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Schizophrenia-like sensorimotor gating deficits in intact inbred and outbred rats: From behavior to brain mechanisms and back

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Schizophrenia-like sensorimotor gating deficits in intact inbred and outbred rats: From behavior to brain mechanisms and back

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El sueño de la razón produce monstruos.

Francisco de Goya

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Abbreviations

5-HT: serotonin

5HT1-7: serotonin receptor subtypes

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

APO-SUS: apomorphine susceptible

APO-UNSUS: apomorphine unsusceptible

CD38: cluster of differentiation 38 / ADP-ribosyl cyclase

COMT: catechol-o-methyl transferase

CSPT: cortico-striato-pallido-talamic circuit

D1-D4: dopamine receptors subtypes

DISC-1: Disrupted in schizophrenia 1

DOI: 2,5-Dimethoxy-4-iodoamphetamine

DSM: Diagnostic and Statistical Manual of Mental Disorders

GABA: gamma-Aminobutyric acid

GABA-A/-B: GABA receptors subtypes

HPC: hippocampus

HS: NIH-HS rats

LSD: Lysergic acid diethylamide

mGlu1-8: glutamatergic receptors subtypes

mPFC: medial prefrontal cortex

MRI: magnetic resonance imaging

NAc: nucleus accumbens

NIH-HS: National Institute of Health genetically heterogenous rat stock

NMDA: *N*-Methyl-d-aspartate

OXTR: oxytocin receptor

PCP: phencyclidine

PFC: prefrontal cortex

PnC: caudal pontine tegmental nucleus

PPI: prepulse inhibition

PSD-95: postsynaptic density 95

PTg: pedunculopontine tegmental nucleus

PV: parvalbumin

qPCR: quantitative polymerase chain reaction

RHA: Roman high-avoidance rats

RLA: Roman low-avoidance rats

SHR: spontaneous hypertensive rats

Abstract

Abstract

Schizophrenia is a debilitating mental disorder that involves several cognitive symptoms, including sensorimotor gating impairments. Sensorimotor gating can be measured via prepulse inhibition (PPI) of the startle response, in which the magnitude of a startle stimulus is attenuated by the presence of a pre-stimulus of lower intensity. Rodent studies evaluating the impact of brain-site specific manipulations on PPI have been very useful to provide insights into this basic schizophrenia-like deficiency. These studies show that PPI deficits are frequently accompanied by other symptoms, including psychomotor agitation, as well as alterations in the cortico-striatal-pallido-thalamic (CSPT) circuit. In particular, treatments that increase or decrease the activity of the medial prefrontal cortex (mPFC), hippocampus (HPC), or nucleus accumbens (NAc) reduce PPI. In this context, a dysfunctional cortical excitatory-inhibitory balance has been proposed as the main neural substrate for cognitive dysfunction in schizophrenia. Moreover, these studies show that PPI deficits can be improved by several antipsychotic drugs, including the neuropeptide oxytocin, which has been suggested as an alternative natural antipsychotic. In contrast to these rodent studies, human studies evaluate the association between natural behavioral differences (diagnosis, symptoms) and neural changes. Thus, in this Doctoral Dissertation, we aimed to contribute to bridge the gap between human and rodent studies by exploring whether spontaneous deficits in PPI in intact inbred and outbred rats are (i) associated with divergences in other schizophrenia-related behaviors, (ii) related to functional and structural differences in the CSPT circuit, and (iii) attenuated by oxytocin. Our subjects of study were the inbred Roman high-avoidance (RHA) and Low-avoidance (RLA) rats, and the outbred heterogeneous stock (HS) rats. RHA rats show lower PPI than RLAs, while HS rats were stratified in sub-groups according to their PPI levels. The present experiments also aimed to provide further face, construct, and predictive validity to our animal models of schizophrenia-relevant symptoms (RHA and HS Low-PPI rats). Regarding behavioral associations, our results show that increased exploration in response to novelty is associated with deficient PPI in HS and Roman rats. Moreover, a high anxious profile was found in rats with increased PPI, while no associations were seen with compulsive-like behavior. In relation to brain structural and functional associations with PPI, we combined structural magnetic resonance imaging and c-Fos expression after PPI in both HS and Roman rats. Our results indicate that lower PPI is associated with decreased mPFC activity in both Roman and HS rats and with increased NAc shell activity in HS rats. Reduced PPI is also associated with decreased mPFC and HPC volumes in Roman and HS rats. Additionally,

using immunofluorescence after PPI, we observed a lower percentage of active inhibitory GABAergic parvalbumin interneurons in RHA than RLA rats. Regarding oxytocin administration, we found that oxytocin increased PPI in HS rats, attenuated PPI deficits in RHA rats, and did not affect PPI in RLAs. Consistent with the differential oxytocin effects on PPI (RHA>RLA), constitutive *CD38* expression (regulator of oxytocin release) was reduced in the mPFC of RHA rats compared to the RLAs, while oxytocin administration increased oxytocin receptor (*OXTR*) expression in both strains. This Doctoral Dissertation shows a consistent pattern of behavioral and neurobiological abnormalities in the HS-Low-PPI rats and RHA rats that increases the face, construct, and predictive validity of these rats as models of schizophrenia-related features. Importantly, our results support the idea that sensorimotor gating is modulated by forebrain structures and highlight the relevance of the mPFC and the cortical excitatory-inhibitory balance in its regulation.

Abstract (Catalan version)

L'esquizofrènia és una malaltia mental incapacitant que involucra diversos símptomes cognitius, com un filtratge sensoriomotor deteriorat. El filtratge sensoriomotor es pot mesurar mitjançant la inhibició prepols (IPP) de la resposta d'ensurt. Les investigacions en rosegadors sobre l'impacte d'alteracions cerebrals específiques sobre la IPP han estat molt útils per augmentar el coneixement d'aquesta deficiència bàsica de l'esquizofrènia. Aquests estudis mostren que els dèficits en IPP apareixen conjuntament amb altres símptomes, com ara l'agitació psicomotora, així com alteracions en el circuit cortico-estriato-pallido-talàmic (CEPT). Específicament, tractaments que augmenten o disminueixen l'activitat en el còrtex prefrontal medial (CPFm), l'hipocamp (HPC) o el nucli accumbens (NAc) redueixen la IPP. És important destacar que la desregulació cortical en el balanç excitació-inhibició s'ha proposat com el principal substrat subjacent als símptomes cognitius de l'esquizofrènia. A més, aquests estudis mostren que diversos fàrmacs milloren la IPP, com ara el neuropèptid oxitocina, el qual s'ha proposat com un antipsicòtic natural alternatiu. A diferència d'aquests estudis en rosegadors, els estudis en humans avaluen l'associació entre diferències naturals en la conducta (diagnòstic, símptomes) i canvis neurals. En aquesta Tesi Doctoral, ens vam proposar contribuir a l'establiment d'un pont entre els estudis en humans i rosegadors i, per això, vam explorar si els dèficits naturals en IPP en rates consanguínies i no-consanguínies intactes (i) s'associaven amb diferències en altres conductes relacionades amb l'esquizofrènia; (ii) es relacionaven amb diferències funcionals i estructurals en el circuit CEPT; i (iii) s'atenuaven per l'administració d'oxitocina. Els nostres subjectes d'estudi van ser les rates consanguínies Romanes d'alta i baixa evitació (RHA i RLA), i les rates no consanguínies de l'estoc heterogeni HS. Les RHA mostren menor IPP que les RLA, mentre que les HS es van estratificar en subgrups segons els seus nivells d'IPP. Els experiments plantejats també pretenien augmentar la validesa aparent, de constructe i predictiva dels nostres models animals de característiques rellevants per a l'esquizofrènia (RHA i HS-baixa-PPI). En relació amb les associacions conductuals, els nostres resultats mostren que l'exploració incrementada en resposta a la novetat s'associa amb dèficits en IPP en rates HS i Romanes. En relació amb les associacions cerebrals estructurals i funcionals amb la IPP, vam combinar l'ús de la ressonància magnètica estructural i l'expressió de c-Fos després de la IPP. Vam trobar que la baixa IPP s'associa amb baixa activitat del CPFm en rates Romanes i HS i amb un augment d'activitat en el NAc en rates HS. La baixa IPP s'associa també amb una disminució del volum cerebral del CPFm i l'HPC en rates Romanes i HS. A més, mitjançant l'ús

