Further characterization of the Roman rats as a model of behavioral, neuroanatomical and neurochemical schizophrenia-relevant features

Ana Sánchez González

Autonomous University of Barcelona

Institute of Neurosciences
Department of Psychiatry and Forensic Medicine
Medical Psychology Unit

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Doctoral Dissertation presented by

Ana Sánchez González

In order to opt for the Degree of Doctor in Neurosciences, under the supervision of

Dr. Albert Fernández Teruel

Institute of Neurosciences
Department of Psychiatry and Forensic Medicine
Medical Psychology Unit

Dr. Susana Aznar Kleijn

Research Laboratory for Stereology and Neuroscience
Bispebjerg and Frederiksberg Hospitals

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Abstract

The present Doctoral Dissertation gathers a series of studies in which we have tried to collect as much evidence as possible in a behavioral, neuroanatomical and molecular level with the aim of validating a new animal model for the study of schizophrenia-relevant symptoms. Such model is constituted by the Roman rats, two inbred strains of rats that have been psychogenetically selected by their good (RHA-I) versus extremely poor (RLA-I) acquisition of the two-way active avoidance task in the shuttle box, giving as a result two strains with well-defined and differential profiles in stress sensitivity, anxiety/fearfulness, impulsivity, vulnerability to drugs of abuse and phenotypes related to the dopaminergic, serotoninergic and glutamatergic neurotransmission systems. In the present work, we have carried out a number of experiments devoted to complete the characterization of the Roman rats in different behavioral, neurochemical and neuroanatomical phenotypes related to schizophrenia.

We have conducted studies on behavioral paradigms relevant for schizophrenia, such as the prepulse inhibition of the acoustic startle response (PPI) and the latent inhibition (LI) effect (studies 1 and 2) in which we have obtained that the RHA-I rats present an impaired PPI as well as LI, which suggest that these two phenotypes are somehow related and may share common underlying mechanisms.

We have also tested the effects of a chronic environmental manipulation such as the isolation rearing of the animals (study 3), in (i) a behavioral level with an extensive battery of tests for schizophrenia-relevant phenotypes, (ii) a molecular level, with the analysis of the 5HT2A receptor density and (iii) an anatomical level with the estimation of the volume of three areas relevant for schizophrenia, such as the prefrontal cortex (PFC), the dorsal striatum (dST) and the hippocampus (HPC). We have observed that, while the behavioral profile has followed our initial expectations of more profound deficits induced by social isolation in the RHA-I rats, we have obtained paradoxical results on the binding studies and volume estimation analysis. Thus, although no differences have been observed in the 5HT2A receptor binding density, we have found between-strain differences in the volume of PFC, HPC and dST (RLA-I>RHA-I in every area), and a global effect of the treatment in the PFC volume.

From the need of transferring the Roman rats from the animal facilities in our lab (where they have been maintained since 1993) to a new SPF animal facility within the Autonomous University of Barcelona, we carried out an embryo transfer

procedure (study 4), and we phenotyped the 5th generation (G5) of animals from this new SPF colony. We observed that this new SPF colony of Roman rats display the typical between-strain differences in the G5 in every phenotype tested, both behavioral and hormonal.

Lastly, according to the results obtained in study 3, in which we observed a difference in the mPFC volume between RHA-I and RLA rats, we decided to study some of the possible causes of this difference in the new SPF colony of Roman rats. In this regard, we studied two parameters that are thought to be altered in schizophrenia, the dendritic spine density and the number of parvalbumin-expressing (PV+) neurons in the PFC (study 5). We observed that there is, indeed, a significant difference in the spine density between the two strains, with a higher percentage of small spines in RHA-I rats and a higher percentage of large spines in the RLA-I rats, while the number of PV+ neurons was not different between the strains.

The data gathered in the present Dissertation adds value to the proposition of the RHA-I rat strain as a useful tool for the study of some relevant symptoms and neurobiological features relevant to schizophrenia.

Resumen

La presente Tesis Doctoral concentra una serie de estudios en los que hemos intentado reunir la mayor cantidad de evidencia posible a un nivel conductual, anatómico y neuroquímico con el objetivo de validar un nuevo modelo animal para el estudio de síntomas relevantes para la esquizofrenia. Este modelo está constituido por las ratas Romanas, dos cepas de ratas que han sido seleccionadas por su rápida (RHA-I) versus extremadamente pobre (RLA-I) adquisición de la tarea de evitación activa en dos sentidos en la "*shuttle box*", lo que ha dado como resultado dos cepas con perfiles marcadamente diferentes en sensibilidad al estrés, ansiedad/miedo, impulsividad, vulnerabilidad a drogas de abuso y fenotipos relacionados con los sistemas de neurotransmisión dopaminérica, serotoninérgica y glutamatérgica. En el presente trabajo hemos realizado una serie de experimentos destinados a completar la caracterización de las ratas Romanas en diversos fenotipos comportamentales, neuroquímicos y neuroanatómicos, relacionados con la esquizofrenia.

Hemos llevado a cabo estudios con dos paradigmas conductuales relevantes para la esquizofrenia, como son la inhibición prepulso de la respuesta de sobresalto acústica (IPP) y la inhibición latente (IL) (estudios 1 y 2), en los que hemos obtenido que las ratas RHA-I presentan una IPP e IL afectadas, indicando que estos dos fenotipos están relacionados de alguna manera y puede que compartan mecanismos subyacentes comunes.

Del mismo modo, hemos testado los efectos de una manipulación ambiental crónica como es la crianza de los animales en aislamiento (estudio 3), a nivel (i) conductual con una extensa batería de tests para fenotipos relevantes para la esquizofrenia, (ii) molecular, con el análisis de la densidad del receptor 5HT2A, y (iii) anatómico con la estimación del volumen de tres áreas relevantes para la esquizofrenia como el córtex prefrontal (PFC), el estriado dorsal (dSt) y el hipocampo (HPC). Hemos constatado que, mientras que el perfil conductual ha seguido nuestras expectativas iniciales de mayores déficits inducidos por el aislamiento social en las ratas RHA-I, hemos obtenido resultados controvertidos en los análisis de densidad de receptor y estimación de volumen. Asi, si bien no se han observado diferencias en densidad de receptores 5HT2A, sí que existen diferencias entre las cepas en volumen del PFC, HPC y dSt (RLA-I>RHA-I en todos los casos), y el aislamiento social induce un incremento global del volumen del PFC.

Además, por la necesidad de transferir los animales desde las instalaciones en nuestro laboratorio, donde han sido mantenidas desde 1993, a una nueva instalación de tipo SPF (libre de patógenos específicos) en la Universidad Autónoma de Barcelona, llevamos a cabo un procedimiento de transferencia embrionaria (estudio 4) y testamos la quinta generación (G5) de animales de esta nueva colonia SPF en los fenotipos más comúnmente usados en la caracterización de las ratas Romanas. De estos análisis observamos que estas nuevas cepas Romanas SPF presentan en la G5 las diferencias típicas en la totalidad de los fenotipos estudiados, tanto conductuales como hormonales.

Por último, dados los resultados obtenidos en el estudio 3 en los que observamos una diferencia en volumen de PFC entre ratas RHA-I y RLA-I, decidimos estudiar algunas de las posibles causas de esta diferencia en las nuevas cepas de ratas SPF. En este sentido, estudiamos dos parámetros que se creen están afectados en la esquizofrenia, la densidad de espinas dendríticas y el número de neuronas que expresan parvalbúmina (PV) en el PFC (estudio 5). De estos experimentos observamos que existe una diferencia significativa en la densidad de espinas entre ambas cepas, con un mayor porcentaje de espinas pequeñas en las RHA-I y mayor porcentaje de espinas grandes en las ratas RLA-I, mientras que el número de neuronas PV no presenta diferencias.

Los datos recogidos en esta Tesis añaden valor a la proposición de las ratas RHA-I como una herramienta útil en el estudio de algunos síntomas comportamentales y características neurobiológicas relevantes para la esquizofrenia.

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Abbreviations

5HT: serotonin

$5HT_{1-7}$: serotonin receptor subtypes

ACTH: Adrenocorticotropic hormone

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

APO-SUS: Apomorphine susceptibility

APO-UNSUS: Apomorphine unsusceptibility

ASR: Acoustic startle response

BAS: Baseline acoustic startle response

CNS: central nervous system

CS: Conditioned stimulus

D1-4: dopamine receptor subtypes

dB: Decibel

dSt: Dorsal Striatum

DLPFC: Dorsolateral Prefrontal Cortex

DOI: 2,5-Dimethoxy-4-iodoamphetamine

ET: Embryo Transfer

FPS: Fear potentiated startle

GABA: Gamma-Aminobutyric acid

HPC: Hippocampus

ITC: Intertrial crossing

ITI: Intertrial interval

LI: Latent inhibition

LSD: Lysergic acid diethylamide

$mGlu_{1-8}$: glutamate receptor subtypes

mPFC: Medial Prefrontal Cortex

MWM: Morris Water Maze

NIH-HS: National Institute of Health N/Nih Genetically Heterogeneous Rat stock

NIMH: National Institute of Mental Health

NMDA: N-Methyl-D-aspartate

NPE: Non pre-exposed

PCP: Phencyclidine

PE: Pre-exposed

PFC: Prefrontal cortex

PPI: Prepulse inhibition

PV: Parvalbumin

RHA: Roman High Avoidance

RLA: Roman Low Avoidance

SIR: Social Isolation Rearing

SPF: Specific Pathogen Free

US: Unconditioned stimulus

WM: Working Memory

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Introduction

1.1 A Brief History of Schizophrenia

Schizophrenia is defined by the National Institute of Mental Health as a chronic, severe and disabling brain disorder that is characterized by an abnormal social behavior [1]. It affects approximately 1% of the world population, with a first episode of psychosis usually in late adolescence or early adulthood [2]. However, this incidence varies depending on the diagnostic definition of schizophrenia used [3] since one of the main characteristic of this disorder relies on the difficulty of its diagnosis.

This disorder was first described by the German physician Emil Kraepelin in 1887 under the name of *"dementia praecox"* [4] to describe a set of disorders with unknown causes but similar symptoms with affected cognitive functioning (dementia) and a premature onset (praecox) [5,6]. Twenty years later, the Swiss Eugen Bleuler coined the term "*schizophrenia"* [7], from the Greek roots *schizo* (split) and *phrene* (mind), to replace Kraepelin's *dementia praecox* [8]. Contrary to the still existing and misunderstood idea of multiple or split personality, Bleuler's term referred to the fragmented thinking or dissociation of thoughts that characterizes people with this disorder [9] and stated that schizophrenia "is not a disease in the strict sense, but a group of diseases" [5]. Since Bleuler, the term schizophrenia has remained but its definition has continued to change to more accurately delimitate the broad field of mental diseases based on observation and classification of symptoms, given the (yet) unknown causes of the disease [8].

1.2 Symptoms

Apart from coining the term, Bleuler also differentiated between fundamental (present in all patients and unique to this disorder) and accessory (could be present in other disorders) symptoms[5]. In his definition, the fundamental symptoms included thought and speech derailment, volitional indeterminacy, affective incongruence and autism, while the accessory symptoms would include delusions and hallucinations (which today are commonly referred to as positive symptoms) [5]. In the following years, a number of clinicians made modifications to the concept of schizophrenia and the criteria to follow to make a diagnosis, from Schneider's "first rank symptoms" (1959) [10] to Leonhard's "endogenous" psychoses (1999) [11]. The work of Kraepelin, Bleuler and many others led to the development of a common diagnostic criteria with the creation and successive editions of the Diagnostic and Statistical Manual of Mental Disorders (DSM) by the American Psychiatric Association that provides a standardized classification

system. According to the last edition published (DSM-V, 2013) two of the five key symptoms of psychotic disorders (delusions, hallucinations, disorganized speech, disorganized or catatonic behavior and negative symptoms) are required, and at least one symptom must belong to one of the first three (delusions, hallucinations and/or disorganized speech) [12]. Furthermore, these symptoms should have a duration of at least 6 months, including one month of active-phase symptoms [13]. The DSM-V diagnostic criteria for schizophrenia is displayed in table 1.

Table 1. Diagnostic Criteria for Schizophrenia.

A. Two (or more) of the following, each present for a significant portion of time during a one-month period (or less if successfully treated). At least one of these must be (1), (2), or (3):
   1. Delusions
   2. Hallucinations
   3. Disorganized speech (e.g. frequent derailment or incoherence)
   4. Grossly disorganized or catatonic behavior
   5. Negative symptoms (i.e. diminished emotional expression or abolition)

B. For a significant portion of the time since the onset of the disturbance, level of functioning in one or more major areas, such as work, interpersonal relations, or self-care, is markedly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, there is failure to achieve expected level of interpersonal, academic, or occupational functioning)

C. Continuous signs of the disturbance persist for at least 6 months. This 6-month period must include at least 1 month of symptoms (or less if successfully treated) that meet Criterion A (i.e., active-phase symptoms) and may include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or by two or more symptoms listed in Criterion A present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences)

D. Schizoaffective disorder and depressive or bipolar disorder with psychotic features have been ruled out because either: (1) no major depressive or manic episodes have occurred concurrently with the active-phase symptoms, or (2) if mood episodes have occurred during active-phase symptoms, they have been present for a minority of the total duration of the active and residual periods of the illness

E. The disturbance is not attributable to the physiological effects of a substance (e.g., a drug of abuse, a medication) or another medical condition

F. If there is a history of autism spectrum disorder or a communication disorder of childhood onset, the additional diagnosis of schizophrenia is made only if prominent delusions or hallucinations, in addition to the other required symptoms of schizophrenia, are also present for at least 1 month (or less if successfully treated)

Nowadays, the most accepted classification divides the symptoms in the next three categories:

1.2.1 Positive symptoms – "adding onto reality"

The positive symptoms of schizophrenia refer to those behaviors that are "acquired but not wanted" and that are not normally found in unaffected individuals. This category includes hallucinations, delusions and disorganized speech [1]. These symptoms tend to relapse and remit with treatment [2].

1.2.2 Negative symptoms – "lacking of something"

These symptoms are associated with a disruption to normal emotions and behaviors. These are characteristics that unaffected individuals present but that are absent in schizophrenia, and include anhedonia (reduced feelings of pleasure), apathy, flat affect (reduced expression of emotion) or difficulties beginning and sustaining activities [1]. These symptoms tend to be chronic [2] and usually lead to withdrawal from society.

1.2.3 Cognitive symptoms

Deficits in cognition are a core feature of schizophrenia that do not derive from the psychosis (positive symptoms) or the negative symptoms, but follow an independent line of development [14]. These deficits include poor executive functioning (ability to understand information and use it to make decisions), attention, working memory (ability to use information immediately after learning it) or impairments in verbal and visual learning [1,15,16]. Cognitive symptoms are the most disabling aspect of the disorder [6] and account for a significant proportion of psychosocial disabilities. Their poor recognition and treatment lead to a poor functional outcome in schizophrenia [17]. For this reason, in 2003, the MATRICS (Measurement and Treatment Research to Improve Cognition in Schizophrenia) initiative was established, sponsored by the NIMH and the US Food and Drug Administration (FDA) [17]. It is aimed to identify the main cognitive domains affected in schizophrenia, improve the evaluation of cognitive deficits and provide a battery of behavioral and pre-clinical tests that supports the discovery, assessment and development of new cognition-enhancing drugs [18,19].

1.3 Risk factors

Even though the etiology of schizophrenia is still unknown, there seems to be a consensus in that a combination of factors can give rise to the disease. It involves the interaction between genetics, environmental factors and developmental processes [6,16].

1.3.1 Genetic factors

Genetics has been shown to play a major role in the development of schizophrenia [2,3,6,13,16,20] as evidenced by the many family, twin and adoption studies that have been carried out [16]. These studies show that the risk of developing schizophrenia increases with the percentage of shared genes (i.e. an increase of 6-17% for first-degree relatives or 50% for identical twins) [6]. Although no crucial gene has been identified as responsible for these rates so far [16], in the past years the contribution of specific DNA variants and different types of risk alleles has been proposed [2]. Genome-wide association studies have been able to identify single-nucleotide polymorphisms (SNPs) and copy number variants (CNVs) that individually confer a relatively high risk of schizophrenia [2], confirming it as a highly polymorphic disorder [2,20].

However, in spite of a family history of schizophrenia being the most significant risk factor [20] there is about 60% of schizophrenic patients that have neither a first- nor a second-degree relative with the disease [6]. This fact gives room for additional nongenetic factors to be required for a person to develop the disorder [6,16,20].

1.3.2 Environmental factors

Apart from genetics, environmental factors have also been shown to play a role in the development of schizophrenia [2,3,6,13,16]. There seems to be two critical periods during which these factors play a major role on the development of the disorder, the perinatal and the (pre)pubertal period [16]. A number of events that take place during early development (in utero or perinatal period) have been associated with an increased risk of developing schizophrenia, such as maternal exposure to viral or parasitic infections, severe malnutrition, maternal stress or pregnancy and birth complications [2,13,16]. Environmental exposures that occur later in life (pre- and pubertal period) such as growing up in an urban environment, cannabis use or minority group position have also been linked to the disorder [6]. Both prenatal and prepubertal factors have been found to

interact, suggesting that the effect of environmental exposures is modified by early events [2].

1.3.3 Developmental processes

Most models of the etiology of schizophrenia suggest additive and/or interactive effects between both susceptibility genes and environmental factors [6], proposing a connection between genetic and environmental factors in a gene-environment interaction [3]. This also fuels the idea that schizophrenia may be a disorder of neural development [6] and should be treated as one of a spectrum of clinical outcomes that result from disruption to the developing brain induced by genetic and/or environmental factors [2].

1.4 Etiology

The wide range of signs and symptoms that can be displayed in schizophrenia but that do not have to be present at the same time or the same individual makes the identification of specific etiological factors in schizophrenia quite a challenge [6]. The disorder is not the result of a single defect in a specific brain area, but it arises from the dysfunction of different neuronal circuits in different brain regions [2,3,21–23].

The attempts to explain the underlying mechanisms of schizophrenia have been based on three main hypotheses: 1) the dopaminergic hypothesis, which focuses on the explanation of positive symptoms through an altered and hyperactive dopaminergic system (e.g. hyperactivity induced by amphetamine or cocaine both in rodents and humans [24]; 2) the serotonin hypothesis, which derives from the psychotomimetic effects of serotonin receptor agonists (e.g. LSD, DOI) and the therapeutic action of the (serotonin antagonist) atypical antipsychotic drugs (e.g. clozapine, olanzapine); and 3) the glutamatergic hypothesis, based on an altered glutamate receptor transmission that can partially explain both positive and negative symptoms [25].

1.4.1 Dopamine

Dopamine is produced in the substantia nigra and the ventral tegmental regions of the brain and its projections are divided into the nigrostriatal, mesolimbic and mesocortical systems [24]. The dopamine receptors are G-protein-coupled receptors that can be divided in four subtypes (D1-4 receptors) [24]. The

discovery of the D2 subtype by Seeman et al. in the 1970s [26,27] meant the discovery of the binding site for antipsychotic drugs and served as a basis for the dopamine hypothesis of schizophrenia [28,29]. This hypothesis focuses on an excessive activity of the subcortical and limbic dopamine D2 receptor system as responsible for the positive or psychotic symptoms of schizophrenia [6,24,30,31], whereas negative and cognitive symptoms would be a result of a dopamine D1 receptor hypofunctionality in the prefrontal cortex [31]. It is based on two clinical observations [6]: 1) dopamine agonists, such as amphetamine, induce psychotic symptoms in healthy individuals and exacerbate them in schizophrenic patients [32], and 2) the potency of an antipsychotic is proportional to the degree of dopamine D2 receptor antagonism [26]. These affirmations are supported by a number of postmortem studies that have been able to identify abnormalities in the pre- and post-synaptic dopaminergic systems in schizophrenia [33]: D1 receptors appear to be decreased in the prefrontal cortex and increased in the parieto-temporal cortex in schizophrenia patients [24], while D2 receptors have been found to be increased in striatal areas [34]. However, these findings seem to be an area of disagreement among researchers and a role of the treatment in these receptor density changes cannot be ruled out [33].

The discovery of the second generation of antipsychotics, the so called atypical antipsychotics, and the fact that their binding profile include receptors other than the D2, introduced the role of different neurotransmitter systems in the etiology of schizophrenia.

1.4.2 Serotonin

Serotonin is a molecule that can be found in the gastroinstestinal tract, blood platelets and the central nervous system (CNS). In the CNS it acts as a neurotransmitter via membrane receptors [35]. Serotonin receptors include both metabotropic (5HT1,2,4-7) and ionotropic (5HT3) receptors that can mediate excitatory or inhibitory neurotransmission by modulating the release of different neurotransmitters (glutamate, GABA, dopamine,…) [36]. The serotoninergic hypothesis emerges from the observation that hallucinogenic effects of LSD might result from an antagonism of serotonin in the CNS [37], acting primarily in the 5HT1A and 5HT2A receptors. This hypothesis is based on an altered serotonin system, although the direction of this alteration is not clear. Postmortem studies have shown increased serotonin levels in subcortical brain regions [38] as well as decreased expression of the 5HT2A receptor and increased expression of 5HT1A receptor in the frontal cortex, [39]. In support of this

hypothesis there is also the fact that atypical antipsychotic drugs (e.g. clozapine, olanzapine, risperidone, among others) are antagonists of the 5HT2A receptors [40–44].

1.4.3 Glutamate

Glutamate is the primary excitatory neurotransmitter in the brain, with glutamatergic neurons utilizing between 60 and 80% of total brain metabolic activity [33]. It is involved in prenatal and childhood brain development, learning and memory [45] and exerts its function through two different types of receptors: ionotropic receptors (NMDA, kainate and AMPA receptors) and metabotropic receptors (mGlu1-8 receptors) [46]. The glutamatergic hypothesis arises from the observation that phencyclidine (PCP) and ketamine (glutamate antagonists) induce negative symptoms and cognitive dysfunction similar to that of schizophrenia (contrary to the dopamine antagonists, that are only able to replicate the positive symptoms) [47,48] and that they function by blocking the NMDA receptor [48]. This hypothesis is supported by postmortem studies that show a disruption in the normal functioning of the glutamatergic system, evidenced as an NDMA receptor dysfunction in schizophrenia [47].

1.4.4 Other considerations

—1.4.4.1 Glutamate-serotonin

As we said before, hallucinogenic effects of drugs like LSD or the atypical antipsychotic clozapine are mediated by the 5HT2A receptor. However, not every drug that activates this receptor have these hallucinogenic effects, and therefore, activation of 5HT2A might not be enough for a suitable treatment of schizophrenic symptoms [42]. In this line, it has been suggested that 5HT2A forms a heterocomplex with another receptor subtype, and this complex would be the target responsible for the neurochemical and behavioral effects induced by hallucinogenic and antipsychotic drugs [42,49]. It has been shown that treatment with drugs targeting the glutamate system exert their function through the mGlu2 receptor, and that this receptor is expressed in the same neurons as the 5HT2A [42,49]. The mGlu2-5HT2A heterocomplex would be the target of both clozapine-like and glutamate antipsychotic drugs [42] and activation of the mGluR2 component of the heterocomplex would eliminate the hallucinogenic-specific component of the signaling responses to LSD-like drugs [49]. This complex integrates serotonin and glutamate signaling, both implicated in

psychotic disorders, to regulate sensory gating functions of the cortex, a process disrupted in schizophrenia [49].

—1.4.4.2 GABA and gamma oscillations

Up until now we have talked mostly about the molecular basis of positive and negative symptoms, but cognitive symptoms of schizophrenia, considered to be a core feature, are probably the most disabling trait of the disorder and are associated with long-term impairment [6]. Cognitive abilities seem to depend on gamma frequency oscillations, that refers to the apparent communication between brain regions that coordinate the firing population of neurons, resulting in a rhythmic input that is reflected in the extracellular field potential as brain oscillations [50].

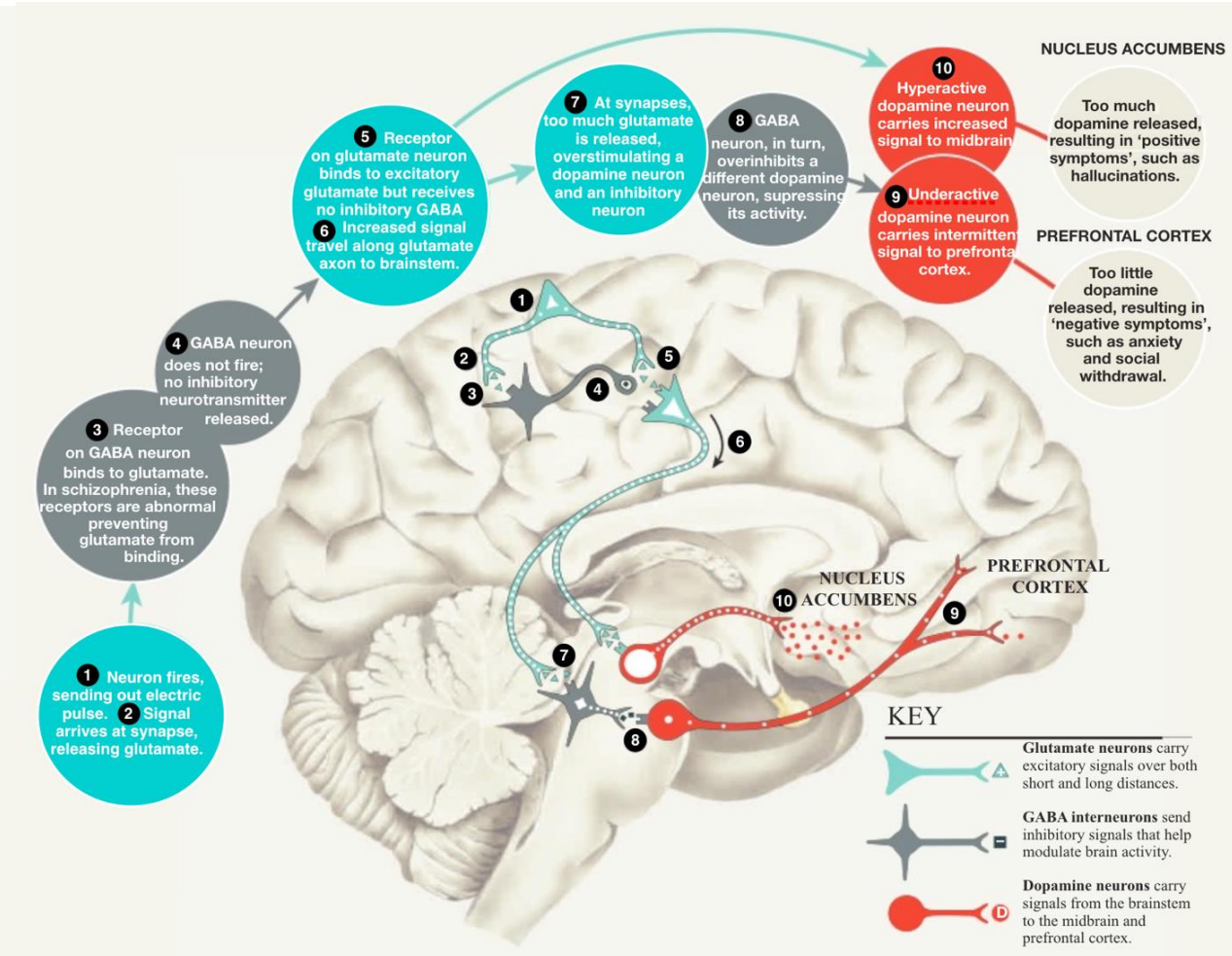


Figure 1. Biochemical cascade of the dopamine, glutamate and GABA systems. A dysregulation of glutamate might explain why there is an excess of dopamine in certain areas of the brain and a deficit in others that give rise to a broad spectrum of symptoms. Taken and adapted from [22].

Individuals with schizophrenia show alterations in cognitive control, altered activation of the prefrontal cortex and reduced gamma oscillation power [6]. Gamma oscillations are thought to be dependent on cortical inhibitory circuitry, and so, cognitive dysfunction in schizophrenia has been suggested to be associated with gamma-aminobutyric acid (GABA) neuron-mediated synaptic inhibition alterations [6,51]. Specifically, there is a subpopulation of GABA neurons that expresses the calcium-binding protein parvalbumin (PV) that provides inhibitory inputs and largely drives cortical gamma oscillations and appear to be decreased in schizophrenia [52].

As we have briefly described, schizophrenia cannot be easily explained, and many participants have to be taken into account. Figure 1 by Elert (2014) [22] shows an integrative schema of three different neurotransmitter systems and their joined action in the etiology of schizophrenia while Table 2 shows a summary of the findings related to the neurotransmitters.

Table 2. Summary of the neurotransmitters involved in schizophrenia and the main findings related to their expression. Taken and adapted from ref. [39].

Transmitter	Main postmortem findings	Other supporting evidence
Dopamine	Increased density of D_2 receptors Decreased cortical DA innervation Increased D_4-like receptor binding Alterations in D_3 receptor splicing	DA-releasing agents produce psychosis All antipsychotics are D_2 receptor antagonists Increased striatal DA release in vivo
Glutamate	Decreased presynaptic markers Decreased HPC AMPA and kainate receptor expression Minor changes in FC NMDA receptor subunits Altered glutamate fibers in cingulate cortex	NMDA receptor antagonists produce schizophrenia-like psychosis Roles of NMDA receptors in development and neurotoxicity Partial NMDA receptor agonists have some therapeutic benefit

Table 2. Summary of the neurotransmitters involved in schizophrenia and the main findings related to their expression. Taken and adapted from ref. [39]. Continued.

Transmitter	Main postmortem findings	Other supporting evidence
5-HT	Decreased FC $5HT_{2A}$ receptor expression	$5HT_2$ agonists (e.g., LSD) are psychotomimetic
	Increased FC $5HT_{1A}$ receptors	$5HT_2$ receptor polymorphisms associated with schizophrenia and clozapine response
	Increased 5-HT transporter affinity	
	Developmental and trophic roles of 5HT	Atypical antipsychotics have high affinity for several 5HT receptors
GABA	Decreased density of FC GABAergic terminals	Roles of GABA in stress and neurotoxicity
	Increased $GABA_A$ receptor binding in limbic areas	
	Altered expression of FC $GABA_A$ receptor subunits	
	Decreased FC expression of GAD	
	Altered density of cingulate GABAergic cells	

5HT=Serotonin; D2-4= Dopamine receptors; DA=Dopamine; FC= Frontal Cortex, HPC= Hippocampus, GABA= Gamma-Aminobutyric acid GAD= glutamate descarboxylase

1.5 Treatment

Once a diagnosis has been made (see *Symptoms*) the first line of treatment is based on the use of antipsychotic drugs (Table 3). This treatment must be chronic and maintained long-term (it can take several months to achieve maximal effect and drop-out often leads to relapse of symptoms [20]), and initial medication should be personalized, taking into account adverse effects profile, dosing and patients preferences [13]. It is aimed to the treatment of the symptoms, not the causes of the disorder [42].

