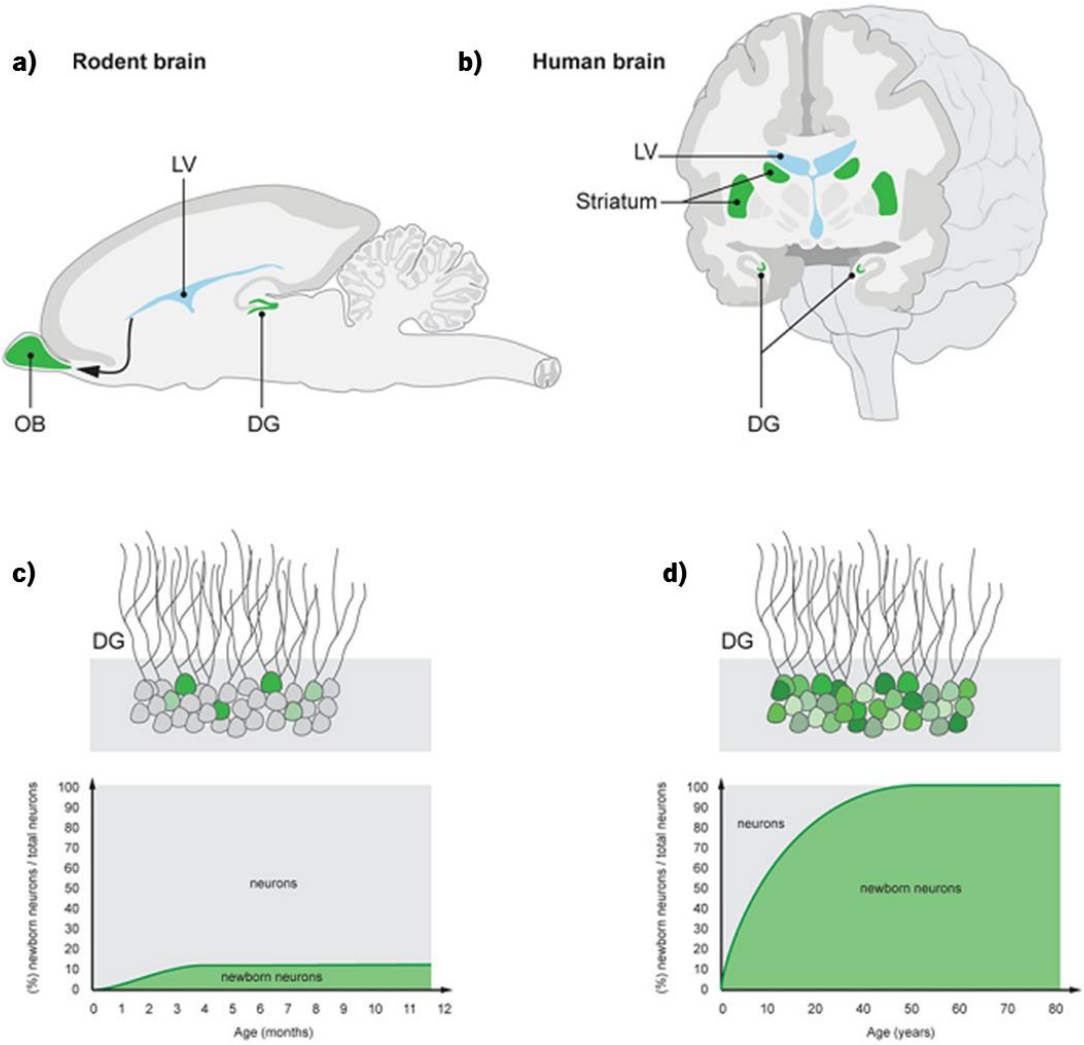


Figure 2 - Schematic representation of adult neurogenesis in the adult rodent and human brain. New neurons are represented in green. **a)** In the rodent brain, neuroblasts that are generated in the subventricular zone (SVZ) travel through the lateral ventricle (LV) until the olfactory bulbs (OB). **b)** In humans, neuroblasts are also present in the subventricular zone, however the new neurons are integrated in the striatum. In the dentate gyrus (DG) of rodents **(a)** and humans **(b)** new neurons are also continuously generated. Nevertheless, the generation of new neurons in the DG is less significant in the adult rodent brain **c)**, compared to the adult human brain **d)**. Adapted from Ernst & Frisén (2015).

1.5.2. Neurogenesis in Schizophrenia

Impairments in neurogenesis have already been reported in different disorders, namely altered hippocampal neurogenesis in Alzheimer's disease (Martinez-Canabal, 2014; Vivar, 2015; Winner & Winkler, 2015), Parkinson's disease (Huisman *et al.*, 2004), stroke and depression (Duman *et al.*, 1999; Madsen et al., 2000; Sahay & Hen, 2007; Sapolsky, 2000).

In 2006, Reif and colleagues studied the proliferation of neural stem cells in the hippocampus in post-mortem brains of patients with unipolar major depression, bipolar disorder and schizophrenia, using Ki-67, an endogenous proliferation marker. Interestingly, this study showed that the proliferation of stem cells is not reduced in patients with unipolar depression, in contrast with a significant reduction of proliferation of neural stem cells in the hippocampus of schizophrenic patients, thus suggesting an involvement of adult neurogenesis and gliogenesis in the etiology of schizophrenia (Reif *et al.*, 2006).

1.6. ASTROCYTES

Astrocytes are the most common type of cell in the brain (Molofsky & Deneen, 2015), performing various key functions essential to the homeostasis of the central nervous system (CNS) such as the modulation of several neurotransmitters, glucose metabolism, glutamate neurotransmission, blood-brain barrier formation and maintenance and synaptogenesis (Rajkowska & Miguel-Hidalgo, 2007). Moreover, astrocytes are also responsible for the regulation of the cortical cerebral blood flow (Takano *et al.*, 2006) and also act in response to central nervous system insults such as ischemia, trauma, infection and neurodegenerative disease through a process designated as reactive astrogliosis, which involves modifications in their morphology and molecular expression (Sofroniew, 2009).

In humans, cortical astrocytes can be distinguished in three different cell types – protoplasmic, interlaminar and varicose projection astrocytes. In summary, protoplasmic astrocytes are the most common cortical astrocytes that are immunostained by glial fibrillary acidic protein, which is an intermediate filament protein (GFAP), revealing a characteristic "bushy" structure. These cells communicate intrinsically through gap junctions via calcium signaling, which ultimately leads to the release of transmitters in the extracellular space that can happen as a result of the neuron-astrocyte communication or transmission with other astrocytes (Mohn & Koob, 2015). Interestingly, these astrocytes are arranged in nonoverlapping territories with independent signaling domains (Oberheim *et al.*, 2009). Interlaminar astrocytes are a particular type of cell described in primates, present in layer I of the cortex (Mohn & Koob, 2015). These astrocytes extend a process into layers III and IV of the cortical molecular layers ending there and contacting with blood vessels and other cells, not following the pattern of independent domains observed in the protoplasmic populations (Mohn & Koob, 2015). Finally, varicose projection astrocytes are present in the layers V-VI and extend processes to layers III, IV and V creating contacts with other cells and blood vessel projections.

1.6.1. Astrocytes in psychiatric disorders

Interestingly, glial pathology has been found in various psychiatric disorders. A study showed decreased glial cell density in layers 3 and 5 of the dorsolateral prefrontal cortex and in deeper layers of the caudal orbitofrontal cortex in major depressive disorder (Rajkowska *et al.*, 1999). Similarly, reductions of glial cell density were also found in the dorsolateral prefrontal cortex in patients with bipolar disorder (Rajkowska, 2000). Likewise, Onguer and collegues reported unaltered neuronal density and neuronal volume in addition to reduced glial cell density in the subgenual part of the anterior cingulate cortex in major depressive disorder, which was also observed in bipolar disorder patients (Öngür *et al.*, 1998). Another study confirmed this observations, showing reduced levels of GFAP in major depressive disorder and bipolar disorder using proteomics (Johnston-Wilson *et al.*, 2000). Furthermore, elevated levels of GFAP in the parietal, superior frontal and cerebellar cortices (Laurence & Fatemi, 2005) have been observed in the brains and cerebrospinal fluid (Ahlsén *et al.*, 1993) of autism spectrum disorder patients implicating a role of glial cells in this pathology.