d'immunofluorescència després de la IPP, vam observar un menor percentatge d'activitat d'interneurones inhibitòries GABAèrgiques de tipus parvalbúmina al CPFm en rates RHA que en RLA. En relació amb l'administració d'oxitocina, vam trobar que l'oxitocina augmentava la IPP en rates HS i RHA, mentre que no afectava la IPP en les RLA. D'acord amb l'efecte de l'oxitocina sobre la IPP (RHA>RLA), els valors constitutius d'expressió de *CD38* (regulador de l'alliberament d'oxitocina) al CPFm eren més baixos en les RHA que en les RLA, mentre que l'administració d'oxitocina va incrementar l'expressió del receptor de oxitocina (*OXTR*) en ambdues soques. Aquesta Tesi Doctoral mostra un patró consistent d'alteracions conductuals i neurobiològiques en rates HS-baixa-IPP i RHA que incrementa la seva validesa aparent, de constructe i predictiva com a models animals de característiques relacionades amb l'esquizofrènia. És important destacar que els nostres resultats donen suport a la idea que el filtratge sensoriomotor està modulat per estructures cerebrals superiors i posen de manifest la rellevància del CPFm i el balanç cortical excitador-inhibidor en la seva regulació.

Abstract (Spanish version)

La esquizofrenia es una enfermedad mental incapacitante que involucra varios síntomas cognitivos, como un filtraje sensoriomotor deteriorado. El filtraje sensoriomotor se puede medir mediante la inhibición prepulso (IPP) de la respuesta de sobresalto. Los estudios en roedores que analizan el impacto de alteraciones cerebrales específicas sobre la IPP han sido muy útiles para aumentar el conocimiento sobre esta deficiencia básica de la esquizofrenia. Estos estudios muestran que los déficits en IPP aparecen junto a otros síntomas, como la agitación psicomotora, y alteraciones en el circuito cortico-estriato-pallido-talámico (CEPT). Específicamente, tratamientos que aumentan o disminuyen la actividad del córtex prefrontal medial (CPFm), el hipocampo (HPC) o el núcleo accumbens (NAc) reducen la IPP. Es importante destacar que la desregulación cortical en el balance excitación-inhibición se ha propuesto como el principal sustrato subyacente a los síntomas cognitivos de la esquizofrenia. Además, estos estudios muestran que la IPP mejora con varios fármacos antipsicóticos, como el neuropéptido oxitocina, el cual se ha propuesto como antipsicótico natural alternativo. A diferencia de los estudios en roedores, los estudios en humanos evalúan la asociación entre diferencias conductuales naturales (diagnóstico, síntomas) y cambios neurales. En esta Tesis Doctoral, nos propusimos contribuir a establecer un puente entre los estudios en humanos y roedores y, para ello, exploramos si los déficits naturales en IPP en ratas consanguíneas y no consanguíneas intactas (i) se asociaban con diferencias en otras conductas relacionadas con la esquizofrenia; (ii) se relacionaban con diferencias funcionales y estructurales en el circuito CEPT; y (iii) se atenuaban por la administración de oxitocina. Usamos las ratas consanguíneas Romanas de alta y baja evitación (RHA y RLA), y las ratas no consanguíneas del stock heterogéneo HS. Las RHA muestran menor IPP que las RLA, mientras que las HS se estratificaron en subgrupos según su IPP. Los experimentos planteados también pretendían aumentar la validez aparente, de constructo y predictiva de nuestros animales modelo de características relevantes para la esquizofrenia (RHA y HS-baja-IPP). En relación con las asociaciones conductuales, nuestros resultados muestran que la exploración incrementada en respuesta a la novedad se asocia con déficits en IPP en ratas HS y Romanas. Respecto a las asociaciones cerebrales estructurales y funcionales con la IPP, combinamos el uso de resonancia magnética estructural y expresión de c-Fos después de la IPP. Encontramos que la baja IPP se asocia con baja actividad del CPFm en ratas Romanas y HS y con un aumento de actividad en el NAc en ratas HS. La baja IPP se asocia también con una disminución del volumen cerebral del CPFm e HPC en ratas Romanas y HS. Además,

mediante el uso de inmunofluorescencia después de la IPP, encontramos un menor porcentaje de actividad de interneuronas inhibitorias GABAérgicas de tipo parvalbúmina en el CPFm en ratas RHA que en RLA. Respecto a la administración de oxitocina, ésta aumentó la IPP en ratas HS y RHA, mientras que no afectó la IPP en las RLA. De acuerdo con el efecto diferencial de la oxitocina sobre la IPP (RHA>RLA), los valores constitutivos de expresión de *CD38* (regulador de la liberación de oxitocina) en el CPFm fueron más bajos en ratas RHA que en las RLA, mientras que la administración de oxitocina incrementó la expresión del receptor de oxitocina (*OXTR*) en ambas cepas. Esta Tesis Doctoral muestra un patrón consistente de alteraciones conductuales y neurobiológicas en ratas HS-baja-IPP y RHA que incrementa su validez aparente, de constructo y predictiva como animales modelo de características relacionadas con la esquizofrenia. Nuestros resultados apoyan la idea de que el filtraje sensoriomotor está modulado por estructuras cerebrales superiores y ponen de manifiesto la relevancia del CPFm y el balance cortical excitador-inhibidor en su regulación.

Introduction

1. Schizophrenia

1.1. Overview

Schizophrenia is a common, severe, chronic, and incapacitating mental disorder that disturbs how a person perceives, thinks, feels, and behaves [1,2]. It typically starts in late adolescence or early adulthood (the usual onset age ranges from 16 to 30 years old) and affects 1% of the population worldwide [2,3]. This disorder represents significant health care costs and is related to a reduction of life expectancy of about 15 years on average [4].

Under the term of "*dementia praecox*", schizophrenia was first identified and studied by Emil Kraepelin in 1893 [5]. It was described as a pattern of chronic and severe social and functional impairments, including psychotic symptoms (i.e. hallucinations and delusions), starting at early adulthood. Later on, in 1908, Eugene Bleuler redefined "*dementia praecox*" as "*schizophrenia*", from the Greek roots σχίζειν "*schizein"* (split) and φρήν "*phrēn"* (mind) [5,6]. According to Bleuler, apart from hallucinations and delusions that are also present in other disorders (e.g. bipolar disorder), there were some fundamental symptoms of schizophrenia, such as thought and speech incoherence, abolition, affective incongruence, and autism. However, nowadays the concept of schizophrenia has changed to a more accurate description of signs and symptoms.

1.2. Symptoms

Following the observations of Kraepelin and Bleuler, several clinicians have made efforts to redefine and better describe the concept and diagnostic criteria of schizophrenia. Their work has led to a standardized classification by the American Psychiatry Association included in their editions of the Diagnostic and Statistical Manual of Mental Disorders (DSM). As shown in Table 1, in the 5th edition of the DSM [7], the diagnosis of schizophrenia requires at least two of the five symptoms of Criterion "A", being one of them delusions, hallucinations, or disorganized speech (known as positive symptoms). Moreover, the signs of disturbance included in criterion "B", involving social and functional impairment, must last for at least six months with one month of symptoms that meet Criterion "A".

Even though these criteria are very helpful to standardize the diagnosis of the disorder, schizophrenia is an extremely complex neuropsychiatric disease with several signs and symptoms that vary from patient to patient and may change in the course of the disorder in the same patient [2,8–11].

Table 1. Diagnostic criteria for schizophrenia [7].