1.5.1 Typical antipsychotics

These drugs, known as the first-generation or typical antipsychotics were developed in the 1950s to treat the psychotic symptoms of schizophrenia [20]. The first antipsychotic, chlorpromazine, was discovered in the early 1950s by Jean Delay (1907-1987) while he was looking for an anti-inflammatory drug for the post-operative traumatic shock [42,53]. He also coined the term neuroleptic to define drugs that are able to mitigate psychotic symptoms [53]. These antipsychotics act by primarily blocking the D2 dopamine receptor, which in the mesolimbic pathway have beneficial effects but in the nigrostriatal pathway can lead to the appearance of extrapyramidal side effects, including dystonia (sustained or repetitive muscle contractions), parkinsonian symptoms (bradykinesia, rigidity) or akathisia (inability to sit still, restlessness) [6,13,20]. Long-term exposure to these drugs can result in the development of tardive dyskinesia, a chronic disorder of the nervous system characterized by involuntary jerking movements such as chewing, protruding of the tongue and facial grimacing [6,20]. Haloperidol, chlorpromazine or fluphenazine are examples of typical antipsychotics.

1.5.2 Atypical antipsychotics

Atypical or second-generation antipsychotics are a newer line of drugs that have been developed over the past 20 years to fight the appearance of motor side effects common to the use of typical antipsychotics [3,20], although they confer a higher risk of metabolic side effects such as diabetes and weight gain [3,13,20]. These drugs generally have lower affinity for the D2 receptor and mainly block the 5HT2A receptor, which was thought to confer them also greater efficacy for negative symptoms in addition to positive symptoms [6], although it has not been yet borne out [54]. The clinical profile of these antipsychotics is yet to be fully defined in terms of the extent of their therapeutic efficacy and adverse effects [55] since it is not clear that they are more effective than first-generation antipsychotic (for review see [54]). Atypical antipsychotics include clozapine, risperidone and olanzapine among others.

Table 3. Antipsychotic medication. Taken and adapted from [13].

Medication	Common side effects	Serious side effects	Comments
Chlorpromazine	Drowsiness, dry mouth, elevated prolactin levels, extrapyramidal symptoms, glucose intolerance, postural hypotension, weight gain	Neuroleptic malignant syndrome, tardive dyskinesia	First drug used to treat psychosis
Haloperidol	Drowsiness, dry mouth, extrapyramidal symptoms, galactorrhea, hypotension, tachycardia	Neuroleptic malignant syndrome, prolonged QT interval, tardive dyskinesia	More effective for treating positive symptoms, but has a high risk of extrapyramidal symptoms
Perphenazine	Drowsiness, dry mouth, extrapyramidal symptoms, galactorrhea, hypotension, tachycardia	Neuroleptic malignant syndrome, tardive dyskinesia	-
Thiothixene	Drowsiness, dry mouth, extrapyramidal symptoms, galactorrhea, hypotension, tachycardia	Neuroleptic malignant syndrome, tardive dyskinesia	-
Second generation			
Clozapine	Constipation, dizziness, headache, metabolic effects, salivation, sedation, tachycardia, weight gain	Agranulocytosis (complete blood count should be obtained weekly for six months, then every two weeks for six months, then monthly), myocarditis, seizures, tardive dyskinesia	Reserved for severe, treatment-refractory schizophrenia

Table 3. Antipsychotic medication. Taken and adapted from [14]. Continued.

Medication	Common side effects	Serious side effects	Comments
Lurasidone	Akathisia, hyperprolactinemia, metabolic changes, nausea, parkinsonism, somnolence	Neuroleptic malignant syndrome, tardive dyskinesia	Higher incidence of adverse effects with higher dosages
Olanzapine	Akathisia, constipation, dizziness, hyperprolactinemia, metabolic changes, postural hypotension, weight gain	Agranulocytosis, neuroleptic malignant syndrome, tardive dyskinesia	More weight gain compared with older second-generation antipsychotics, lower discontinuation rate
Paliperidone	Hyperprolactinemia, metabolic changes, orthostatic hypotension, priapism, somnolence, weight gain	Neuroleptic malignant syndrome, prolonged QT interval, tardive dyskinesia	Active metabolite of risperidone
Quetiapine	Agitation, dizziness, dry mouth, headache, metabolic changes, postural hypotension, somnolence, weight gain	Agranulocytosis, neuroleptic malignant syndrome, tardive dyskinesia	High discontinuation rate compared with other second-generation antipsychotics
Risperidone	Anxiety, hyperprolactinemia, hypotension, insomnia, metabolic changes, nausea, weight gain	Agranulocytosis, neuroleptic malignant syndrome, tardive dyskinesia	Higher incidence of extrapyramidal effects, increased prolactin levels
Ziprasidone	Agitation, hypotension, metabolic changes, nausea, somnolence, tachycardia, weight gain	Agranulocytosis, neuroleptic malignant syndrome, tardive dyskinesia	Least amount of weight gain compared with other second-generation antipsychotics
Third generation			
Aripiprazole	Anxiety, constipation, dizziness, headache, insomnia, metabolic changes, nausea, vomiting	Agranulocytosis, neuroleptic malignant syndrome, tardive dyskinesia	Smaller effect on lipids compared with other second-generation antipsychotics

Both typical and atypical antipsychotic drugs accomplish their function by binding to their target receptor. It has been postulated that ligands can be characterized by the effects elicited by their interaction with their target, meaning that a ligand can be classified as a full or a partial agonist [56]. Given the differential role that dopamine seems to play in the development of positive and negative symptoms, it has been hypothesized that a partial dopamine agonist, with dual effects according to the dopamine levels might have an increased efficacy in the treatment of schizophrenia [56]. In this regard, the antipsychotic aripiprazole has been studied. It is a relatively new approved drug with a dual action on dopamine: it acts on postsynaptic D2 receptors and presynaptic autoreceptors. This "dopamine stabilizer" acts as a partial antagonist when dopamine activity is high (for example, in the mesolimbic pathway) competing with dopamine and diminishing positive symptoms. When dopamine activity is low (mesocortical pathway) it acts as a partial agonist and occupy additional receptors, causing partial activation that results in improvement of negative and cognitive symptoms [56].

Table 4. Receptor affinities for the most common antipsychotics at therapeutic doses. Taken and adapted from [55].

	First generation	**Second generation**				**Third generation**
Receptor	*Haloperidol*	*Clozapine*	*Risperidone*	*Olanzapine*	*Quetiapine*	*Ziprasidone*	*Aripiprazole*
D1	+	+	+	++	-	+	-
D2	++++	+	+++	++	+	+++	++++
D3	+++	+	++	+	-	++	++
D4	+++	++	-	++	-	++	+
5HT1A	-	-	-	-	-	+++	++
5HT1D	-	-	+	-	-	+++	+
5HT2A	+	+++	++++	+++	++	++++	+++
5HT2C	-	++	++	++	-	++++	+
5HT6	-	++	-	++	-	+	+
5HT7	-	++	+++	-	-	++	++
α1	+++	+++	+++	++	+++	++	+
α2	-	+	++	+	-	-	+
H1	-	+++	-	+++	--	-	-
m1	-	++++	-	+++	++	-	-
DA trp		++		++			-
NA trp		+		++		++	-
5HT trp						++	-

-= minimal to none; += low; ++= moderate; +++= high; ++++= very high, D1-4=dopamine receptors, 5HT1-7=serotonin receptors, α1-2= adrenergic receptors, H1=histamine receptor, m1=cholinergic receptor, DA=dopamine, NA=noradrenaline, 5HT=serotonin, trp=transporter

Although it is considered as an atypical antipsychotic, aripiprazole is the first one of a new developing line of third generation antipsychotics. Table 4 shows the receptor affinities for some of the most common antipsychotic drugs at a therapeutical doses.

1.5.3 Other treatments

In addition to medication, adjunctive therapies such as cognitive behavior therapy, family intervention and social skills training should be offered [2,6,13]. The UK National Institute for Health and Care Excellence (NICE) offers a guideline for the correct management of schizophrenia and recommend that every patient should be given cognitive behavioral therapy and family intervention [58]. Psychosocial treatment interventions help in enhancing medication compliance, self-steem and coping strategies for life stressors, which reduce the risk of relapse and rehospitalization, enhance medication adherence, and lead to a higher level of functioning [6].

1.6 Neurobiology of schizophrenia

As we said before, the causes and underlying mechanisms of schizophrenia are far from being clarified, but a number of changes in an anatomical, biochemical and behavioral level have been observed in an increasing number of studies on the pathophysiological changes of schizophrenia [3]. Abnormal brain structure and neurochemical composition lead to abnormal function as evidenced by an abnormal network response to cognitive tasks, with both hyperactivity or hypoactivity of different brain regions depending on the specific task [3].

1.6.1 Neuroanatomy

With the development and improvement of neuroimaging techniques the number of studies on the anatomical changes in schizophrenia increases by the minute. The most common, universal changes found in structural brain imaging studies seem to be increases in ventricular size, decrease in brain volume, cortical surface abnormalities and focal alteration of white matter tracts [3,59]. However, it is not clear whether this loss of volume is accompanied also by a loss of neurons [60] or a reduced neuropil (reduced dendritic arborization and dendritic spine density) [59,61]. If we talk about a specific brain area the focus is on the prefrontal cortex (PFC), involved in specific aspects of the disorder related to cognitive deficits

(working memory and executive functions) [2], although less consistent findings also implicate different brain areas such as the hippocampus [60], amygdala, thalamus or nucleus accumbens [62].

Back to seemingly the most interesting area, the PFC is considered a "motor executive", while also playing a role in memory. Lesions in certain areas of the PFC result in deficits in attention, working memory and planning, and an inability to initiate and carry out goal-directed behaviors, features that are reminiscent of either positive or negative symptoms of schizophrenia [59]. Neuropathological studies have revealed several alterations in some aspects of the PFC such as a marked loss of dendritic spines of pyramidal neurons in layer III [61,63] and lower expression of parvalbumin in GABA interneurons (but not in the number of cells) [51,52]which might account for the overall reduction of PFC volume observed in schizophrenic individuals when compared to control samples. Cognitive deficits of schizophrenia might be explained by an imbalance in these two parameters that are involved in the generation of gamma oscillations (see *GABA and gamma oscillations*). In this line, two different hypotheses have been described: 1) an alteration of PV neurons results in a weaker inhibition to pyramidal cells, which are disinhibited and fire in an asynchronous wave or 2) an alteration of pyramidal neurons with a weaker excitatory drive to PV neurons due to a reduced number of dendritic spines that results in a compensatory reduction of inhibition feedback from the PV neurons. Either one of these hypothesis would explain the alterations in gamma oscillation leading to the appearance of cognitive deficits [51].

1.6.2 Neurochemistry

As we outlined in the *Etiology* section there seems to be an important imbalance of molecules in schizophrenia and the development of new techniques has allowed the observation of biochemical disturbances in the brain. The most investigated hypothesis of schizophrenia implicates the participation of different neurotransmission systems, such as dopamine, glutamate, serotonin or GABA and alterations therein (Table 2). Apart from the differences in dopamine receptor densities, evidence for changes in pre-synaptic dopamine has been suggested. There seems to be a greater release of dopamine in the striatum, and control of dopamine release seems to be disinhibited in schizophrenia. It has also been shown a dysfunction in the PFC that could be involved in the negative and cognitive symptoms [64]. The evidence for disturbances in the glutamate system are focused on deficits in the temporal cortex, medial temporal lobe and striatal

regions, with losses of uptake sites and increases in NMDA receptors in a compensatory manner. These abnormalities may underlie deficits of cortico-subcortical innervation responsible for cognitive and negative symptoms. In the GABA system, there is evidence for cortical and hippocampal losses of GABA-containing neurons. Emerging studies pose a role for non-neurotransmitter molecules such as the enzyme COMT (cathecol-O-methyltransferase), involved in the metabolism of dopamine and noradrenaline or N-acetylaspartate (NAA), a marker of neuronal integrity. Lower levels of COMT lead to increased dopamine, which is associated with improved cognitive function while NAA appears to be reduced in the medial temporal lobe in schizophrenia, consistent with suggestions of neuronal deficits in the hippocampus and amygdala, and in the cortex, correlating with an increase in dopamine release in the striatum [64].

The understanding of the neurochemical pathology that underlies negative and cognitive symptoms has to be improved and translated into more effective strategies and treatments.

1.6.3 Endophenotypes

The concept of endophenotype refers to a sign or symptom that is heritable, co-segregates with the disease, also occur in healthy relatives and is also present in patients in remission [65]. These traits are more easily quantifiable and are more related to specific brain areas and neurotransmitter systems, which makes them more accessible for studying in animal models, and therefore, increasing their translatability [16]. A number of endophenotypes have been identified in schizophrenia (see Table 5) but we will only review some of them.

—1.6.3.1 Sensorimotor gating

Sensorimotor gating refers to the ability of discarding irrelevant or redundant stimuli and filter it out from the rest of meaningful sensory inputs [66]. It can be measured with the prepulse inhibition (PPI) of the acoustic startle response test that measures the ability of a prepulse to diminish the startle caused by a subsequent pulse, and the P50 suppression test that uses two auditory stimuli presented at 500 ms intervals, expecting a lower response to the second stimulus than the first [65]. Both these measures appear to be affected in schizophrenic patients and alterations in inhibitory neural circuits have been found, suggesting that the inhibitory mechanisms in these individuals are not capable of adequately adjusting to distinct or repetitive inputs [65]. Another valid measure is the latent

inhibition (LI) that refers to the retardation of associative conditioning between a conditioned stimulus (CS) and an unconditioned stimulus (US) resulting from pre-exposure of the CS alone prior to conditioning [67]. In normal conditions the CS is identified as a "safe" one during the pre-exposure and therefore the retardation in learning the CS-US association. Given the inability of schizophrenic patients to ignore irrelevant stimuli they are unable to learn the CS-US association and thus, present a low or impaired latent inhibition [68].

—1.6.3.2 Eye-tracking dysfunction

The "smooth pursuit" movement of the eye that takes place when a subject is following an object moving at a constant velocity (a pendulum for example) is also affected in schizophrenia, manifested as corrective saccades that follow smooth pursuit eye movements that are slightly slower than the target. It involves integration of functions of the PFC eye fields, visual and vestibular circuitry, thalamus and cerebellum [65].

—1.6.3.3 Working memory and executive cognition

The term working memory refers to the type of memory that is active and relevant only for a short period of time (i.e. keeping in mind a phone number until is dialed) [69]. It depends on the dorsolateral area of the PFC, that is known to be altered in schizophrenic patients, resulting in a compromised working memory and executive cognition in these individuals [65].

Table 5. Candidate endophenotype markers in schizophrenia. Taken and adapted from [5].

Neurophysiological markers and endophenotypes	Cognitive markers and endophenotypes
Electrodermal deviance	Verbal dysmnesic cognitive subtype
Prepulse inhibition of the startle reflex (PPI)	Attention and vigilance-based cognitive subtype
Deficient gating of the auditory evoked response (P50)	Continuous performance tests (CPT, signal/noise ratio)
P300 amplitude reduction and latency delay	Verbal memory deficit, cortical or subcortical cognitive type
N400 amplitude reduction (semantic context underutilization)	Prefrontal executive/working memory phenotype
Mismatch negativity (MMN)	Dysexecutive cognitive subtype
Smooth pursuit eye movement dysfunction (SPEM)	Generalized (diffuse, pervasive) cognitive deficit, CD)
Antisaccade error rate (AS)	Spatial working memory
Multivariate electrophysiological endophenotype (MMN, P50, P300, AS)	Frontal/abstraction deficit profile

Neuroimaging markers and endophenotypes	Other markers and endophenotypes
Fronto-thalamic-cerebellar gray matter deficit	Neurological soft signs
Fronto-striato-thalamic gray matter deficit	Composite laterality phenotype
MRI whole-brain non-linear pattern classification	Nailfold plexus visibility
Frontal hypoactivation in response to cognitive tasks (hypofrontality)	Minor physical anomalies
Atrophic and static (neurodevelopmental) schizophrenia endophenotypes	

1.7 Animal models

The development of animal models constitutes a basic tool to increase the understanding of the neurological basis of psychiatric disorders and the development of new, improved treatments [70]. In order for an animal model to be considered a useful tool it must fulfill different validity criteria: face validity (analogy of symptoms with the human condition), construct validity (replication of the theoretical neurobiology mechanisms underlying the condition) and predictive validity (similarity of known treatment effects and potential for the discovery of new targets) [70,71].

This triad of validities is independent, so that, for instance, an animal model can present construct and predictive validity with no face validity (Fig. 2). Although the development of animal models for several neurological disorders has been quite successful, schizophrenia presents itself with a higher difficulty due to several reasons. Firstly, the fact that there is a lack of knowledge about the etiology and pathology of the disorder implies that most animal models to date rely on hypothesis rather than facts, and secondly, only the cognitive symptoms can truly be incorporated to an animal model, since the other categories of symptoms require the subjective participation of the patient in psychiatric interviews (description of visual and auditory hallucinations, delusions, thought disorder) [16,70]. Also, the fact that virtually every symptom can occur in other disorders and may or may not be present in a specific individual forces a model to model more than one symptom [16].

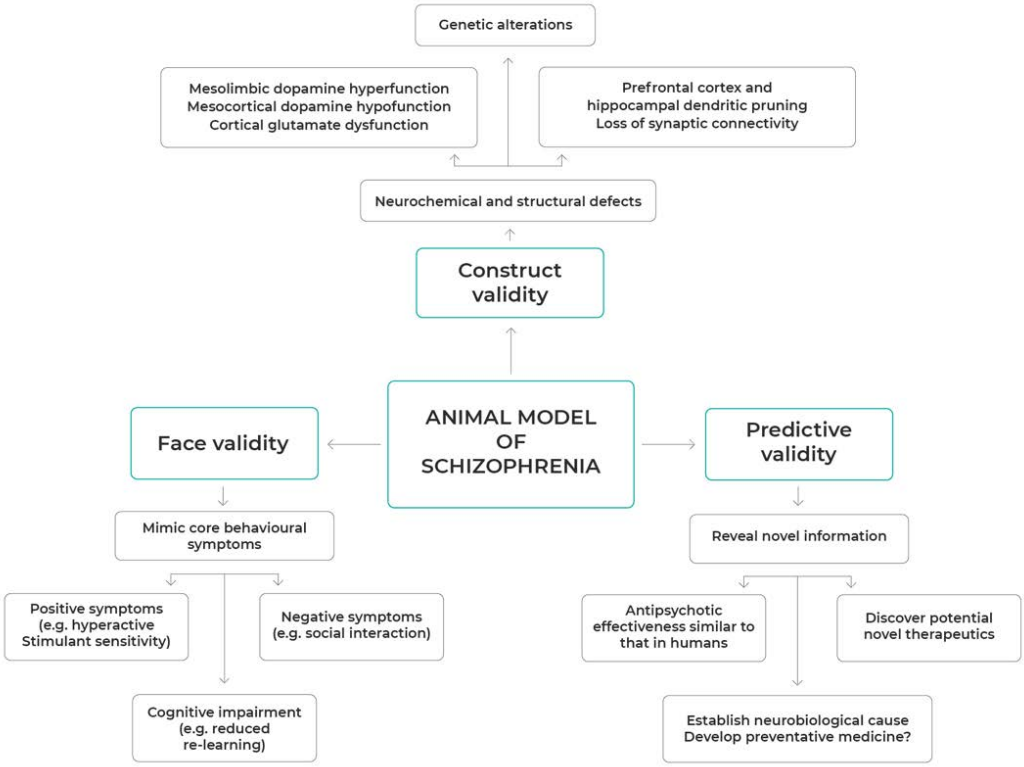


Figure 2. Schematic diagram of the key abnormalities expected to be present in an animal model of schizophrenia with sufficient construct, face and predictive validity. Taken and adapted from [70].

Despite these inconveniences, there is a number of animal models available that present sufficient behavioral and neurochemical abnormalities for the study of schizophrenia or schizophrenia-relevant symptoms (Fig. 2).

It has been estimated that over 20 different animal models have been developed and they all fit into one of four different categories: neurodevelopmental, pharmacological, lesion and genetic models. For the sake of extension in this dissertation we will only focus on the neurodevelopmental and genetic models (for review of the remaining models see [70,72,73].

1.7.1 Neurodevelopmental models

These models are based on the thought that schizophrenia results from an abnormal development of the brain that starts years before the onset of the illness [74]. Exposure of neonates with a genetic predisposition to adverse environmental inputs such as maternal stress, malnutrition, viral insult or exposure to neurotoxins, to mention a few, can increase the risk of developing schizophrenia [70]. These models use an early environmental manipulation that produce irreversible changes in the developing CNS, disrupting the normal neurogenesis. These manipulations are able to reproduce some of the core symptoms, that appear during the post-pubescent period, following the natural course of the illness, and therefore, adding face validity to the models [70]. These animal models include viral exposure to influenza virus [75,76], the viral mimic polyriboinosinic–polyribo-cytidilic acid (PolyI:C) during gestation [77] or lesions in the neonatal ventral hippocampus [78].

Another widely used environmental manipulation is the post-weaning social isolation rearing of the animals, that causes alterations in the brain in development and behavioral deficits by depriving rat pups (known to be social animals) of social interaction with their littermates [74]. The consequences of this manipulation include behavioral, cognitive, neurochemical and neuroanatomical alterations that resemble some of the core symptoms of schizophrenia [79,70,78,80–91]. Some of these changes are summarized in Table 6.

Table 6. Alterations induced by the social isolation rearing of the animals.

Behavioral changes		
Locomotor activity	Hyperactivity in a novel arena	Varty et al. (2000) [81]
Sensorimotor gating	Impairment of PPI	Varty et al., (2000) [81] Bakshi et al., (1998) [82] Day-Wilson et al., (2006) [83]
Social interaction	Increased total social interaction and aggression	Wongwitdecha et al (1996) [84] Möller et al. (2011) [86]
Cognitive changes		
Novel object	Deficits in novel object discrimination	Bianchi et al. (2006) [90]
Spatial learning	Improvements in water-maze	Wongwitdecha et al. (1995) [85]
Attention	No impairments in attentional set-shifting task	Dalley et al. (2002) [87] Weiss et al. (2004) [80]
Anxiety	Anxiogenic on elevated plus-maze Neophobia Reduced cage escape latency	
Neurochemical changes		
	Reduced PFC D1 binding	Heidbreder et al. (2000) [88]
	Behavior sensitivity to DA agonists	Powell S. (2010) [78]
	Reduced response to DA antagonists	Jones et al. (1992) [89]
	Elevated DA release in nucleus accumbens Altered DA turnover in PFC	
	Increased density of 5HT-2A1R in hippocampus Decreased basal 5HT turnover in nucleus accumbens	
	Decreased synaptophysin in hippocampus	
	Decreased BDNF in hippocampus	
Anatomical changes		
	Reduced PFC volume	Silva-Gomez et al. (2003) [79]
	Reduced dendritic spine density	Day-Wilson et al. (2006) [83]
	Loss of PV-containing neurons	Schubert et al. (2009) [91]
	Cytoskeletal alterations in hippocampus	

1.7.2 Genetic models

These models arise from the observation that schizophrenia has an important genetic component as seen in twin and first-relative studies (see *Risk factors*). Given the high homology between human and rodent genes, these models allow the screening of susceptible schizophrenia genes and to test if alteration in these genes lead to abnormalities related to the disorder [72]. Currently, the improvement of genetic engineering techniques have allowed the development of animal models (mice first and rats later) based on deletion (knock-out), reduction of the expression (knock-down) of single genes or insertion/substitution of specific DNA sequences (knock-in) (for review see [70,72,92]). However, before this progress, these genetically-oriented models were based on selective breeding, a process in which animals are bred for a particular trait or phenotype (usually but not always a certain type of behavior or behavioral response) [72,92]. There are several models that have been developed following this strategy for the study of schizophrenia, such as the Low- and High-PPI rat lines, selected by their low/high sensorimotor gating measured by the PPI of acoustic startle response (ASR) [93]; APO-SUS (apomorphine susceptible) and -UNSUS (unsusceptible) rats, that are selected by their extreme gnawing responses to the dopaminergic agonist apomorphine [94]; Brattleboro rats (BRAT), that are Long Evans-derived rats with a single mutation that impairs vasopressin release and display an innate deficit of PPI [95]; or the Spontaneous Hypertensive Rats (SHR), derived from Wistar Kyoto rats (WKY) that have been proposed as a model for Attention Deficit Hyperactivity Disorder (ADHD) since they show hyperactivity, impulsivity, attention deficits and PPI impairments [72,96]. For review see [72].

—1.7.2.1 Roman rats

One of these selectively bred models with special importance for this dissertation is the Roman rats, which have been psychogenetically selected for their good vs extremely poor acquisition of the active avoidance task, resulting in two well differentiated strain/lines: the Roman High Avoidance (RHA) and Roman Low Avoidance (RLA) rats [97–106]. These strains originated in 1965 from a pool of albino Wistar rats that were subjected to the two-way active avoidance test in the shuttle box [107]. They have been maintained in Switzerland since 1972 as an outbred colony (RHA-Verh and RLA-Verh), and in Cagliari (Sardinia, Italy) since 1994 as another outbred colony, maintaining their differences in the two-way avoidance and other phenotypes [98,99,108]. In 1993, a subset of the Swiss colony was transferred to Spain to develop inbred strains (RHA-I and RLA-I) in order

to try to identify the genetic bases responsible for the divergent profiles seen in these rats [99,101,109]. These inbred lines are maintained to date in the lab of Dr. Albert Fernández-Teruel.

These rats have been extensively profiled on stress sensitivity and anxiety/fearfulness traits, concluding that RLAs show elevated levels of hormonal responses (ACTH, corticosterone and prolactin) to stress, enhanced levels of anxiety/fear in different novelty, conflict (unconditioned and conditioned) and mild stress tests [102,104], increased "frustration" responses to tests with reduction of expected rewards [110,111]. The RHAs display a proactive behavioral/coping profile with low levels of anxiety, evidenced in their active, impulsive and novelty seeking behavior in conflict/stress situations [104,112,113], and show a higher preference for some addictive drugs and an increased locomotor sensitization after the repeated administration of dopaminergic agonists and other drugs of abuse [114,108,115].

On a neurochemical level these rats also differ. For example, studies on the dopaminergic system have revealed that RHA rats show an increased dopamine release in the PFC under stress situations [116], enhanced dopaminergic sensitization after repeated administration od psychostimulants and have a reduced density of D2 autoreceptor in the nigrostriatal pathway [108,114]. Differences on the serotoninergic and glutamate systems have also been found in a study by Klein et al. [117] that observed RHAs show higher expression levels of 5HT1A and 5HT2A in the frontal cortex and decreased binding levels of mGlu2/3 receptors (further studies showed no detectable levels of mGluR2 protein in frontal cortex, hippocampus and striatum) [117]. These findings might account to a certain extent for the between-strain differences in impulsivity and vulnerability to addiction [108,117] and they resemble the receptor pattern observed in treatment-naïve schizophrenic patients. This pattern is essential for attentional filtering processes and might increase the vulnerability to psychotic disorders [49].

These neurochemical characteristics combined with the other phenotypical between-strain differences mentioned above (summarized in Table 7) and some others such as novelty-induced locomotor activity [118], deficits in latent inhibition [119] and deficits in PPI [72], that resemble some of the signs/symptoms seen in schizophrenic patients, leads us to propose the RHA rats as a new animal model for the study of schizophrenia-relevant symptoms.

Table 7. Profile of Roman rats in schizophrenia-relevant phenotypes. Selected references between brackets.

Schizophrenia-relevant behavioural models/symptoms or neural phenotypes	RHA vs RLA
I.- Positive Signs/Symptoms [108,120–122]	
A. Locomotor activity in response to novelty B. Sensitivity to psychotomimetic/psychostimulant drugs	RHA: higher locomotor responses to novelty (also compared to the Sprague Dawley strain) and higher locomotor sensitization to psychostimulants
II.- Negative Signs/Symptoms [72,123]	
A. Decreased nesting behaviour	RHA: decreased nesting behaviour
B. Social behaviour/aggression: resident-intruder test	RHA: increased aggression latency in resident-intruder test
C. Decreased social interaction	RHA: decreased social interaction in the two-cage two-hole test (unpublished results from our lab).
III.- Cognitive Signs/Symptoms [102,112,117,119,124,125]	
A. Decreased working memory in the place paradigm (compared to the HS rat stock)	RHA: impaired working delayed-matching to place paradigm in the MWM (also compared to the HS rat stock)
B. Deficits in attention/sensorimotor gating/executive functions	RHA: impaired PPI (both vs RLA and outbred NIH-HS rats)
C. General cognitive deficits	RHA: impaired latent inhibition threshold (compared to SD rats), impaired place learning and spatial memory, and impaired spatial reversal learning in the Morris water maze.
IV.- Neurochemical/neuroanatomical phenotypes linked to schizophrenia [108,114–117,120,126–128]	
A. Central DAergic function	RHA: increased mesolimbic and mesocortical dopamine responses to DAergic agonists or stress (respectively).
B. Hippocampal function and neuromorphology	RHA: decreased hippocampal function and reduced neuronal density in hippocampal CA fields/layers.
C. Serotoninergic and glutamate function	RHA: enhanced expression of 5HT2A and absence of expression of mGluR2 expression in the hippocampus
D. 5HT2A/mGluR2 complex in prefrontal cortex	In comparison with the RLAs, the 5HT2A/mGluR2 complex in RHA resembles the profile of schizophrenic patients and mGluR2 knockout mice

—1.7.2.2 NIH-HS rats

Apart from the Roman rats, in our lab we work with a second animal model, the heterogeneous rat stock "*National Institute of Health N/Nih Genetically Heterogeneous Rat stock"* (NIH-HS) developed by Hansen and Spuhler in 1984 [129]. This stock derives from the rotational crossing of 8 different parental strains (Fig. 3) for more than 80 generations, resulting in a pool of animals in which each individual has a unique combination of the parental genome.

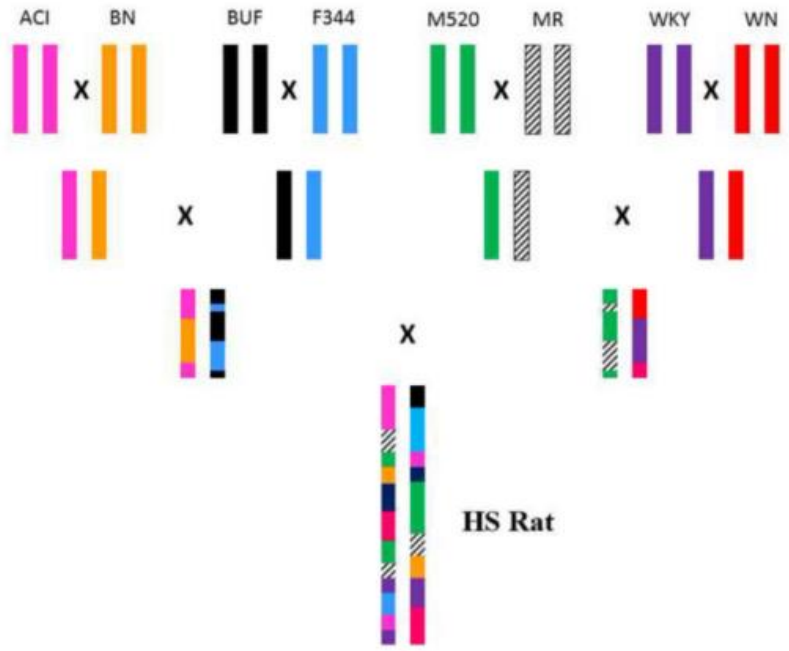


Figure 3. Diagram of the rotational breeding program followed to develop the NIH-HS rat stock from 8 different inbred parental lines: Agouti (ACI/N), Brown Norway (BN/SsN), Buffalo (BUF/N), Fischer 344 (F344/ N), M520/N, Maudsley Reactive (MR/N), Wistar-Kyoto (WKY/N) and Wistar-Nettleship (WN/N). Taken from [130].