1.6.2. Evidences of astrocytic involvement in schizophrenia

Initially, several studies using the histochemical Holzer staining approach reported increased densities of astrocytes in the hippocampus, the periaqueductal grey matter and in multiple cortical areas of schizophrenic patients (Casanova *et al.*, 1990; Stevens *et al.*, 1988; Stevens, 1982). However, when other techniques were used such as the Nissl staining or GFAP immunostaining, no evidence for astrogliosis in schizophrenia was found (Damadzic *et al.*, 2001; Falkai *et al.*, 1999; Garey, 2010; Rajkowska *et al.*, 2002; Schmitt *et al.*, 2009; Williams *et al.*, 2013a; Williams *et al.*, 2013b). Instead, some studies have shown loss of astrocytes in diverse cortical and subcortical areas in schizophrenia (Rajkowska *et al.*, 2002; Steffek *et al.*, 2008; Williams *et al.*, 2013a), which is particularly pronounced in the white matter (Williams *et al.*, 2013a). Likewise, reduced levels of GFAP mRNA have been found in white matter regions of brains of schizophrenic patients (Webster *et al.*, 2005). However another study failed to find altered densities of GFAP in frontal or temporal association cortices (Garey, 2010). Moreover, increased density of S100B immunoreactive astrocytes was reported in post-mortem brains of paranoid but not residual schizophrenic patients (Steiner *et al.*, 2008) as well as increased levels of S100B in bodily fluids of schizophrenic patients (O'Connell *et al.*, 2013; Rothermundt *et al.*, 2004). However, other studies have failed to replicate these findings in serum levels of S100B in schizophrenia (Uzbay *et al.*, 2013; van der Leeuw *et al.*, 2013). Finally, a polymorphism of the excitatory amino acid transporter 2 (EAAT2), an important glutamate transporter primarily expressed in astrocytes, was associated to impairments in executive functions and working memory in schizophrenia (Spangaro *et al.*, 2012). In summary, taking in account the wide range of

functions of astrocytes in the CNS as well as clues provided by studies in patients, it is plausible to suggest that astrocytes may be involved in the pathophysiology of schizophrenia.

1.7. OBJECTIVES

Schizophrenia is a highly complex, debilitating psychiatric disorder that is characterized by positive, negative and cognitive impairments that impact the quality of life not only of patients, but also of their caretakers. Over the last decades, adult neurogenesis has been proposed to be involved in the pathophysiology of several neuropsychiatric disorders. However, glial pathology has been suggested as a relevant factor to understand the etiology of schizophrenia. Therefore, using a well-established neurodevelopmental model of schizophrenia this work aimed to target the modulation of antipsychotics with different pharmacological profiles in the formation and morphology of astrocytes. More specifically, this study aimed to:

1. Evaluate the effect of MAM and the possible modulation of different antipsychotics in behavioral traits that are impaired in patients with schizophrenia;
2. Investigate possible impairments in gliogenesis and astrocyte morphology induced by MAM and the effects of antipsychotics in these measures;
3. Assess the expression of astrocytic-related genes in the hippocampus of MAM and antipsychotic-treated animals.

2
Materials and Methods

2. MATERIALS AND METHODS

2.1. ANIMALS

Experiments were carried out in male Wistar rats (Charles River Laboratories, Barcelona, Spain), weighing 300–400 g, aged four months. These animals were housed (three per cage) under standard laboratory conditions (12h light/ dark cycle, at 22 °C, relative humidity of 55%, food and water provided ad libitum). All experiments have been approved by the Institutional Ethical Commission and were performed in accordance with the European Community Council Directive 86/609/EEC and 2010/63/EU regarding the use of animals for scientific purposes. The experiments were designed to reduce animal suffering, as well as the number of animals used. Prior to the behavioral experiments, all animals were daily handled (for 10 minutes) and also habituated to experimental room at least 30 minutes before the performance of the tests.

2.2. PRENATAL EXPOSURE TO MAM

To evaluate the impact of different antipsychotics in the methylazoxymethanol (MAM) model of schizophrenia, animals were assigned to two different experimental groups: controls (CTRL) and MAM, in which the offspring of pregnant Wistar rats were injected subcutaneously with saline (1 ml/kg) or MAM (20 mg/kg; National Cancer Institute, Midwest Research Institute, Kansas City, MO, USA) at gestational day 17 (GD17). Subsequently, the MAM group was subdivided into five experimental groups treated at the age of three months for five weeks with vehicle, clozapine, haloperidol, risperidone or aripiprazole.

2.3. DRUGS

In order to evaluate the effects of different antipsychotic drugs in the MAM model of schizophrenia, the offspring of female rats injected with MAM were daily injected intraperitoneally (i.p.; 1 ml/kg) for five weeks with clozapine (CLOZ, atypical antipsychotic; 2,5 mg/kg in ultra-pure water with HCl (2M; Panreac, Barcelona, Spain); Kemprotec, Middlesborough, UK), haloperidol (HAL, classical antipsychotic; 0,05 mg/kg in ultra-pure water; Sigma-Aldrich, St Louis, MO, USA), risperidone (RIS, atypical antipsychotic; 0,25 mg/kg in ultra-pure water and glacial acetic acid (Carlo Erba, Barcelona, Spain); Kemprotec, Middlesborough, UK), aripiprazole (ARIP, atypical antipsychotic; 1 mg/kg in ultra-pure water and Tween 80 (1%, Barcelona, Spain); Kemprotec, Middlesborough, UK).

2.4. BEHAVIOR TESTS

2.4.1. Prepulse Inhibition Test

Prepulse inhibition test (PPI) consists in exposing the animal to a non-startle auditory stimulus before subjecting the individual to a strong startling stimulus. This test measures the acoustic startle reflex, reflecting the sensorimotor gating, as the exposure to the pre-stimulus inhibits the startle response to a strong auditory stimulus. To perform this test, the animals were placed in Plexiglas cylinders with 16 cm length and a diameter of 9 cm. The cylinders were set onto a horizontal plate equipped with a transducer that allows the detection of startle response in a sound attenuated chamber. After an acclimatization period of five minutes with white noise [70dB(A)], five startle trials of 120 dB bursts of white noise were delivered, during 40 ms. The session consisted in the presentation of ten startle trials of 120 dB, followed by prepulse intensities of 2,4,8 and 16 dB(A) above background level, respectively PP72, PP74, PP78 and PP86 with a duration of 20 ms. The startle amplitude was measured as the mean of ten startle trials applied, considering the prepulse intensity of 74 dB, that represents the only prepulse intensity in which another animal model of schizophrenia, DISC1 transgenic mice, present deficits in prepulse inhibition (Hikida *et al.*, 2007). PPI (in percentage) was calculated as follows: 100 − (Mean of all startle amplitudes on prepulse trials/Basal startle amplitude) x 100.

2.4.2. Tumble and Play

Tumble and play is a behavioral test that allows the evaluation of social play behavior, which was assessed according to the protocol previously described by Borges and colleagues (Borges *et al.*, 2013). Briefly, two strange rats from the same experimental group were placed in an unfamiliar housing cage for 10 minutes after 3,5 hours of social isolation. The behaviors of the animals were recorded using a video camera and subsequently analyzed by a blind observer. The performance of the animals was scored per pair of animals by investigating the frequency of pouncing, which is an indicator of play solicitation, as it is an attempt of an animal to rub or nose the back of the neck of the other animal, and the frequency of pinning, that is the response of the to the solicitation, in which the solicited animal fully rotates on its dorsal surface, with the other animal standing on top of it and also the amount of time spent in social exploration, which is considered as the time spent sniffing any part of the body of the test partner, including the anogenital area.