A. Two (or more) of the following, each present for a significant portion of time during a 1-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 avolition)
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.
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 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)

Apart from the diagnostic criteria, the symptoms of schizophrenia are typically divided into three main categories, including positive, negative, and cognitive symptoms [10,12]:

- **Positive symptoms:** represent a loss of touch with reality, such as hallucinations, delusions, disorganized thinking, and psychomotor agitation.
- **Negative symptoms:** involve disrupted emotions and social behaviors, such as flattened affect, reduced speech, abulia, lack of initiative, and social withdrawal.
- **Cognitive symptoms:** denote a reduction in intellectual functioning and cause the most severe consequences in some patients in the long term. Of note, these symptoms usually remain when the active-phase of the disease (Criterion “A”) has vanished [6]. Examples of these symptoms are deficient working memory, difficulties to pay attention or focusing, and poor executive functioning.

On the other hand, schizophrenia also shows comorbidity with anxiety and obsessive-compulsive symptoms [13,14]. In this regard, it is estimated that 38.3% of people with schizophrenia spectrum disorders show comorbid anxiety disorders, such as social phobia, posttraumatic stress disorder, and obsessive-compulsive disorders [14]. The presence and severity of anxiety and obsessive-compulsive symptoms in schizophrenic patients are related to more severe clinical features and poorer outcomes.

1.3. Risk factors

Although the origin of schizophrenia remains unknown, it is demonstrated that both genetic risk and environmental factors interact to cause the disorder [2,15]. Accordingly, it is not just a genetic history or a disrupted environment that leads to the development of schizophrenia, but a complex interaction of factors throughout lifespan.

1.3.1. Genetic factors

Many genetic studies, including family, twins and, adoption studies, have highlighted that the genetic factor plays a key role in the risk of suffering from schizophrenia [11,16–18]. As displayed in Fig. 1, the closer the relative with schizophrenia are, higher is the risk of developing this disorder (from general population to identical twins). Of note, it has been calculated that the genetic heritability of schizophrenia may reach up to 80% [19,20], which means that most of individual differences that cause vulnerability to develop this disorder are related to genetics. However, 63% of schizophrenic patients with no first- or second-degree relatives diagnosed with schizophrenia [21]. This evidence opened the door to study environmental factors associated with increased odds of suffering schizophrenia.

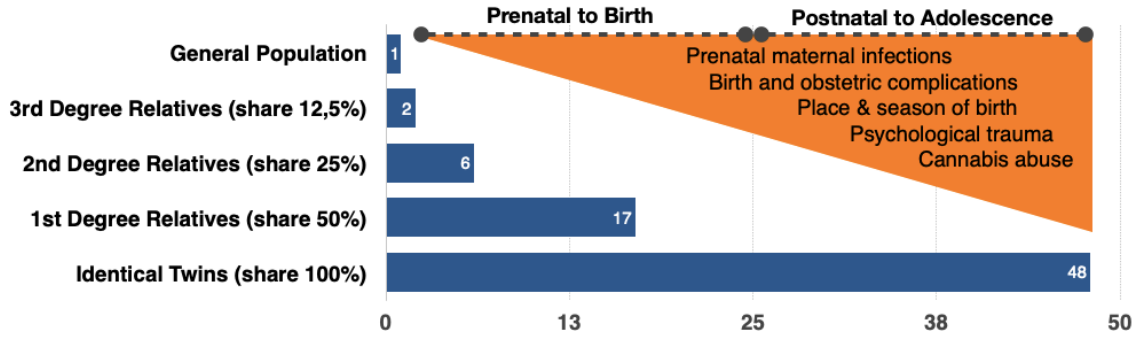

Figure 1. Genetic and environmental risk factors in schizophrenia. (Adapted from [21]). The figure illustrates the increasing risk of suffering from schizophrenia when genetic and environmental risk factors interact. Blue bars indicate the increasing genetic risk of developing schizophrenia according to the percentage of shared genes (e.g. from general population to identical twins). The triangle shows some examples of environmental factors during two neurodevelopmental critical periods (i.e. prenatal to birth and postnatal to adolescence periods) that increase the risk of suffering from schizophrenia.

1.3.2. Environmental factors

As shown in Fig. 1, there are two main critical periods in the neurodevelopment when some environmental factors can increase the risk of developing schizophrenia [2]:

- **From prenatal period to birth:** some factors can disrupt the normal brain maturational processes during this period, such as prenatal maternal viral infection, prenatal malnutrition, advanced parental age, season of birth (winter), or obstetrical complications [22–25].

- **From postnatal period to the end of adolescence:** some insults can increase the vulnerability to schizophrenia during this period, such as childhood trauma or adversity, famine, urban dwelling, or cannabis smoking [26–29].

Finally, it is important to mention that both environmental and genetic factors can compensate each other. For instance, a genetic risk of schizophrenia can be attenuated by suitable environmental inputs, as well as an environmental risk of schizophrenia can be prevented by low genetic risk [2,15,25,30].

1.4. Neuroanatomy

Several approaches, from postmortem to magnetic resonance imaging (MRI) studies, have allowed mapping several brain regions that are affected in schizophrenia. These brain areas go from brain stem and midbrain to forebrain regions, with special interest to forebrain areas that modulate our behavior and cognition, such as the prefrontal cortex (PFC), the hippocampus (HPC), and the striatum [3,31].

1.4.1. Prefrontal cortex

Following the work of Kraepelin, Alois Alzheimer systematically investigated the postmortem brain of patients with "*dementia praecox*". Importantly, in his first publication in 1983, Alzheimer described the thinning of the neocortical layers in patients with "*dementia praecox*", which he interpreted as a sign of a destructive process [6]. Conversely, recent studies postulate that there is an impairment in the regeneration process, rather than a destructive process [6]. Either way, more than a century later, it is still well accepted that neocortical alterations, particularly in the prefrontal cortex (PFC), play a major role in the psychotic profile and are one of the main targets in the treatment of schizophrenia [31].

The PFC is a prominent region in primates that regulates various higher-order executive functions that are impaired in schizophrenia, such as perception, attention, motivation, language, reasoning, memory, consciousness, response inhibition, decision-making, etc. [32,33]. In general, the main function of the PFC is to integrate sensory and motor information from subcortical brain regions, along with past experiences, to provide appropriate responses [33]. Even though the PFC is more prominent in primates, lesion studies in rodents have shown that the rodent medial PFC (mPFC) may be involved in some cognitive functions similar to the primate PFC, such as working memory, attentional shifting, and the regulation of emotional responses [33–36]. In this sense, and in agreement with the observations of Dr. Alzheimer, MRI studies have reported structural and functional abnormalities in the PFC of patients with schizophrenia [37–39].

1.4.2. Hippocampus

The HPC is located at the medial temporal lobe in primates and is present in all mammalian species [33]. This brain area plays a relevant role in the regulation of several behaviors and cognitive functions that are impaired in schizophrenia, such as spatial navigation, and learning and memory [40–42]. The HPC regulates these behaviors and cognitive functions in intimate connection with the PFC [33]. In this sense, it has been observed that the connectivity between the PFC and the HPC is disrupted in schizophrenic patients [33]. Moreover, as for the PFC, several MRI studies have revealed abnormalities in the HPC of schizophrenic patients [43].

1.4.3. Dorsal and ventral striatum

The striatum is a nucleus located in the subcortical basal ganglia of the forebrain and is involved in various components of cognition that are dysfunctional in schizophrenia. The striatum is divided into the dorsal and ventral parts. The dorsal striatum mediates cognitive processes, such as stimulus-response learning (habit formation), motor and action planning, decision-making, and executive functions (e.g. inhibitory control and impulsivity), whereas the ventral striatum (so-called nucleus accumbens, NAc) mediates reward perception, salience processing, reinforcement, and motivation [44–46]. Of note, this brain area modulates these behaviors and cognitive functions through afferent and efferent pathways from and to the PFC [47]. In this regard, several reports have revealed that the striatum shows structural and functional abnormalities in schizophrenia and is responsible for the positive symptomatology of the disorder [47].