The importance of this animal model is that they represent better than the other typically used laboratory rat/strains/lines the general population of rats, and probably the human population, being a useful tool for studying the genetic basis of biological, behavioral and disease-related complex traits [104,105,131–133] and help make connections between phenotypes and genotypes [133].

These rats have also been extensively characterized in profiles of timidity and defensive flight [132], coping styles and stress hormone responses [104,105], levels of unlearned anxiety, learned fear [103,134] and PPI [72]. They have also been successfully used for high-resolution genetic mapping of quantitative trait loci (QTL) to identify genes contributing to fear/anxiety-related behaviors [131,133], multiple sclerosis [133,135], bone fragility [130] and other disease-related complex traits [135,136].

NIH/HS rats show passive coping behavior and levels of anxiety similar to those shown by the RLA rats, characterized by freezing reactions (or immobility) in conflictive or stressful situations [104]. In cognitive tasks NIH-HS rats also resemble RLA rats and show a good performance in spatial learning and long-term spatial memory tasks in the Morris Water Maze [125].

02 /

Objectives

The main objective of this Doctoral Thesis is to continue with the characterization of the Roman rats on a behavioral, neuroanatomical and biochemical level to add more evidence in support of the proposal of the RHA rat strain as an animal model for the study of schizophrenia-relevant symptoms/features.

For this purpose, five different studies/goals have been set:

Study 1- *Association between prepulse inhibition of the startle response and latent inhibition of two-way avoidance acquisition: A study with heterogeneous NIH-HS rats.*

- To characterize the NIH-HS rat stock in two behavioural paradigms related to schizophrenia such as the prepulse inhibition of the acoustic startle response and the latent inhibition of the two-way active avoidance response.
- To define, for the first time in heterogeneous (outbred) rats, whether or not these two processes are somehow related or associated.

Study 2- *Prepulse inhibition and latent inhibition deficits in Roman high-avoidance vs. Roman low-avoidance rats: Modeling schizophrenia-related features.*

- To determine if the same association between PPI-LI found in NIH-HS rats (study 1) is shown in RHA-I and RLA-I rats.

Study 3- *Effects of social isolation rearing in the Roman rats in behavioral tasks and neuroanatomy of brain areas relevant for schizophrenia*

- To determine if RHA-I animals reared in social isolation show a higher PPI deficit, among other behavioral alterations typical of the "Social Isolation Syndrome" (i.e. anxiety, hyperactivity, spatial learning deficits), than RLA-I animals.
- To determine if the social isolation treatment causes differences in volume in areas of interest.

Study 4- *Conservation of phenotypes in the Roman rats after embryo transfer*

- To test if the generations of Roman rats born after an embryo transfer procedure present the typical between-strain differences in several representative phenotypes.

Study 5- *Characterization of dendritic spine density in pyramidal neurons of the PFC and quantification of parvalbumin neurons*

- To investigate if the differences in volume in the PFC found in study 3 are associated to structural differences in dendritic spine density in pyramidal neurons or to differences in the number/density of parvalbumin+ neurons.

03 /
Studies

3.1.- Study 1. Association between prepulse inhibition of the startle response and latent inhibition of two-way avoidance acquisition: A study with heterogeneous NIH-HS rats.

As we mentioned before, attention-related processes appear to be impaired in schizophrenia. Two of these processes are the prepulse inhibition of the acoustic startle response (PPI) and latent inhibition (LI). PPI, as defined in the *Endophenotypes* subsection, is a cross-species phenomenon [137–139] that can be measured in both, mammals and humans, by using the same procedure, providing a very applicable paradigm for translational research [138]. The LI phenomenon, also defined in *Endophenotypes,* has also been shown to be impaired in schizophrenic patients [68,140], reflecting dysfunctions in attentional and/or cognitive filtering mechanisms of the brain [68,140,141].

Several studies have been carried out to unravel the neural basis and structures involved in both PPI and LI processes. In the case of the PPI, Koch and Schnitzler proposed in their study from 1997 [139] that the ASR is triggered by excitatory input from the auditory pathway to the midbrain inferior colliculus (IC). In turn, the IC activates the superior colliculus (SC), with important projections to the pedunculopontine tegmental nucleus (PPTg) that inhibits the pontine reticular nucleus (PnC). The inhibition of the PnC leads to a downregulation of the startle response, resulting in the measurable PPI effect [139].

Likewise, the brain circuit for the LI has been established, with structures like the entorhinal cortex, the basolateral amygdala or the nucleus accumbens playing a role in the latent inhibition effect [68]. Furthermore, some studies have suggested that modulation of both PPI and LI may share some limbic structures, including the hippocampus, the basolateral amygdala and the nucleus accumbens (e.g. [68,142]). The fact that both processes share common brain structures reinforce the idea that they are somehow related.

The assessment of sensorimotor gating and attentional filtering in animal models has increasingly become an important tool for the understanding of the neurobiological basis of the disorder and the development of novel drugs with improved efficacy [67,68,70,143]. Associations between PPI and LI have been found in some selectively-bred rat strains (e.g. APO-SUS and APO-UNSUS rats, RHA-I and RLA-I rats), but not in other cases of either genetically- selected or outbred rats (e.g. see review by Del Río et al. [72]). The high genetic variability

shown by the NIH-HS rat stock is paralleled by a wide range of scores in many quantitative phenotypes/traits, which makes those rats a unique resource for the study of "normal" (i.e. unforced, unselected) variability in many traits of interest.

To the best of our knowledge no study has thus far addressed whether PPI and LI are associated in outbred (genetically heterogeneous) rats. The present study will focus on the characterization of the profile of the NIH-HS rats based on their performance on these two behavioral tests (PPI and LI), and the relationship, if any, between both. No study has still evaluated their sensorimotor gating profiles, hence the present study intends to shed light on the relationships among different attention-related (attentional filtering) processes that are relevant for schizophrenia and may provide indications on whether selection based on "normal" PPI variation can be a useful rat model for studying clusters of schizophrenia-relevant symptoms.

Material and methods

Animals

The subjects of this study have been 107 NIH-HS rats with an average age of 3-4 months at the beginning of the experiment (weight, 330+9.8 gr, mean + SEM). They were housed in pairs of the same sex and family in macrolon cages (50 x 25 x 14 cm) and maintained with food and tap water *ad libitum*.

We received 40 pairs of NIH-HS rats from Dr. Eva Redei (Center for Comparative Medicine, Northwestern University, Chicago, USA) in 2004. These animals are bred and grown at the Autonomous University of Barcelona (UAB) and kept in standard conditions of temperature (22±2°; 50-70% relative humidity) and a 12h light-dark cycle (lights on at 08:30h).

Tests were performed under the light phase of the cycle and all the protocols were approved by the Committee of Ethics of the Autonomous University of Barcelona in accordance with the European Communities Council Directive (86/609/EEC) regarding the care and use of animals for experimental procedures.

Apparatus and Procedure

—Prepulse inhibition

Four acoustically isolated boxes of 90 x 55 x 60 cm were used (Sr-Lab Startle Response System, San Diego Inst., San Diego, USA). Each box consists of a plexiglas tube (8.2 x 25 cm) where the rat is placed, situated on top of a platform with a sensor that detects the strength caused by the movements of the rat when it is subjected to the acoustic stimulus (startle response). These data are transduced by an accelerometer into a voltage which is amplified, digitized and saved into a computer for further analysis. The boxes are constantly lit by a 10w lamp and the acoustic stimuli are delivered by two speakers placed 15 cm from each side of the plexiglas tube. A white noise generator provides background noise of 55 dB. The startle session started with 5 minutes of habituation to the box. Then, 10 "pulse-alone" trials with the startle stimulus (105 dB, 40 ms) were administered to obtain a basal measure of the ASR (BAS1). After this, the different types of trials were randomly administered in blocks of 6 trials that were repeated 10 times (60 trials in total):

- "Pulse-alone" trials (105 dB), that constitutes the BAS2 measure used to calculate the percentage of PPI (see formula below).

- Prepulses of 65, 70, 75, and 80 dB (20 ms) followed by the startle stimulus (105 dB, 40 ms). These trials are "PPI" trials used to calculate the percentage of PPI (see formula below).

- Trials with no stimulus (only the background noise of 55 dB).

Following these 10 repetitions of 6 trials (60 trials), 5 "pulse-alone" trials were administered, this making the BAS3 measure.

The interval between stimulus (prepulse and pulse) was of 100 ms, while the intertrial interval between two trials varies between 15 and 45 seconds, with a mean of 30 seconds.

The rats' startle responses were recorded during 200 ms following the presentation of the acoustic (startling) stimulus (i.e. the 105 dB pulse).

The percentage of PPI (%PPI) for each prepulse trial and for each intensity of the acoustic prepulse, as well as the mean %PPI was calculated according to the following formulas:

$$\%PPI = 100 - \left(\frac{startle\ response\ amplitude\ on\ prepulse\ trials}{startle\ response\ amplitude\ on\ BAS2\ pulse\ trials}\ x\ 100\right)$$

$$Total\ PPI\ = \frac{\%PPI65 + \%PPI70 + \%PPI75 + \%PPI80}{4}$$

—Latent Inhibition

Rats underwent LI testing 2 weeks after PPI testing. The experiment was carried out in three identical shuttle boxes (Letica, Panlab, Barcelona, Spain) placed inside independent acoustically isolated boxes. Each box is slightly illuminated by a fluorescent bulb placed behind the shuttle box and kept in a dark room.

Each shuttle box consisted of two equally-sized compartments (25 x 25 x 28 cm) connected by an opening. The conditioned stimulus (CS) is administered in the form of an acoustic tone of 63 dB. The unconditioned stimulus (US) was a scrambled electric shock of 0.5 mA delivered through a grid floor. The session consisted of a 5 min habituation period followed by pre-exposure to 15 trials of 5s CS (administered at variable intervals, with a mean of 30 seconds intertrial interval) for the pre-exposed (PE) rats, while the non-pre-exposed (NPE) animals spent the equivalent time in the shuttle box without receiving stimulus (8 minutes 30 seconds). Immediately following this phase, the second part of the session - learning phase- started. It consisted of 40 training trials, in which the US was delivered during the last 30 seconds of a 35s CS (i.e. CS and US overlapped). The CS or US was terminated when the rat crossed to the opposite compartment, considering the crossing during the first 5 seconds of the CS as an avoidance and the crossing during the US an escape response. If the animal did not change compartments during the whole US it was considered as a response failure. Following termination of each CS or US an intertrial interval (ITI) of 15-45 seconds was presented. Intertrial crossings (crossings during the intertrial intervals) were also scored and will be further on referred to as ITC.

Statistical Analysis

All the analyses were performed with the Statistical Package for Social Science software (SPSS). Pearson correlations were performed separately for NPE and PE conditions. From the range of %PPI values obtained for 80 dB intensity a division was made from percentiles 33 and 66, dividing the sample into three different subgroups, HighPPI, MediumPPI and LowPPI. The division of

subgroups on the basis of any other of the three prepulse intensities did not lead to significant effects (data not shown). One way ANOVAs were performed on BAS1-3 scores as a function of PPI subgroup, followed by Duncan's multiple range tests. For the analyses of LI variables (avoidances and ITCs), repeated measures 2 x 3 x2 ANOVA were performed, with condition (PE or NPE) and PPI subgroup (high, medium or low) as between-subject factors, and trial blocks 1-20 and 21-40 as within-subject factor. Also, factorial ANOVAs ("2 conditions x 3 PPI subgroups") were performed for the 1-20 trial block of the three LI variables. Post-hoc multiple range Duncan's tests were performed following significant ANOVAs.

Results

Table 1 shows the descriptive statistics of the different variables measured in the PPI session for the whole NIH-HS rat sample (n=107). The highest ASR values are obtained in BAS1. BAS2 and BAS3 scores are lower as a consequence of habituation. Concerning %PPI, progressively higher scores are observed as a function of the increasing prepulse intensities (see means %PPI65, %PPI70, %PPI75 and %PPI80 in Table 1).

Table 1. Mean and standard error of PPI test variables. Values in bold correspond to the ones used to divide the animals in Low-, Medium- and High-PPI groups.

	Mean	Standard error
Body weight (g)	330	9,85
BAS1	1431,68	131,10
BAS2	764,42	70,67
BAS3	633,26	65,13
%PPI 65	17,72	12,36
%PPI 70	38,54	9,49
%PPI 75	52,34	7,58
%PPI 80	**65,39**	**5,26**
Total PPI	43,49	8,54

Table 2. Descriptive statistics (mean±SEM) of PPI and LI variables and main group comparisons are shown.

	Low PPI		***Medium PPI***		***High PPI***	
	NPE (n=19)	**PE (n=16)**	**NPE (n=20)**	**PE (n=17)**	**NPE (n=15)**	**PE (n=21)**
	Mean (sem)	**Mean (sem)**	**Mean (sem)**	**Mean (sem)**	**Mean (sem)**	**Mean (sem)**
PPI variables						
BAS1	732,8 (135,4)	1322,4 (285,6)	1193,7 (1188,1)	1317,1 (1569,1)	1679,4 (354,5)	2284,3 (351,7)
BAS2	304,6 (70,0)	531,4 (129,5)	657,3 (409,4)	724,5 (783,3)	964,1 (184,6)[a]	1363,1 (207,8)[a]
BAS3	378 (78,0)	444,3 (107,3)	546,9 (407,8)	615,6 (912,7)	802,3 (234,5)	983,2 (162,6)
%PPI 65	-73,6 (43,3)	-66,2 (51,9)	52,7 (15,7)	44,1 (32,8)	73,5 (5,8)	71,1 (2,9)
%PPI 70	-35,8 (39,2)	-13,5 (31,9)	64,2 (12,1)	52,2 (32,4)	83,6 (3,1)	78,4 (2,1)
%PPI 75	-10,7 (28,7)	0,3 (27,8)	74,3 (6,8)	73,8 (13,0)	89,2 (1,5)	85,4 (1,3)
%PPI 80	**12,1 (20,2)**	**31,5 (16,5)**	**81,8 (3,5)**	**81,6 (3,3)**	**93,3 (0,9)**	**91,5 (0,7)**
PPI Total	-27,0 (32,4)	-12,0 (31,6)	68,2 (8,0)	63,0 (18,5)	84,9 (2,4)	81,6 (1,6)
Shuttle box variables						
Trials 1-20						
Avoidances	0,7 (0,3)	0,8 (0,4)	1,4 (1,7)	1,1 (1,8)	**3,5 (1,1)**	**0,9 (0,2)***
ITCs	5,2 (0,8)	6,4 (0,8)	8,3 (4,9)	8,3 (8,8)	**13,3 (1,9)**	**5,2 (0,7)***
Trials 21-40						
Avoidances	3,4 (0,9)	3,1 (0,8)	5,5 (4,7)	4,7 (4,3)	7,4 (1,7)	4,4 (1,1)
ITCs	9,6 (1,7)	7,9 (1,6)	11,4 (8)	9,4 (8,6)	15,7 (3,5)	6,52 (1,0)
Trials 1-40						
Avoidances	**4,1 (1,2)**	**3,9 (1,1)**	7,0 (5,5)	5,8 (5,5)	10,9 (2,7)	5,29 (1,3)
ITCs	**14,8 (2,1)**	**14,2 (1,2)**	19,7 (11,4)	17,7 (16,5)	29,1 (5,1)	11,8 (1,5)

a, $p<0.05$ vs the corresponding LowPPI group; *, $p < 0.05$ vs the respective NPE sub-group (Duncan's tests following significant ANOVA effects). NPE: non-preexposed animals; PE: preexposed animals; ITCs: intertrial crossings.

For the LI test the animals that had previously undergone the prepulse inhibition assay were divided into three subgroups, HighPPI, MediumPPI and LowPPI, according to the scores obtained in the %PPI80 (see "statistical analyses"). These three groups were each divided again into two approximately equal groups: preexposed (PE) and non-preexposed (NPE) animals. PPI and LI data of these 6 subgroups are shown in Table 2.

Correlation analyses among variables from the PPI and LI procedures were separately performed for the NPE and PE conditions and are shown in Table 3A-B. In both cases there appear high positive correlations among most PPI variables (ranging from 0.94 to 0.99). Similarly, there are mostly high positive correlations among BAS (BAS1, BAS2, BAS3) variables (ranging from 0.47 to 0.84), but there are only very low (although significant) positive correlations among BAS2 scores and %PPI at the different intensities (ranging from 0.29 to 0.34, $p<0.05$) (see Table 3A-B). Moreover, there were essentially no significant correlations among measures from the PPI test and the LI test (Table 3A-B). Finally, as expected, mostly high positive correlations among ITCs and avoidances in the shuttle box LI task were observed (0.59 and 0.67 between ITCs and total Avoidances in NPE and PE conditions, respectively; Table 3A-B).

To test for the effects of PPI levels on LI, a repeated measures ANOVA (2 x 3 x 2) was performed for the avoidances in the two trial blocks, from 1-20 and 21-40, showing significant effects of "block" [($F(1, 101)= 83.61$, $p<0.001$)] and "PPI group" factor [$F(1,101)=3.66$, $p=0.029$]. The effect of the condition (NPE or PE) and the interaction between the two factors show a positive tendency which did not reach significance [$F(1,101)=3.6$, $p=0.061$; $F(1,101)=1.79$, $p=0.172$), respectively] (Fig. 1A). Also, a 2 x 3 ANOVA was performed on avoidances during the first (1-20) trial block, resulting in significant values of the three effects: "condition" [$F(1,101)=5.40$, $p=0.022$], "PPI group" [$F(1,101)=3.93$, $p=0.023$] and the interaction between both [$F(1,101)=4.12$, $p=0.019$)] (Fig. 1A). The previous "PPI group" effects indicate that the higher the %PPI the higher the number of avoidances (it can be seen very clearly in Fig 1B), while "condition" and "condition x PPI group" " effects mainly refer to the fact that PE induces a reduction of avoidances (i.e. latent inhibition) essentially in the HighPPI subgroup (Fig. 1A-B). ANOVA analysis of the total avoidances (i.e. in the whole 40-trial session) led to a "PPI group" effect [$F(1,101)=3.66$, $p=0.029$; see post hoc Duncan's tests in Fig. 1B] again confirming that the higher the %PPI the higher the number of avoidances.

Table 3. Pearson correlation matrix for the PPI and LI variables. (A) NPE group; (B) PE group.

A									Trials 1-20		Trials 1-40	
	BAS1	**BAS2**	**BAS3**	**%PPI65**	**%PPI70**	**%PPI75**	**%PPI80**	**TOTAL PPI**	**Avoidances**	**ITCs**	**Avoidances**	**ITCs**
LB1	1											
LB2	0,60**	1										
LB3	0,47**	0,84**	1									
%PPI65	0,23	0,32*	0,21	1								
%PPI70	0,21	0,29*	0,19	0,98**	1							
%PPI75	0,23	0,31*	0,20	0,97**	0,99**	1						
%PPI80	0,23	0,34*	0,21	0,95**	0,94**	0,96**	1					
TOTAL PPI	0,23	0,32*	0,21	0,99**	0,99**	0,99**	0,97**	1				
Trials 1-20												
Avoidances	0,04	0,11	-0,02	-0,01	-0,02	0,00	0,08	0,00	1			
ITCs	0,08	0,32*	0,19	0,18	0,18	0,22	0,23	0,20	0,50**	1		
Trials 1-40												
Avoidances	0,02	0,17	0,02	0,03	0,00	0,04	0,13	0,04	0,88**	0,53*	1	
ITCs	0,03	0,26	0,14	0,07	0,06	0,08	0,12	0,08	0,62**	0,87*	0,67**	1

B

									Trials 1-20		Trials 1-40	
	BAS1	**BAS2**	**BAS3**	**%PPI65**	**%PPI70**	**%PPI75**	**%PPI80**	**TOTAL PPI**	**Avoidances**	**ITCs**	**Avoidances**	**ITCs**
LB1	1											
LB2	0,77**	1										
LB3	0,60**	0,69**	1									
%PPI65	0,23	0,29*	0,25	1								
%PPI70	0,25	0,31*	0,24	0,96**	1							
%PPI75	0,27	0,32*	0,28*	0,99**	0,95**	1						
%PPI80	0,24	0,32*	0,25	0,94**	0,96**	0,95**	1					
TOTAL PPI	0,25	0,31*	0,26	0,99**	0,98**	0,99**	0,97*	1				
Trials 1-20												
Avoidances	0,04	-0,01	0,13	0,00	0,05	0,02	0,08	0,03	1			
ITCs	-0,01	-0,08	-0,01	0,03	0,02	0,03	0,02	0,03	0,26	1		
Trials 1-40												
Avoidances	0,21	0,04	0,16	0,08	0,10	0,09	0,11	0,10	0,75	0,37*	1	
ITCs	0,06	0,00	0,10	0,04	0,06	0,03	0,03	0,04	0,44**	0,89*	0,59**	1

*p ≤ 0.05 (two-tailed significance).

**p ≤ 0.01 (two-tailed significance).

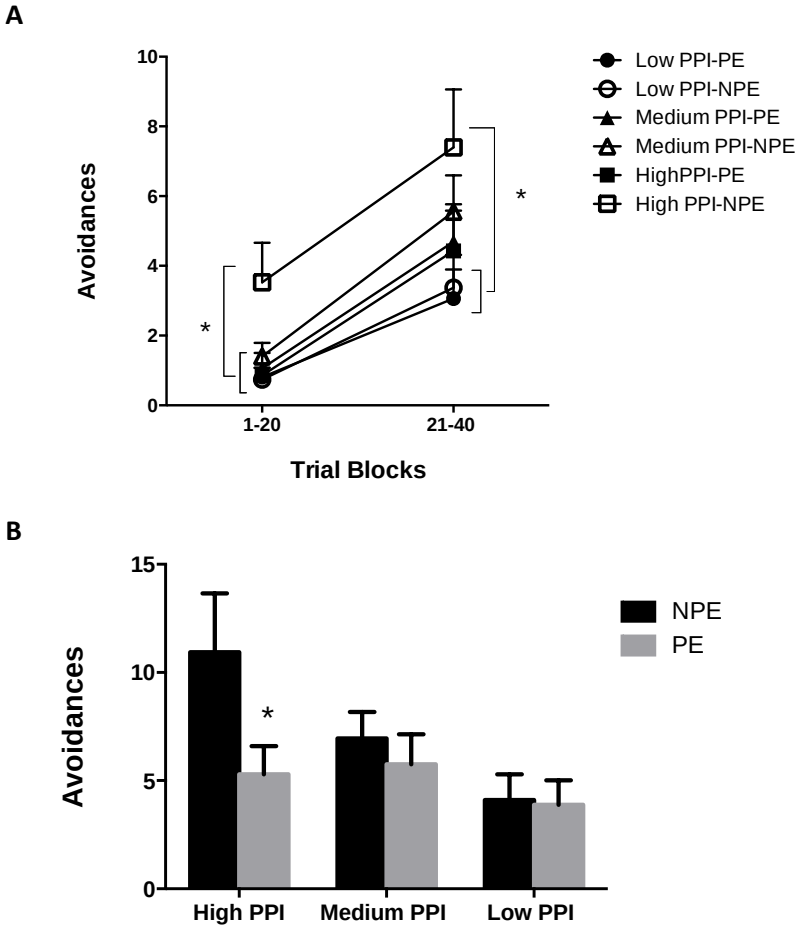


Figure 1. Mean ± SEM of avoidances in different trial blocks. (A) Accumulated avoidances in 1–20 and 21–40 trial blocks. *, $p < 0.05$ among the groups indicated (Duncan's multiple range test). (B) Accumulated avoidances in the whole 40-trial session (trials 1–40).*, $p<0.05$ vs HighPPI-NPE group (Duncan's multiple range test following significant ANOVA). See "n" in Table 2.

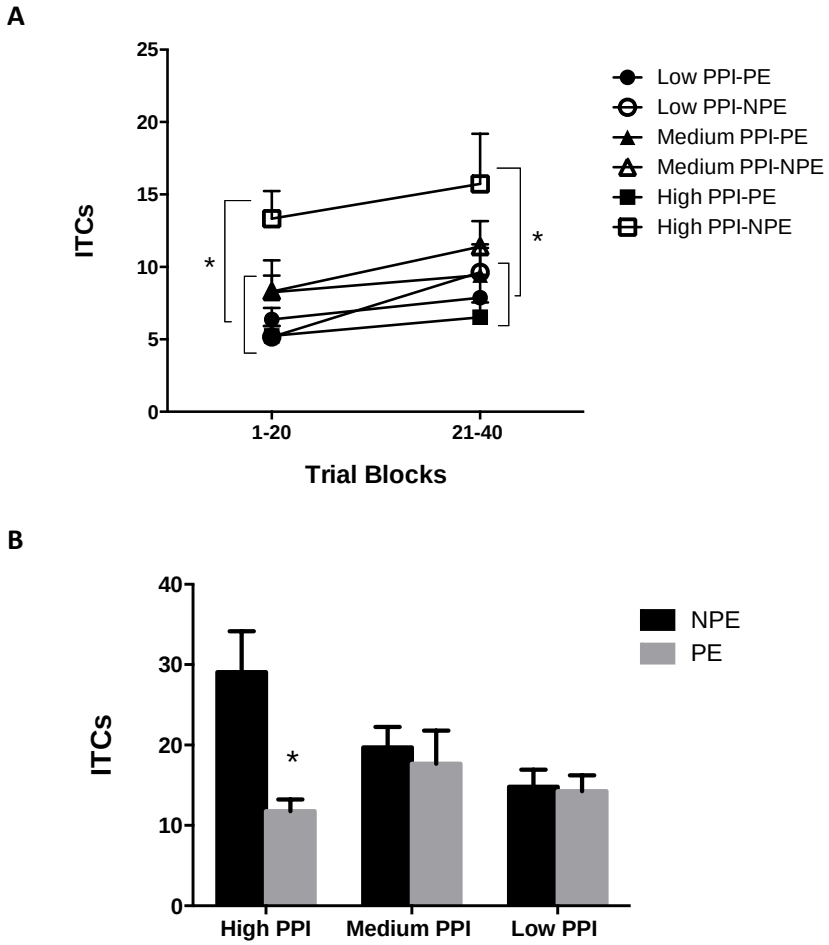


Figure 2. Mean ± SEM of ITCs in different trial blocks. (A) Accumulated ITCs in 1–20 and 21–40 trial blocks. *, $p < 0.05$ among the indicated groups (Duncan's multiple range test). (B) Accumulated ITCs in the whole 40-trial session (trials 1–40). *, $p<0.05$ vs HighPPI-NPE group (Duncan's multiple range test). See "n" in Table 2.

The same analyses were performed on ITCs (Fig. 2). In this case, the two factor repeated measures ANOVA showed significant values of "block" [(F(1, 101)= 13.97, p<0.001)], the "condition" [F (1,101)=7.51, p=0.007], and the interaction between both factors [F (1,70)=4,91 p=0,009)], but no for the "PPI group" [F (1,101)=2.09, p=0.129]. The ANOVA for ITCs in the 1-20 trial block revealed significant values for the three effects: "condition" [F (1,101)=4.78, p=0.031], "PPI group" [F (1,70)=3,90 p=0,023] and "condition x PPI" [F (1,101)=7.67,

p=0.001)]. Post-hoc Duncan's tests showed that the HighPPI-NPE group is the only which is significantly different from the five other subgroups (Fig. 2).

Discussion

This study presents the first characterization of PPI-LI associations in outbred (genetically heterogeneous) rats, specifically in the NIH-HS rat stock. The main result is that latent inhibition (LI) of two-way active avoidance acquisition appears only in the HighPPI group, as shown by the significant difference between the respective NPE and PE subgroups, i.e. HighPPI-PE rats show impaired acquisition of two-way avoidance compared with HighPPI-NPE rats (see Fig. 1A–B). This result is consistent with the literature, since NPE rats learn the CS-US association better than PE animals, which have been pre-exposed to the CS and therefore have identified this stimulus as a "safe" one [67,119,142,140]. In contrast with that, what is new in the present results is the absence of LI in subgroups of rats presenting low or mediumPPI levels (Fig. 1A–B). It might be argued that this absence may be due to the fact that they make too few avoidance responses (i.e. a possible floor effect). However, this explanation seems unlikely for the following reasons:

1) the HighPPI group shows LI already in trials 1–20, as the NPE sub- group shows only 3.5 avoidances and (in spite of it) the corresponding PE subgroup makes 0.9 (thus showing LI; see Table 2, square in bold);

2) if we match the LowPPI and HighPPI groups by their avoidance levels we can see that the LowPPI-NPE subgroup makes 4.1 avoidances in the whole 40-trial session (which are comparable to the 3.5 avoidances of the HighPPI-NPE subgroup during the 1–20 trial block), but their respective LowPPI-PE subgroup – with 3.9 avoidances – does not show latent inhibition (see Table 2, squares in bold).

When taking into account the variables of the latent inhibition test we observed that intertrial crossings (ITCs - changes of compartments between trials that predict two-way avoidance acquisition) are positively correlated with avoidances, and both behavioral responses load always in the same factor (following factor analyses), thus indicating that they are part of the same behavioral dimension, i.e. acquisition of the task in the shuttle box [97,103,104,132,134,144]. ITCs are related to fear to the whole experimental situation, i.e. contextual fear, which in turn is known to depend on the strength of the CS-US association ([97,145] and

references therein). ITCs tend to decrease as acquisition progresses (and CS-US-induced fear declines) and they are reduced by massive exposure to the (prospective) conditioned context in parallel to contextual fear reduction [97]. Aguilar et al. [97] have addressed this issue with Roman High and Low Avoidance rats (RHA-I vs RLA-I), obtaining that RHA-I rats, which acquire the avoidance response much more efficiently than RLA-Is, respond to contextual fear by moving impulsively back and forth and making many intertrial crossings [97]. Thus, if pre-exposure to the CS reduces the strength of the CS-US association through the LI process, this may lead to a weakened contextual fear conditioning and, as a consequence, to a reduction of ITCs (other than avoidances) in CS-preexposed (PE) animals.

Accordingly, the results from this study show that ITCs follow a trend parallel to avoidances, as indicated by the significant differences between HighPPI-NPE and HighPPI-PE subgroups (Fig. 2A–B) and the absence of differences between LowPPI-NPE vs LowPPI-PE and between MediumPPI-NPE vs MediumPPI-PE subgroups (Fig. 2A–B). This supports the results obtained by Aguilar et al. based on Mowrer's hypothesis that ITCs are related to fear to the context [97,145], since pre-exposure to the CS is presumably reducing the strength of the CS-US association (i.e. the LI process), which in turn may (hypothetically) lead to a weakened contextual fear conditioning and, as a consequence, to a reduction of ITCs (other than avoidances) in the PE group.

The fact that the same group of animals, in our case the LowPPI group, appears to have a deficit in both paradigms tested, as evidenced by the low scores of prepulse inhibition and the inability to learn the CS-US association in the LI test, prompt us to think that both measures are somehow related and may share common mechanisms.

3.2.- Study 2. Prepulse inhibition and latent inhibition deficits in Roman high-avoidance vs. Roman low-avoidance rats: Modeling schizophrenia-related features.