2.5. BRAIN PROCESSING

Animals were anaesthetized by an i.p injection of sodium pentobarbital (Eutasil, 60 mg/Kg, i.p.; Ceva Saúde Animal, Portugal). For molecular analysis (RT-PCR) the animals were transcardially perfused with 0,9% saline and the brains were excised and macrodissected. For the analysis of the astrocytic morphology (immunofluorescence) the animals were transcardially perfused with 0,9% saline and 4% PFA. Brains were collected and embedded in O.C.T (Tissue-Tek O.C.T. compound, Sakura Finetek Europe, Netherlands) and posteriorly kept at -20 °C. Serial coronal sections (20 μm) of the prefrontal cortex (PFC; Bregma 3.20) and covering the length of the hippocampus (Hipp; Bregma -5.080) were sliced in a cryostat and collected to slides that were kept at -20 C°.

2.6. IMMUNOFLUORESCENCE

2.6.1. GFAP/Ki-67 Immunohistochemistry

In order to assess astrocytic morphology and astrogliogenesis, an immunofluorescence protocol using antibodies against GFAP and Ki-67 (Table 1) was used in the hippocampus. GFAP is an intermediate filament protein highly present in astrocytes; therefore, an immunostaining against this protein allows the specific labelling of astrocytes (Hol & Pekny, 2015). Also, Ki-67 is a protein expressed during the cell cycle except in G0, representing an endogenous proliferation marker (Kokoeva et al., 2007). Therefore, the combination of antibodies against GFAP and Ki-67 will allow the tracking of new astrocytes.

After selection of the slides (Bregma: -3.24) and posterior defrost, the samples were permeabilized for 10 minutes in a PBS-T (0.5%) solution, in order to potentiate the binding of the antibody to the antigen. Then, samples were washed with PBS and submerged in boiling citrate buffer (10mM, Sigma Aldrich, St Louis, MO, USA) and held at sub-boiling for 20 minutes for antigen retrieval. After cooling at room temperature (RT) for 15 minutes, slides were washed three times with PBS and 4% bovine serum albumin (BSA, Sigma Aldrich, St Louis, MO, USA) was added for 30 minutes at RT to decrease unspecific antibody bounds. The samples were incubated overnight with primary antibodies (Table 1) diluted in PBS, in a humid chamber at 4°C. On the consecutive day, the slides were washed with PBS and incubated with the corresponding secondary antibodies (Table 2) diluted in PBS, at RT for 2 hours in humid chamber. Subsequently, samples were washed again and incubated with DAPI (1:1000; Invitrogen, California, USA) for 10 minutes. After being washed, the slides were mounted with Immu-mount (ThermoFisher Scientific, USA) and stored at 4°C.

Table 1. Dilutions and specificities of the primary antibodies (AB) used in the immunofluorescence protocol.

Primary AB	Specie	Dilution	Company
anti-GFAP	Mouse	1:200	Sigma, Missouri, USA
anti-Ki-67	Rabbit	1:300	AbCam, Cambridge, UK

Table 2. Dilutions and specificities of the secondary antibodies (AB) used in the immunofluorescence protocol.

Secondary AB	Specie	Dilution	Company
Alexa Fluor® 594 anti-mouse	Goat	1:1000	ThermoFisher Scientific, USA
Alexa Fluor® 488 anti-rabbit	Goat	1:1000	ThermoFisher Scientific, USA

2.6.2. GFAP/Ki-67

Gliagenesis was measured as a quotient of double positive GFAP/Ki-67 positive cells and the total number of Ki-67-positive cells in the subgranular zone of the dentate gyrus (SGZ; defined as a 3-cell layer-thick region on the inner side if the granule layer of the dentate gyrus), by counting the corresponding cell numbers using FV10-ASW 2.0 Viewer software (Olympus, Germany) to visualize the images obtained in the confocal microscope (600x magnification (oil), Olympus FV1000, Germany). The proliferation density was evaluated in the SGZ, and estimated as a quotient between the total number of Ki-67-positive cells and the area of the SGZ, which was determined on a motorized Olympus BX51 (Olympus, Germany) and Stereo Investigator 10 Software® (MicroBrightField, Williston, VT, USA).

Using the images obtained in the confocal microscope, astrocytic morphology was evaluated with the help of Simple Neurite Tracer software which allowed the 3D reconstruction of astrocytes. For each animal (n=5 per group), six astrocytes were selected in the DG and reconstructed. Total length of the astrocytic processes, total number of ramifications and also Sholl analysis was determined for each astrocyte. The measurements from single astrocytes were averaged from each animal and compared among experimental groups.

2.7. MOLECULAR ANALYSIS

2.7.1. RNA extraction, cDNA conversion and real-time PCR analysis

Total RNA was isolated from the macrodissected hippocampal tissue using the Direct-zol RNA miniPrep kit (Zymo Research, Irvine, CA, USA). Briefly, tissue was mechanically homogenized using a 20 G needle and a syringe in 600 µl of Qiazol Reagent (Qiagen, Valencia, CA, USA) and then total RNA was prepared according to the instructions of the manufacturer. Total RNA (500 ng) was reverse transcribed into cDNA using qScript™ cDNA SuperMix (Quanta Biosciences™, Gaithersburg, Md, USA).

Using PrimerBlast software (NCBI, USA), primers for Glutamate transporter-1 isoform-b (GLT-1), glial fibrillary acidic protein (GFAP), S100 calcium binding protein B (S100-B), signal transducer and activator of transcription 3 (STAT3) and Notch were designed (Table 3). RT-PCR plates were analyzed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, LLC, CA, USA) using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX; Solis Biodyne, Tartu, Estonia). Considering the expression levels of the housekeeping gene Beta-2-Microglobulin (B2M), the expression levels target genes were normalized and the relative expression was calculated applying the ΔΔCt method. Results are expressed as fold-change of mRNA levels between the corresponding experimental groups after normalization to B2M expression levels.

Table 3 . Sequences of primers used for real time RT-PCR and the corresponding product size.

Gene	Sequence	Product Size (bp)
GLT-1	**Fw** 5' AATGTGTCTATGCCGCACAC 3' **Rv** 5' GCAGGGGATGGTGCTTTT 3'	128
GFAP	**Fw** 5' GACCAGCTTACTACCAACAGTGCC 3' **Rv** 5' TGGTTTCATCTTGGAGCTTCTGCCT 3'	98
S100B	**Fw** 5' AGTCCTTGGACACCGAAGCCA 3' **Rv** 5' CTTCCTGCTCTTTGATTTCCTCCA 3'	219
STAT3	**Fw** 5' TGGACCGTCTGGAAAACTGGATAAC 3' **Rv** 5' CTCCACCACGAAGGCACTCTTCATTA 3'	194
Notch	**Fw** 5' CCACAGGCTGGCAAGGTCAAAC 3' **Rv** 5' ATGGGTTGGGTCGGCAGTCATC 3'	174

2.8. EXPERIMENTAL DESIGN

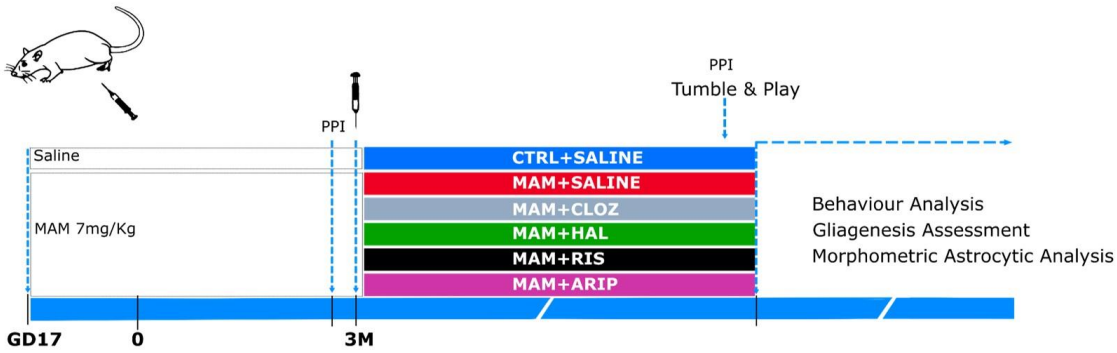


Figure 3 – Schematic representation of the experimental design. Pregnant rats were injected either with saline or MAM at gestational day 17. One week preceding the beginning of the antipsychotic treatment, and a week before the sacrifice, the prepulse inhibition test (PPI) was performed to assess sensorimotor gating. At the age of three months, animals were injected with saline or with an antipsychotic, for five weeks. One week before the sacrifice the animals performed the tumble and play test, to investigate social behavior.