Thus, experimental evidence is generally consistent with the idea that alterations in the PFC, HPC, or striatum underlie schizophrenia symptoms [31,37–39,43,47]. However, it has to be taken into account that psychiatric disorders, such as schizophrenia, show subtle neuroanatomical differences compared to neurological disorders, where the causes are clearly due to alterations of specific brain regions. Therefore, regarding the causes of psychiatric disorders, it is important to consider impairments at the level of synapses, connectivity between brain regions, and neurotransmission [16].

1.5. Etiology

It is difficult to establish a specific dysfunctional brain pattern in schizophrenia, as symptoms differ from patient to patient and may change in the same patient [8–11]. Moreover, as mentioned before, schizophrenia is not the consequence of a dysfunction of specific brain regions, but an impairment at the level of complex neuronal circuits among various brain areas [16,17,48,49]. In this sense, as summarized in Fig. 2, four main hypotheses have been postulated to try to explain the neural mechanisms underlying schizophrenia symptoms. These hypotheses are based on the alteration of four different neurotransmitters: dopamine, serotonin, glutamate, and GABA.

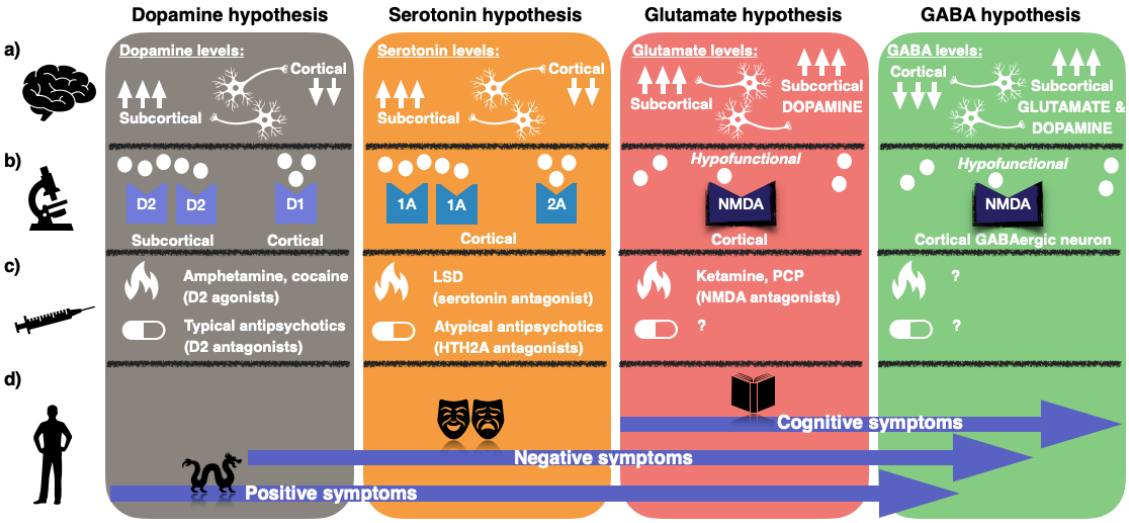

Figure 2. Summary of dopamine, serotonin, glutamate and GABA hypotheses of schizophrenia. Representation of four levels of evidence for the four neurotransmitter hypotheses of schizophrenia: **a)** neurotransmitter levels in subcortical or cortical brain regions (↑ or ↓); **b)** receptor abnormalities in cortical and subcortical brain areas; **c)** pharmacological action of probed enhancer or reducer antipsychotic drugs; **d)** symptoms explained by the hypothesis. For details see sections 1.5.1-4.

1.5.1. Dopamine hypothesis

Dopamine is a neurotransmitter that plays a central role in developing appropriate goal-directed behaviors [50], and it binds to four subtypes of metabotropic receptors (D1, D2, D3, D4) all over the brain [51]. Importantly, this neurotransmitter has three main projections from the substantia nigra and ventral tegmental area to: (i) the dorsal striatum (nigrostriatal pathway), (ii) ventral striatum (mesolimbic pathway), and (iii) PFC

(mesocortical pathway); and dopamine has also a fourth endocrine pathway from the hypothalamus to the pituitary gland (tuberoinfundibular pathway) [50,51]. In this regard, the dopamine hypothesis postulates that there is a hyperdopaminergic activity in both the nigrostriatal and mesolimbic pathways, which can explain positive symptoms, and reduced dopaminergic activity in the mesocortical pathway, which can explain part of the negative symptoms [3]. This hypothesis stems from the observations that dopamine agonists (amphetamine or cocaine) induce psychotic symptoms in healthy subjects and aggravate them in schizophrenic patients [52]; and that the potency of an antipsychotic drug to reduce positive symptoms is proportional to the degree of dopamine D2 receptor antagonism (e.g. chlorpromazine or haloperidol; [53–56]). Moreover, evidence from postmortem studies shows abnormalities in the pre- and post-synaptic dopaminergic receptors in schizophrenic patients, such as increased D2 receptors in striatal areas [57], and reduced D1 receptors in the fronto-parieto-temporal cortex [51] (see Fig. 2). However, these dopaminergic abnormalities in schizophrenic patients may be explained as a consequence of antipsychotic treatments, as it has not been confirmed in untreated patients [58].

The discovery of the second-generation (atypical) antipsychotics that act on other receptors, apart from the D2 receptor, suggests that the etiology of schizophrenia would need alternative hypotheses to explain its underlying mechanisms.

1.5.2. Serotonin hypothesis

Serotonin is a neurotransmitter that mediates excitatory and inhibitory transmission by modulating the release of other neurotransmitters, such as glutamate, GABA, or dopamine [59]. It binds to metabotropic (5HT1, 2, 4-7) and ionotropic (5HT3) receptors in several brain regions. The serotonin hypothesis suggests that schizophrenia is a consequence of a dysfunction in the serotoninergic system. It is based on the fact that serotonin agonists of the 5HT1A and 5HT2A receptors (e.g. LSD or DOI) cause psychotomimetic effects, such as hyperactivity or hallucinations [60]; and the discovery that the antagonism of the 5HT2A receptor is the basis of the therapeutic action of the second-generation (atypical) antipsychotic drugs (e.g. clozapine, risperidone or olanzapine; [61,62]). Unlike the first-generation antipsychotic drugs, the atypical antipsychotics have revealed improvements not only in positive symptoms, but also in

negative symptoms [63,64]. In this regard, it seems that the 5HT2A antagonism would result in a specific increase in dopamine transmission in the mesocortical pathway [61,62]. In line with pharmacological studies, postmortem studies have shown increased expression of the 5HT1A receptor and decreased expression of the 5HT2A receptor in the frontal cortex [43], as well as elevated serotonin levels in subcortical brain areas and reduced cortical levels [65] (see Fig. 2).

Nevertheless, as serotonin cannot explain the whole complexity of signs and symptoms of schizophrenia and it regulates other neurotransmitter systems, such as glutamate and GABA, other alternative hypotheses have been proposed.

1.5.3. Glutamate hypothesis

Glutamate is the main excitatory neurotransmitter in the brain. It is involved in several kinds of learning and memory processes, and its function is crucial during prenatal and early childhood brain development [31,57]. It acts on both ionotropic (NMDA, kainate, and AMPA) and metabotropic (mGlu1-8) receptors. The glutamate hypothesis suggests that excessive glutamatergic release from cortical to subcortical brain regions would cause an increase of dopamine in the mesolimbic system [31,48]. This is supported by the evidence that glutamate NMDA antagonists increase subcortical dopamine and cause positive, negative, and cognitive symptoms in healthy subjects, while pro-dopaminergic drugs only replicate positive symptoms [66,67]. In line with this idea, postmortem studies highlight several alterations in the normal functioning of the glutamatergic system in schizophrenia, such as the presence of hypofunctional NMDA receptors in inhibitory cortical neurons that would lead to increased subcortical glutamatergic release [66] (see Fig. 2 and 3).