A large amount of studies has been carried out with the Roman rat lines/strains (i.e. outbred or inbred, respectively) along the past four decades, and several traits have been seen to differ between these lines/strains, other than their differential ability to acquire the two-way avoidance task. RLA rats are anxious/fearful and show passive coping when facing conflict and/or stressful situations, whereas RHA rats are less anxious/fearful, display proactive coping responses, which in turn allow them to quickly acquire the two-way avoidance task. Schizophrenic patients show deficits in sensorimotor gating and attentional processes, like PPI and latent inhibition (LI), which are considered endophenotypes of the disorder, and thus, a rat model of schizophrenia-related features with good validity would be expected to present impairments of both PPI and LI [94]. In a preliminary study from our laboratory we observed that RHA-I rats had some deficits in latent inhibition of the two-way avoidance response in comparison with Sprague-Dawley rats [119], but RHAs could not be compared with RLA-I rats due to the incapability of the latter to acquire the two-way avoidance task. Therefore, in the present study we aimed at measuring LI in a task that could allow direct comparison between both the RHA-I and RLA-I strains, i.e. LI of fear-potentiated startle (FPS; [141,142]). In the procedure of fear-potentiated startle (FPS), animals are submitted to a classical conditioning training by performing several pairings of a neutral stimulus (the prospective CS) with an aversive one (US, electric footshock). Once this is done, when the CS is presented, the acoustic startling stimulus (pulse) elicits a bigger startle response - ASR - in the rat, i.e. the subject shows greater ASR following "CS-pulse" presentation than following "pulse alone" presentation. Pre-exposure of rats to the prospective CS (prior to classical conditioning training) produces the LI phenomenon, i.e. a decrease of ASR following "CS-pulse" presentation due to a decrease of the strength of the classical conditioning association [141,142]. Following the results obtained in study 1, in which we observed impairments of both PPI and LI in the same group of HS rats (LowPPI group), we hypothesized that, relative to RLA-Is, RHA-I rats would display PPI and LI impairments.

Material and methods

Animals

A total of 42 male RHA-I and 42 male RLA-I rats were used in this study. At the beginning of the experiment the animals were experimentally naive (they had not been used in any other experimental or testing procedure), they were approximately 5 months old, weighing 410.3±7.0 g (RHA-I) and 412.8±8.8 g (RLA-I) (means ± SEM). Rats were housed in pairs of the same sex and strain in macrolon cages (50×25×14 cm) and maintained with food and water *ad libitum* under standard conditions of temperature (22±2 °C), humidity (50– 70%) and a 12 h light-dark cycle (lights on at 08:30 h). The experiments were carried out during the light phase of the cycle (from 9:00 to 19:30 h) and the procedures were approved by the Ethics Committee of the Autonomous University of Barcelona in accordance with the European Communities Council Directive (86/609/EEC) on the use of laboratory animals.

Apparatus and procedures

—PPI

See *Materials and methods* in study 1.

—Latent inhibition of fear-potentiated startle (LI)

In this case, the LI test was conducted in the same apparatus as the PPI test (see Materials and methods in study 1) with a light located on the ceiling of the box as the CS. The test was conducted on two consecutive days. Pre-exposure, baseline (preconditioning phase) and conditioning sessions were consecutively performed on the first day, while a second baseline (postconditioning phase) and the LI test took place the following day. Background noise intensity was 55 dB. For the LI test half of the rats from each strain (RHA-I, n=42; RLA-I, n=42) were randomly allocated to the "Pre-exposure" (PE) or "Non- pre-exposure" (NPE) subgroups to form a total of 4 experimental groups. Thus, the final "n" for the 4 groups were: NPE-RLA, n = 21; PE-RLA, n = 21; NPE-RHA, n = 21; PE-RHA, n = 20 (one rat of this group was excluded due to technical problems).

Pre-exposure (day 1)

After the 5-min period of familiarization to the startle chamber, rats from the "pre-exposure" (PE) groups received 15 repetitions of the prospective conditioned stimulus (CS, light which lasted for 3.7 s with an interstimulus interval of 45 s). Non pre-exposed (NPE) rats spent an identical period of time in the box without receiving the prospective CS.

Baseline ASR: preconditioning phase (day 1)

This first baseline ASR (first day, preconditioning phase) took place immediately after the pre-exposure phase and before conditioning, and consisted of 30 trials. Each trial consisted of an acoustic stimulus of 95 dB and 50 ms, with an interstimulus interval of 30 s. The performance (i.e. the startle response) of the rat was recorded during 200ms following the presentation of the 95 dB pulse.

Conditioning (day 1)

This phase was carried out immediately following the previous ("preconditioning phase" baseline) one. Each rat was trained with 15 pairings of the CS (light) with the US (shock). They received the CS alone during the first 3.2 s, after which a 0.7mAshock (US)was administered through a grid on the floor of the tube where the rat is placed while the CS continued for another 0.5 s. Inter-trial interval was 30 s.

Baseline ASR: postconditioning phase (day 2)

This phase took place 24hafter the conditioning session. This second baseline ASR (second day, postconditioning phase) was performed following the 5-min period of familiarization to the box and consisted of 35 trials (identical to those of the "preconditioning phase" baseline).

LI test (day 2)

The "LI test" phase started immediately following the previous ("postconditioning phase" baseline) one. It consisted of 20 acoustic stimulus alone (95 dB, 50ms; "pulse-alone" trials) and 20 of the same stimulus preceded by the CS (3.7 s light; "CS+pulse" trials), presented in pseudorandom order. These trials were separated by 30s of inter-trial interval. The difference of ASR between pulse-alone trials with respect to "CS + pulse" trials would indicate the degree of (cue-conditioned) fear-potentiated startle (FPS). The difference between pre-exposed (PE) and non-pre-exposed (NPE) rats in their level of FPS

would represent the phenomenon of latent inhibition (LI). In both the PPI and LI experiments cage floors were always thoroughly cleaned between consecutive rats, using a 10% EtOH solution, and dried with paper towel.

Statistical analyses

Statistical Package for Social Science (SPSS) software was used to analyze the data. Regarding prepulse inhibition data, a repeated measures ANOVA analysis was performed taking into account different prepulses (within subject factor) and strains. The same procedure (repeated measures ANOVA) served to analyze different baselines of the PPI session and to analyze the different phases of the LI procedure. One-way ANOVA was chosen to analyze the significant differences between strains. To measure the fear potentiated startle, repeated measures ANOVAs were also used to see the difference between trial types (within subjects), and strain and exposure (pre-exposed or not) were the between-subject factors. Again, one-way ANOVA was used to see the significant effects of strain and pre-exposure (ANOVA). Post hoc Duncan's tests were applied when necessary and following significant repeated measures ANOVAs.

Results

Prepulse inhibition

Figure 1 shows the baseline ASR for both rat strains and the habituation effect ["block" effect, $F(2, 162)=69.58$, $p<0.001$]. ANOVA also displays a significant effect of the "strain" [$F(1, 81) = 5.046$, $p<0.05$; see Duncan's tests in Fig. 1], as RLA-Is show significantly in- creased responses in BL1 and BL2. The percentages of prepulse inhibition (%PPI) for each prepulse intensity are shown in Fig. 2A. The difference between strains is highly significant for each of the four prepulse intensities: 65 dB, $F(1, 81)= 11.345$, $p<0.001$; 70 dB, $F(1, 81)=24.21$, $p<0.001$; 75 dB, $F(1, 81)= 18.992$, $p<0.001$; 80 dB, $F(1, 81) = 12.056$, $p<0.001$. It shows the deficit of RHA-I rats with respect to RLA-Is in the whole PPI test. Thus, total PPI (averaged across the 4prepulse intensities) presents a significant difference between strains [$F(1, 81)=23.173$, $p<0.001$], as it is shown in Figure 2B.

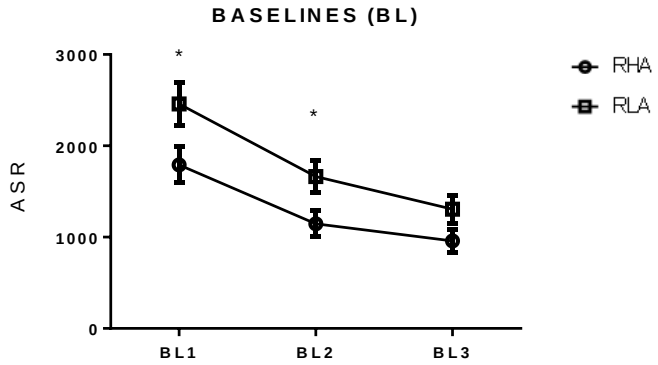


Figure 1. The three trial blocks (BL1, BL2, BL3; see "Materials and methods") of baseline ASR are shown for RLA-I (N=42) and RHA-I (N=41) strains. Results are shown as mean and standard error. *p<0.05 between both groups in the same block (BL) (Duncan's tests).

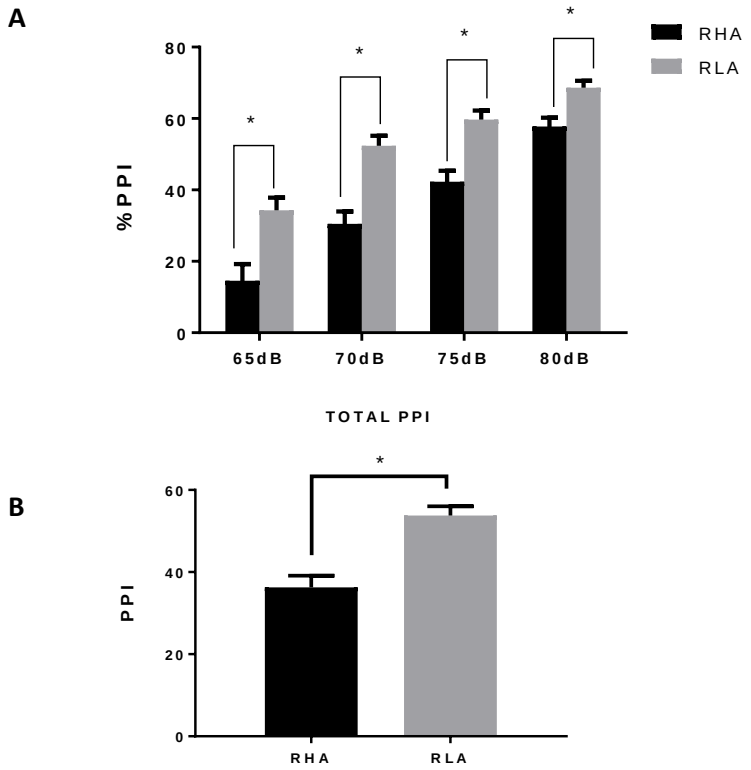


Figure 2. (A) Percentages of PPI in RLA-I (N=42) and RHA-I (N=41) rats are shown for the four prepulse intensities (65–80 dB). (B) Total PPI (averaged across the four prepulse intensities). *p<0.05 (Duncan's tests). Results are presented as mean and standard error.

Latent inhibition

Figure 3A shows the baseline ASR from day 1 (preconditioning phase). No conditioning has been established yet, so all the subjects from the same strain have a similar habituation curve, and again there are global differences between the RHA-I and RLA-I groups ["strain" effect, $F(1, 81)=8.210$, $p<0.05$] showing that RLA-Is display overall increased baseline ASR (see also Student's t-tests in Fig. 3A). In the second baseline ASR phase, carried out the following day (postconditioning phase, day 2; Fig. 3B), after the pre-exposure and the conditioning phases which were carried out on day 1, there is a clear tendency in pre-exposed (PE) RLA-I rats to show less startle response than their non-pre-exposed (NPE) counterparts. The ANOVA test revealed "strain" [$F(1, 81)=4.79$, $p=0.032$] as well as "trial block" [$F(6, 480) = 7.05$, $p<0.001$] effects. Posthoc Duncan's tests showed that non pre-exposed RLA-I rats (NPE-RLA) differ from the other three groups (including pre-exposed RLA-I rats) in several trial blocks, thus highlighting the increasing effect of pre-exposure on ASR habituation to the conditioned context in RLA-I rats (but not in RHA-I rats) (Fig. 3B).

Concerning the "LI test" phase (Fig. 4), the ANOVA for the 4 blocks of 5 trials (with 2 within-subjects factors, i.e. "trial block" and "trial type", and 2 between-subjects factors, i.e. "pre-exposure program" and "strain") shows a "trial type" ("CS+pulse" vs. "pulse-alone") effect [$F (1, 81)=7.26$, $p=0.009$] and a "trial block" effect [$F (1, 81)=72.35$, p b 0.001]. It also shows a significant effect for the "trial type x strain" interaction [$F(1, 81) = 11.34$, $p<0.001$]. To dissect this interaction "strain" and "trial type" were analyzed separately. First, we performed an analysis of the "pulse-alone" trials taking both strains into account. This ANOVA shows a significant "strain" effect [$F(1, 81)=11.49$, $p<0.001$], "trial block" effect [$F(1, 81) = 7.59$, $p=0.007$], "trial block x strain" effect [$F(1, 81)=5.75$, $p=0.019$] and "pre-exposure program x strain" effect [$F(1, 81)=5.12$, $p=0.026$]. Next we analyzed each strain by pulse-alone trial type. In this case, although the analysis for the RHA-I strain shows no significance for any of the effects, the analysis for the RLA-I strain shows a significant "trial block" effect [$F(1, 40)=8.23$, $p=0.007$] and a "pre-exposure program x trial block" effect [$F(1, 40)=5.12$, $p=0.029$), indicating that pre-exposure has differential effects depending on the strain, i.e. PE-RLA rats show decreased responses as compare to NPE-RLA rats, an effect that is not present between both RHA-I groups (see Duncan's tests in Fig. 4A and C).

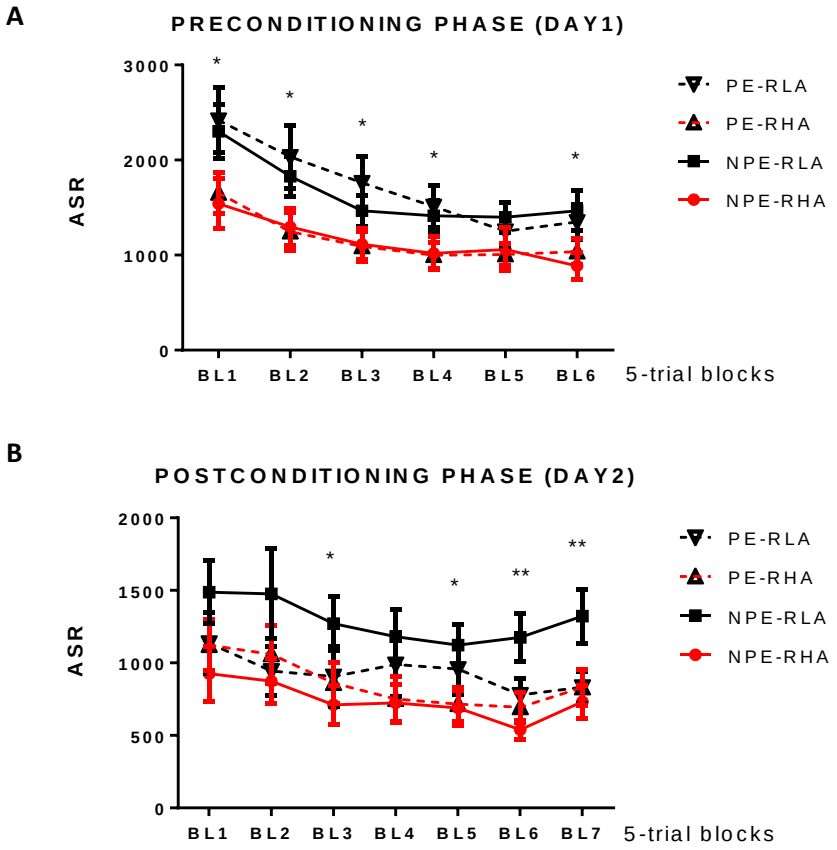


Figure 3. Baseline ASR from (A) day 1 (preconditioning) and (B) day 2 (postconditioning). Four groups are displayed, depending on the "strain" (RHA-I, RLA-I) and "pre-exposure" (PE, NPE) conditions Groups: PE-RHA, pre-exposed RHA-I (n=20); NPE-RHA, non-pre-exposed RHA-I (n=21); PE-RLA, pre-exposed RLA-I (n=21); NPE-RLA, non-pre-exposed RLA-I (n=21). Results are shown as mean and standard error. A) Baseline ASR (preconditioning phase, day 1) is shown for the six blocks. No group has still been submitted to conditioning. *$p < 0.05$ between both strains (pooling NPE and PE groups, Student's t-test). B) Baseline ASR (postconditioning phase, day 2) is shown 24 h after the pre-exposure and conditioning sessions. It is shown divided in seven blocks. *$p<0.05$ vs. NPE-RHA; **$p<0.05$ vs. the other 3 groups (Duncan's tests following significant ANOVA for each trial block).

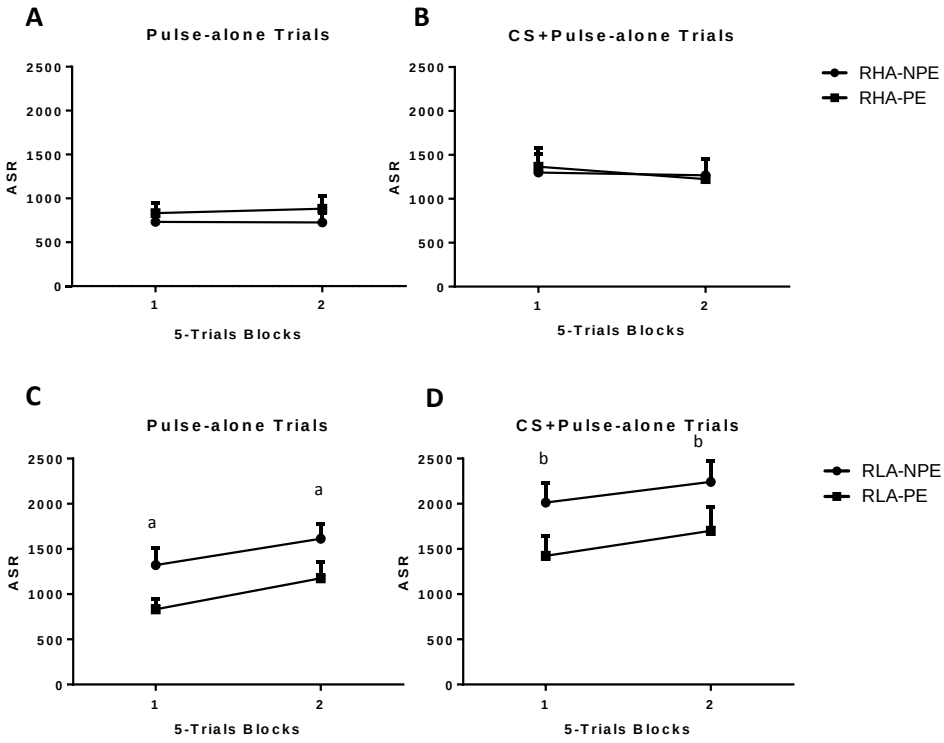


Figure 4. ASR amplitude in the "LI test" phase (day 2) is shown (means ±SEM). It appears divided in two different graphs, separately for both types of trials (i.e. "pulse alone" and "CS+pulse") and for both rat strains. "n" as in Fig. 3. (A), (C) Average ASR for each 5-trial block of pulse-alone during the "LI test" phase. (B), (D) Average ASR for each 5-trial block of "CS+pulse" during the "LI test" phase (see "Materials and methods"). a, $p<0.05$ vs. PE-RLA, NPE-RHA and PE-RHA groups; b, $p<0.05$ vs. NPE-RHA and PE-RHA groups (Duncan's tests).

Then we performed the same analyses for the "CS +pulse" trials. The ANOVA for both strains shows significant "strain" [$F(1, 81) = 6.87$, $p=0.010$] and "trial block x strain" [$F(1, 81)=5.77$, $p=0.019$] effects. The meaning of this interaction is clarified by post-hoc Duncan's tests revealing that the NPE-RLA group is significantly different from the two RHA-I groups, whereas the PE-RLA group is not different from any of the RHA-I groups (see Fig. 4B and D).

Figure 5 shows the mean ASR differences between pulse-alone trials before (i.e. "preconditioning phase") and after (i.e. "postconditioning + LI" phases) conditioning. The ANOVA (2 "strain" x 2 "pre-exposure program" x 2 "trial

block") shows an effect of "strain" [F(1, 81)=8.09, p=0.006] as well as a "trial block" (i.e. "pre- conditioning" vs. "postconditioning+LI") effect [F(1, 81)=58.88, p<0.001]. It also shows a triple "strain x pre-exposure program x trial block" interaction [F(1, 81)=5.48, p=0.022]. Post hoc Duncan's test shows that there is a significant difference between the PE-RLA and NPE-RLA groups in the "postconditioning+LI" trials, thus indicating that pre-exposure reduces ASR during these pulse-alone trials specifically in RLA-I rats (Fig. 5).

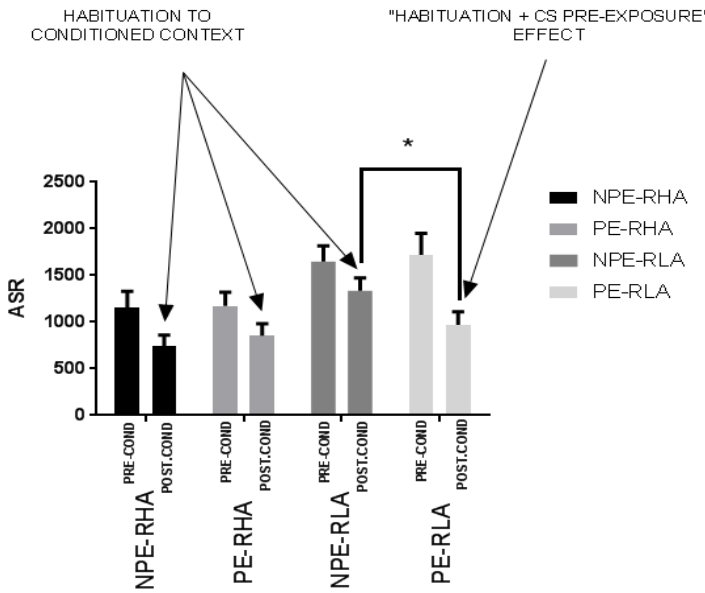


Figure 5. Mean ± SEM of ASR differences between pulse-alone trials before conditioning and after conditioning. Results for each group (i.e. each pair of bars) are divided in: left bar, "PRE-COND", which represents the averaged baseline ASR during the preconditioning phase (day 1); right bar, "POST-COND", which represents the averaged baseline ASR during the postconditioning phase (day 2) plus the pulse-alone trials of the "LI test" phase (day 2). *p<0.05 (Duncan's test). Group symbols and "n" as in Fig. 3.

Discussion

The present results confirm that RLA-I rats show increased unconditioned acoustic startle responses (ASR), giving further support to the idea that such a difference is a consistent characteristic of both Roman rat strains [103,146]. Likewise, the results of the PPI experiment corroborate our previous finding that PPI is more robust in RLA-I than in RHA-I rats [72,147], thus supporting the contention of worsened sensorimotor gating processes in the latter. Baseline ASR

has been related to PPI, suggesting that subjects (humans or mice) with lower response to the unconditioned acoustic stimulus display higher percentage of PPI [148]. Similar results were also observed in a study conducted with the APO-SUS and APO-UNSUS rat lines, which differ in both their vulnerability to apomorphine and PPI levels [94]. Conversely, our results show that RHA-I rats display impaired PPI and lowered baseline ASR, which is in line with results obtained in study 1, in which subjects with more robust PPI showed higher baseline ASR too [147]. Therefore, according to these different studies, the sign (direction) of the relationship between baseline ASR and PPI is unclear, so further research is warranted on that issue. In a previous study on LI of the two-way active avoidance we compared RHA-I rats with Sprague-Dawley rats [119], since RLA-Is display a floor effect and do not learn the avoidance task (their high fear/anxiety levels lead to freezing responses; e.g. [104]). In the present study we used a different LI procedure that could allow a direct comparison between RHA-I and RLA-I rats, i.e. the LI of fear potentiated startle -FPS- (see *Materials and Methods* and ref. [141,142]).

Interestingly, in the LI test phase, "trial block", "trial type" ("pulse-alone" vs. "CS+pulse") and "strain x trial type" effects were found, indicating that startle responses to pulse-alone and/or CS-pulse trials depend on (or vary as a function of) the strain of rat although, globally, startle responses are higher during CS-pulse trials (i.e. "trial block" effect). Remarkably, RLA-I pre-exposed rats showed a latent inhibition of fear to the context, i.e. pre-exposure to the prospective CS in RLA-I rats induced a reduction of ASR during the "postconditioning phase" (pulse-alone trials) and during the "pulse-alone" trials of the "LI test phase". To the best of our knowledge, such a phenomenon, i.e. LI of fear to the context in an acoustic startle procedure, has been found for the first time in the present study. It was possible to measure this kind of contextual fear due to the comparison between the baselines (of startle response) carried out on both days (i.e. "preconditioning phase", day 1, and "postconditioning phase" in day 2), which other authors have not used [141,142]. It is known that there is a relationship between the CS-US association and this type of contextual fear, i.e. the stronger the CS-US association the greater the fear to the context. Thus, RLA-I pre-exposed (PE) rats display a weakened CS-US association due to pre-exposure to the prospective CS and, as a consequence, they exhibit less fear to the context than the non-pre-exposed (NPE) RLA-I group. This phenomenon is not observed in the RHA-I strain. Both NPE- and PE-RHA-I groups present similar startle responses, so pre-exposure to the prospective CS has no effect on this rat strain. It has to be highlighted that such a LI to the context, observed in

PE-RLA rats, is evidenced as an accelerated habituation of the startle responses during postconditioning. Indications of LI to the CS were only observed in pre-exposed RLA-I rats (PE-RLA), as this group did not show any difference vs. both RHA groups in CS-pulse trials, while, as indicated by their significantly increased ASR responses, NPE-RLA rats were actually different from NPE-RHA and PE-RHA rats (see Fig. 5). These differences (i.e. the effect of pre-exposure on the responses of RLA rats) were not as pronounced as in the pulse-alone trials (i.e. fear to the context), but this is still consistent with the idea that pre-exposure of RLA-I rats to the prospective CS weakens the associative process of classical conditioning in that strain (producing LI), while leaving it unaffected in the RHA-I strain. However, further parametrical studies under different experimental conditions are required to assess the LI to the cue (CS) process and to add more evidence on its differential expression in both rat strains. To summarize, LI to the context is observed in pre-exposed RLA-I rats as an acceleration of startle habituation, and LI to the CS in PE-RLA rats is evidenced as a partial reduction of the increased ASR observed during "CS-pulse" trials.

Therefore, the present findings of LI to the context in RLA-I (but not RHA-I) rats lend some support to those from study 1, i.e. the association between good PPI and improved LI, while they also constitute further evidence that RHA-I rats present an altered attentional profile that adds to their profile of schizophrenia-relevant features.

3.3.- Study 3. Effects of social isolation rearing in the Roman rats in behavioral tasks and neuroanatomy of brain areas relevant for schizophrenia

It is well known that manipulations in early life can affect the adult behavior of the individual. Social isolation has been described as an environmental manipulation that produces a disturbance in the expression of normal behavior [149,150]. The post-weaning isolation of animals has deep effects on the adult individual at a psychological, behavioral and neurochemical level [83,86–89,91,150,151]by causing a detrimental effect on the development of the brain. Social isolation has been described to cause alterations that mimic certain features present in neurodevelopmental disorders, such as schizophrenia [150], in an enduring, robust and replicable way [150].

Social isolation has been widely described to cause and deepen some of the features characteristic of schizophrenia such as: reduction in prepulse inhibition (PPI) [70,86,152–155], hyperactivity [152,153], alterations in working memory as measured in the Morris water maze (MWM) [150,152], altered dopamine and serotonin levels in limbic regions [83,153], reduced volume of cortical regions, most importantly, medial prefrontal cortex (mPFC) [83,91], or different alterations in hippocampus [83,155] and striatum [150]. Therefore, the fact that this manipulation is able to mimic some of the most important characteristics of schizophrenia, makes it possible to suggest social isolation as an animal model for these kind of disorders with sufficient construct and predictive validity [149,150,155].

In this study we evaluated the effects of social isolation rearing in both strains of the Roman rats, RHA-I and RLA-I (Roman High- and low-avoidance rats) at a behavioral level by testing them in PPI (sensorimotor gating), elevated zero-maze (anxiety), locomotor activity and spatial working memory and long-term reference memory in the MWM. We also evaluated the effects at a neuroanatomical level by investigating volume differences in mPFC, dorsal striatum (dSt) and dorsal hippocampus (dHPC) with stereology, and at a neurochemical level by assessing binding levels of the serotonin receptor 5HT2A also in mPFC, dSt and HPC. We expect to confirm the results obtained by Oliveras et al. [152] in the behavioral profile and hypothesize that RHA-I rats should present a more profound reduction in volume in the proposed areas of interest, particularly in the mPFC, and an increased expression of the serotonin receptor when compared to their RLA-I counterparts.

Material and Methods

Animals and housing conditions

In this study we used male animals from both strains of the Roman rats, RHA-I (n=26) and RLA-I (n=26), that were randomly assigned to one of the two experimental groups (control and isolated). Control animals (n=10 per strain) were housed in pairs of the same strain in macrolon cages (50 x 25 x 15 cm) while the isolated ones (n=16 per strain) were individually housed at PDN 21 also in macrolon cages (35 x 25 x 15 cm) having visual, olfactory and auditory but not physical contact with their littermates. Both experimental groups were reared under the same conditions: 12 h light-dark cycle (lights on at 8:30 am), controlled temperature (22 $\pm$ 2°C) and humidity (50-70%) with food and water *ad libitum*. Experimental phase started after 14 weeks of isolation and all the experiments or tests were conducted during the light phase of the cycle, with housing conditions remaining the same during the whole study. The experiments were approved by the committee of Ethics of the Autonomous University of Barcelona in accordance with the European Communities Council Directive (86/609/EEC) regarding the care and use of animals for experimental procedures.

Experimental design

14 weeks after isolation (animals aged 17 weeks), a battery of several behavioral tests was conducted (see below). All the animals were subjected to the same tests and in the same order, which was carefully settled according to the literature in order to avoid confusing results regarding overlapping effects. The animals first underwent two PPI sessions (weeks 17-18), followed by testing in the elevated zero maze (week 19), actimetry (week 21), and several tasks in the Morris water maze (weeks 24-25). Three weeks after the behavioral phase was concluded, the animals were sedated with inhaled isofluorane 5%, sacrificed by decapitation and their brains immediately removed, frozen in liquid nitrogen and stored at -80° for further structural and binding analyses (see below).

Behavioural test battery

—PPI

For this study, two sessions of PPI were administered elapsing one week between the two. In the first session the intensities of the prepulse were 65, 70, 75 and 80

dB, while in the second session we narrowed it down to two intensities of 59 and 63 dB.

For further details on the PPI protocol see *Material and methods* in study 1.

—Elevated zero-maze

Two weeks after PPI testing the animals were tested in an elevated zero-maze, similar to that described by Shepherd et al. [156]. The maze is composed of an annular platform (a circular corridor 105 cm diameter and 10 cm width) made of black plywood and elevated 65 cm above ground level. It has two open sections or quadrants and two closed ones with walls 40 cm high.

The maze is situated in a room slightly illuminated by a red fluorescent light. The trials are videotaped and the behavioral measures are taken outside the testing room by a trained observer. The session began with the animal placed in an enclosed quadrant facing the wall. The animals were tested for 5 minutes to measure the following variables: latency of the first entry to an open section, number of entries in open sections, time spent in open sections, head dips over the edge of the platform and line crossings among 8 different defined zones.