2.9. DATA ANALYSIS

Statistical analysis was performed using SPSS (IBM, New York, USA). To investigate differences in the Sholl analysis of the astrocytes a repeated measures ANOVA was used and One-way ANOVA was used to analyze the remaining results. Differences between groups were measured by Tukey's honestly significant difference test (Tukey HSD) post hoc analysis. Statistical significance was accepted for $P< 0.05$. Results are expressed as mean± standard error of the mean (SEM).

3

Results

3. RESULTS

3.1. PREPULSE INHIBITION

Impaired prepulse inhibition (PPI) of the acoustic startle reflex illustrates a deficit in sensorimotor gating, which is a minor neurological symptom in schizophrenic patients. Since this reflex is highly conserved across species, PPI is widely used in rodent models of schizophrenia (Jones et al., 2011). PPI was evaluated one week before the beginning of antipsychotic treatment and after five weeks of antipsychotic treatment. The percentage of prepulse inhibition before the beginning of the treatment was not different between CTRL and MAM animals ($F_{1,15}$ = 0.661, p=0.429) or between MAM animals treated with saline, clozapine, haloperidol, risperidone or aripiprazole ($F_{4,45}$=0.066, p=0.992) (Figure 4). Likewise, prenatal exposure to the cytostatic agent MAM did not induce alterations in PPI at the end of the experiment ($F_{1,16}$=1.754, p=0.204) and this effect was not altered by treatment with antipsychotics with different pharmacological profiles ($F_{4,46}$=0.202, p=0.936).

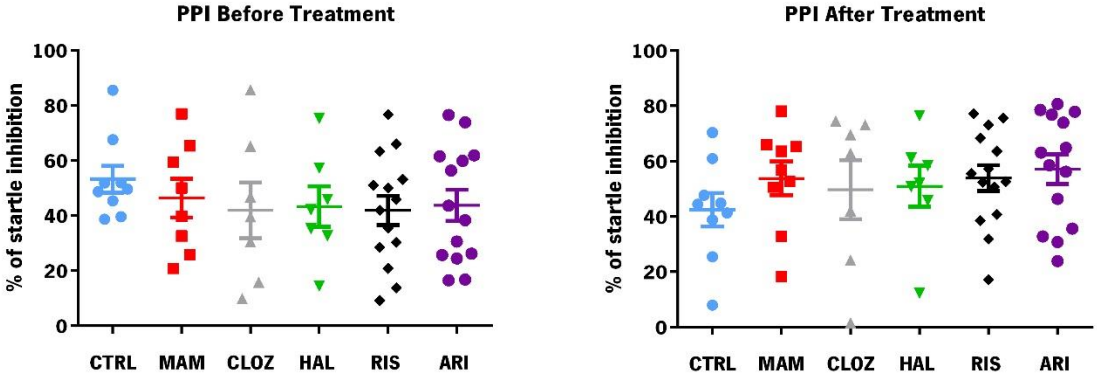


Figure 4 – Percentage of inhibition of the startle reflex measured before and after chronic treatment with antipsychotic drugs of different classes. The results are represented as mean ± SEM. N=9-14 animals per group.

3.2. EFFECTS OF ANTIPSYCHOTIC TREATMENT IN SOCIAL BEHAVIOR

To determine if MAM animals present deficits in playful behavior and if treatment with antipsychotics exerted any effect in this dimension, the tumble & play test was performed. As shown in figure 5a)), no differences were found in the request of play solicitation between control and MAM animals ($F_{1,10}$=0.335, p=0.576) or between MAM and treated animals ($F_{4,24}$=1.907, p=0.142). The response to playful behavior

was not different between control and MAM animals ($F_{1,10}$=3.107, p=0.108) or between MAM and any of the antipsychotic treated animals ($F_{4,24}$=0.847, p=0.509; figure 5b)). However, the probability of an animal responding to play solicitation (Figure 5c)) is significantly higher in control than MAM animals ($F_{1,10}$=10.703, p=0.008) and also significantly different between MAM and treated animals ($F_{4,24}$=2.360, p=0.05). Post hoc tests indicated that aripiprazole was the only group in which play acceptance was significantly different compared with MAM animals (p=0.044). Finally, no differences were found regarding social exploration between control and MAM groups ($F_{1,10}$=0.078, p=0.786) or between MAM and animals treated with antipsychotics ($F_{4,24}$=2.217, p=0.097; Figure 5d)).