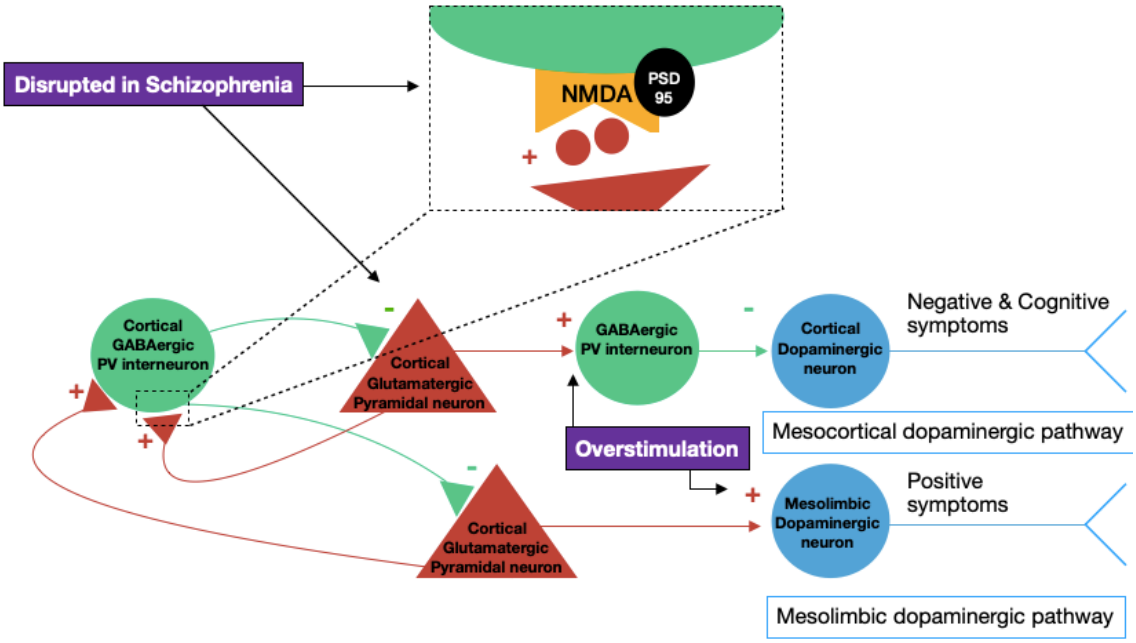

Figure 3. Schematic representation of the GABA/Glutamate balance in the PFC. In normal physiological conditions, cortical glutamatergic pyramidal neurons activate cortical GABAergic parvalbumin (PV) interneurons that, in turn, inhibit the pyramidal neurons. In the magnification, there is a glutamatergic NMDA receptor with the scaffolding protein PSD-95 on a PV interneuron. This feedback between cortical GABAergic and glutamatergic neurons regulates the stimulation of dopaminergic cortical and mesolimbic neurons. In schizophrenia, reduced PV interneurons activation due to a dysfunctional glutamatergic drive (measurable as reduced PSD-95 puncta on PV interneurons) leads to overactivation of glutamatergic projections. This disruption in the cortical GABAergic and glutamatergic regulation leads to higher glutamatergic transmission that overstimulates: i) mesolimbic GABAergic neurons that overinhibit dopaminergic neurons that project to the PFC, causing negative and cognitive symptoms; and ii) mesolimbic dopaminergic neurons, responsible for positive symptoms.

1.5.4. GABA hypothesis

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain and plays a crucial role in the modulation of several cognitive processes in the PFC [68,69]. It binds to ionotropic (GABA-A) and metabotropic (GABA-B) receptors in several brain regions [68]. Following the glutamate hypothesis, the GABA hypothesis postulates that GABA-mediated cortical inhibition is dysfunctional in schizophrenia, leading to higher excitatory neural transmission to subcortical brain areas [48,68,70]. In this regard, as illustrated in Fig. 3, it has been suggested that dysfunctional NMDA receptors in GABAergic neurons do not activate these inhibitory neurons, and it leads to overactivation of glutamatergic projections [48,67]. This higher glutamatergic

transmission overstimulates (i) mesolimbic dopaminergic neurons, responsible for positive symptoms; and (ii) mesolimbic GABAergic neurons that, in turn, overinhibit dopaminergic neurons that project to the PFC, causing negative and cognitive symptoms ([48]). This hypothesis is based on the evidence that schizophrenic patients show poor gamma oscillation frequency in the PFC, a pattern of neuronal firing critical for cognitive abilities, such as attention and working memory [67,71,72]. Gamma oscillations depend on cortical inhibitory circuitry and it seems that dysfunctional cortical GABA-mediated synaptic inhibition would explain cognitive impairment in schizophrenia [69]. Particularly, a subpopulation of GABA neurons that expresses the calcium-binding protein parvalbumin (PV) seems to be essential to provide inhibitory inputs and to drive cortical gamma oscillations [72]. In this regard, postmortem studies show altered PV neurons in the PFC of schizophrenic patients [73–77].

1.6. Pharmacological treatments

Antipsychotic drugs are the first line of treatment for schizophrenia. These pharmacological treatments must be personalized and maintained in the long-term, as they aim at the symptoms but not the causes of the disorder [18]. Once the patient is stabilized, psychological approaches can be introduced in the treatment of schizophrenia [18,78].

1.6.1. Typical antipsychotics

The first drug used to treat the psychotic symptoms of schizophrenia was chlorpromazine, which was discovered by mistake in the early 1950s [79]. Chlorpromazine gave rise to the first-generation (typical) antipsychotic drugs that have in common the D2 receptor antagonism [53,54]. This pharmacological action of blocking the D2 receptor has beneficial effects in the mesolimbic pathway (see "*1.5.1. Dopamine hypothesis*"), as it reduces positive symptoms [53–55,62]. However, it causes severe motor side effects when acting in the nigrostriatal pathway, including dystonia, parkinsonism, and akathisia, or endocrine abnormalities when acting on the tuberoinfundibular pathway, and it aggravates negative and cognitive symptoms via the mesocortical pathway [11,18,47,62,80]. Some examples of typical antipsychotics are chlorpromazine, haloperidol, and thiothixene.

1.6.2. Atypical antipsychotics

With the idea to reduce the motor and cognitive side effects, over the past 30 years a new generation of antipsychotics has been developed. These antipsychotics are called the second-generation (atypical) antipsychotic drugs. They have in common lower affinity to block the D2 receptor than the typical antipsychotic drugs and the ability to block the 5HT2A receptor (see "*1.5.2. Serotonin hypothesis*"). In this sense, the atypical antipsychotics are less effective with positive symptoms than the typical ones, but they improve negative and cognitive symptoms by increasing dopamine in the mesocortical pathway [63,64]. However, the beneficial effects of the atypical antipsychotic drugs over the negative and cognitive symptoms are a matter of controversy [81,82]. Moreover, these drugs still cause severe side effects, including weight gain, tardive dyskinesia, and metabolic disorders [18,81]. Some examples of atypical antipsychotics are clozapine, lurasidone, olanzapine, paliperidone, quetiapine, risperidone, and ziprasidone.

On the other hand, a more recent pharmacological approach has tried to aim at the differential role that dopamine seems to play in the development of positive and negative symptoms. According to the dopaminergic hypothesis, positive symptoms are due to excessive dopamine in the mesolimbic pathway and lower dopamine in the mesocortical pathway (see "*1.5.1. Dopamine hypothesis*"). In this sense, the third-generation (atypical) antipsychotic aripiprazole shows dual actions according to dopamine levels, as it acts as a partial antagonist when dopamine activity is high (blocking the receptor in the mesolimbic pathway) and acts as partial agonist when dopamine activity is low (activating dopamine receptors in the mesocortical pathway) [64]. However, it should be considered that aripiprazole is less effective with positive symptoms, does not work in all patients, and causes serious side effects, including agranulocytosis, neuroleptic malignant syndrome, and tardive dyskinesia [18,64].