—Locomotor activity

Two weeks later the rats were tested in activity boxes to assess horizontal locomotor activity. This test took place in 3 identical Plexiglas activity cages (40 x 40 x 40 cm) equipped with a frame of photocell beams that are interfaced by a computer software (Acti-Track; PANLAB, Barcelona, Spain) that records the horizontal activity of the rats. To begin the test the rat is placed in the center of the cage and its behavior is recorded for 30 minutes. The testing room is illuminated with a 60W white fluorescent light. Locomotor activity was measured as total photocell breaks for each of the six 5-min intervals.

—Morris water maze

Lastly, after 24 weeks of isolation, the animals were tested in four different tasks in the Morris water maze (MWM). This maze consists of a circular pool (150 cm diameter, 60 cm height) filled with water at 23 $\pm$ 1°C to a depth of 30 cm. The water was made opaque with non-toxic white paint and there were no visible cues available in the pool.

Four points equally spaced around the perimeter of the tank served as arbitrarily designed starting points and platform locations (N, S, E, W and NE, NW, SE, SW respectively). The sessions were videotaped by a camera mounted above the center of the pool with a tracking system (Smart v.2.5.14; PANLAB, Barcelona, Spain) to obtain measures of latency to reach the platform, distance travelled and speed.

—Delayed matching to place (DMTP) task

The animals were tested on 4 consecutive days when they were allowed to swim in the pool for 90s or until they found a platform located on a fixed position each day. Each animal went through 2 trials per day: a sample/acquisition trial and a retention trial with an intertrial interval of 30s (the rat was allowed to spend 15 seconds on the platform and 15s on its homecage). If the rat was not able to find the platform in the 90s it was gently guided by the experimenter to the platform. The platform locations and starting points were pseudo-randomly chosen and varied each day. Several room cues were constantly visible from the pool.

Escape latencies, path lengths and swimming speed for each animal and trial were provided by the tracking system.

—Place learning and transfer test

The place task (training) session consisted of 3 trials per day on 4 consecutive days (12 trials total). Each trial started from one of the 4 starting positions randomly determined for each trial with the rat placed into the water facing the wall of the pool. If the rat failed to escape within 90s it was gently guided to the platform and once the animal got to the platform it was allowed to stay there for 15s. The intertrial interval within the session was 25-30 min approximately. The measure obtained from this test was the distance travelled to reach the platform.

Reference memory was tested 3 hours after the last learning trial by having the animal recall the position of the platform (removed) in 60s. This test started with the rat placed in the "S" starting point facing the wall. The parameters measured were: latency to reach the platform position, distance travelled, number of entries into the platform position (annulus) and time spent in the annulus.

—Cued task

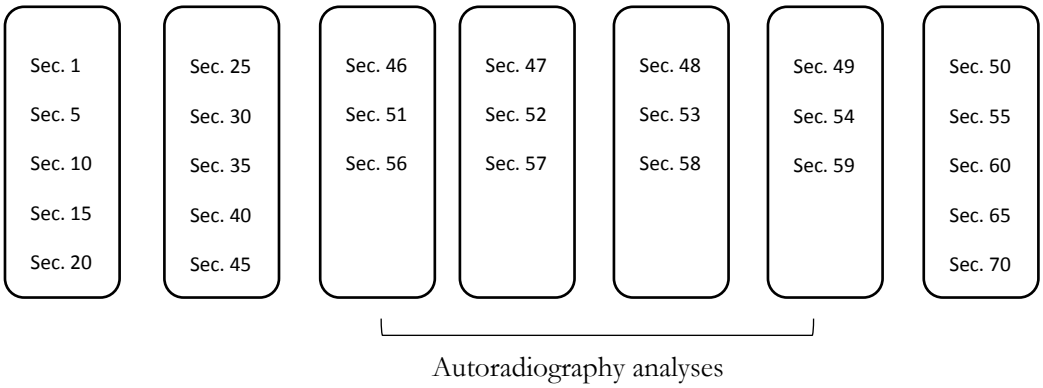
This test took place 7-8 days after the transfer test and consisted of 4 consecutive trials with four different starting positions and an intertrial interval of 25 minutes. The platform situated in the NW quadrant was visible (1cm above water) and cued with a small flag (blue and white stripes). The performance in this test was assessed by the distance travelled to reach the platform.

Sacrifice and brain extraction

Three weeks after the last behavioural test was carried out the animals were sedated with inhaled isofluorane 5% and they were sacrificed by decapitation. Immediately after the whole brain was extracted, frozen in liquid nitrogen and stored at -80°C until further processing. For the volume estimation analysis the brains were shipped to the lab of Dr. Susana Aznar in Copenhagen (Research Laboratory for Stereology and Neuroscience, Bispebjerg Hospital, Copenhagen, Denmark).

Tissue preparation

Each brain was weighted before being cut in 15 µm coronal sections in the cryostat. The brain was sectioned from Bregma 5.64 to -4.44 approximately according to Paxinos and Watson's rat brain atlas [157] and 1 out of every 5 sections was picked for volume estimation. When a region of interest was reached (mPFC- Bregma 3.24mm, dSt- Bregma 1.80mm and dHPC- Bregma -2.40 mm) the sections discarded for volume estimation were picked for binding analyses until 4 series of 3 sections for each structure were completed (see scheme below).



For convenience purposes the mPFC and dSt sections for binding analyses were mounted on the same slides following the corresponding order. Once the brain was sectioned, all the slides were kept at -20°C until they were processed (see below).

Autoradiography

To assess 5HT2A receptor binding, two of the four collected (consecutive) slides for each region and animal were used for autoradiography. The protocol was modified from [158] and performed using 3H-MDL100907 [R(+)-a-(2,3-dimethoxyphenyl)-1-[2-(4-fluorphenyl)-ethyl]-4-piperidin-methanol] (specific activity; 64 Ci/mmol) and non-specific binding was determined using 10 lM ketanserin tartrate (3-[2-[4-(4-fluorobenzoyl)- 1-piperidinyl]-ethyl]-2,4[1H,3H] quinazolinedione tartrate). Sections were thawed for 30 min at room temperature (RT) and preincubated in Tris-HCl buffer (for the total binding slides) and buffer + 10 μM ketanserin (for the non-specific binding slides) for 20 min at RT under constant gentle shaking. Then, the slides were incubated in the same buffer containing 2nM of 3H-MDL100907 (+10μM ketanserin for the non-specific binding slides) for 60 min. Following incubation the slides were washed 2x 5 min in ice-cold buffer, 1x 20s in ice-cold dH_2O and dried in a cool stream of air for 1h. Finally, the slides are placed in a fixator for overnight coating in paraformaldehyde vapor at 4°C. The next day, the fixator was taken to RT for 30 min before opening and then the slides were transferred to an exicator and left to dry for 3h at RT. Then, the slides were exposed to a BAS- TR2040 Imaging Plate (Science Imaging Scandinavia AB, Nacka, Sweden) for 3–14 days together with 3H-microscales (GE Healthcare, UK) at 4°C. Finally, the imaging plate was scanned on a BAS-2500 scanner (Fujifilm Europe GmbH, Dusseldorf, Germany) and specific and non-specific binding was determined in the prefrontal cortex, dorsal striatum and dorsal hippocampus using ImageJ (NIH) and expressed as fmol/mg tissue equivalents (TE).

Volume estimation

The sections to be evaluated were selected in a consistent interval (random uniform sampling). The total number of sections containing a given structure was considered so that 10-12 sections total were chosen, providing an unbiased measure. The volume of the areas of interest was estimated by using the Cavalieri principle [159]. Briefly, the region of interest (ROI) was outlined in each selected section and a point grid was placed on top of the section so the number of points

within the ROI could be counted. The size of the grid and the distance between points was given by the software and it was set so no more than 150-200 points per ROI were counted. These parameters among others (interval of sections, thickness, total sections) were taken into account to obtain the volume estimation of each region by applying the corresponding mathematical formulae [159]. The volumes of three areas of interest were estimated (see Fig. 1): mPFC (Bregma 4.20 - 2.52 mm), dSt (Bregma 2.76 - -1.44 mm) and dHPC (Bregma -1.80 - -4.36 mm). The estimates were obtained according to the Cavalieri principles [159]. The accuracy of the stereological measure is represented by the coefficient of error (CE), which is defined as the standard error of the mean of repeated estimates divided by the mean [159].

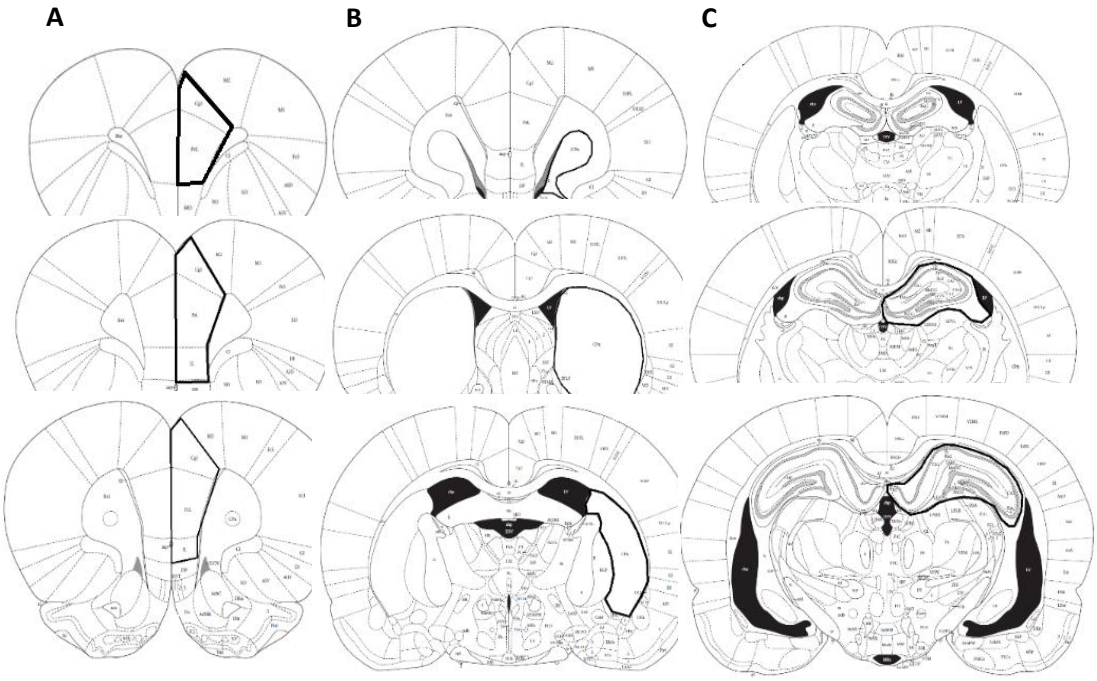


Figure 1. Delimitation of the areas of interest for the estimation of volumes for A) mPFC (Cg1, PrL, IL); B) dSt (CPu) and C) HPC (CA1, CA2, CA3, Rad, MoDG, LMol, GrDG, PoDG, SLu, Py). Cg1= cingulate cortex, area 1; PrL=Prelimbic cortex; IL= Infralimbic cortex; CPu= Caudate Putamen (striatum); CA1-3= field CA1-3 of the hippocampus; Rad= radiatum layer of the hippocampus; MoDG= molecular layer of the dentate gyrus; LMol= lacunosum moleculare layer of the hippocampus; GrDG= granular layer of the dentate gyrus; PoDG= polymorph layer of the dentate gyrus; SLu= stratum lucidum of the hippocampus; Py= pyramidal cell layer of the hippocampus.

Statistical analysis

Statistical analysis was performed using the "Statistical Package for Social Science" (SPSS, version 17). Factorial ANOVA analysis with strain (RHA-I and RLA-I) and treatment (control or isolated) as between-subject factors were applied to all test measures with one variable. For PPI analysis, a repeated measures ANOVA was applied with strain and IR treatment as between-subject factors and the pre- pulse intensities (59 dB and 63 dB) as within-subject factor. For activity results a repeated measures ANOVA was performed with the time intervals (6 interval of 5 min each) as a within-subject factor and strain and IR treatment as between-subject factor. In the DMTP task a repeated measures ANOVA was also performed with the 2 trials as a within-subject factor and the strain and IR treatment as between-subject factors. To analyze place-learning a repeated measures ANOVA was conducted with strain and IR treatment as between-subject factors and the 12 trials (3 trials for 4 days) as a within-subject factor. Finally, a repeated measures ANOVA was conducted for the cued task, with strain and IR treatment as between-subject factors and the 4 trials as within-subject factor. Further analysis with one-way ANOVAs within each strain were applied when the previous overall ANOVAs led to significant "strain × treatment", "strain × time interval" or "strain × trial" interaction effects.

For the analysis of binding and volume estimation ANOVA analyses with strain and treatment as between-subject factors and the three areas measured as within-subject factor were performed. Following significant overall ANOVA results, separate ANOVAs within each strain and area were performed.

Results

—PPI

Repeated measures ANOVA with the 4 or 2 prepulse intensities (for session 1 and 2, respectively) as within-subject factor and "strain" and "treatment" as between-subject factors revealed only a significant effect for strain in PPI session 1 (Fig. 1A) [$F(1,47) = 20.47$, $p<0.001$] while the analysis for session 2 (Fig. 1B) yielded a significant "strain x treatment" effect [$F(1,47) = 6.96$, $p=0.011$] in addition to the "strain" effect [$F(1,47) = 15.71$, $p<0,01$]. Separate one-way ANOVAs for each strain and prepulse intensity revealed a significant effect only for the 59 dB intensity in PPI session 2 (Fig. 1B) [$F(1,47) = 13.94$, $p<0.01$]. The analyses for the total PPI only showed a significant effect in the RHA rats in

session 2 (Fig. 1C) [F (1,24) = 8.56, p<0.01]. The analyses for the baseline startle (Fig. 1D) only showed a significant effect for "strain x treatment" in PPI session 2 [F (1,47) = 4.64, p=0.036].

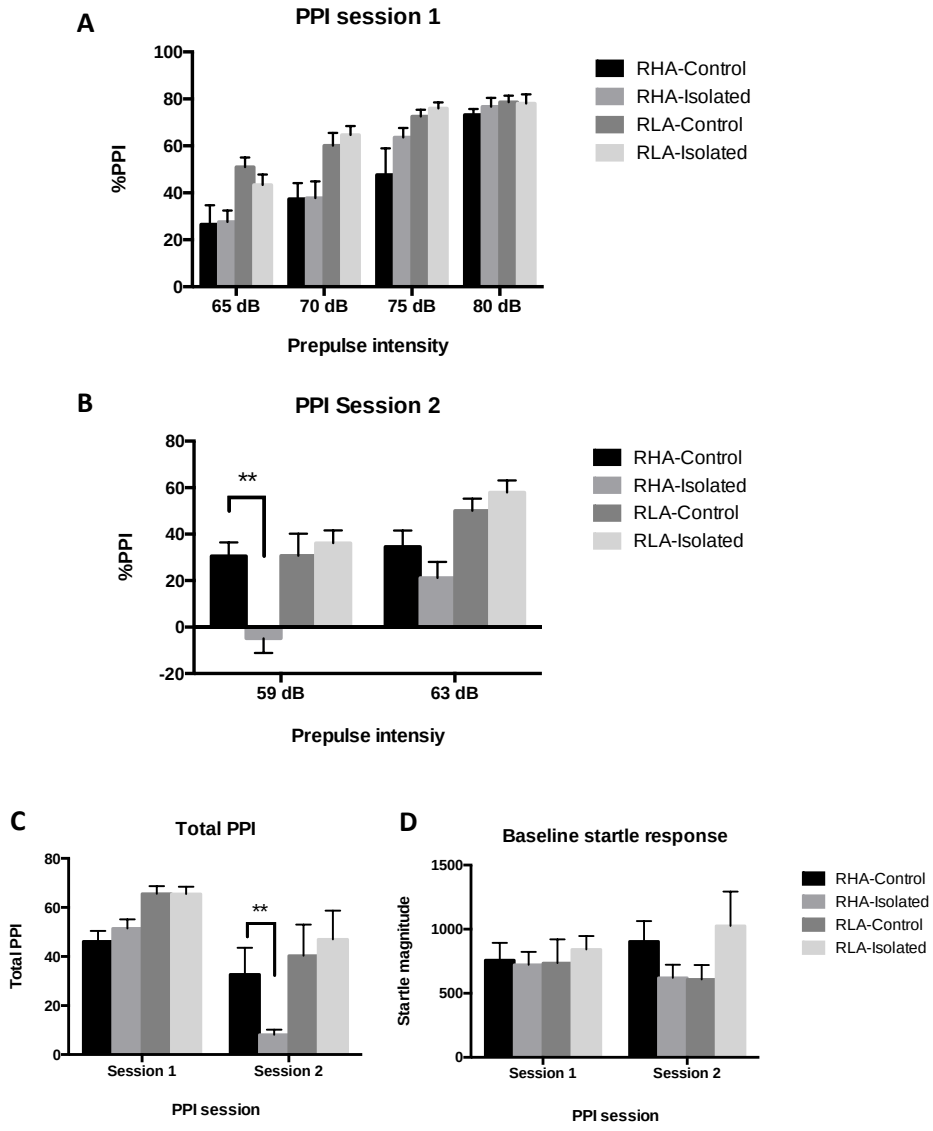


Figure 1. Mean ± SEM for the PPI measures. (A) Values for PPI session 1 for both strains and treatment groups; (B) values for session 2; (C) values for the total PPI in both sessions for the four experimental groups; (D) Baseline startle values for the 10 pulse-alone trials during BAS 2 phase. *p<0.05, **p<0.01. n=9 RHA-C, n=10 RLA-C, n=16 RHA/RLA-I.

—Elevated zero-maze

ANOVAs with "strain" and "treatment" as between-subject factors and the different measures of the elevated zero maze were performed (Fig. 2). For the "latency of entry into the open sections" (Fig. 2A) we obtained a significant "strain" effect [$F(1,47) = 8.46$, $p=0.006$] and "strain x treatment" effect [$F(1,47)=4.13$, $p=0.048$]. Separate ANOVAs for each strain showed no significant "treatment" effect but a tendency to significance could be seen in the RLAs [$F(1,25) = 3.76$, $p=0.061$].

Both these effects, "strain" and "strain x treatment" were also observed in "time spent in open sections" (Fig. 2B) [$F(1,47) = 6.52$, $p=0.012$, $F(1,47) = 7.36$, $p=0.009$, respectively]. The separate ANOVAs for each strain revealed a significant "treatment" effect only for the RHA-I rats [$F(1,24) = 5.46$, $p=0.029$], indicating that isolated RHA-Is spend significantly less time in the open sections than their controls. For the "entries in the open sections" (Fig. 2C) we only found a significant "strain effect" [$F(1,47) = 7.25$, $p=0.010$].

The ANOVA for "head dips" (Fig. 2D) showed significant effect for "strain" [$F(1,47)=19.63$, $p<0.001$], "treatment" [$F(1,47)= 23.06$, $p<0.001$] and the interaction "strain x treatment" [$F(1,47)=16.13$, $p<0.001$]. Separate ANOVAs showed significant "treatment" effect for the RHAs [$F(1,24)= 31.10$, $p<0.001$].

Lastly, the analysis for the "line crossings" (Fig. 2E) yielded only a significant "strain" effect [$F(1,47)=4.74$, $p=0.035$], but separate ANOVAS did not reveal a significant effect of the treatment in any strain.

—Horizontal locomotor activity

Repeated measures ANOVA with "strain" and "treatment" as between-subject factors and the six time intervals (5 min) as within-subject factor, revealed significance only for the "strain" effect [$F(1,47)=7.39$, $p=0.009$]. The "treatment" effect showed a tendency to significance [$F(1,47)=3.55$, $p=0.066$]. We then performed separate ANOVAs for each strain because we had a directed hypothesis that RHA-I rats would be more affected by isolation rearing than their RLA-I counterparts (i.e. isolated RHA-I rats would show increased hyperactivity). Thus repeated measures ANOVA for each strain showed a significant effect of the treatment only for the RHA-I animals (Fig. 3B) [$F(1,24)=5.91$, $p=0.023$], meaning that the isolated RHA-I animals travelled longer distances during the session (30 min). ANOVAs for each 5-min interval showed that the isolated

RHA-I rats travelled longer distances in 2 of the intervals (3rd and 5th) [$F(1,24)=4.99$, $p=0.035$].

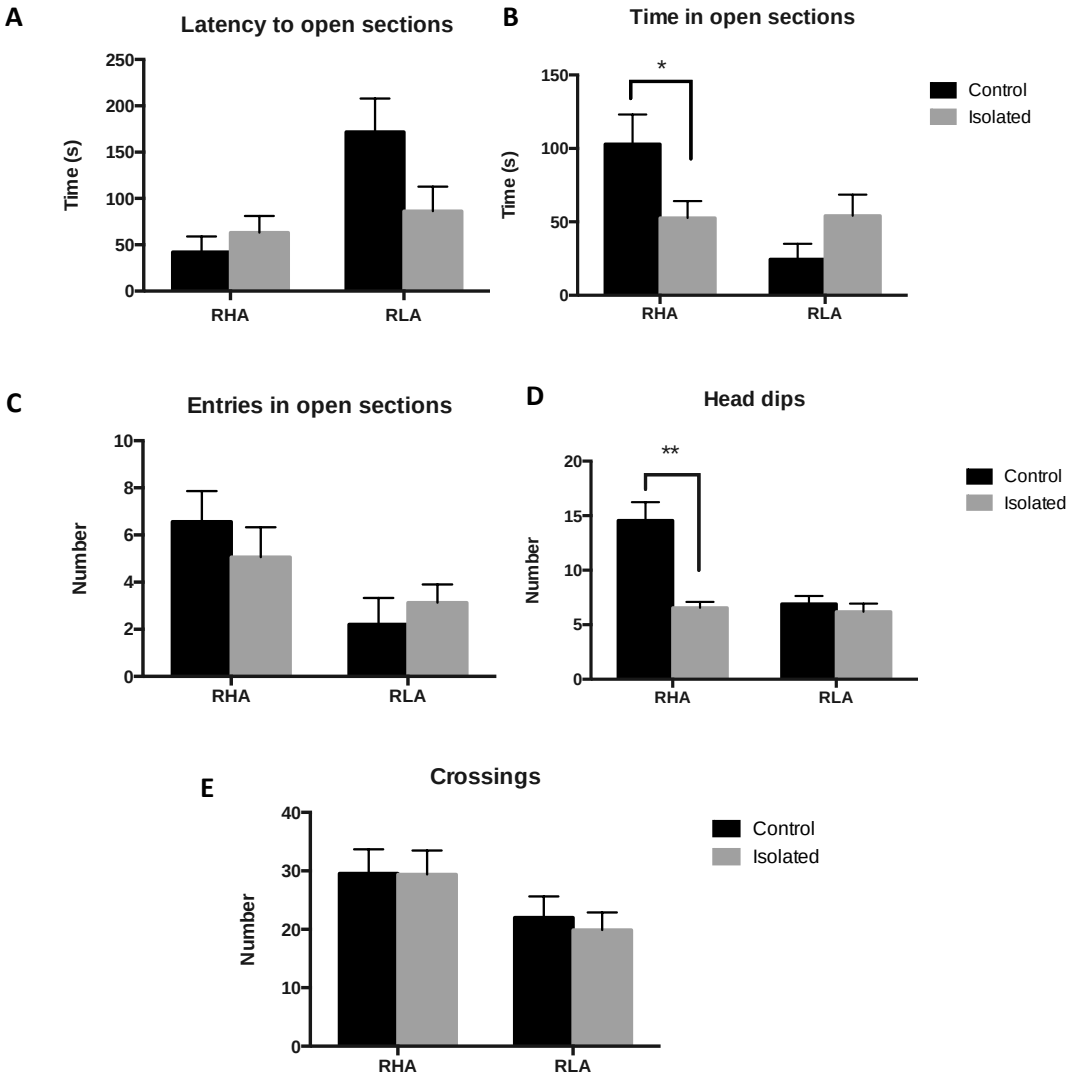


Figure 2. Mean ± SEM of the elevated zero maze variables: (A) latency to open sections; (B) time spent in open sections; (C) entries into open sections; (D) head dips and (E) line crossings. *$p<0.05$, **$p<0.01$, n=9 RHA-C, n=10 RLA-C, n=16 RHA-I/RLA-I.

A two-way ANOVA for the "total distance" (Fig. 3A) travelled in the 30 min session revealed a significant "strain" effect [F (1,47)=7.39, p=0.009] and a tendency to significance of the "treatment" effect [F (1,47)=3.55, p=0.066]. Again, separate ANOVAs for each strain revealed a significant "treatment" effect only for the RHA-I rats [F (1,24)=5.91, p=0.023].

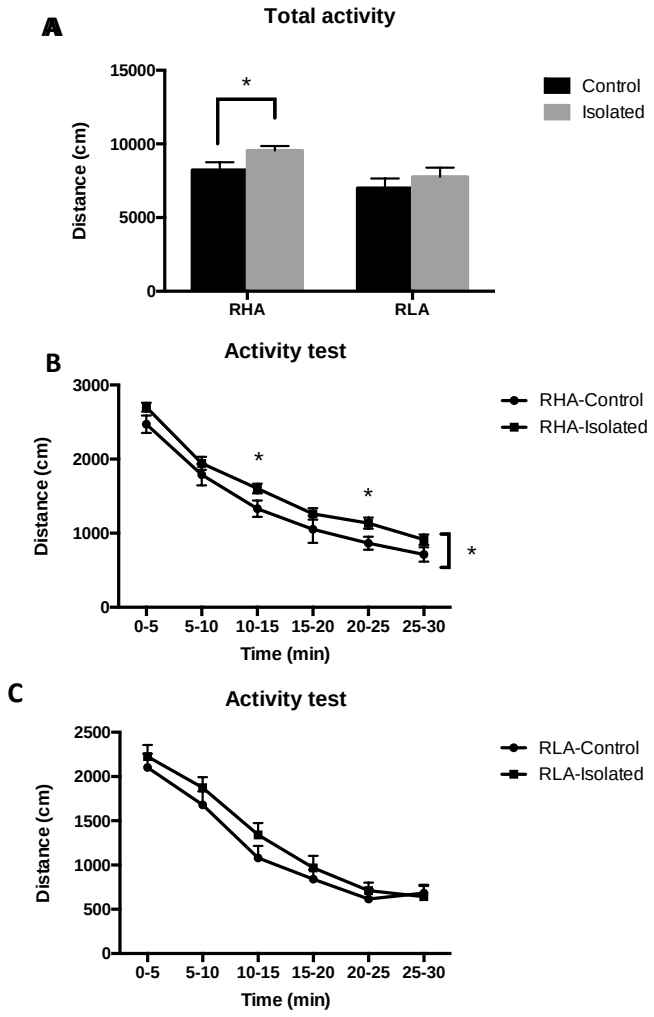


Figure 3. Mean ± SEM of the distance travelled by the animals in the activity test . (A) Total distance travelled in the 30 min session; (B) distance travelled by the RHA rats (control and isolated) in 6 5-min intervals; (C) distance travelled by the RLA rats (control and isolated) in 6 5-min intervals. *p<0.05, **p<0.01, n=9 RHA-C, n=10 RLA-C, n=16 RHA-I/RLA-I.

—Morris water maze

Delayed matching to place

The mean distance travelled in the first and second trials (Fig. 4A) was analyzed with a repeated measures ANOVA that revealed a significant effect for "strain" and "treatment" [F (1,47)=16.83, p<0.001, F (1,47)=4.16, p=0.047, respectively]. The analysis for each strain showed a significant effect of the trial in both RLA-I and RHA-Is [F (1,25)=69.37, p<0.001, F (1,24)=9.45 p<0.05, respectively] and the interaction "trial x treatment" only in the RLA-Is [F (1,25)=5.12, p=0.033]. Separate ANOVAs for each trial and strain revealed a significant effect of the treatment only for the RLA-Is [F (1,25)=4.98, p=0.035] meaning that the isolated RLA-I rats travelled longer distances in the second trial than their controls.

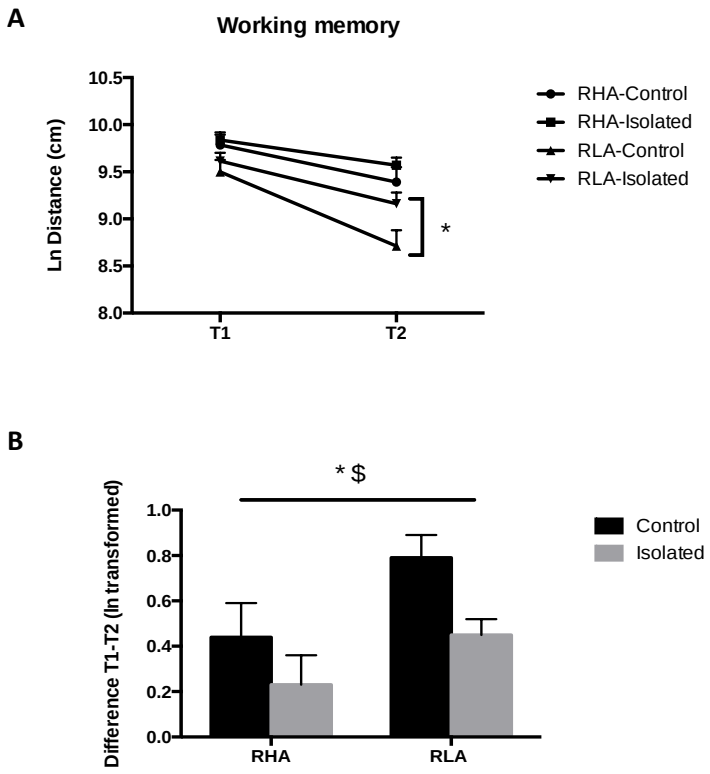


Figure 4. (A) Mean ± SEM of the distance travelled by the rats in the first (T1) and second (T2) trials. (B) Mean ± SEM of the difference between first and second trial of the four days of working memory task. * strain effect, $ treatment effect, p<0.05, n=9 RHA-C, n=10 RLA-C, n=16 RHA-I/RLA-I.

Two-way analysis of covariance (ANCOVA) was performed for the difference between the distance travelled in the first and second trials, taking the distance in the first trial as a covariate (Fig. 4B). This analysis yielded a significant effect for "strain" [F (1,47)=8.17, p=0.006] and "treatment" [F (1,47)=6.87, p=0.012]. Separate ANOVAs revealed a significant "treatment" effect only for the RLA-I rats [F (1,25)=6.32, p=0.019].

Place learning

Repeated measures ANOVA for the distance travelled in each of the 12 trials (3 trials/day) (Fig. 5 A-B) revealed no significant effect in any of the strains. The separate analysis for the RLA-I rats showed a tendency to significance for the "treatment" effect [F (1,24)=3.43, p=0.076].