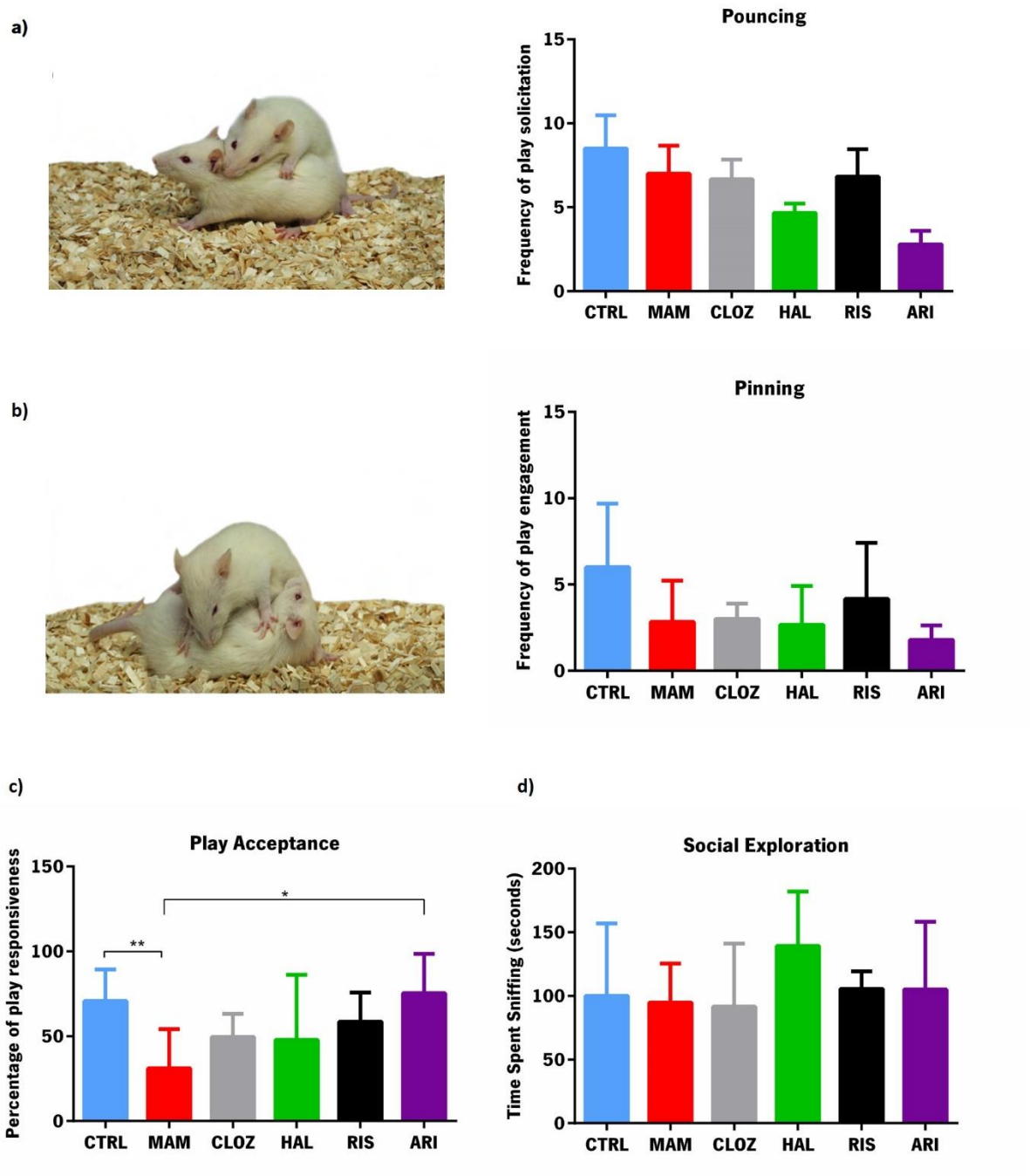


Figure 5 - Evaluation of playful behavior in control, MAM and treated animals. **a)** Number of solicitation of playful behavior; **b)** Frequency of engagement in playful behavior, as a response to the play solicitation; **c)** Percentage of responses to play solicitation. **d)** Time spent in social exploration, namely sniffing the body of the test partner. Data represented as mean±SEM. *p≤0.05, p≤0.01. n= 5 animals per group. Behavior examples adapted from Trezza *et al.* (2010).

3.3. GLIAGENESIS

To investigate the possible role of the formation of glial cells in the context of schizophrenia, the evaluation of recently formed astrocytes in this animal model was performed (in animals treated and untreated with antipsychotics of different classes) using an immunofluorescence protocol to mark proliferating cells with Ki-67, an endogenous proliferation marker, and GFAP that is a classical glial cell marker.

The density of proliferating cells in the SGZ was unaltered in MAM animals ($F_{1,8}$=2.358, p=0.164; Figure 6b)), and antipsychotic treatment did not induce any effects ($F_{4,19}$=0.590, p=0.674). However, the percentage of ki-67-positive cells that co-labelled with GFAP-immunopositive cells was significantly lower in MAM animals ($F_{1,8}$=23.018, p=0.001; Figure 6c)). Moreover, treatment with antipsychotics reverted the insult implemented by exposition to MAM ($F_{4,20}$=4.184, p=0.013). Specifically, post hoc tests revealed a tendency of clozapine to revert this effect (p=0.06) and that aripiprazole had a significant effect in this parameter (p=0.02).

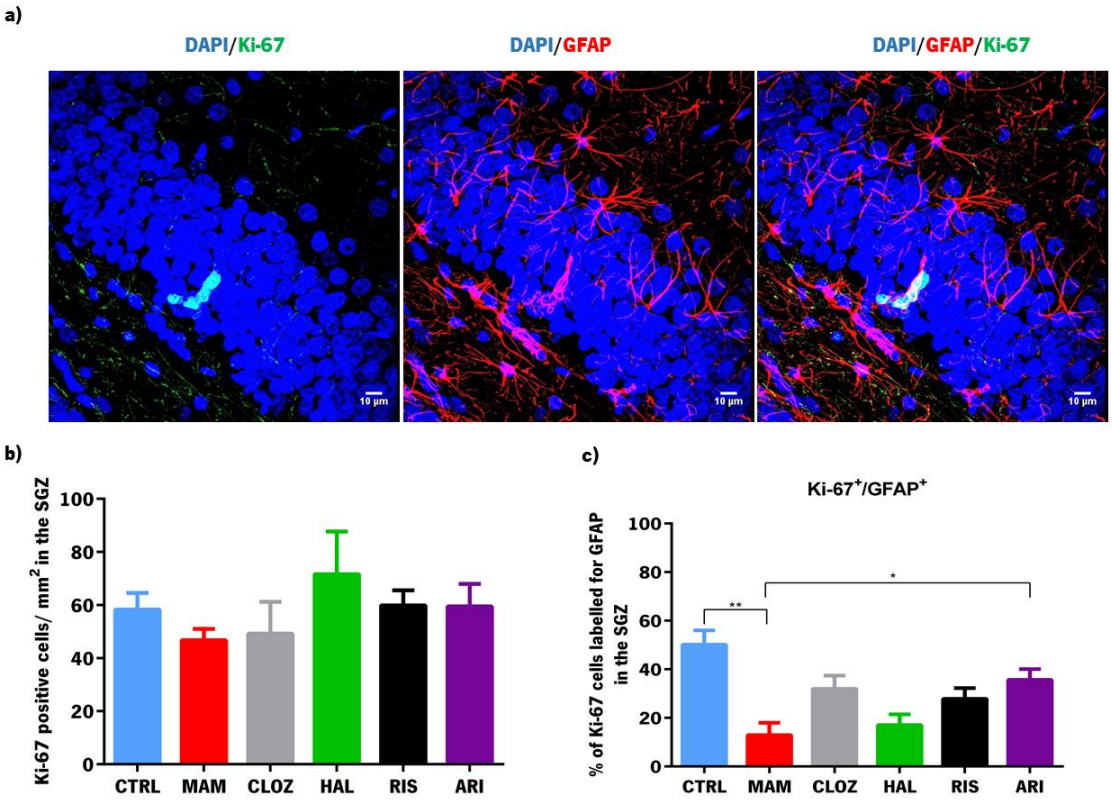


Figure 6 - Analysis of proliferating glial cells in the subgranular zone of the dentate gyrus of the hippocampus. a) Image of a niche of recently formed glial cells in the SGZ obtained by confocal

microscopy. **b)** Density of recently-mitotic cells immunopositive for the endogenous marker of proliferation Ki-67. **c)** Percentage of Ki-67 immunopositive cells that co-labelled with cells marked with GFAP. Results are presented as mean ±SEM. *p<0.05, **p≤0.01, ***p≤0.001. n= 5 animals per group.

3.4. ASTROCYTIC REMODELLING

The morphological evaluation of astrocytes revealed that MAM animals present a significant atrophy of the astrocytic processes, as measured by the total length ($F_{1,8}$=15.504, p=0.004; Figure 7b)). Additionally, antipsychotic treatment clearly reverted the insult established by the administration of the cytostatic agent MAM ($F_{4,20}$=5.621, p=0.003). Administration of the classical antipsychotic haloperidol (p=0.003) and the atypical antipsychotics clozapine (p=0.01) and risperidone (p=0.04) reverted the atrophy induced by prenatal MAM administration as measured by total length of the astrocytic processes.

The number of astrocytic processes was also affected by the exposition to MAM during gestation ($F_{1,8}$=13.547, p=0.006) as represented in Figure 7c)). The chronic treatment with antipsychotics inflicted a positive effect on the number of astrocytic processes ($F_{4,20}$=2.884, p=0.04). Furthermore, the classical antipsychotic haloperidol was the only drug that successfully reverted this effect (p=0.03).

Sholl analysis of the astrocytic processes revealed significantly less intersections in MAM animals, which is translated into a lower degree of complexity ($F_{1,9}$=7.994, p=0.02; Figure 7d)). The changes imposed by the anti-proliferative agent MAM were significantly affected by treatment with antipsychotics ($F_{4,21}$=3.292, p=0.03). Post hoc tests revealed that clozapine displayed a tendency to revert this effect of MAM (p=0.06) and haloperidol was able to restore astrocytic complexity (p=0.04).