Finally, it is noteworthy that about 50-70% of first-episode schizophrenic patients respond to antipsychotic drugs, with this response rate decreasing to 20% for those who suffer a second psychotic episode [83]. Thus, about a 30% of patients diagnosed for schizophrenia suffer from treatment-resistant schizophrenia, as they never respond to any antipsychotic drugs. Moreover, as mentioned before, most antipsychotic medications are effective with positive symptoms but worsen or have no effect on negative and cognitive symptoms [81,82]. Additionally, antipsychotics are known to

cause other serious physiological side effects, such as weight gain, diabetes, movement disorders, tardive dyskinesia, prolactin elevation, and sedation [18]. For all these reasons, there is an urgent need to find better and more effective treatments for psychotic disorders.

1.6.3. Oxytocin

With the idea to reduce side effects and act on other relevant brain targets, such as glutamate or GABA, other alternative pharmacological drugs have been proposed to treat schizophrenia. One of the possible candidates meeting these two criteria is oxytocin [84–86], which is an endogenous neuropeptide synthesized in the paraventricular and supraoptic nuclei of the hypothalamus. Traditionally, oxytocin was associated with uterine contractions and breastfeeding. Nevertheless, it has been recently linked to social and cognitive-related behaviors [87,88]. Specifically, increased oxytocin levels in blood plasma are related to various positive events, such as trust, physical contact with a partner, reduced hormonal response to stressors, or reduced anxiety. In contrast, reduced levels of oxytocin in blood plasma have been related to several psychiatric conditions, such as autism spectrum disorders, depression, and schizophrenia [89]. Particularly, oxytocin has been proposed as an alternative natural antipsychotic, as the exogenous administration of oxytocin increases trustworthiness in healthy subjects [90,91], reduces positive and negative symptoms in schizophrenia [92], increases eye gaze in schizophrenia [93], and improves several schizophrenia-like behaviors in rodents [84,94–96]. In this regard, findings are generally consistent with the idea that oxytocin has an inhibitory profile, as it reduces glutamate and increases GABA release [97–99]. Moreover, human functional MRI studies have proved that peripherally administered oxytocin modulates the PFC activity [100]. Importantly, following the glutamate and GABA hypotheses, the administration of oxytocin might help to reduce the activity of the glutamatergic and dopaminergic systems in subcortical areas and increase dopamine in the PFC by increasing cortical inhibition (see "*1.5.3. Glutamate hypothesis*" and "*1.5.4. GABA hypothesis*"). Thus, oxytocin could reduce positive, negative, and cognitive symptoms [92,101]. Although oxytocin is not yet an approved drug for the treatment of schizophrenia, findings are encouraging and indicate that it deserves further investigation.

2. Animal models of schizophrenia:

An animal model is a more manageable and simplified form of a human clinical condition [102,103]. The most used model organisms in neurosciences are rats and mice, which share very similar genetic bases with humans [104]. Moreover, these models allow the use of more invasive procedures to study the molecular and structural bases of the human clinical condition [105].

2.1. Validity criteria

The development of animal models constitutes a necessary tool to increase our knowledge about the neurobiological basis of psychiatric disorders and the development of novel treatments [105]. However, as illustrated in Fig. 3, in order to consider an animal model as a useful tool to study a specific psychiatric disorder, it should meet the three independent validity criteria [105–107]:

- **Face validity:** the animal model must replicate most symptoms of the human clinical condition (i.e. analogy with human symptoms). However, some symptoms of schizophrenia cannot be mimicked in rodents, such as hallucinations or delusions.
- **Construct validity:** the animal model must replicate the theoretical neurobiological mechanisms underlying the human clinical condition.
- **Predictive validity:** the animal model must respond to the same treatments that improve or worsen the symptoms of the human clinical condition.

Thus, and according to Jones and colleagues [105], an ideal animal model of schizophrenia must show deficits after puberty, loss of connectivity and functionality between the HPC and the PFC, dopaminergic and glutamatergic dysfunctions, vulnerability to stress, reward-related impairments, attentional/cognitive deficits, and reduced social behavior and/or anhedonia.

2.2. Schizophrenia-relevant symptoms in rodents

As summarized in Fig. 4, there are several rodent behaviors that are relevant to positive, negative, and cognitive signs and symptoms of schizophrenia [103]. These schizophrenia-like symptoms represent the “face validity” of an animal model. However, these symptoms must be accompanied by alterations in schizophrenia-relevant brain circuits (“construct validity”), as well as significant responses to effective schizophrenia-relevant treatments (“predictive validity”).

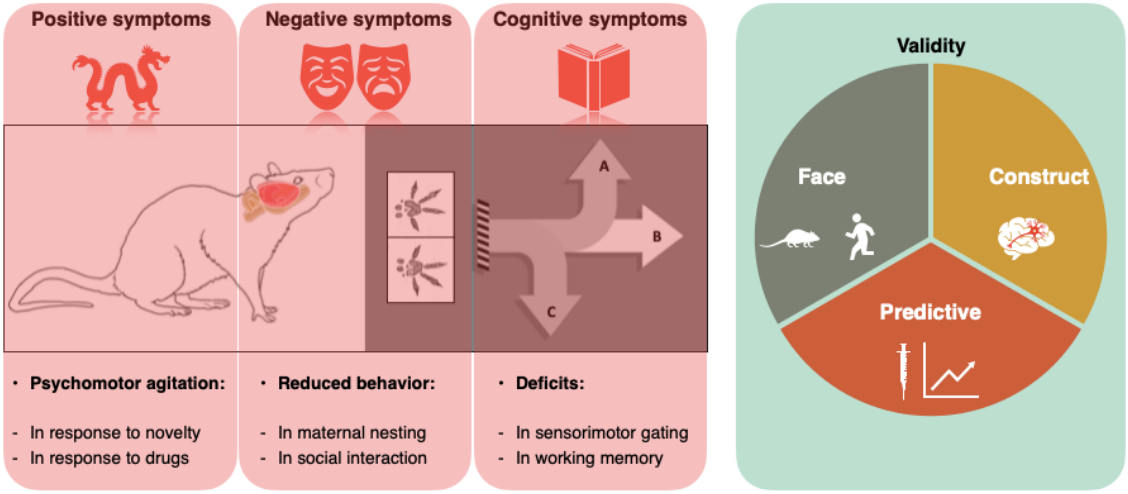

Figure 4. Summary of rodent behaviors that are relevant to positive, negative, and cognitive symptoms of schizophrenia, and validity criteria of animal models. Analysis of rodent behavior goes from alterations in motor responses (psychomotor agitation relative to positive symptoms) and social preference (reduced behavior in social contexts relative to negative symptoms) to cognitive alterations (deficits in cognitive-related tasks relative to cognitive symptoms). An animal model is considered a useful tool to study a specific psychiatric disorder when it meets the three independent validity criteria: face validity (similar symptoms in rodents and schizophrenic patients), construct validity (similar brain mechanisms involved in rodents and schizophrenia), and construct validity (similar drug effects in rodents and schizophrenia).

2.2.1. Positive symptoms

Since the main positive symptoms of schizophrenia cannot be measured in rodents (i.e. delusions, hallucinations, or thought disorder), the primary positive symptom mimicked in rodents is psychomotor agitation (relative to grossly disorganized behavior in schizophrenia). This psychomotor agitation is measured as spontaneous increased locomotor activity in the home-cage, in response to novelty (e.g. in the open field test),

or in response to psychotomimetic drugs (e.g. increased effects of amphetamine, cocaine, phencyclidine (PCP), ketamine, etc. compared to control group) [103].