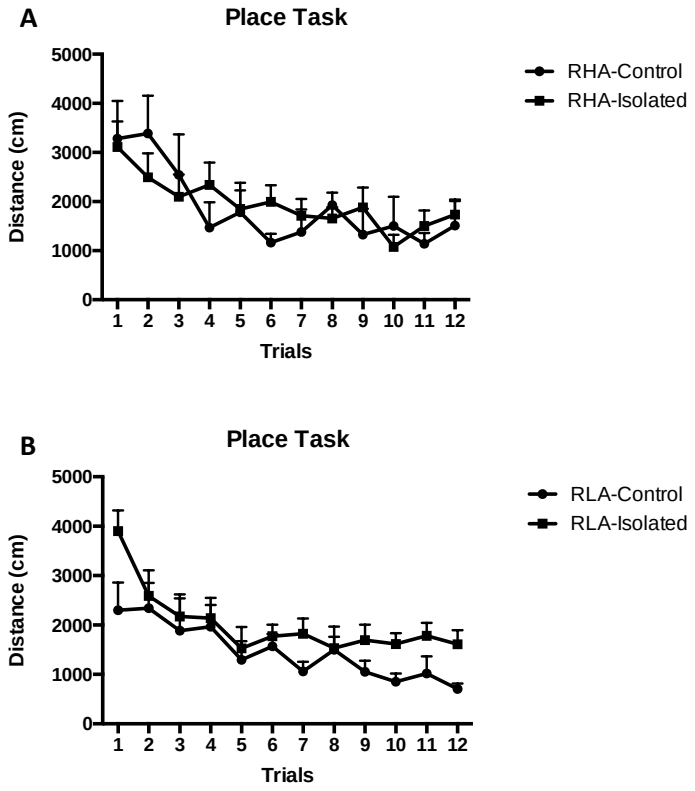


Figure 5. Mean values ± SEM of the distance travelled by the (A) RHA and (B) RLA rats in the 12 trials of the place task in the MWM, n=9 RHA-C, n=10 RLA-C, n=16 RHA-I/RLA-I.

Transfer test

The two-way ANOVA analysis with the annulus crossings, with "strain" and "treatment" as between-subject factors, revealed a "strain x treatment" effect [$F(1,47)=4.60$, $p=0.037$], while the separate ANOVAs for each strain revealed a significant "treatment" effect only for the RHA-I rats [$F(1, 24)=22.99$, $p<0.001$]. In the case of the variable "time spent in the annulus" the same analysis yielded a significant overall "treatment" effect [$F(1,47)=5.25$, $p=0.026$] and a "treatment" effect also for the RHA-I rats [$F(1,24)=10.96$, $p=0.003$].

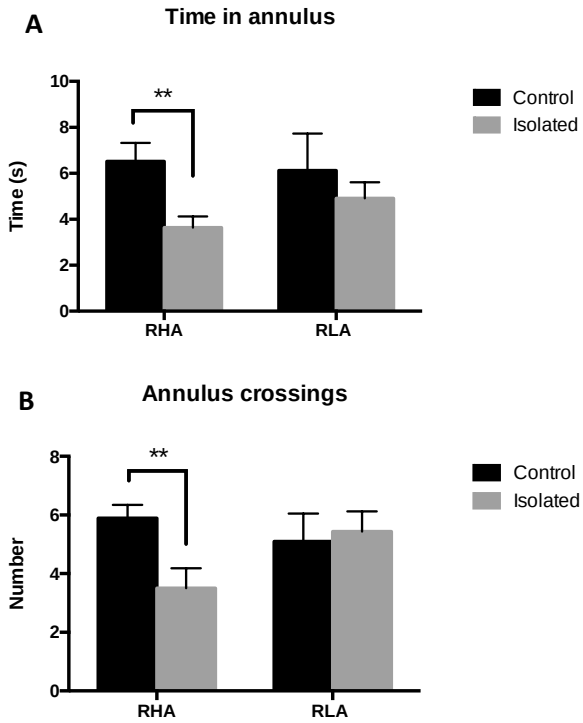


Figure 6. Mean ± SEM of (A) annulus crossings and (B) time spent in the annulus for both strains and treatment. **p<0.01, n=9 RHA-C, n=10 RLA-C, n=16 RHA-I/RLA-I.

Cue task

A repeated measures ANCOVA for the distance travelled on trials 2 and 4, taking the measure of the first trial as a covariate yielded no significant results for any of

the effects. This can indicate that there was no motivational, visual or motor problems in any of the groups.

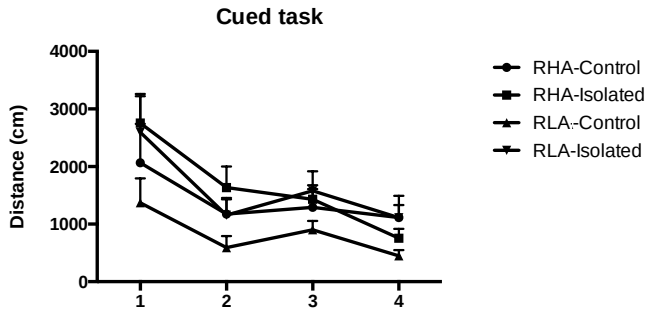


Figure 7. Mean ± SEM distance travelled by the rats in each of the four trials of the cued task, n=9 RHA-C, n=10 RLA-C, n=16 RHA-I/RLA-I.

Binding results

ANOVA analyses with strain and treatment as between-subject factors and the receptor density in terms of [^{3}H]MDL100907 binding of the three areas measured as within-subject factor showed no significant effect of the strain or treatment in in any of the three areas analyzed.

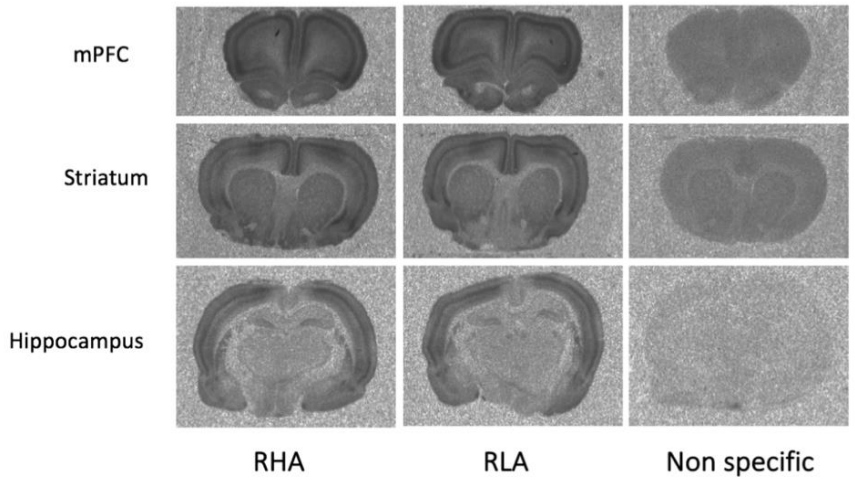


Figure 8. 5HT2A receptor binding detected by [3H]MDL100907 in the mPFC, striatum and hippocampus. Non-specific binding was determined in the presence of ketanserin.

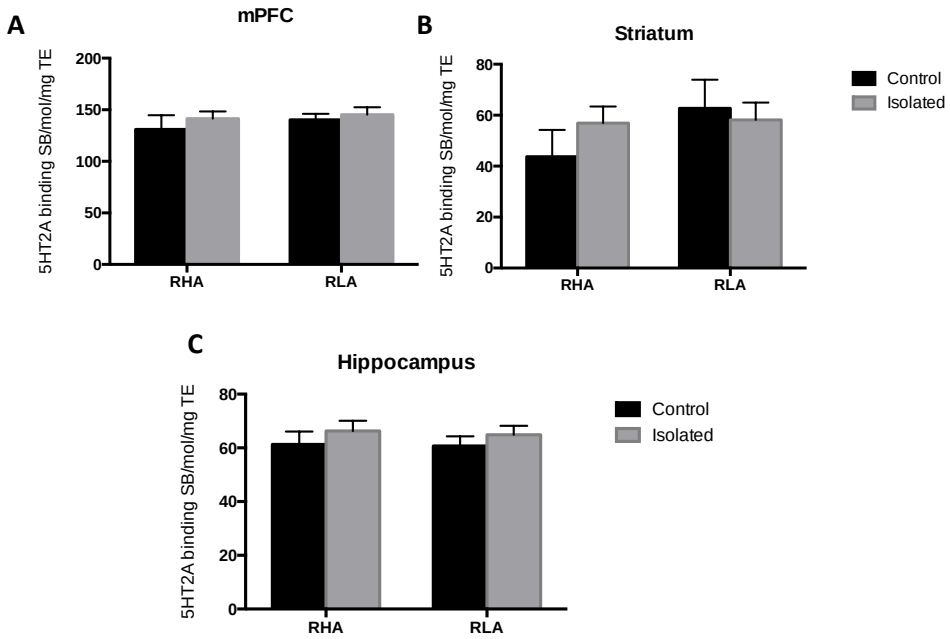


Figure 9. Mean values ±SEM of the receptor density in the three areas measured. (A) Medial prefrontal cortex, (B), dorsal striatum (C) dorsal hippocampus. RHA-C n=8, RHA-I n=16, RLA-C n=7, RLA-I n=14 (the differences in the 'n' between groups are due to damages in the tissue during its manipulation).

Stereological results

Table 1. Volume estimation of the brain areas studied.

			mPFC			dSt			HPC	
		N	Volume (mm^3)	CE	N	Volume (mm^3)	CE	N	Volume (mm^3)	CE
RHA	Control	8	5.34 (0.19)	0.02	8	24.29 (0.152)	0.02	8	17.89 (0.31)	0.03
	Isolated	15	6.08 (0.20)	0.02	15	25.04 (0.161)	0.02	15	18.56 (0.26)	0.03
RLA	Control	7	5.94 (0.24)	0.02	7	25.26 (0.204)	0.02	7	18.52 (0.21)	0.02
	Isolated	12	7.04 (0.15)	0.02	12	26.23 (0.125)	0.02	12	19.52 (0.28)	0.02

CV=Coeficient of variation (values represented in parenthesis); CE= coeficient of error.

Factorial ANOVAs with "strain x treatment" as between-subject factors showed significant effects of the strain in the three areas (Fig. 10 A-C) [F (1,41)=4.27, p=0.045, (F (1,41)=6.85, p=0.013 and (F (1,41)=5.23, p=0.028, respectively], indicating higher volumes in RLA-I rats. The treatment effect was only significant for the mPFC [F (1,41)=5.96, p=0.019].

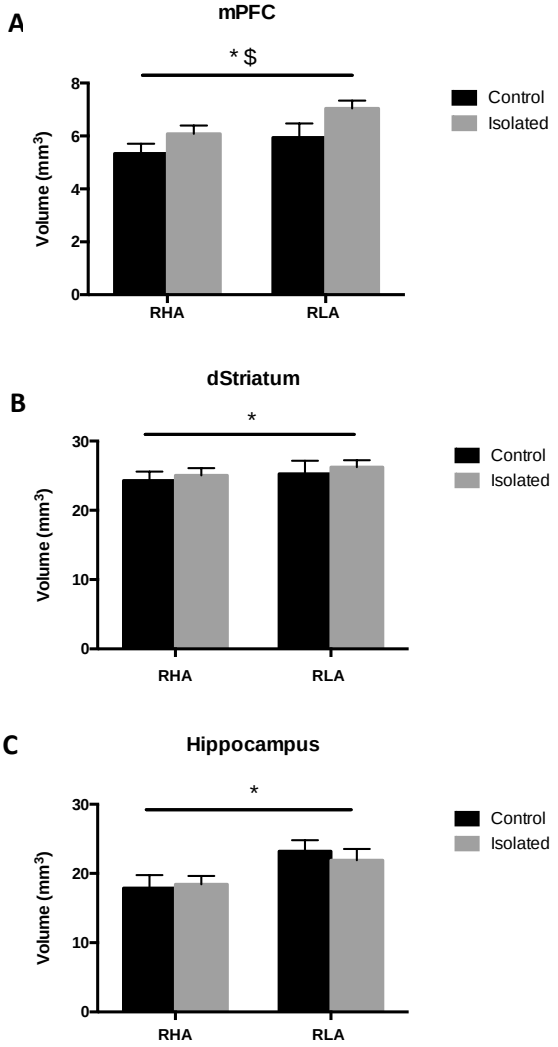


Figure 10. Mean values ±SEM of the volume of the three areas measured. (A) Medial prefrontal cortex, (B) dorsal hippocampus, (C) dorsal striatum. *, indicates the strain effect, $ indicates treatment effect, $p<0.05$.

Discussion

The behavioral results from the present study are in line with those obtained by Oliveras et al. [152] regarding the behavioral effects of isolation rearing (IR) in the Roman rats in tasks that measure schizophrenia-related phenotypes. In this context, it has been confirmed that IR affects RHA-I rats, as it induces an impairment of PPI, an increase of anxiety-related responses in the elevated zero maze, such as head dips or time spent in the open sections, increased hyperactivity (as measured in the activity cages, where isolated RHA-I rats travelled more distance in average) and a deficit of spatial working and reference memory (transfer test). On the other hand, we did not observe any effects of IR in the RLA-I rats. The fact that there were no significant treatment and/or strain effects in the cued learning task confirms that the differences obtained in learning/memory tasks were not due to any physical, sensory or motivational discrepancies among groups. These results are consistent with the already described divergent profiles of the Roman rats, being the RHA-I rats less anxious and novelty seekers when compared to the RLA-Is, that display better PPI and working memory performance [72,104,147,152,160]. They are also mostly in line with what can be found through the literature regarding IR behavioral effects [81,149,150,153,154].

However, we have obtained controversial results in the volumetric and receptor binding analyses. Contrary to what we expected, isolation rearing did not produce a reduction in volume in the areas investigated. Conversely, isolated rats of both strains showed consistently larger volumes in the mPFC. According to the literature, IR has been shown to produce a systematic and significant reduction in volume in the mPFC [83,91] and other regions such as hippocampus [155], in line with what has been described for schizophrenic patients. To estimate the volumes of the areas studied we used a stereological approach, as has been described before [83,155,161] and the precision of the volume estimates was provided in the coefficient of error (CE) according to [159] (see Table 1). The sampling is considered optimal when CE is approximately half or less of the observed CV (=standard deviation/mean), which was the case in this study (see Table 1). To the best of our knowledge, the technique was carried out properly, but a human error cannot be ruled out as an explanation for the controversial results obtained. Similarly, the 5HT2A binding analysis yielded no significant results, as we could not detect any difference in the expression of this receptor between strains or isolated v. control animals in any of the areas investigated, in contradiction with

the results obtain by Klein et al. [117], despite having followed the same protocol and technique.

At variance with the work by Klein et al. [117], the rats from the present study were submitted to extensive behavioral testing/training until they were about 7 months old (and tissue sampling was carried out at about 8 months of age). Thus, such a repeated testing (and/or the age of the animals) could be responsible for the absence of 5HT2A binding differences (between RHA-I and RLA-I control rats) in the present study. Due to time limitations, the study could not be repeated within the timeframe of this dissertation but a replication of the study is already in our agenda to try to discard or confirm our first results, in which case further research should be carried out.

3.4.- Study 4. Conservation of Phenotypes in the Roman High- and Low- Avoidance Rat Strains after Embryo Transfer

The need to transfer the RHA-I and RLA-I rat strains to a barrier facility prompted us to set up a re-derivation through embryo transfer (ET), to eliminate the possible pathogens that are not allowed in the new barrier facility and to establish a specific pathogen-free (SPF) colony of Roman rat strains. Following embryo transfer we evaluated whether some of the most characteristic between-strain phenotypical differences (e.g. in two-way active avoidance acquisition, conditioned fear, anxiety, novelty seeking, sensorimotor gating and stress-induced neuroendocrine responses, see Table 1) are preserved in the generation 5 (G5) after the embryo transfer.

Therefore, the aim of the present study was to evaluate whether the 5th (G5) generation post-ET presents the typical between-strain differences in several representative phenotypes, such as (1) the acquisition of two-way active avoidance in the shuttle box (i.e. the criterion used for the bidirectional selection of the strains), inter-trial crossings and context-conditioned freezing/fear (related to conditioned anxiety and fear and to the acquisition of two-way avoidance, e.g. [101,103,104,134,144]), (2) novel object exploration (behavioral inhibition/disinhibition, curiosity toward a novel/unknown object, related to unlearned anxiety and novelty seeking; e.g. [160]), (3) open field activity and self-grooming (related to behavioral inhibition/fearfulness in the face of a novel/aversive situation; e.g. [101,106]), (4) baseline startle (unconditioned emotional reflex/ reactivity) and prepulse inhibition (PPI; sensorimotor gating, a pre-attentional process that is impaired in schizophrenic patients; e.g. [147]), and (5) stress- induced corticosterone response (differences in plasma corticosterone concentrations in baseline conditions and after an acute stressful situation in both rat strains; e.g. [104,162]). We hypothesized that, if genetic factors are the main determinants of the RHA-I versus RLA-I phenotypic differences in two-way active avoidance acquisition (RHA-I > RLA-I), context-conditioned fear (RHA-I < RLA-I), behavioral inhibition/fearfulness under novelty (RHA-I < RLA-I), novelty-induced self-grooming (RHA-I < RLA-I), novelty seeking (RHA-I > RLA-I), startle response (RHA-I < RLA-I), sensorimotor gating (RHA-I < RLA-I) and stress-induced neuroendocrine response (RHA-I < RLA-I), then it follows that these trait differences should also be observed already in embryo-transferred generations of RHA-I and RLA-I rats.

Table 1. Typical behavioral and (post-stress) hormonal differences between the Roman rats in some of the most representative tests/measures.

	Measurements	**Sense of differences**	**Selected references**
2-way active avoidance	Avoidances	RHA>RLA	[98–101,104,113,160,163]
	ITC	RHA>RLA	
	Context-conditioned freezing	RHA<RLA	
Prepulse inhibition (PPI)	%Total PPI	RHA<RLA	[72,147]
Open field	Distance travelled	RHA>RLA	[101,102,106]
	Rearings	RHA>RLA	
	Grooming latency	RHA>RLA	
	Grooming	RHA<RLA	
Elevated plus maze	Open arms entries	RHA>RLA	[101,118]
	Distance in open arms	RHA>RLA	
	Time spent in open arms	RHA>RLA	
Elevated zero-maze	Entries into open sections	RHA>RLA	[104,105,125,152,160,163]
	Time spent in open sections	RHA>RLA	
	Head dips through the edge	RHA>RLA	
Shock-induced suppression of drinking (conflict)	Number of punished licks	RHA>RLA	[164,165]
Cue-and context-conditioned fear	Time spent freezing	RHA<RLA	[103,146]
Baseline startle and Fear-potentiated startle	Acoustic startle response	RHA<RLA	[103,146,166]
Hole board	Head-dipping	RHA>RLA	[101,167,168]
Novelty-induced hyperactivity	Distance travelled	RHA>RLA	[106,152,168]

Table 1. Typical behavioral and (post-stress) hormonal differences between the Roman rats in some of the most representative tests/measures. Continued.

	Measurements	Sense of differences	Selected references
Spatial working memory	Distance savings to reach the platform	RHA<RLA	[124,147,152]
	Transfer test (annulus crossings)	RHA<RLA	
	Transfer test (time in annulus)	RHA<RLA	
Spatial reference memory	Distance to reach the platform	RHA>RLA	[124,125,152, 169]
Hormones (post-stress)	Corticosterone	RHA<RLA	[104,118,162, 109]
	Prolactin	RHA<RLA	
	ACTH	RHA<RLA	

Materials and methods

Embryo transfer (ET)

Female Sprague–Dawley (OFA) rats from a colony in our facility were used (n=40; see Table 2) to set-up the rat ET procedure. After determination of their oestrus cycle phase by measure of vaginal impedance with an estrogenic monitor (model MK-11, Bionic Iberica SA, Spain), 8–16 week old OFA females were naturally mated with 2–6 month old males to be used as embryo donors. Positive matings were detected by checking the presence of spermatozoa in the vaginal smear. The next day pseudo-pregnant females were obtained by mating with two vasectomized males (5–6 month old). In a preliminary study, embryos were collected from OFA females the day after mating and incubated overnight in KSOM medium [EmbryoMax® KSOM Medium (1×) w/ 1/2 Amino Acids & Phenol Red, Merck Chemicals and Life Science SA, Spain]. Only two-cell embryos were transferred to pseudo-pregnant females. During the real transfers, embryos were collected 48 h after mating. The pregnant females were anesthetized with isoflurane and euthanized by cervical dislocation. The abdominal cavity was opened by a transverse incision, the uterine horn was located and a cut was made between the oviduct and ovary and then through the uterus near the oviduct. The

oviduct, with some adjacent uterine and ovarian tissue was then transferred into a 35-mm dish with M2 (EmbryoMax M2 Medium kit with phenol red, Merck Chemicals and Life Science SA, Spain) where the embryos were released tearing the oviduct. The embryos were washed in EmbryoMax M2 medium ten times before and another ten times after transporting the embryos into the barrier facility. To transfer RHA-I and RLA-I embryos, natural mating was set-up in the original colony. The oviducts removed from pregnant RHA-I (n=21) and RLA-I (n=19) rats were placed in dishes with M2 medium and transported to the barrier facility in less than 20 min. There, upon five to ten additional washes in M2 medium, the embryos contained in 100 μl drops of the medium covered by mineral oil and placed in a Petri dish were transferred inside the barrier facility. Once inside, embryos were washed another five to ten times and transferred to a pseudo-pregnant female. Inhalatory (isoflurane) anesthesia was used during the surgical procedure. A dorsal incision was done in each rat to reach the left ovary. A drop of epinephrine was used to minimize bleeding when opening the bursa ovarica and embryos were placed into the oviduct through the infundibulum of the Fallopian tube. The three layers of the surgical wound (muscular, subcutaneous and skin) were closed separately. The recipient females were maintained in pairs during the first 2 weeks after the embryo transfer procedure.

Experimental subjects

The animals used were 10 RHA-I and 11 RLA-I rat strains males for the behavioral assays, and 10 RHA-I and 10 RLA-I males for the hormonal study. They were obtained from our colony at the Autonomous University of Barcelona (Medical Psychology Unit, Department of Psychiatry and Forensic Medicine). Animals were housed in same-sexed pairs and were maintained with freely available food and water freely, with a 12:12 h light–dark cycle (lights on at 08:30 h) under con- trolled temperature (22 ± 2 °C) and humidity (50–70%). For the behavioral experiments we used males from generation 5 (G5) after ET, born from crossing ♀RHA-I × ♂RHA-I and ♀RLA-I × ♂RLA-I from generation 4, and for the hormonal study we used males from generation 3 (G3), born from crossing ♀RHA-I × ♂RHA-I and ♀RLA-I × ♂RLA-I from generation 2. Every experimental group was composed of rats from seven to ten different litters. Rats were approximately 2 months old at the beginning of testing in the "novel object exploration test" (see below) and 3 months old at the beginning of the remaining behavioral tests (i.e. open field, pre pulse inhibition of startle, two-way active avoidance; see below). The behavioral tests were performed at 2–4 days intervals. For baseline and stress-induced corticosterone (see below) measures we used

naïve 5-month-old rats. All testing was carried out between 0800 and 1400h and the procedures were in accordance with the Spanish Royal Decree (RD 53/2013) for the protection of experimental animals and with the European Communities Council Directive (2010/63/EU).

In the behavioral study we carried out a battery of tests to all the different groups of RLA-I and RHA-I in order to characterize the main phenotypes of the strains after ET. Each animal was submitted to all behavioral tests in the order described below. Experimental procedures were conducted counterbalancing strain across time of the day and across days of testing.

—Novel object exploration test (NOE)

The test evaluated the exploratory response when a novel/ unknown object was introduced in the home cage. The food was removed from the home cage before starting the test. One hour later, the unknown object (a graphite pencil Staedtler Noris, HB no. 2) was perpendicularly introduced in their cage through the grid cover, until the tip made contact with the cage bedding. To facilitate the observation of the animals each cage was pulled out from the rack about 15 cm, and the latency of the first exploration and the total time spent exploring the novel object were recorded for 3 minutes. An observer standing at about 40 cm from each homecage recorded the behavior of each rat using a stopwatch.

—Open field test (OF)

The apparatus was a circular arena (Ø: 83 cm) surrounded by white walls (height, 34 cm) and divided into 19 equal sectors by lines drawn on the floor. Total number of crossings, grooming activity and latency to start grooming were measured during the 5 min of test for each rat.

—Baseline startle and pre-pulse inhibition (PPI) of the acoustic startle response

See *Materials and methods* in study 1.

—Two-way active (shuttle box) avoidance acquisition (SHAV)

The experiment was carried in out with three identical shuttle boxes (Letica, Panlab, Barcelona, Spain) each placed within independent sound-attenuating boxes made of plywood. A dim and diffuse illumination was provided by a fluorescent bulb placed behind the opaque wall of the shuttle box. The experimental room was kept dark. The shuttle boxes consisted of two equally sized compartments (25 × 25 × 28 cm), connected by an opening (8 × 10 cm). Training consisted of a single 40-trial session. A 2400-Hz, 63 dB tone pulse and a light (from a small 7 W lamp) functioned as the CS (conditioned stimulus). The US (unconditioned stimulus) which started at the end of the CS, was a scrambled electric shock of 0.7 mA delivered through the grid floor. Once the rats were placed into the shuttle box, a 4 min habituation period (without administering any stimulus) was allowed before starting the training session. Each one of the 40 trials consisted of a 10 s CS, followed by a 20 s US. The CS or US was terminated when the animal crossed to the other compartment, with crossing during the CS being considered as an avoidance response and crossing during the US as an escape response. Once a crossing had been made or the shock (US) discontinued, a 60 s inter-trial interval (ITI) was presented during which crossings between compartments (ITC) were scored. Freezing behavior, defined as the complete absence of movements except for breathing, was also scored during the 60 s ITI of trials 2–5 as an index of context-conditioned fear.

—Plasma corticosterone concentration measurements in basal and post-stress conditions

Both rat strains from G3 were evaluated for their pre- and post-stress hormone responses at 5 months of age. Blood samples were taken between 9:30 and 12:30 h using the tail-nick procedure in resting conditions, in order to evaluate baseline hormone levels. One week after basal blood sampling, rats were individually exposed to a novel environment (plexiglas cage, 40 cm × 40 cm × 40 cm) for 20 min in a novel room (white-painted walls, fluorescent illumination), and post-stress blood samples (for post-stress hormonal measurements) were taken by tail-nick immediately following the 20 min exposure to the novel cage. Care was taken to get each animal's blood sample in less than 2 min after removal from its home cage (baseline sampling) or from the novel cage (post-stress sampling). The animals were returned to their home cage in the animal room after each blood sampling. The tail-nick consisted of gently wrapping the animals with a cloth, making a 2 mm incision at the end of the tail arteries and then massaging the tail

while collecting approximately 200 ml of blood into ice-cold safe-lock 2.0 ml tubes (Eppendorf, Hamburg, Germany). Plasma obtained after centrifugation was stored at −80 °C until processing. An enzyme-linked immune sorbent assay (competitive ELISA), was used to determine plasma corticosterone concentrations. EMS Reader MF V.2.9-0 was the reader used. Corticosterone EIA (Immunodiagnostic System Ltd, IDS Ltd; Boldon, United Kingdom) was the corticosterone reagent used. Finally, the Immute® 1000 (Siemens) apparatus was used to perform the ELISA measurements.

Statistical analyses

Student's t test for independent samples was used for all comparison measures. For the plasma corticosterone concentrations analysis, a repeated measures factorial ANOVA ("2 strains × 2 generations × pre-/post-stress") was applied, followed by independent Student's t tests within each generation because we had a directed hypothesis that within each generation RLA-I rats should display higher stress-induced plasma corticosterone concentrations than RHA-I rats.

Results

Embryo transfer

The results of the ET procedure are shown in Table 2. Collecting the embryos of 21 RHA and 19 RLA females and transferring them into 40 SD females we obtained a total number of 145 pups born in a SPF colony.

Table 2. Summary of the results obtained after the embryo transfer procedure, including the total number of pups born in generation 1 (G1, born from Sprague Dawley (SD) rats)

	Embryo donor	Recipient (SD)	Pregnant (SD)	Nº of litters	Pups born (G1)
RHA-I	21	21	16	15	93
RLA-I	19	19	15	13	52

Novel object exploration test (NOE)

Results on "latency" to explore for the first time the novel object (Fig. 1) showed a "strain" effect [G5, $t(19) = 2.3$, $p < 0.035$], as RLA-I animals displayed a longer "latency" to explore for the first time the novel object compared to RHA-I rats.

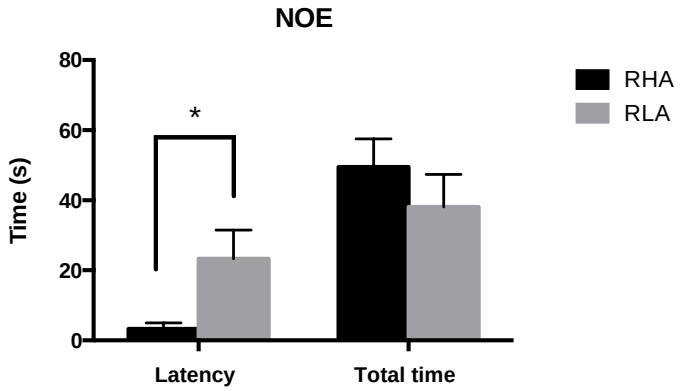


Figure 1. Mean±SEM of (A) latency to start exploring and "total time" spent in the NOE test. *p<0.05, Student's t-test.

Open field test (OF)

The results of the OF test (Fig. 2) showed a "strain" effect on the number of "crossings" [G5, $t(19) = 5.5$, $p<0.001$], as RHA-I rats showed a more robust ambulation than their RLA-I counterparts. Regarding self-grooming behavior, results showed "strain" effects on "latency to start grooming" [G5, $t(19) = 3.9$, $p<0.001$], as well as on "time spent grooming" [G5, $t(19) = 5.8$, $p<0.001$], indicating that RLA-I rats started sooner and spent more time self-grooming than RHA-I rats. In summary, in the OF test there were clear "strain" effects on "latency to start grooming" and on "time spent grooming", with RLA-I rats spending more time grooming and a shorter latency to start it than the RHA-I rats.

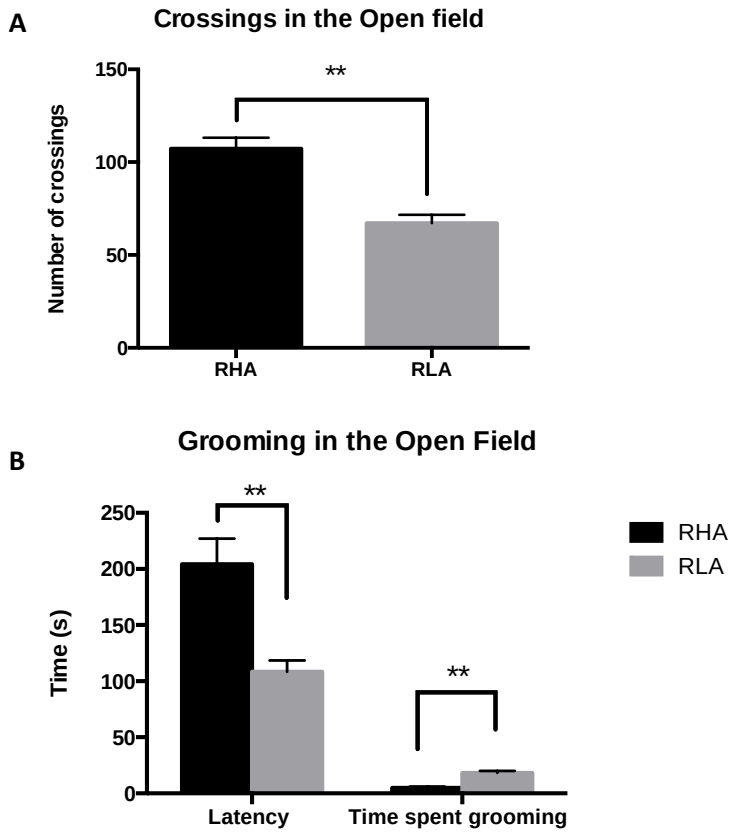


Figure 2. Mean±SEM of (A) number of crossings of open field sections and (B) latency to start grooming and time spent grooming in the OF test. *p<0.05, Student's t-test.