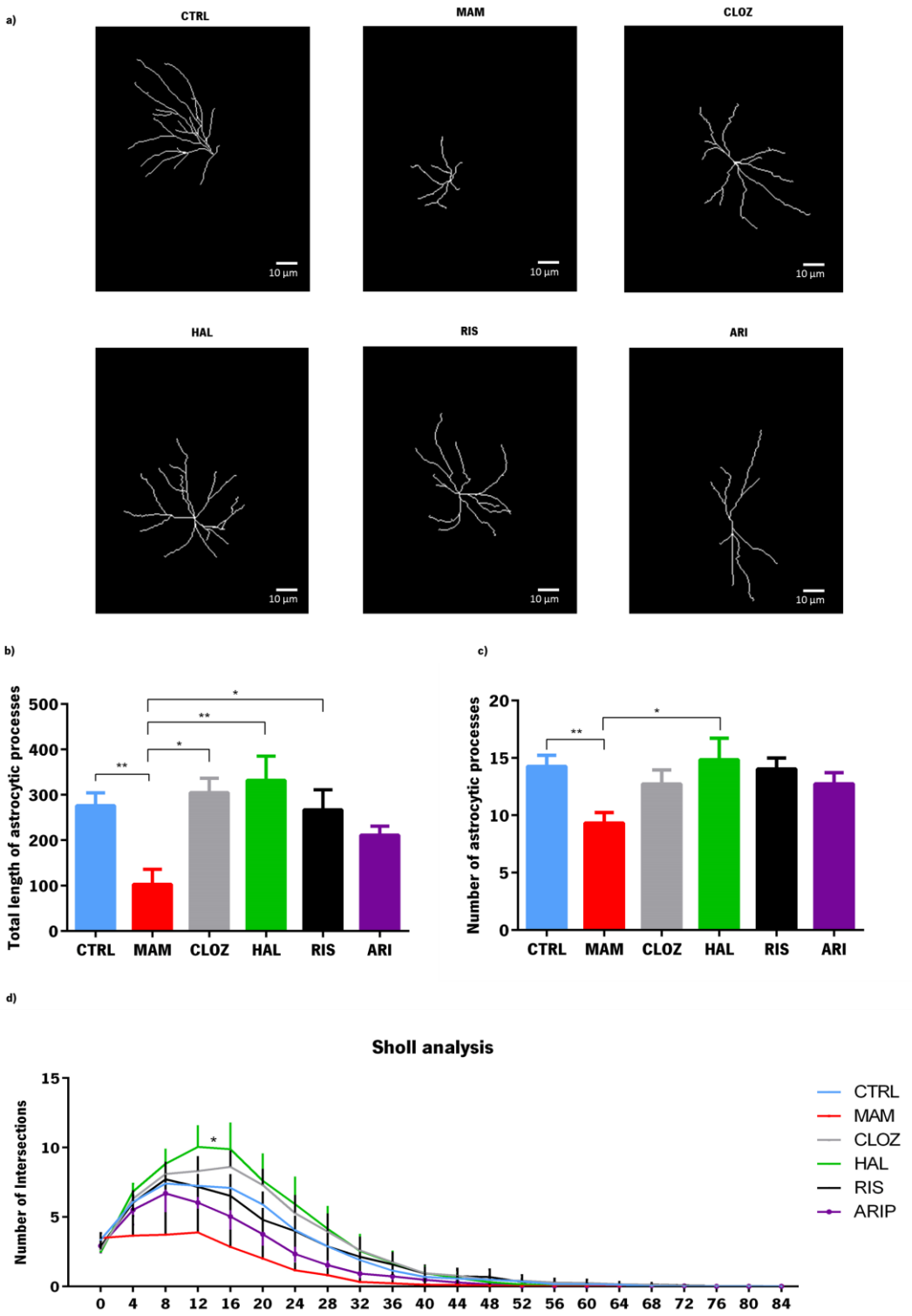


Figure 7 - Morphometric analysis of GFAP immunopositive astrocytes using Simple Neurite Tracer to reconstruct astrocytes in the dentate gyrus. **a)** Representative astrocytes of control, MAM, clozapine, haloperidol, risperidone and aripiprazole animals. **b)** Total length of astrocytic processes in the SGZ. **c)** Number of astrocytic processes in the SGZ. **d)** Sholl analysis of the distribution of astrocytic processes in the SGZ. Data represented as mean ±SEM. *p<0.05, **p≤0.01. n= 5 animals per group.

3.5. GENE EXPRESSION

In order to assess possible molecular pathways affected by MAM as well as possible effects of antipsychotics, the hippocampal mRNA expression of Notch, S100B, GFAP, GLT-1 and STAT3 was quantified by quantitative real time (RT)-PCR analysis.

The expression of Notch in the hippocampus of MAM animals was unaltered ($F_{1,8}$=1.269, p=0.297) and the antipsychotic treatment did not impact the expression of this gene ($F_{4,21}$=0.335, p=0.851).

In the hippocampus, neither MAM admnistration ($F_{1,8}$=0.099, p=0.763) or treatment with antipsychotics had an impact in the levels of S100B mRNA ($F_{4,21}$=1.431, p=0.267).

The admnistration of MAM did not interfere with gene transcript levels of GFAP ($F_{1,11}$=0.001, p=0.976), and no effect was also observed with treatment with antipsychotics ($F_{4,27}$=0.889, p=0.486).

Similarly, the levels of GLT-1 transcript levels were not affected by cytostatic agent MAM ($F_{1,9}$=0.956, p=0.838) or by chronic treatment with any of the antipsychotics used in the study ($F_{4,26}$=0.355, p=0.838).

Likewise, STAT3 mRNA expression in the hippocampus was not significantly different neither in MAM ($F_{1,11}$=0.372, p=0.555) or treated animals ($F_{4,27}$=1.535, p=0.255).

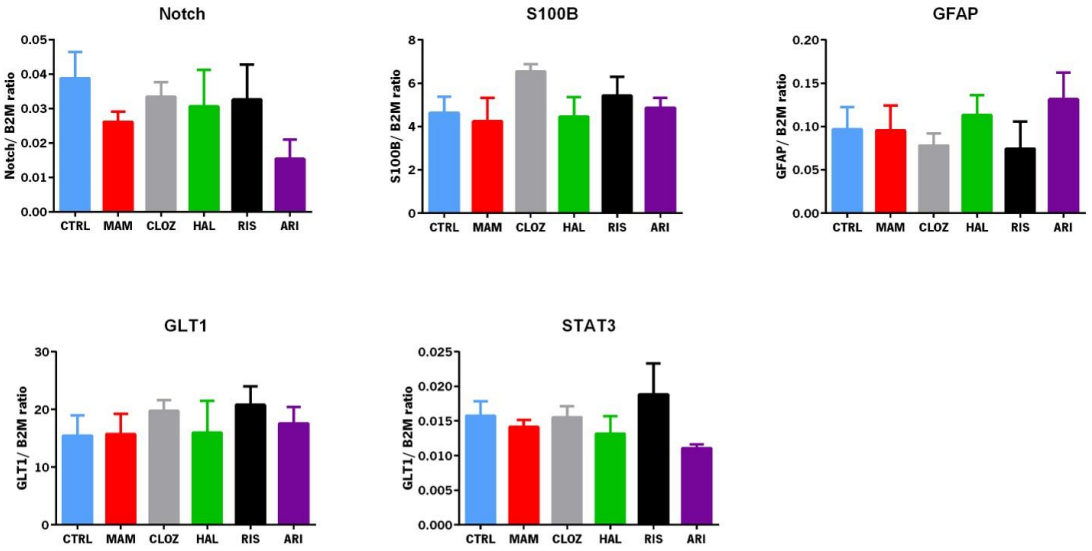


Figure 8 - MAM insult did not affect hippocampal mRNA expression levels of Notch, S100B, GFAP, GLT1 and STAT3. Data represented as mean ±SEM. n= 5 animals per group.