2.2.2. Negative symptoms

In general, negative symptoms are measured in rodents as decreased social behavior and anhedonia. Examples of these symptoms are [103]:

- Social withdrawal.
- Decreased interaction with a juvenile conspecific.
- Decreased place preference for a caged peer conspecific.
- Decreased preference for social novelty.
- Altered social dominance on tube test.
- Altered aggression behavior on resident intruder assay.
- Decreased nesting behavior.
- Reduced home-cage social interaction.

2.2.3. Cognitive symptoms

Cognitive symptoms would be inferred in rodent by gathering behavioral samples in three main domains, such as impairments in [103]:

- Working memory (e.g. impaired working memory performance in the T-maze, the 8-arm radial maze, or the Morris water maze).
- Attention, sensorimotor gating, and executive functions (e.g. decreased sensorimotor gating, latent inhibition, 5-choice serial reaction time test, or set-shifting ability, i.e. cognitive flexibility).
- Spatial learning (e.g. decreased spatial learning in the Morris water maze or the 8-arm spatial maze).

2.3. Sensorimotor gating impairments

Sensorimotor gating is defined as the ability of a sensory event to suppress a motor response, and it can be operationally measured via prepulse inhibition (PPI) of the startle response in both humans and rodents [108,109]. As illustrated in Fig. 5, in PPI the magnitude of the startle response is attenuated by the presence of a pre-stimulus of lower intensity [110]. This attentional and cognitive ability is impaired in several clinical conditions, such as schizophrenia, Tourette's syndrome, and obsessive-compulsive disorder [111–113]. Experimentally-induced PPI deficits in rodents are used as a common endophenotype to model this basic schizophrenia-like deficiency [114–116].

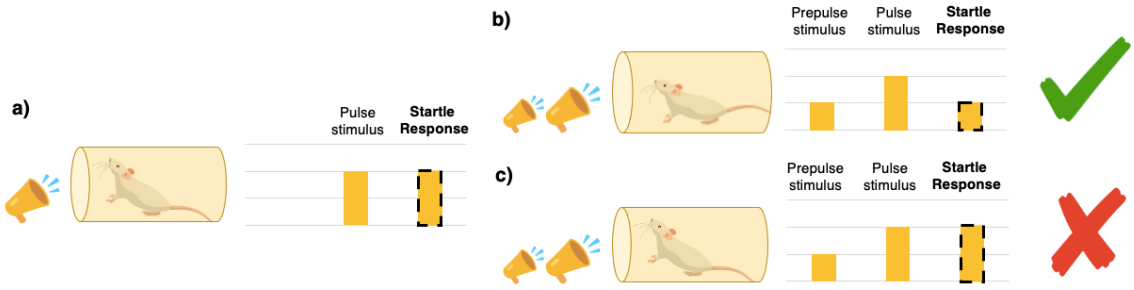

Figure 5. Representation of prepulse inhibition (PPI) of the acoustic startle response. a) A high-intensity acoustic stimulus elicits a startle response. **b)** PPI refers to the ability of a low-intensity stimulus (prepulse) to reduce the startle response triggered by the high-intensity stimulus. **c)** When PPI deficits are present, subjects show poor ability to diminish the startle response.

The study of PPI has gained relevance since it was first demonstrated in 1978 that it is reduced in schizophrenic patients [117]. Since then, PPI deficits in schizophrenia have been replicated in almost 40 reports in the literature [118,119]. Moreover, PPI is a useful and objective measure to study the mechanisms underlying schizophrenia, as it is characterized by:

- **High test-retest stability:** PPI shows stable individual differences [120,121].
- **Response to antipsychotic drugs:** PPI deficits are mitigated by effective antipsychotic treatments in schizophrenic patients [122], as well as in animal models [123].
- **Genetic and environmental influences:** PPI deficits are also found in unaffected relatives of schizophrenia patients [124] and can be experimentally induced by environmental treatments in rodents, such as social isolation [125–127].

Regarding the neural bases of PPI, the startle reflex would be elicited by an excitatory input from the auditory pathway that activates the caudal pontine tegmental nucleus (PnC) and produces a motor startle response; while the PPI of the startle reflex is triggered by excitatory projections from the auditory pathway that via inferior and superior colliculus trigger the activation of the pedunculopontine tegmental nucleus (PTg) and inhibit the PnC [123]. Nevertheless, rodent and human studies have revealed that PPI is modulated by the cortico-striatal-pallido-thalamic (CSPT) circuit involving efferent pathways from the PFC, thalamus, HPC, amygdala, and dorsal and ventral striatum to the PTg [39,113,123,128,129]. In this sense, both animal and human temporal lobe epilepsy studies have involved the HPC and the amygdala in the PPI dysfunction [113]. It seems that the HPC and the amygdala act via NAc on the PPI circuit, even though other studies suggest that it is through the mPFC [130,131]. The NAc and the mPFC, through glutamatergic projections, would regulate the PTg, which in turn would reduce or increase the startle reflex. In this regard, as described for schizophrenia (see "*1.4. Neuroanatomy*" and "*1.5. Etiology*"), findings generally agree that PPI deficiencies are accompanied by dysregulation between cortical and subcortical brain areas [37–39,123,132].

2.4. Experimental strategies

Animal models of schizophrenia have been developed with the idea to better understand the underlying mechanism of the disorder, as well as to find novel and improved treatments. In order to do so, several experimental strategies have been developed. These strategies can be divided into two main categories: (i) from brain mechanisms to behavior and (ii) from behavior to brain mechanisms.

2.4.1. From brain mechanisms to behavior

A usual approach in rodent studies of schizophrenia has been to assess the impact of specific brain manipulations on behavior. These manipulations can be classified into four main groups, namely lesion, pharmacological, genetic and neurodevelopmental studies, and are summarized in Table 2.

Table 2. From brain mechanisms to behavior experimental strategies.

	Manipulate specific	***Focused on***	***Examples***
Lesion studies	Brain regions	The role of neurodegeneration in schizophrenia	Lesion of the mPFC [133,134], HPC [78,133,135], NAc [136]
Pharmacological studies	Neurotransmission systems	The role of dysfunctional neurotransmission in schizophrenia	Propsychotic [58,123,137] and antipsychotic [138,139] drugs
Genetic studies	Neural targets	The genetic susceptibility to develop schizophrenia	Dopamine [140] and glutamate [106,141] receptors; DISC-1, COMT, Neuregulin, etc. [19]
Neurodevelopmental studies	Neurodevelopmental periods	The environmental risk to develop schizophrenia	Neonatal viral exposure [142–144] or post-weaning social isolation [125,126,145]

All these experimental strategies have in common the use of invasive procedures to cause or reverse schizophrenia-like symptoms. Thus, these approaches go from brain differences to behavioral features. Moreover, these animal models allow the use of invasive procedures, such as immunohistochemistry of brain tissue, to study or confirm the impact of these manipulations. These studies provide fundamental knowledge about the etiology of schizophrenia. However, these hypothesis-driven approaches do not allow to assess the contribution of unexpected processes that may be relevant for the disorder.

2.4.2. From behavior to brain mechanisms

In contrast to the hypothesis-driven approaches, other studies have used intact rodents that spontaneously differ in illness-related symptoms to study brain alterations [146–150]. These studies are based on stratifying or selectively breeding animals for spontaneous differences in specific behavioral phenotypes to look at brain mechanisms. Examples of selectively bred animal models of schizophrenia are (i) the Low- and High-PPI rat strains [116,151]; (ii) the APO-SUS/APO-UNSUS, which are High- and Low-susceptible to the dopaminergic agonist apomorphine rat strains, respectively [152]; and (iii) the Spontaneous Hypertensive rat strain (SHR) that shows hyperactivity, attention deficits, and PPI impairments [153]. Of note, this approach is similar to human studies, as they generally evaluate the correlation between behavioral differences (diagnosis, symptoms) and neural changes (e.g. using MRI after PPI [39,154]). However, the rodent models allow the use of invasive procedures that are not applicable to humans (e.g. immunohistochemistry after a behavioral test). This approach is less specific than the hypothesis-driven approaches, but it does not present the limitation of assuming a given mechanism.