Zero maze (ZM)

Figure 3 shows the results of the analysis of the total time spent in the open sections of the zero maze by both strain show no significant effect but the analysis of both, total number of entries in the open sections and head dips performed by the animals show a significant strain effect [$t(19)= -2.2$, $p<0.05$, $t(19)=-3.2$, $p=0.004$] respectively.

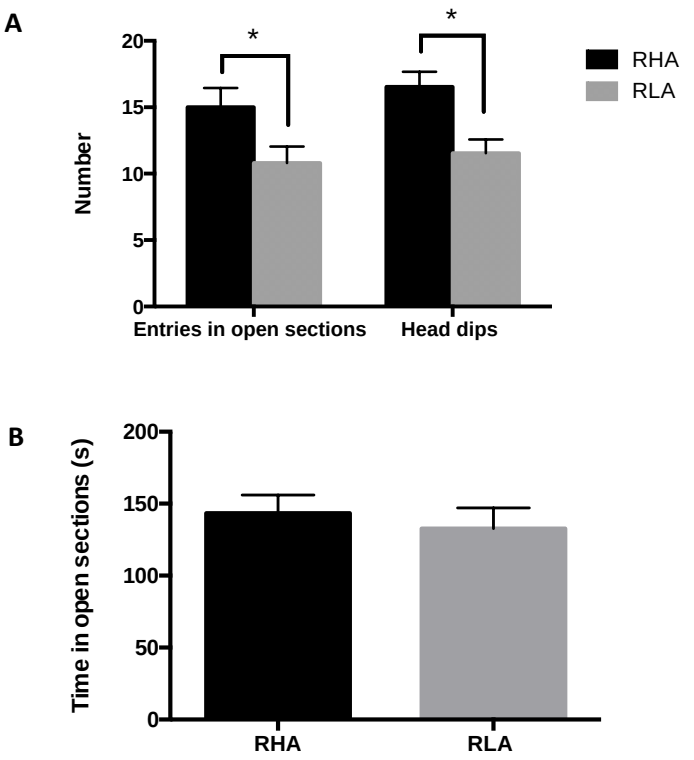


Figure 3. Mean +SEM of (A) total time spent in the open sections of the zero maze and (B) entries in open sections and head dips performed by RHA-I and RLA-I rats. *p<0.05, **p<0.01, Student's t-test.

Prepulse inhibition test (PPI)

The %PPI is shown in Figure 4. Student's t-test revealed significant group effects at prepulse intensities 65, 70 and 75 dB [$F(19) = 3.1$, $p<0.05$; $t(19) = 3.0$, $p<0.06$; $t(19) = 3.0$, $p<0.06$; respectively], in all cases indicating that RLA-I showed higher PPI levels than RHA-I rats. Analysis of the "total %PPI" revealed a significant "strain" effect [$t(19) = 3.3$, $p<0.04$]. In summary, the "strain" effect on "total %PPI" indicates that RHA-I rats display impaired PPI as compared to RLA-I rats.

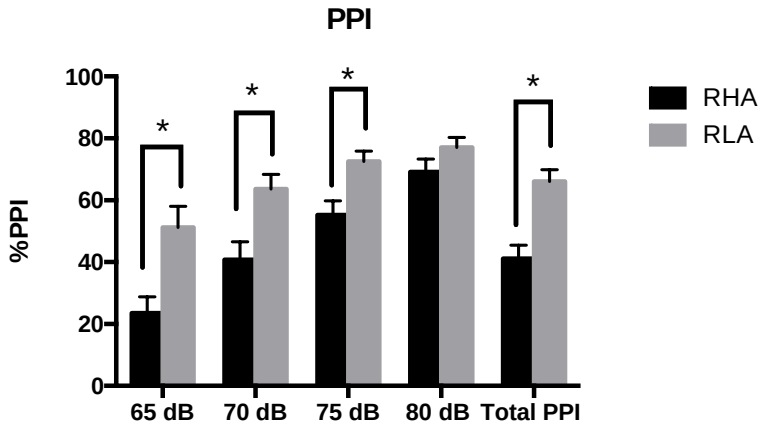


Figure 4. Mean±SEM. %PPI for RHA-I rats in the four prepulse intensities tested and the PPI. *p<0.05, Student's t-test following overall repeated measures ANOVA (2 strains x 4 prepulse intensities).

Two-way active (shuttle box) avoidance acquisition (SHAV)

Figure 5 shows the results of two-way active (shuttle box) avoidance acquisition. The ANOVA applied to the results showed the expected "strain" effect on avoidances, as RHA-I rats performed significantly more "avoidance" responses than RLA-I rats [$t(19) = 8.9$, $p<0.001$]. Moreover, a "strain" effect on "time spent freezing" was observed, showing that RLA-Is spent a longer time freezing than RHA-I rats [$t(19) = 8.8$, $p<0.001$]. "Strain" effects were observed on "inter-trial crossings" [ITC, $t(19) = 4.4$, $p<0.05$], as RHA-Is showed ITCs than RLA-I rats. Summing up, we observed clear cut and consistent "strain" effects on all the variables that are related with the criterion of selection of the Roman strains: thus, compared to RHA-I rats, their RLA-I counterparts show a markedly impaired avoidance acquisition, fewer intertrial crossings and more intense context-conditioned fear/freezing.

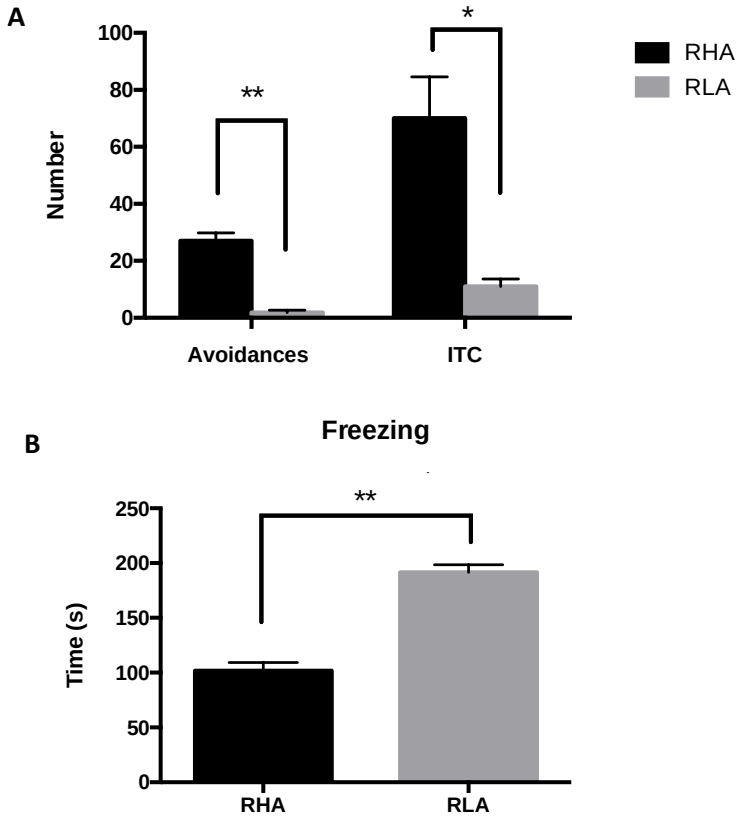


Figure 5. (A) Mean± SEM of avoidances and ITC performed by RHA-I and RLA–I rats in the two-way active avoidance task. (B) Mean± SEM of the time spent in freezing during the 60s intertrial intervals of trials 2-5 in the two-way avoidance task. *p<0.05, Student's t-test.

Hormones

Figure 6 shows the plasma corticosterone concentrations of the Roman strains from G3 in basal and post-stress conditions. Repeated measures ANOVA analysis revealed significant effects of "strain" [$F(1,18) = 34.8$, $p<0.001$] and "strain x pre-/post-stress" [$F(1,18)=44.6$, $p<0.001$] effects on plasma corticosterone concentrations. No significant strain-dependent differences in the basal corticosterone concentrations were observed, but following stress RLA-I rats display higher concentrations of corticosterone than RHA-Is [$t(18)=8.13$, $p<0.001$].

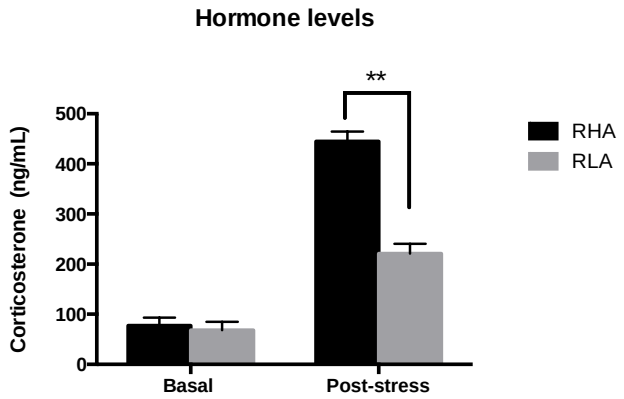


Figure 6. Mean±SEM of the concentration of corticosterone in plasma in RHA-I and RLA-I rats in basal and post-stress conditions. **p<0.001, Student's t test.

Discussion

The present is a comparative study of some of the basic behavioral and hormonal profiles of the RHA-I and RLA-I rats after ET, specifically, in G5 (born from RHA-I and RLA-I of G4), in relation with the typical differences between both strains (see Table 1). The purpose of the ET procedure to establish a new, pathogen free RHA-I/RLA-I breeding colony into a barrier (SPF) facility was fully accomplished.

Some of the most characteristic phenotypic differences between both strains are shown in Table 1, including clear cut differences in anxiety/fearfulness, behavioral disinhibition, novelty seeking and sensorimotor gating (PPI) among others. Table 1 summarizes the results of a sample of representative studies (including selected references) comparing phenotypes of both Roman strains. These studies (and many others) consistently indicate that, besides showing very good two-way avoidance acquisition (the selection criterion for RHA-I rats) the RHA-I strain also displays less conditioned fear (as evidenced by conditioned freezing and fear-potentiated startle), less anxiety, worse PPI, worse spatial learning and lower hormonal responses to stress than RLA-I rats (e.g. [99,101,103,104,113,125,146,147,152,160]).

G5 post-ET also show the typical strain-based phenotypic characteristics related with the selection trait, i.e., two-way active avoidance responses, intertrial crossings and context-conditioned freezing (e.g. [98,104,160,170]). Behavioral and genetic studies have indicated that these three behavioral responses are

related and have high heritability, i.e. they are strongly linked to strain-related genetic factors (e.g. [98,104,170] and references therein). Therefore, the present results agree with these studies by showing that, despite having been derived through ET, G5 RHA-I and RLA-I rats show the typical between-strain differences in the three above mentioned phenotypes. Thus, this indicates that, any possible maternal influence (of SD dams) [e.g. 167] on the phenotypes of embryo-transferred RHA-I and RLA-I rats, is already undetectable by G5. These results provide indirect support to the contention that those three behavioral phenotypes are strongly dependent on genetic factors (see also [98,170]). This is also consistent with findings from Driscoll and Bättig (1982) [98], who carried out a cross-fostering study with the Swiss sublines of Roman rats, from which the present RHA-I and RLA-I strains were derived, and reported no foster-mother influences on either two-way avoidance behavior or on freezing responses [98].

Between-strain differences in near all the tested co-selected phenotypes are observed in the present G5 suggesting that the possible environmental influences that could have operated on the initial generations after ET are in some way overcome by selection pressure along subsequent generations of selective breeding. It is also noteworthy that the stress-induced corticosterone response in G3 was more robust in RLA-I than in RHA-I rats (this measure was not taken in G5 because of breeding limitations), in agreement with the typical difference observed between the strains in previous studies (see [104,118,162]).

The present study only provides evidence on phenotypical between-strain differences at G5 and G3. Whether some of these phenotypes might be to some extent influenced by environmental (pre- and/or post-natal) factors is an issue that cannot be solved by a simple (and single-generation) study such as the present one. Appropriate answers on the "weight" of genetic and non-genetic factors influencing these co-selected traits can only be provided by studying the hybrids of non-segregating (RHA-I, RLA-I and F1) and segregating (F2 and the two backcrosses, "F1 × RHA-I" and "F1 × RLA-I") populations [170].

This study will not be discussed any further in the G*eneral Discussion* section.

3.5.- Study 5. Characterization of dendritic spine density in pyramidal neurons of the PFC and parvalbumin expression.

As we have mentioned in the *Introduction*, it has been widely acknowledged in the literature that schizophrenia characterizes by a reduction in volume in certain brain areas, including the PFC. We have approached this matter in study three, and following the results obtained in which we have seen a difference in volume between the two strains (RHA<RLA) in PFC, we wanted to address the rationale behind it. In this regard, the quantification of both, spine density in pyramidal neurons and parvalbumin neurons (see below), constitute an important aspect to be taken into account in the characterization of the Roman rats. Spines are small protrusions from the dendritic shaft of cortical and hippocampal pyramidal neurons that receive inputs from excitatory axons, performing a significant role in regulating neuronal excitability [171,172]. Each spine typically receives one glutamatergic synapse, reflecting the amount of excitatory drive a neuron receives. Spine density seems to be lower in multiple cortical areas, especially in neurons located in layer III, a major site for cortico-cortico and thalamo-cortical integration and deficits in spine density have been associated with working memory, attention and sensorimotor gating impairments, which suggest that such a deficit might contribute to the clinical features of schizophrenia [172]. Spines are commonly classified in three different types according to their morphology: thin spines, with a thin, long neck and a small bulbous head; mushroom spines, with a larger head, and stubby spines, which have no neck [171,173].

On the other hand, parvalbumin neurons are a subtype of calcium-binding GABAergic interneurons that selectively expresses parvalbumin and provide inhibitory input to excitatory pyramidal cells [51]. Postmortem studies have shown that there is a reduction of parvalbumin levels in layers III and IV of the dorsolateral prefrontal cortex in schizophrenic patients, as well as a reduction in the expression of GAD67, the primary enzyme for the synthesis of GABA. The inability to reach normal GABA expression levels during development or failure to maintain these levels during adulthood appears to be implicated in the development of schizophrenia [174]. Furthermore, both measures, dendritic spine quantification and PV+ interneurons are involved in the generation of gamma oscillations (see *Etiology* and references therein) which makes their characterization still more important for our goals.

Hence, the aim of the present study was to measure, in the PFC of RHA-I and RLA-I rats, the absolute and relative amount and density of dendritic spines in

pyramidal neurons, the relative presence of small (i.e. thin) and large (i.e. mushroom and stubby) spines, and the number of neurons expressing parvalbumin. According to the latest findings from schizophrenic patients [52,172], if RHA-I rats are a good model of schizophrenia-like phenotypes, they should show no changes in the number of parvalbumin neurons and a reduced number (or density) of pyramidal dendritic spines.

Material and methods

Spine density quantification

—Animals

For a pilot of this study 8 animals of each strain were shipped to the lab of Dr. Javier González-Maeso in Richmond (Department of Physiology and Biophysics, Virginia Commonwealth University, Virginia, USA), where the technique was learnt. Once the technique was mastered, we repeated the experiment with 8 male rats of each strain from the recently established (through ET, see study 4) SPF colony of inbred RHA-I and RLA-I rats at the age of 4 months approximately.

—Stereotaxic Surgery

All surgical procedures were approved by the Universitat Autònoma de Barcelona Animal Care Committee and followed the guidelines of the European Commission on Animal Care. Viral vector HSV-GFP was injected into the frontal cortex by stereotaxic surgery according to standard methods (for a more detailed protocol see [175,176]. Rats were anesthetized with inhalatory isofluorane (4% for induction and 2.5-3% for maintenance) during the surgery. The virus was delivered bilaterally with a Hamilton syringe at a rate of 0.1 μL/min for a total volume of 0.5 μL on each side. The following coordinates were used: +1.6 mm rostrocaudal, −3.8 mm dorsoventral, +2.6 mm mediolateral from bregma (relative to dura) with a 10° lateral angle. The coordinates were taken according to a rat brain atlas [157].

Three days after surgery, when the virus reaches its maximum expression, the animals were deeply anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) and perfused transcardially with PBS+PFA 4%. Brains were removed, immersion-fixed in 4% PFA in PBS at 4 °C for 24h and stored at 30%

sucrose for at least 48h. Then, the brains were sectioned in 40 μm thick coronal sections using a vibratome (Leica VT1000S) from frontal cortex. The free-floating sections were transferred to 24-well dishes containing PBS. For the immunohistochemistry assay the coronal brain sections were washed with PBS and incubated in 10% normal goat serum with 0.1% Triton X-100 in PBS for 60 min at 4 °C. The sections were then incubated overnight in the same solution containing the primary antibody anti-GFP (Invitrogen A-11122, 1:1000). The sections were rinsed three times in PBS for 10 min each time and incubated for 1 h with Alexa Fluor-488 conjugated goat anti-rabbit antibody (1:2000). Following incubation, the sections were washed three times with PBS, and the immunostained sections were mounted and examined by confocal fluorescence microscopy (Zeiss LSM700, Carl Zeiss).

—Spine density analysis

For spine analysis, apical dendritic segments 50–150 μm away from the soma were randomly chosen from HSV infected neurons expressing GFP. Images were taken of pyramidal neurons, characterized by their triangular shape, in frontal cortical layers II/III. The dendritic segments to analyze have to follow some requirements: (i) the segment has to be filled completely, (ii) the segment must be at least 50 μm from the soma and (iii) the segment cannot overlap with other dendritic branches. Dendritic segments were imaged using a 63× lens (numerical aperture 1.46; Carl Zeiss) and a zoom of 2.0. Pixel size was 0.03 μm in the xy plane and 0.01 μm in the z plane. Images were taken with a resolution of 1,024 × 1024, the pixel dwell time was 1.27 μm/s and the line average was set to 1.

We analyzed an average of 2-3 dendrites per neuron on 8-10 neurons per animal. For quantitative analysis of spine size and shape, NeuronStudio was used with the rayburst algorithm described previously. NeuronStudio classifies spines as stubby, thin or mushroom on the basis of the following values: (i) aspect ratio, (ii) head-to-neck ratio and (iii) head diameter. Spines with a neck can be classified as either thin or mushroom, and those without a neck are classified as stubby. Spines with a neck are labeled as thin or mushroom on the basis of head diameter.

Parvalbumin neurons quantification

—Animals

A total of 24 male rats (12 from each strain) from the same SPF colony (see above) were perfused with PFA 4% (following the same protocol as described above) at the age of 5 months approximately. Following extraction of the brains, they were postfixed in PFA 4% and sucrose 30% and then frozen in isopentane. The 24 brains were shipped to the lab of Dr. Susana Aznar for the analyses described below.

—Colorimetric immunohistochemistry

Brains were cut at 80 μm coronal sections using a cryostat. Following systematic random sampling principles [159], sections were collected in six parallel series through the most frontal part of the brain until Bregma level +0.02 mm (obtaining 5-6 sections containing the entire mPFC). The free -floating 80 μm sections were rinsed for 2 x 10 min in PBS, incubated for 30 min in 3% H_2O_2 in PBS to block endogenous peroxidase activity, rinsed in distillated H_2O (dH_2O) and subsequent incubated for 30 min at 90-95 °C in Target Retrieval Solution (Agilent DAKO, pH 6 (S 1699)) diluted 1:10 in dH_2O. After 20 min at room temperature (RT) for cool down, sections were washed in PBS with 1% Triton X-100 (TX) 3 x 10 min and subsequently incubated for 60 min in PBS with 10% fetal calf serum (FCS). The sections were then incubated at 40 °C for 2 days with a monoclonal mouse anti-parvalbumin antibody (Sigma P3088) diluted 1:10.000 in 10% FCS/PBS. After incubation in primary antiserum, the sections were kept at RT for 15 min and then washed in PBS with 1% TX (PBS-TX) 3 X 10 min and incubated for two days at 40 °C with the secondary horseradish peroxidase (HRP) anti-mouse antibody (Agilent (DAKO) Envision-HRP) diluted 1:10 in PBS-TX. On the third day, sections were transferred to PBS and washed 5 x 10 min, after they were pre-incubated in a solution of 0.01% diaminobenzidine (DAB, Sigma Aldrich) in PBS for 7 min, and incubated in a solution of 0.01% DAB + 0.2 μl 30% H_2O_2 in PBS for 10 min. Sections were finally rinsed in 0.1 M Phosphate Buffer 2 x 10 min and mounted on superfrost slides and air-dried for 50-60 min. Once dried, the sections were immersed in a cresyl violet (Sigma Aldrich) solution for counterstaining and after the dehydration steps coverslipped.

—The stereological design and precision of estimates

The total number of Parvalbumin positive neurons was estimated bilaterally for the mPFC using the optical fractionator sampling design as described previously [177]. Estimates of the reference volume (Vref) for mPFC were obtained by employing a point-counting method based on Cavalieri's principle combined with systematic random sampling [159,178]. The counting procedure was performed using the New-CAST software (Visiopharm, Hoersholm, Denmark) superimposing counting frames on the image and a Nikon Eclipse 60i microscope (Nikon Nordic AB, Copenhagen, Denmark). The microscope was equipped with a Heidenhain electronic microcator measuring the z-axis (section thickness) with a precision of 0.1 μm. The x-y position was monitored with a ProScanTM II motorized stage system (Prior Scientific Instruments Ltd., Cambridge, UK). Sections were analysed using a 60X oil immersion objective (NA = 0.5) allowing focus in a thin focal plane within a thick section (Olympus, Ballerup, Denmark). Digital live micro-scope images were visualized by a high-resolution Olympus DP72 camera (Olympus).

Statistical analysis

All the analyses were performed with the Statistical Package for Social Science software (SPSS). For the analysis of the dendritic spine quantification we performed a repeated measures ANOVA with the strain as between-subject factor and the three types of spines as within-subject factors. Following significant oerall ANOVA we performed Student's t-test analysis for each variable (i.e. for each spine type).

For the analysis of number PV+ neurons we also performed unpaired Student's t-test for independent groups.

Results

Dendritic spines

For the analysis of the dendritic spine quantification we created the variable "number" (Fig. 1 A-D), with the total number of spines quantified in a constant dendritic length of 50μm, "density" (Fig. 2 A-D) with the number of spine per μm of dendrite quantified and "percentage" (Fig. 3 A-D) for each of the three types of spines (stubby, thin and mushroom) taking the total number of spines as

the 100%. We performed a repeated measures ANOVA for each variable and revealed an interaction "type x strain" effect in the three of them [$F(1,9)=8.75$, $p=0.01$; $F(1,9)=7.68$, $p=0.015$; $F(1,9)=9.21$, $p=0.009$, respectively] and a "strain" effect in "number" and "density" [$F(1.9)=11.016$, $p=0.009$; $F(1,9)=7.021$, $p=0.026$, respectively]. We then performed Student's t-test analysis for each variable and revealed a significant effect of the "thin" type of spines in "number" [$t(9)= 3.77$, $p=0.004$] and "density" [$t(9)=3.89$, $p=0.004$] and the three types in the percentage variable [stubby: $t(9)=-3.82$, $p=0.008$; thin: $t(9)=3.15$, $p=0.012$; mushroom: $t(9)=-2.75$, $p=0.022$].

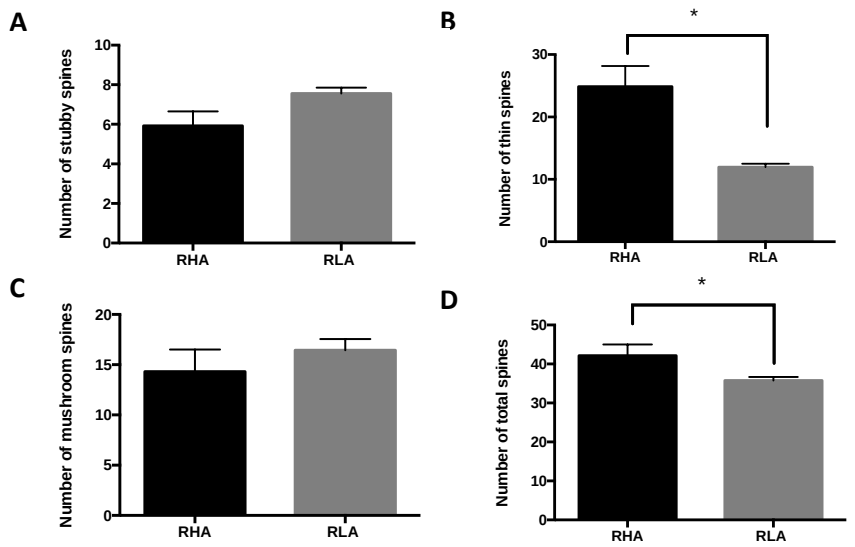


Figure 1. Mean ±SEM of the number of dendritic spines in a constant length of 50 µm. (A) Stubby spines, (B) Thin spines, (C) Mushroom spines and (D) Total spines. *$p<0.05$, Student's t-test.

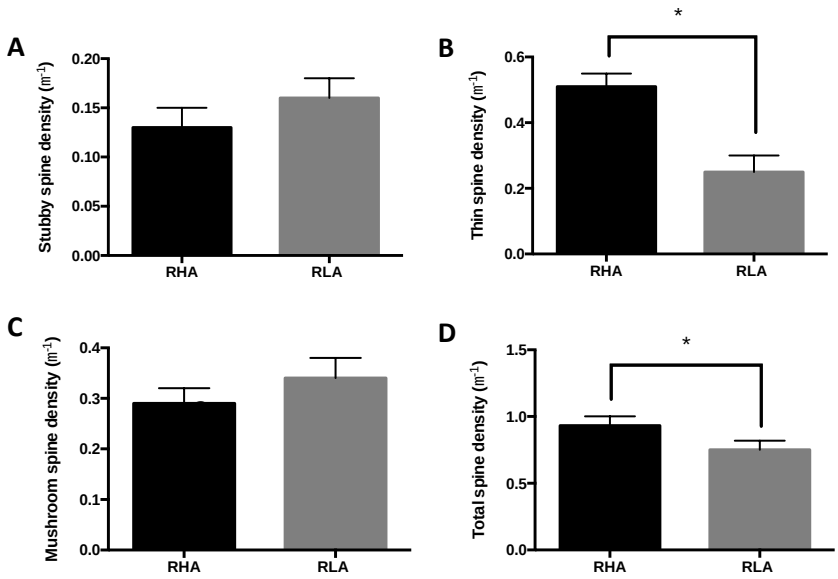


Figure 2. Mean ±SEM of the spine density for each spine type. (A) Stubby spines, (B) Thin spines, (C) Mushroom spines and (D) Total spines. *p<0.05, Student's t-test.

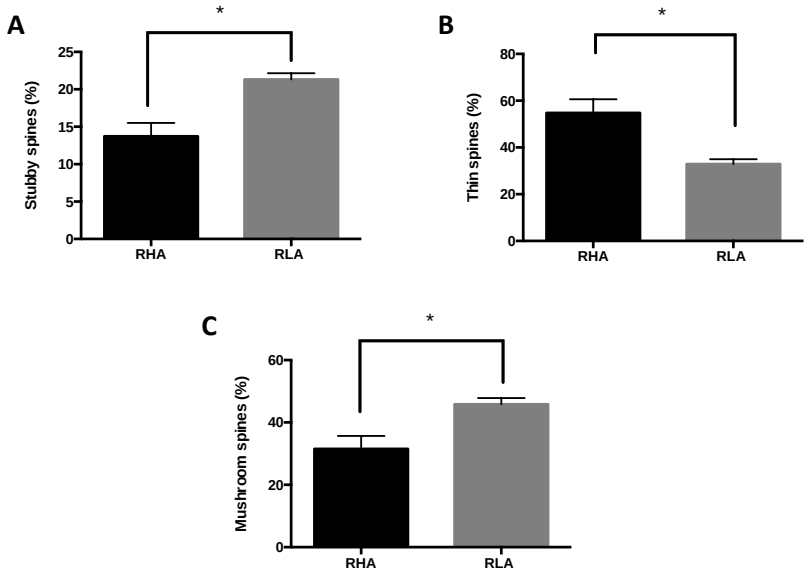


Figure 3. Mean ±SEM of the percentage for each type of spine. (A) Stubby spines, (B) Thin spines, (C) Mushroom spines and (D) Total spines. *p<0.05, Student's t-test.

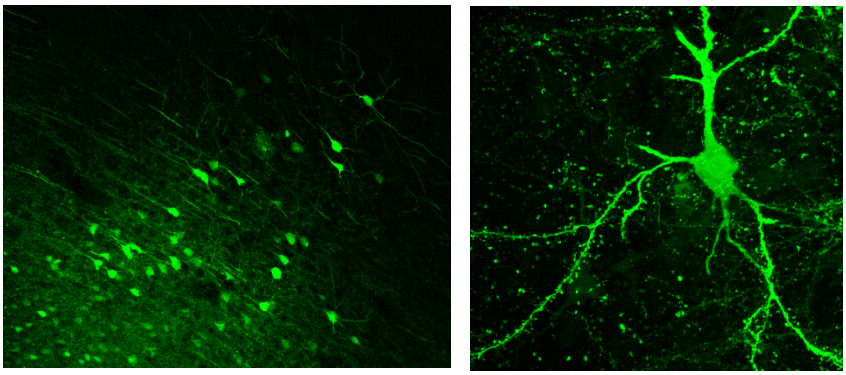


Figure 4. Left panel: field of the site of infection at 10x magnification. Right panel: infected pyramidal neuron at 63x magnification.

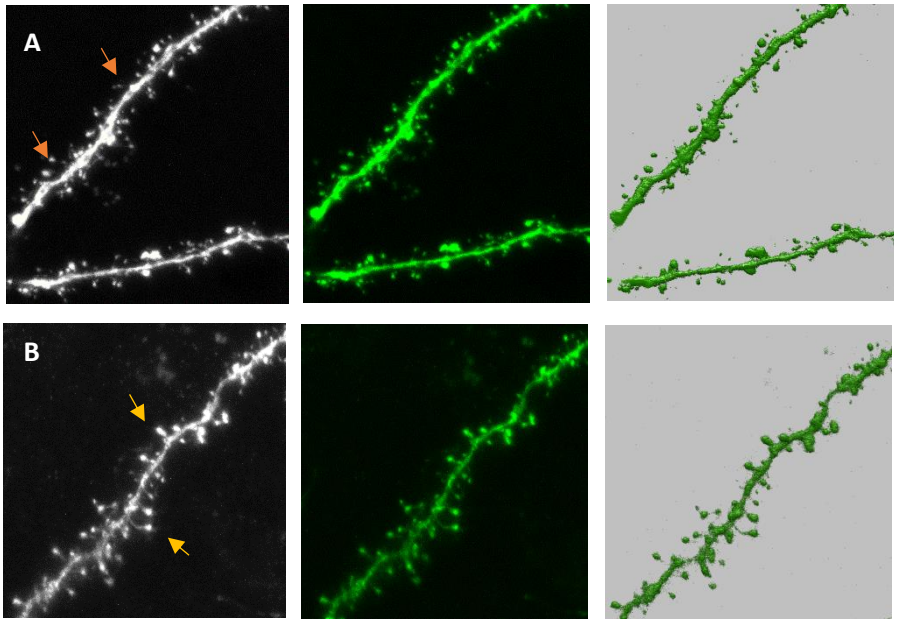


Figure 5. (A) Dendrite and dendritic spines from a pyramidal neuron in the PFC of a RHA-I rat and (B) a RLA-I rat. The orange arrows indicate thin spines and the yellow arrows indicate mushroom spines. The right panel corresponds to a 3D reconstruction of the dendrite and the spines.