4

Discussion

4. DISCUSSION

Animal models are highly valuable in the study of complex heterogeneous psychiatric disorders, representing valuable preclinical tools to explore the underlying mechanisms of disorders such as schizophrenia. However, some of the core symptoms of psychiatric disorders such as verbal learning and thoughts are unique human features, thus impossible to assess in animals (Jones *et al.*, 2011). In this study, a neurodevelopmental model of schizophrenia – the administration of cytostatic agent methylazoxymethanol acetate (MAM) at gestational day 17 – was used. As previously referred, neonatal exposure to environmental insults represents a major risk factor of the development of schizophrenia (Matheson *et al.*, 2011). Also, *in utero* exposure to MAM impairs embryonic neurodevelopment, i) leading to behavioral dysfunction at cognitive and sensorimotor gating levels; ii) inducing anatomical and histological disturbances, such as increases in neuronal density (medial prefrontal cortex, parahippocampal cortex, hippocampus) and decreases in cortical thickness; iii) neurophysiological impairments, as striatal hyperdopaminergia, corticocortical synaptic transmission and impaired glutamatergic neurotransmission in the hippocampus; iv) pharmacological impacts, such as quick onset of antipsychotic drug effects on dopaminergic neurons and increased sensitivity to psychostimulants such as phencyclidine (PCP) and amphetamine (Modinos *et al.*, 2015). Nonetheless, it has been discussed whether cortical dopaminergic dysfunction and sensorimotor gating are a cause (reflecting symptoms of the disorder) or a consequence of impaired development of frontal cortical-limbic circuits in the MAM model, along with other models such as chronic phencyclidine (PCP) administration and neonatal hippocampal lesion (Jones *et al.*, 2011).

In the present study, MAM animals did not reveal deficits in the prepulse inhibition test (PPI), which is a widely used test in rodent models of schizophrenia. This paradigm is very popular as a result of the clinical observations that patients with schizophrenia are not able to filter irrelevant sensory stimuli, which resulted in the construct of the gating deficits of schizophrenia (Swerdlow *et al.*, 2008). However, PPI is not used in the clinics as a diagnostic measure. Besides, in addition to the considerable variability, there is also a vast overlap in PPI distribution among affected and normal populations, apart from the fact that PPI deficits are also observable in other disorders (Swerdlow *et al.*, 2008). Interestingly, in animal models, although most groups describe PPI impairment in the offspring of mothers injected with MAM at GD17 (Hazane *et al.*, 2009; Moore *et al.*, 2006), one group only found PPI impairments in GD10 or GD11 MAM rats (Talamini *et al.*, 2000).

Nonetheless, negative symptoms such as anhedonia, blunted affect, poverty of speech and lack of motivation to engage in social interaction have acquired increased relevance in the field of schizophrenia

(Millan *et al.*, 2014). Particularly, social withdrawal is one of the first signs of psychosis that predicts the development of schizophrenia (Maki *et al.*, 2014; Velthorst *et al.*, 2012). Likewise, social withdrawal is part of a constellation of behavioral impairments that must be present in animal models of schizophrenia to consolidate their face validity (Jones *et al.*, 2011). In fact, it has been shown that well established animals models such as chronic phencyclidine administration (PCP), a non-competitive NMDA antagonist which aggravates psychotic symptoms and induce psychomimetic symptoms in healthy subjects (Modinos *et al.*, 2015), causes long-lasting deficits regarding social withdrawal (Wilson & Koenig, 2014) as well as reduced active social interaction in young male offspring of pregnant rats injected with MAM at GD17 (Flagstad *et al.*, 2004). Social play behavior is one the first manifestations of mammalian interaction that is directed not to the mother, but at peers, representing an index of healthy development (Trezza *et al.*, 2010). Likewise, social deficits has been shown to represent a core symptom in early-onset schizophrenia (Møller & Husby, 2000). In this study, the Tumble & Play test was used to assess playful behavior. A remarkable advantage of employing this test is the fact that it is a very natural behavior of the animals, performed in a regular housing cage, thus reducing stress. Interestingly, play responsiveness was found to be reduced in MAM animals, confirming once again social deficits in this animal model. Furthermore, aripiprazole was the only antipsychotic drug able to revert this effect in play acceptance induced by prenatal MAM administration. Curiously, despite the low levels of play solicitation observed in animals treated with aripiprazole, they frequently engaged in playful behavior in response to solicitations, resulting in higher percentage of play acceptance. The most interesting characteristic of aripiprazole is that it acts as a high affinity partial agonist of D2 receptors. Considering the beneficial effects of dopamine in social behavior (Krach *et al.*, 2010), it is feasible to hypothesize that the blockage of dopamine receptors by the remaining antipsychotic drugs used in this study is preventing dopamine from exerting a favorable effect. Therefore, in the future it would be interesting to perform microdialysis in freely moving animals during the performance of the tumble and play test, to measure the levels of dopamine. Additionally, each measure could have been evaluated for each individual animal, and the latency to make the first contact could be evaluated to get a more extensive readout of this paradigm. As an alternative, social conditioned place preference test could have been utilized as it is a widely used test to assess social rewarding, which might enlighten the role of antipsychotics in social behavior since conditioned place preference is directly related to the dopaminergic system (Assar *et al.*, 2016).

Interestingly, several studies have linked social environment to cell plasticity, particularly cell proliferation and adult neurogenesis in the dentate gyrus of the hippocampus (Dranovsky *et al.*, 2011; Leasure & Decker, 2009; Lu *et al.*, 2003; Stranahan *et al.*, 2006). In 2006, a remarkable post-mortem

study by Reif and colleagues reported that neural stem cell proliferation is significantly reduced in the dentate gyrus of the hippocampus in brains of schizophrenic patients, suggesting an involvement of adult neurogenesis or adult gliogenesis in the etiology of schizophrenia (Reif *et al.*, 2006). As previously referred, despite the increasing amount of evidence that suggests that astrocytes are much more than a sidekick to neurons in the central nervous system, there is still a great gap regarding knowledge on astrocytes, particularly on possible involvement of the formation of new glial cells in the etiology of psychiatric disorders. In the present study, cell proliferation in the dentate gyrus of the hippocampus was found unaltered on animals exposed to MAM during gestation. More interestingly, the percentage of recently mitotic glial cells was dramatically reduced by MAM and improved by chronic administration of the antipsychotic drug aripiprazole. Thus, in this dimension the modulation of dopamine D2 receptors seems to also exert a possible effect on the formation of new glial cells since aripiprazole partially activates D2 receptor output, and this effect is not observed with treatment with the other antipsychotics tested in this study. A recent study in a widely used genetic mouse model of the disease, DISC1 knockdown mice, revealed that these animals present a deficit in gliogenesis that is reversed by the increase of expression levels of DISC1 (Wang *et al.*, 2016), confirming that gliogenesis is a potential candidate target to study the basis of schizophrenia.

Neuroplasticity is the capacity of the brain to alter and adapt in response to modifications in its input, by creation of new circuits, adjusting the number of neurons or glia, synaptic remodelling, alteration of the number of dendritic spines, among many other mechanisms (McCullumsmith, 2015). This process has already been reported to be altered in the hippocampus of schizophrenic patients, namely in the form of synaptic remodelling, by means of increased spine density on CA3 pyramidal cell apical dendrites (Li *et al.*, 2015) and antipsychotic drugs have been proven to modulate neuroplasticity (Konradi & Heckers, 2001). Another study has found evidence of glial pathology on the prefrontal cortex of schizophrenic patients, illustrated by the reduction of the area occupied by GFAP-immunoreactive cell bodies and processes (Rajkowska *et al.*, 2002). However, there is still a lack of information regarding astrocytic plastic events in the hippocampus in the context of schizophrenia. In this study, morphometric analysis of astrocytes was performed using a novel informatics technique that allows the reconstruction of GFAP immunopositive astrocytes. A decrease in the number of astrocytic processes of astrocytes in the dentate gyrus of animals exposed to MAM was observed. Remarkably, treatment with haloperidol completely reverted this deficit induced by the prenatal administration of MAM. This effect could be explained as a compensatory mechanism to balance the observed decrease of newly formed glial cells in the dentate gyrus of these animals, thus attempting to establish more connections by means of increasing the number