PV+ neuron quantification

For the analysis of the number of PV+ interneurons we performed an unpaired Student's t-test for the mPFC volume and the total PV+ neuron number in the mPFC. The analysis revealed an almost significant difference in the mPFC volume [$t(21)=1.96$, $p=0.06$] and no significant differences in the total number of PV+ neurons [$t(19)=0.37$, $p=0.7$)].

Table 1. Area volume and numerical estimation of the mean number of Parvalbumin (PV) immune-positive neurons and in the mPFC of RHA-I and RLA-I rats

	RLA-I (n=11)		RLA-I (n=12)		
	Mean values	CE	Mean values	CE	Group differences
Volume (mm^3)	10 (0.12)	0.02	9 (0.15)	0.02	P=0.06 (p<0.03 one-tailed)*
PV number	73.642 (0.32)	0.07	70.034 (0.32)	0.08	p=0.7

Coefficient of variation (CV) values represented in parenthesis. CE, coefficient of error. * We considered the t-test one-tailed because we had a directed hypothesis that RLA-I rats would have a higher PFC volume (see [169]).

Discussion

In the present study we have evaluated two parameters that have been potentially involved in psychotic and cognitive symptoms in schizophrenia by multiple lines of evidence (e.g. [52,179]). Alterations in cortical pyramidal neurons and cortical PV+ GABAergic interneurons have been consistently observed in postmortem studies of individuals with schizophrenia and extensively described in the literature [63,180–183].

Dendritic spines are dynamic structures [184], and their morphology is known to change during development, with thin spines transitioning to mushroom spines, which are usually more stable in shape and density [172,185] and so, spine density and their morphology reflect neuronal development, plasticity and connectivity [172,186]. We have studied for the first time in the Roman rats these two parameters in order to add further molecular and structural evidence to the construct validity of our model (see Fig. 2 in *Introduction*). We have observed that RHA-I rats show a higher density of thin spines when compared to their RLA-I

counterparts. We found no significant between-strain differences in the absolute number or density of stubby and mushroom spines. Interestingly, however, the percentage of "large" (i.e. stubby and mushroom types) was higher in RLA-I rats than in their RHA-I counterparts.

The majority of papers reviewed highlight a decrease in the dendritic spine density of the DLPFC in schizophrenia [61,63,79,171,172,180], but only a few of them address the different types of spines. It has been suggested that an over-representation of small spines and/or a decreased proportion of large spines in the PFC, would imply a decreased proportion of stable synapses and may be related to conditions of mental retardation or impaired cognitive functions [184,186]. Small spines (as the "thin" type) have been described as "learning" spines, since they are transient and are involved in rapid plasticity [172,184]. They seem to correspond with "silent synapses", expressing NMDA but not AMPA receptors [184,186]. In contrast, "large" spines, represented by the "stubby" and "mushroom" types, are proposed as "memory" spines, which are more stable than the small ones and are involved in long-term synaptic plasticity, associated with AMPA receptors [172,186], that activates the change from small to large spines [184–186].

There is evidence from previous reports indicating that the RHA-I rats have, compared to their RLA-I counterparts, several PFC-related deficits, such as a less active PFC ([127] and unpublished results from our lab), reduced PFC volume [169], greater impulsive behavior [117], latent inhibition deficits ([119] and present Dissertation) and impaired spatial working memory and reference learning/memory [125,147,152].

On the basis of that evidence, it is tempting to suggest that the over-proliferation of small (thin) spines, as indicated by the greater amount and percentage of them in the PFC of RHA-I rats vs RLA-I rats, and the fact that RLA-I rats present a higher percentage of large spines than their RHA-I counterparts, may be factors underlying the impaired general learning ability of RHA-I rats [102,112,124,125,146,147,152]. Further studies are warranted to relate these differences in spine density and proportion with the known between-strain differential cognitive abilities, as well as to evaluate expression of NMDA and AMPA receptors in the PFC of both rat strains.

From the analysis of PV+ GABAergic interneurons in the PFC we found no significant differences between both strains, which is in line with our initial hypothesis, since the alterations described in the literature do not include total number of PV+ interneurons in schizophrenia, but deficits related to PV mRNA expression and the density of PV-immunoreactive puncta [187]. Accordingly, future studies at our laboratory will address whether PV mRNA and synaptic glutamate markers (e.g. PSD95) at the PFC are different between both Roman rat strains.

04 /

General Discussion

Brief summary of results

In **study 1** we have shown that PPI levels are associated with LI and, particularly, that LowPPI (and MediumPPI) NIH-HS rats show an impaired LI process.

Following the results of this study we evaluated whether PPI and LI are associated in the Roman rats and in **study 2,** and we confirmed that RLA-I rats show more robust PPI in RLA-I than RHA-I rats. Also, in regard to the LI test, pre-exposed (PE) RLA-I rats display a weakened CS-US association due to pre-exposure to the prospective CS and, as a consequence, they exhibit less fear to the context than the non-pre-exposed (NPE) RLA-I group. That is, RLA-I rats show LI to the acoustic startle response (to the conditioned context). This phenomenon is not observed in the RHA-I strain since both NPE- and PE-RHA-I groups present similar startle responses, so pre-exposure to the prospective CS has no effect on this rat strain.

Given that the RHA-I rat strain seems to display some spontaneous behavioral features compatible to those shown by schizophrenic patients, in **study 3**, we tested the effects of an environmental treatment such as social isolation rearing, which has been shown to induce several schizophrenia-like symptoms. We observed that RHA-I rats showed increased PPI deficits, increased anxiety, hyperactivity and long-term reference memory deficits. Besides, neuroanatomical studies showed that RHA-I rats present a reduction in volume of the PFC in comparison with their RLA-I counterparts, while social isolation induced an increase in the volume of that area in both rat strains.

Study 4 arose from the need to transfer the Roman rat colony to a new SPF facility. After carrying out an embryo transfer procedure we proved that the typical phenotypical differences between both strains were maintained already in generation 5 of the "new" colony.

Lastly, we took the characterization of the Roman rats a step further (using the newly generated SPF rat colony), and in **study 5** we studied differences in dendritic spine density and parvalbumin neurons in PFC of both strains. We observed significant differences between the three types of spines analyzed in the two strains but no difference when analyzing the number PV+ neurons.

The main objective of this Doctoral Dissertation was to complete the characterization of the Roman rats, and more specifically of the RHA-I strain, to gather as much evidence as possible to support their proposition as an animal model for the study of schizophrenia-relevant features. To achieve this goal, we have carried out a number of studies focused on the behavioral, neuroanatomical and molecular profiles of the Roman rats, both in a basal state and after being subjected to an environmental treatment known to potentiate or exacerbate traits associated with schizophrenia.

Associations between PPI and LI in genetically heterogeneous rats, and profiles of RHA-I and RLA-I rats

We first started the storyline of this dissertation by establishing a starting point with the study of a heterogeneous rat stock, the NIH-HS rats, which, by being the result of a rotational crossing of 8 different parental lines, are closer to the heterogeneity of the general population than other typically used laboratory rat strain. We tested these animals in two attention-related paradigms relevant for schizophrenia, such as PPI and LI, and we observed that there seems to be an apparent association between the two, since the animals that displayed a deficit in PPI (low PPI scores) also showed an impaired LI effect, as shown by the fact that these animals were not able to learn the association between the CS and the US in neither condition, pre-exposure to the CS or non-preexposure. This suggests that a relative impairment of sensorimotor gating (i.e. PPI) may be related to deficits in higher attentional processes (i.e. LI, [119]). Whether such a relationship may extend to other attentional and cognitive functions will need further research. Importantly, however, we have recently reported that relatively high PPI levels are related to (and predictive of) enhanced spatial working memory in NIH-HS rats [147,188], thus linking PPI with higher cognitive processes.

Some previous studies have evaluated PPI-LI associations in genetically-based rat models [72] such as the Roman rats. RHA-I rats have been shown to display decreased acquisition and retention of fear in different paradigms, worsened working memory, and impaired PPI and LI, thus being considered a putative model for certain schizophrenia-related features [72,147]. An example is constituted by the APO-SUS (apomorphine susceptible) and APO-UNSUS (apomorphine unsusceptible) rats, two Wistar-derived rat lines that have been bidirectionally selected for extreme gnawing responses to apomorphine, a dopamine agonist [94]. The APO-SUS line shows impaired PPI and LI [72,94].

Likewise, Freudenberg et al. [189] have shown that selectively bred low-PPI rats display some cognitive deficits (e.g. perseveration, cognitive inflexibility) that are common in some psychiatric disorders, including schizophrenia [189], although the authors have not reported any LI deficit in these rats. The above studies suggest, therefore, some relationship between PPI and LI at least in some genetically selected strains. However, as far as we know the present study represents the first time in which PPI-LI relationships have been investigated and demonstrated in genetically heterogeneous rats.

On the other hand, consistent with the habituation of baseline startle responses, the average ASR scores obtained in the three BAS trial blocks diminish as the session progresses (i.e. fromBAS1 to BAS3 phases). BAS responses are higher in the HighPPI group (especially in the BAS2 phase; see Table 2, study 1) than the LowPPI group. In this connection, although it has been proposed that PPI levels can be independent from baseline startle responses, a relationship between BAS and PPI has been demonstrated by Csomor et al. [148]. In studies conducted in humans and mice a negative association between PPI and BAS has been shown. Those groups showing high percentage of PPI display lower baseline startle measures [148], at odds with the results obtained in this study with NIH-HS rats, as the HighPPI group shows also increased BAS levels. Furthermore, and also in contrast to the present results, studies with other rats strains that have been either selectively bred by their low- and high-PPI levels or by sensitivity to apomorphine (i.e. APO-SUS and APO-UNSUS), show that rats displaying lower PPI exhibit enhanced baseline of the acoustic startle response [94]. Conversely, RHA-I rats display impaired PPI (compared with the RLA-Is) and decreased BAS [147], which is in line with the results from present study 1. Therefore, such qualitative differences in BAS-PPI response associations observed in different rat strains, mice and humans suggest that the relationships between these processes (i.e. baseline startle and PPI) are more complex than proposed by Csomor et al. [148].

PPI is altered in several human neuropsychiatric disorders including schizophrenia. It has been hypothesized that impaired PPI might be associated with, or predictive of, cognitive deficiency in such diseases (e.g. see Singer et al., [188]). In this regard, in previous studies from our laboratory, comparing NIH-HS with RHA-I/RLA-I rats, we found that those animals with high PPI scores, i.e. RLA-I rats and NIH-HS rats as a population (see [147]), also display better spatial working memory (WM) than RHA-I rats [147]. Moreover, High-PPI NIH-HS rats were found to display better spatial working memory than Low-PPI NIH-HS rats [147]. These findings, jointly with the present results from study 1, suggest

that there may be some shared underlying mechanisms in PPI, WM and LI. In particular, the PFC and the hippocampus [138–140,190–192]) might be structures involved in these processes. Partially consistent with this, we have found that neural activity (as measured by c-Fos activation) in PFC of Low-PPI NIH-HS rats is lower than in High-PPI NIH-rats (Tapias-Espinosa et al. unpublished results).

Schizophrenias usually present (or are associated with) complex clusters of symptoms. Knowing which of them are inter-related or which are orthogonal is important for both, progress in neurobiological research and for the development of novel treatments addressed to particular symptoms or clusters of symptoms. Thus, research with laboratory animals with translational value may be crucial for that progress. Using genetically heterogeneous NIH-HS rats (which are the most heterogeneous – i.e. outbred – laboratory rats in existence) has the possible advantage that the obtained results may have more translational value than those obtained with standard laboratory rat strains. We have shown that PPI levels are associated with LI and, particularly, that LowPPI (and MediumPPI) NIH-HS rats show an impaired LI process. Jointly with our previous study showing PPI-working memory associations in NIH-HS (and RHA-I/RLA-I) rats, the present findings suggest that by focusing on extremes from a heterogeneous rat stock, it is possible to detect a useful (perhaps "at risk") phenotype for neurobehavioral studies of attentional anomalies that have been linked to schizophrenia.

Given the results obtained in study 1 and the apparent association between attention-related processes (such as PPI and LI), the second step in our research was to provide further evidence on the validity of RHA-I vs. RLA-I rats as a genetically-based model of schizophrenia-relevant features by jointly assessing PPI and LI of the startle response. The results from study 2 suggest that RHA-I rats exhibit deficits in pre-attentive processes (PPI, sensorimotor gating) and in attention/information processing (LI) as compared to their RLA-I counterparts. This contention is further supported by the fact that the PPI deficit of RHA-I rats is also evident when compared with the genetically heterogeneous NIH-HS rat stock [147], derived from eight rat strains among which there were three Wistar strains (it is also important to remind here that RHA-I and RLA-I rats were originally derived from Wistar; see [99,113,147]). Thus, when taking NIH-HS rats as a reference control group, PPI levels of RHA-Is are much worse than those from the NIH- HS stock, while the latter show PPI levels identical to RLA-I rats (see [147]). Thus, at least with regard to sensorimotor gating (PPI), the selection of RHA-I and RLA-I rats has apparently resulted in a unidirectional selection, i.e.

selection for poor PPI levels in RHA-I rats, as PPI levels from RLA-Is are identical to those from NIH-HS rats [147].

Apart from that, and further completing the profile of RHA-I rats, other studies have shown that they present deficits in executive/cognitive functions [112,124,125,147,169]. Therefore, RHA-I rats show several required traits to be considered a valid model of schizophrenia-relevant features. Further support to that profile is provided by pharmacological and neurochemical studies. Thus as said earlier in this Dissertation, RHA rats (both from the outbred lines and from the present inbred strains) display higher locomotor sensitization and mesotelencephalic dopaminergic activation than RLA rats when repeatedly treated with (dopamine-agonist) psychostimulants or opiates [108,114,120,122]. Studies with anti-psychotic and pro-psychotic drugs have shown, among other differential pharmacological effects, that haloperidol improves and apomorphine impairs PPI more markedly in RHA-I than in RLA-I rats [193]. Recent pharmaco-neurochemical studies also suggest a higher central dopaminergic tone in RHA-I rats than in their RLA-I counterparts [114,120,194].

Neurotransmitter systems other than dopamine are thought to be also involved in schizophrenia and schizophrenia-related symptoms. Thus, serotonin and glutamate neurotransmission have been involved in psychotic (and in negative and cognitive) symptoms of schizophrenia [49]. In fact, some antipsychotic drugs work through (serotonin) 5HT2A receptors (2AR), while (glutamate) mGluR2-related drugs induce schizophrenia-like symptoms. Recent studies have suggested that these two receptors would establish physical and physiological interactions through specific transmembrane domains, forming functional complexes in the brain. It has been shown, that when one of them is altered the other one is modified [49]. In this regard, an overexpression of 2AR and a lowered expression of mGluR2 receptors have been observed in postmortem brains of schizophrenic patients. This has been associated to psychosis as 2AR activation by drugs is related to hallucinogenic symptoms and mGluR2 activation reverses those symptoms. In this context, a recent study has shown that the RHA-I rat strain has a "psychotic" profile regarding the expression of these receptors: a high expression of 2AR and undetectable levels of mGluR2 expression in prefrontal cortex, hippocampus and striatum [117]. Therefore, this 2AR-mGlu2 receptor profile lends further neurochemical support to the RHA-I as a model of schizophrenia-related features with construct validity. The present PPI results, and especially the novel LI differences between RLA-I and RHA-I rats, add to their known differences in executive functions (for review see [147]), reduced

volume of the hippocampus (see also [128]) and medial prefrontal cortex (as well as increased volume of the lateral ventricles; [169]) in RHA-I rats, and to the fact that the PPI deficit of RHA-I rats can be reversed by the antipsychotic drug haloperidol (while the drug does not affect PPI in RLA-I rats; [193]). There are, in addition, differences in social interaction (considered as a model of negative symptoms of schizophrenia), as RHA-I rats are impaired compared to RLA-Is, and the NMDA antagonist MK801 further impairs social behavior in the former strain (being devoid of effects in RLA-I rats) (unpublished results from our laboratory).

Collectively, however, the above strain-related profiles and the present results are consistent with the idea that the RHA-I rat strain may be a valid model of some schizophrenia-relevant features or symptoms.

Long term social isolation rearing effects on behavioral/cognitive phenotypes, neuroanatomical and neurochemical serotonin-related phenotypes

For the next step we tested the effects of social isolation rearing (SIR) of the animals as an environmental treatment known to potentiate features relevant for schizophrenia. In study 3, we evaluated the behavioral, neurochemical and neuroanatomical effects of SIR on the Roman rats.

Firstly, when compared to the RLA-I rats, we have observed that RHA-I rats show a PPI deficit (as seen in study 2 and previous studies from the lab; see [72]), as well as lower anxiety levels (as measured in the elevated zero maze, see [72,109]), higher locomotor activity and worsened spatial working memory. These results are consistent with the typically observed between-strain differences. On the behavioral level, SIR has been shown to produce a deeper effect on the RHA-I rats. RHA-I isolated rats displayed PPI deficits (at the 59 dB prepulse intensity), hyperactivity, increased anxiety levels in the elevated zero maze and worsened long-term spatial reference memory. Social isolation impaired spatial working memory in both RHA-I and RLA-I rats. However, the fact that no effect whatsoever was observed in the cued task (seen as a "guided learning") of the MWM, points out that the differences obtained in the other tasks were not due to physical, sensory or motivational deficits. Therefore, in line with previous results [152] and consistent with the idea that the RHA-I strain is more vulnerable to the induction of schizophrenic-like symptoms, these animals display an almost complete behavioral "isolation syndrome" that is not seen in the RLA-I rats [150].

A deficit in PPI is one of the most widely described effects of long-term SIR [81,149,153]. In contrast with other studies with a more robust effect [149,153], we found that PPI was only impaired at the lowest prepulse intensity (59 dB). This may be due to the different strains of animals used across studies [150,154] (for review of differences among strains see [72]).

The results obtained in both, locomotor activity and anxiety levels, are consistent with the literature. RHA-I rats show a significant increase of locomotor activity, a feature related to the positive symptoms of schizophrenia [150]. The effects of the SIR on anxiety levels make that the scores obtained by isolated RHA-I rats resemble those of the control RLA-I rats, and so, the typical differences between both strains (as described in [103,104,125,160]) are lost.

Lastly, regarding MWM measures of spatial learning and memory, isolated RHA-I rats show an impairment of long-term spatial memory, while social isolation impairs spatial working memory in both rat strains. The induction of cognitive deficits (similar to those present in schizophrenia) seems to be related to changes in PFC (e.g. [152] and references therein) and hippocampal activity [169,195]. In this regard, as we have mentioned earlier, RHA-I rats appear to have a more vulnerable hippocampus [126,128,127], as well as less PFC volume and activity [127,169], which may explain the cognitive deficits that "naïve" RHA-I rats present and the fact that they are more vulnerable to the effects caused by the SIR.

When taking the neuroanatomical studies into account, controversial results have been obtained. On one hand, there are overall strain effects on PFC and HPC volumes, which are consistent with MRI results from our lab showing an enlargement of these areas in RLA-I rats [163,169]. On the other hand, it has been described throughout the literature that SIR produces a decrease in volume in several brain areas relevant for schizophrenia, particularly in PFC and hippocampus [83,150,155], similar to the volume differences seen between postmortem samples of individuals with schizophrenia (see *Introduction* in study 3 and references therein). However, contrary to all these previous results, ours show an overall increase in the volume of the PFC of isolated animals of both strains.

Despite an extensive screening of the literature, it is the first time that an increase in volume has been described as an isolation rearing effect, i.e. long term isolation rearing induced an increase in the volume of PFC in both rat strains. This is an interesting but paradoxical phenomenon, given that isolated rats, particularly RHA-Is, show a number of behavioral deficits that are typical of the "social

isolation rearing syndrome" [80,86,90,150,196]. The protocol used for the isolation of the animals in study 3 has been considerably longer than those usually described in the literature, i.e. we have used an approximately 30-week isolation period, while 8-12 weeks is the most usual isolation period reported in the literature [81,86,154]. One might argue that the extensive isolation and the intensity of the behavioral testing (two PPI sessions, activity testing, elevated zero-maze test, several learning tasks in the Morris Water Maze) the animals received were significantly larger than the usual published procedures, and therefore, unexpected effects may have taken place (including possible compensatory mechanisms). If we only consider the neuroanatomical effect of isolation, i.e. the increase in volume of the PFC, it may be seen as reminiscent of a sort of "sensitization" effect. That is, the environmentally impoverished PFC of long-term isolated Roman rats may have developed neural plasticity and/or compensatory neural processes (e.g. synaptic reorganization, involving reorganization of the equilibrium among neurotransmitters) that place it in a sort of "sensitized" state that "over-reacts" with other plasticity processes when the subject (and thus its brain and its PFC) is "stimulated" by the repeated behavioral testing (that also involves increased handling and "stimulation" by the experimenter). This (supposedly) sensitized "over-reaction" (or "hyper-reactivity") of PFC plasticity mechanisms/processes might hypothetically lead to an increase in volume above the levels of the effects that the same stimulation (of repeated behavioral testing) would have in control animals (as these already have their normal social stimulation in their home cages during the whole period, and thus, hypothetically, would not have a PFC in such a "sensitization-like" hyper-reactive state). Neural sensitization processes of dopaminergic (and other) limbic mechanisms have been described as a consequence of isolation rearing (e.g. [197–199]. Thus, it may not be unconceivable that some similar sensitization process could occur in the PFC, although there is no published evidence on that so far. It is also true that, to the best of our knowledge, there is no previous published work using a 30-week isolation period in rats showing brain (particularly PFC) volumetric measures, and thus the neural plasticity processes that may occur during such a long environmentally impoverished period remain to be explored.

In this context, of neural plasticity, one of the factors receiving more attention in recent years is the brain-derived neurotrophic factor (BDNF). It has been proposed BDNF might play a role in the pathogenesis of various neuropsychiatric diseases through its participation in cellular proliferation, migration, neuronal survival and maintenance of neuronal functions, structural integrity and neurogenesis in the mature brain [200]. In this regard, it might be noteworthy that

the RHA-I rats have been shown to present higher expression levels of BDNF in the PFC, but lower protein levels [Aznar et al. unpublished results], while a reduction of hippocampal BDNF/trkB signaling has been found in outbred RLA vs. RHA rats [201].

Isolation rearing (of only a few weeks), as a paradigm of chronic stress, has been shown to produce a decreased expression of BDNF in the hippocampus, but its effects on the prefrontal cortex have not been properly addressed [200]. However, some studies have shown that following a resocialization of animals reared in isolation there is an increase in BDNF expression in the PFC [202,203]. Our isolated rats have not been resocialized during the whole procedure, but from week 14 until week 27 of isolation they received frequent stimulation related to behavioral testing (room changes, different testing apparatus, stimulation by experimenter handling, etc.). It remains to be investigated whether the present "paradoxical" isolation rearing-induced increase in volume of the PFC in the Roman rats may be related to the above arguments, or to the particular rat strains used here, or to other unknown factors. The isolation-induced increase of the PFC volume is difficult to reconcile with the present behavioral findings, as these are completely in line with the typical "isolation rearing syndrome" that is commonly observed after 8-12 weeks of isolation [86,90,150,154,196]. Thus, all the speculative arguments proposed above await experimental testing. The first approach should be to transversally compare the Roman rats reared in isolation for 12 weeks (from weaning) with other groups reared in isolation for 30 weeks, and to evaluate brain volumes besides a minimum of behavioral phenotypes.

Similar arguments could also be used to explain the results obtained in the 5HT2A binding analysis, in which we could not detect any differences between both strains in any of the areas analyzed. Some previous studies on the Roman rats have described significant differences between RHA-I and RLA-I rats in the PFC in 5HT1A receptor and serotonin transporter (SERT) [117], and in the 5HT2A receptor in both tissue sections [117] and membrane homogenates [204]. However, following training of the animals in the 5-choice serial reaction time task, the differences in the 5HT1A receptor binding levels were lost [117]. Thus, the even more intense training received by our animals during the isolation rearing treatment might account for the loss of 5HT2A binding differences.

Taking these facts into account, as said above, a replication of these experiments (which has not been possible in the course of this dissertation due to time limitations) in order to try to clarify the "paradoxical" results obtained in the present study 3.

Dendritic spines and parvalbumin neurons in the PFC of RHA-I and RLA-I rats

The volumetric and functional differences observed between the RHA-I and the RLA-I rats in the PFC ([127,169] and findings from study 3) prompted us to evaluate whether two structural parameters, namely dendritic spine density and number of PV+ neurons in the PFC would differ between both rat strains. Regarding dendritic spine density analysis, we found that RHA-I rats present a higher amount of thin spines when compared to the RLA-I rats. As we mentioned before, thin spines are considered to be "learning" spines [172,184,186] involved in the acquisition of new memories that will be later consolidated through the transition of thin spines to mushroom spines, considered to be the stable, long-term storage of memory [186]. On the other hand, thin spines have been recently described to be positively correlated with working memory in monkeys [205], suggesting that impairments in working memory function common to schizophrenia reflect a specific deficit in thin spines [172]. Conversely, there is also evidence suggesting that over-proliferation of small spines (i.e. thin spines), relative to the proportion of large spines (such as mushroom and stubby spines) in pyramidal neurons of the PFC, may lead to a decreased proportion of stable synapses and may be related with conditions of mental retardation or impaired cognitive functions [183,184,186]. However, working memory not only depends on the PFC, but a number of other structures, including the hippocampus and its connections with the PFC [206–208]. The RHA-I rats have been shown to have a reduced volume [163] and lower activation of the hippocampus and PFC [126,128,127,206–208] which could imply that the PFC-HPC connectivity may be also impaired at some level, thus supporting the cognitive impairments which characterize RHA-I rats.

Long-term potentiation (LTP) phenomenon, which plays a key role in learning and memory, is related to cognitive deficits [209] and appears to be impaired in schizophrenia [209-211]. LTP-like plasticity activates the transition from small (i.e. thin) to large (e.g. mushroom) spines [172,185]. According to the dynamics of spines and the role they play in structural and functional plasticity (i.e. LTP, for instance), a logical proposal would be that the fact that small (thin) spines predominate in RHA-I rats could mean that late-phase LTP is impaired in this strain [184,186]. This would be compatible with the formation of lesser long-term memories (and, thus, impaired memory performance) in that strain than in RLA-I rats. It would really be interesting to evaluate LTP in PFC of the Roman strains in association with measures of memory and spine types.

From the quantification of PV+ interneurons in the PFC we found no significant differences between RHA-I and RLA-I rats. This is in line with our initial hypothesis and what is described in the literature, since a number of studies have shown that, rather than a reduced number of PV+ neurons, the alterations found in schizophrenia include lower PV levels, which is thought to reflect a lower glutamatergic drive to the PV neurons, but not a loss of PV neurons itself [52,184,187]. This sets the way for the next experiments in which we will look at the expression of PV.

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Conclusions

The main conclusions we have drawn from the present Doctoral Dissertation are:

1. There is a positive association between PPI and LI in outbred (genetically heterogeneous) NIH-HS rats. The LowPPI group is deficient in sensorimotor gating (PPI) and, in parallel, it displays an impairment of the latent inhibition effect.

2. The RHA-I rats show a deficit in PPI as well as no sign of latent inhibition of fear-potentiated startle when compared to their RLA-I counterparts.

3. The impairments in PPI and LI presented by both, LowPPI NIH-HS and RHA-I rats are similar to those observed in schizophrenia and might be associated to some, still unknown, common underlying mechanisms.

4. When subjected to social isolation rearing (SIR), the Roman rats, and specially the RHA-I rats display the well-known "isolation syndrome" at the behavioral level, including deficits in PPI, hyperactivity, increased anxiety and decreased long-term spatial and working memory.

5. The SIR effects produced in the anatomical and molecular level, although controversial, might find explanation in the intensity and extension of the isolation treatment and the testing received by the rats. In any case, a replication of this study must be carried out in order to clarify the results obtained.

6. The newly generated SPF RHA-I and RLA-I colonies, derived through embryo transfer, continue to exhibit the typical between-strain phenotypical differences that have been well stablished in numerous previous studies.

7. The fact that RHA-I rats present more "small" (thin) spines and lower proportion of "large" (mushroom) spines than RLA-I rats in pyramidal neurons of the PFC, could be related with the impaired learning/memory ability of RHA-I rats. The failure to find differences in the number of PV+ neurons matches our initial hypothesis and the literature.

8. Taking together all the findings of this Dissertation we find ourselves in a good position to keep considering that RHA-I rats constitute a good tool to study certain relevant aspects of schizophrenia.

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Appendix I

List of publications

Tapias-Espinosa, C., Río-Álamos, C., Sampedro-Viana, D., Gerbolés, C., Oliveras, I., **Sánchez-González, A**., et al. (2018). Increased exploratory activity in rats with deficient sensorimotor gating: a study of schizophrenia-relevant symptoms with genetically heterogeneous NIH-HS and Roman rat strains. Behav. Processes. Accepted.

Río-Álamos C, Gerbolés C, Tapias-Espinosa C, Sampedro-Viana D, Oliveras I, **Sánchez-González A**, et al. (2017). Conservation of Phenotypes in the Roman High- and Low-Avoidance Rat Strains After Embryo Transfer. Behav Genet. 47:537–51

Oliveras, I., **Sánchez-González, A**., Sampedro-Viana, D., Piludu, MA., Río-Álamos, C., Giorgi, O., Corda, MG., Aznar, S., González-Maeso, J., Gerboles, C., Blázquez, G., Cañete, T., Tobeña, A., Fernández-Teruel, A. (2017). Differential effects of antipsychotic and propsychotic drugs on prepulse inhibition and locomotor activity in Roman High- (RHA) and Low-Avoidance (RLA) rats. Pshychopharmacol. 234:957-975

Oliveras, I., **Sánchez-González, A**., Piludu, MA., Gerboles, C., Río-Álamos, C., Tobeña, A., Fernández-Teruel, A. (2016). Divergent effects of isolation rearing on prepulse inhibition, activity, anxiety and hippocampal-dependent memory in Roman high- and low-avoidance rats: A putative model of schizophrenia-relevant features. Behav Brain Res. 314, 6-15

Esnal, A.[1], **Sánchez-González, A**.[1], Río-Álamos, C., Oliveras, I., Cañete, T., Blázquez, G., Tobeña, A., Fernández-Teruel, A. (2016). Prepulse inhibition and latent inhibition deficits in roman high-avoidance vs. roman low-avoidance rats: Modeling schizophrenia-related features. Physiology and Behavior, 163, 267-273

[1]Equal contribution

Sánchez-González, A., Esnal, A., Río-Álamos, C., Oliveras, I., Cañete, T., Blázquez, G., Tobeña, A., Fernández-Teruel, A. (2016). Association between prepulse inhibition of the startle response and latent inhibition of two-way avoidance acquisition: A study with heterogeneous NIH-HS rats. Physiology and Behavior, 155, 195-201