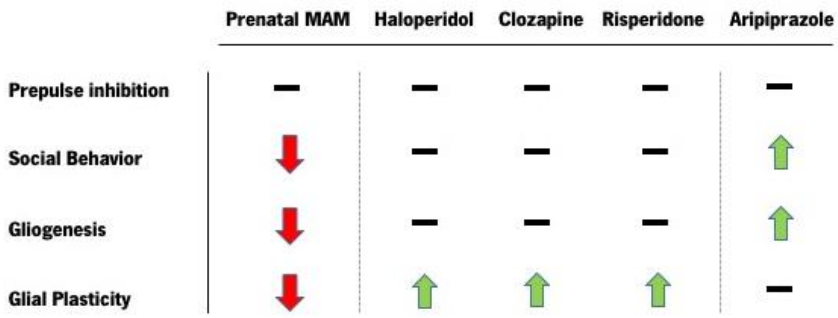
of astrocytic processes. Likewise, the total length of astrocytic processes is considerably diminished in the hippocampal dentate gyrus of MAM animals. The treatment with clozapine, risperidone and haloperidol reverted the deficiency imposed by the administration of MAM, which implicates a possible involvement of dopamine D2 receptors. The common link between these drugs is the D2 antagonism along with the fact that haloperidol, that revealed the higher levels of total length of astrocytic processes, presents higher affinity for these receptors. This suggests that even though the blockage of dopamine receptors is not boosting the formation of new glial cells, the high affinity for D2 receptors is in fact enhancing the remodelling of mature astrocytes, by increasing the number of its processes and their total length. Moreover, Sholl analysis which is a measure that indicates the degree of astrocytic complexity, revealed once more that astrocytes are in fact less complex in the hippocampus of MAM animals and that this effect is only reverted by treatment with haloperidol, reflecting again a possible compensatory mechanism dependent on the modulation of dopamine receptors.

Additionally, this study explored multiple astrocytic-related genes since an increasing amount evidence suggest an involvement of glial pathology in this disorder, as previously referred. Notch signaling is important for the regulation of asymmetric cell division, cell fate specification and cell morphogenesis in development (Kopan & Ilagan, 2009). Furthermore, this pathway is also involved in the formation of myelin (Hu *et al.*, 2003), and interestingly some evidence suggest myelin abnormalities in the context of schizophrenia (Davis *et al.*, 2003). However, in this study the levels of mRNA expression of Notch in the hippocampus of MAM animals were found unaltered, though a recent study in an animal model of demyelination that exhibits behavioral characteristics of schizophrenia suggests an involvement of Notch signaling pathway in the behavioral amelioration induced by an atypical antipsychotic, quetiapine (Wang *et al.*, 2015). Also, S100B is a calcium binding protein, expressed by astrocytes, that has been found to be altered in schizophrenia (Yelmo-Cruz *et al.*, 2013). In this study, the levels of expression of S100B mRNA failed to reveal any differences in the hippocampus of MAM animals. Even though several studies have linked increased levels of S100B with schizophrenia (O'Connell *et al.*, 2013; Rothermundt *et al.*, 2004), other reports failed to reveal significant changes in the levels of S100B (Uzbay *et al.*, 2013; van der Leeuw *et al.*, 2013), which is in line with the results obtained in this study. Likewise, levels of GFAP were found unaltered in MAM animals – although levels of GFAP mRNA have been found reduced in the white matter of the anterior cingulate cortex of schizophrenic patients (Webster *et al.*, 2005), indicating a region-specific effect, other reports failed to identify altered densities of GFAP in frontal or temporal association cortices (Garey, 2010). In this study, the levels of GLT-1 mRNA were also found unaffected by the admnistration of MAM during gestation which is line with a report from Catalano that failed to find

an association between a genetic polymorphism of EAAT2 (the human homologue of GLT-1) and schizophrenia (Catalano *et al.*, 2002). Nonetheless, recent studies have linked polymorphisms of EAAT2 to deficits in working memory in schizophrenic patients (Poletti *et al.*, 2014; Spangaro *et al.*, 2012). Also, STAT3 is involved in the neurogenesis to gliogenesis switch, activating the pathway, promoting the expression of GFAP (Miller & Gauthier, 2007). As one of the main goals in this study was to assess the role of gliogenesis in this animal model, the mRNA levels of STAT3 in the hippocampus of these animals was measured, revealing that the expression of these gene is unaltered. Nevertheless, further studies are needed evaluating the expression of this gene in other brain regions, such as the subventricular zone, where the proliferation of neural stem cells also occurs, and even if the fact that the RNA was isolated from the whole hippocampus, may be confounding region-specific patterns of expression on the different hippocampal subfields.

In summary, the present study revealed for the first time that the prenatal exposure to the alkylating agent MAM induced significant behavioral deficits in social behavior and impairments in gliogenesis and glial plasticity in adulthood (Table 4). Interestingly, the effects of different antipsychotic drugs were clearly related with their pharmacological mechanism of action. While the dopamine partial agonist aripiprazole reversed the social behavior deficits and potentiated adult gliogenesis, the dopamine receptor antagonists haloperidol, clozapine and risperidone had no significant behavioral effects but were able to restore glial morphology in this model. These observations suggest that adult gliogenesis may play a key role in social behavior in the context of schizophrenia and that the development of new drugs that potentiate the generation of new astrocytes may pave a new way in the treatment of the negative symptoms of schizophrenia. Furthermore, the modulation of glial morphology by dopamine antagonists, apart from being a possible compensatory mechanism for the impairments in gliogenesis, offer a new perspective regarding the role of pre-existing astrocytes in the onset and recovery from schizophrenia.

Table 4 - Prenatal exposure to MAM induced deficits in social behavior, gliogenesis and glial plasticity. Adult rats exposed in utero to the cytostatic agent MAM do not show deficits in the prepulse inhibition of the acoustic startle (PPI), but reveal however deficits in social play behavior, evaluated by the Tumble & Play test, and also impairments at the level of the formation of glial cells, as well as in the morphology of adult astrocytes in the hippocampus. This novel study shows that a third-generation antipsychotic, aripiprazole, which is a partial dopamine agonist, is the only drug used in this study that reverts the impairments imposed by the administration of MAM on social behavior and gliogenesis. On the other hand, it was also the only drug that did not revert the impact of MAM at astrocytic remodeling, and interestingly haloperidol, which is a high affinity dopamine antagonist seems to be the most effective drug at the level of astrocytic plasticity, suggesting that this might be a mechanism to compensate the low levels of gliogenesis observed.



5

Conclusions

5. CONCLUSIONS

Cumulative evidence has shown that the size and burden of mental disorders, such as schizophrenia, is becoming a major health and socioeconomic challenge of this century, thus emphasizing the need for discovery of diagnostic markers and better treatments to deal with these disorders and provide better quality of life for patients and their beloved ones.

The results of this study suggest that antipsychotics with different pharmacological profiles exert a differential effect on social behavior, formation of glial cells and glial morphology in an animal model of schizophrenia. However, the role of astrocytes on social behavior and its modulation by antipsychotics should be further explored using optogenetics to silence astrocytes in the hippocampus, for example, in order to evaluate the astrocytic involvement in the observed social behavior impairments, that interestingly are only reverted by treatment with a third-generation antipsychotic.

This study shows, for the first time, that prenatal exposure to the cytostatic agent MAM is an interesting model to study the astrocytic involvement in schizophrenia, since this agent clearly impairs the number of recently formed glial cells and also astrocytic plasticity. Furthermore, the results of this study show that even though aripiprazole reverted the deficits induced by prenatal exposure to MAM on the formation of new glial cells, it was also the only drug unable to revert impairments at the level of astrocytic complexity, suggesting a compensatory mechanism. It would be interesting to perform the same evaluations, using an exogenous proliferation marker such as bromodeoxyuridine (BrdU) to assess the long-term effects of these pharmaceutical compounds.

As the assessment of astrocytic-related genes was not conclusive, future studies should target the expression of these genes specifically in the dentate gyrus of the hippocampus and also in other affected brain regions in schizophrenia.

Finally, the results of this study clearly state that the modulation of astrocytes may be important in the effects of antipsychotics and further studies should consider the involvement of astrocytes to improve the development of new treatments for schizophrenia.

6

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