2.5. Roman and HS rats

Among the animal models of schizophrenia based on an approach that goes from behavior to brain mechanisms, the inbred Roman high-avoidance (RHA) and low-avoidance (RLA) rats, as well as the "*National Institute of Health N/Nih Heterogeneous Stock of rats*" (HS rats), have provided useful insights into schizophrenia-like symptoms.

2.5.1. Roman rats

The RHA and RLA rats were bidirectionally selected and bred for their very good (RHA) vs. extremely poor (RLA) ability to acquire the two-way active avoidance task [155–158]. Importantly, several studies have highlighted that the RHA rats, compared to the RLAs, show several phenotypes that might be relevant for positive, negative, and cognitive symptoms of schizophrenia:

- **Positive symptoms:** the RHA show higher locomotor responses to novelty and higher locomotor sensibilization to psychostimulants [138,159–162].
- **Negative symptoms:** the RHA show decreased nesting/nursing behavior, increased aggression latency in resident-intruder test, and decreased social interaction ([107,163] and unpublished results from our laboratory).
- **Cognitive symptoms:** the RHA show impairments in PPI, working memory, latent inhibition, and spatial learning and memory [127,164–166].

On the other hand, apart from these behavioral differences that show the face validity of the model, neurobiological studies have also provided construct and predictive validity for the schizophrenia-like RHA model. In this sense, among many other phenotypes that differentiate them from RLAs and other rat strains, the RHA rats show: i) increased mesolimbic and mesocortical dopamine responses to dopaminergic agonists; ii) altered behavioral responses (e.g. PPI, locomotor activity) to dopaminergic agonists, antagonists, and atypical antipsychotic and pro-psychotic (5-HT- or NMDA-interacting)drugs; iii) decreased HPC function and neuronal density; iv) increased expression of 5HT2A in the PFC; v) decreased expression of mGlu2A receptors in the PFC, HPC, and striatum; vi) enlarged lateral ventricles; and vii) reduced volume and function of the mPFC and the HPC ([138,159,174,175,160,167–173] and unpublished results from our laboratory). Thus, as the RHA show behavioral (face validity),

neuroanatomical and neurofunctional (construct validity), and drug-response (predictive validity) features that resemble schizophrenia, it suggests that these rats may be a valid model for this disorder.

2.5.2. HS rats

The HS rats were developed to obtain a stock of laboratory rats as genetically heterogeneous as possible [176,177]. To this aim, a crossing of eight parental inbred strains was carried out for more than 80 generations (see Fig. 5).

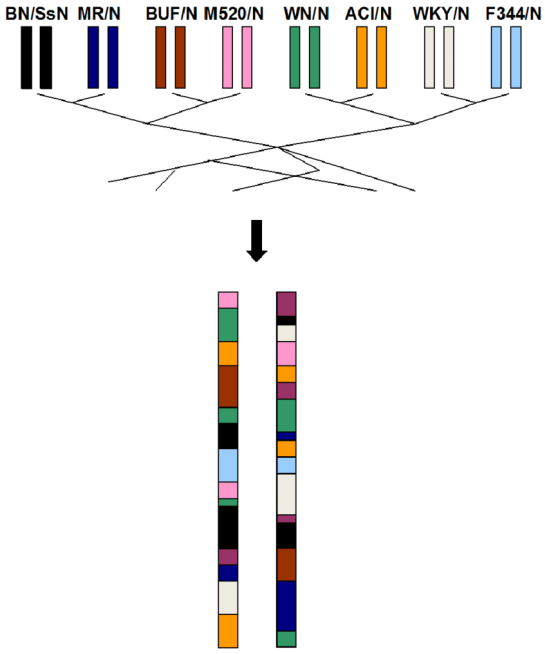

Figure 6. Diagram of the rotational breeding program followed to develop the HS rat stock from eight different inbred parental lines: Brown Norway (BN/SsN), Maudsley Reactive (MR/N), Buffalo (BUF/N), M520/N, Wistar-Nettleship (WN/N), Agouti (ACI/N), Wistar-Kyoto (WKY/N) and Fisher 344 (F344/N) (from [178]).

This rotational breeding program generated rats with a higher genetic recombination pattern and phenotypic variability than those shown by the most commonly used laboratory rat strains [176]. Importantly, since HS rats appear to show genetic variabilities more similar to human population, they constitute an excellent tool to study the neurobiological and genetic basis of both normal and abnormal (illness-related) complex traits [146,177]. Furthermore, the stratification of HS rats by Low-, Medium, and High-PPI has shown significant associations among impaired PPI and other

schizophrenia-related symptoms, such as deficient working memory [165] or latent inhibition [179]. In this sense, gene expression studies in HS rats have revealed associations between low PPI and differential expression of several pre- post-synaptic markers that resemble schizophrenia [180]. Thus, this suggests that the stratification of HS rats by low PPI may constitute a putative model of some schizophrenia-relevant phenotypes.

Objectives

The general aim of this Doctoral Dissertation was to explore the behaviors and underlying neurobiological mechanisms associated with decreased sensorimotor gating in both intact inbred Roman and outbred HS rats, as well as to use them to test the PPI-enhancing effects of novel treatments for schizophrenia. Moreover, it aimed to provide further face, construct, and predictive validity to our two rat models of schizophrenia (i.e. RHA and HS Low-PPI). The specific aims of this Doctoral Dissertation were to:

- Evaluate behavioral associations among PPI and other schizophrenia-related symptoms, such as psychomotor agitation (increased exploratory activity), compulsive-like, and anxious-like behaviors.
    - Study 1: "*Increased exploratory activity in rats with deficient sensorimotor gating: a study of schizophrenia-relevant symptoms with genetically heterogeneous NIH-HS rats and Roman rat strains*".

- Explore structural and functional brain differences in the CSPT circuit associated with PPI deficits in HS and Roman rats.
    - Study 2: "*Schizophrenia-like reduced sensorimotor gating in intact inbred and outbred rats is associated with decreased medial prefrontal cortex activity and volume*".
    - Study 3: *"Decreased activity of parvalbumin interneurons in the medial prefrontal cortex in intact inbred Roman rats with reduced sensorimotor gating"* (Annex 1).

- Assess the PPI-enhancing effects of oxytocin in HS and Roman rats:
    - Study 4: "*Oxytocin attenuates sensorimotor gating impairments in inbred Roman rats in line with strain differences in CD38 gene expression"* (Annex 2).

Studies

<u>Study 1</u>: "Increased exploratory activity in rats with deficient sensorimotor gating: a study of schizophrenia-relevant symptoms with genetically heterogeneous NIH-HS rats and Roman rat strains".

Tapias-Espinosa, C., Río-Álamos, C., Sampedro-Viana, D., Gerbolés, C., Oliveras, I., Sánchez-González, A., Tobeña, A., Fernández-Teruel, A. (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. Behavioural Processes, 151, 96–103.
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Increased exploratory activity in rats with deficient sensorimotor gating: a study of schizophrenia-relevant symptoms with genetically heterogeneous NIH-HS and Roman rat strains

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ABSTRACT

Schizophrenia involves positive, negative and cognitive symptoms, as well as comorbidity with anxiety and obsessive-compulsive disorder. Prepulse inhibition (PPI) of the startle response is a measure of sensorimotor gating that is impaired in schizophrenia and animal models of the disease. Remarkably, impaired PPI has been related to other schizophrenia-like features in rodent models, such as cognitive deficits and hyperactivity. However, it remains to be investigated whether deficient PPI and increased exploratory activity are associated in genetically heterogeneous (outbred) naïve animals. This study was undertaken to evaluate the relationships among PPI and other schizophrenia-related symptoms, such as augmented exploratory activity, anxiety and compulsivity in the genetically heterogeneous (outbred) NIH-HS rat stock (HS) and in the genetically-selected inbred Roman High-Avoidance (RHA) and Low-avoidance (RLA) rats. Animals underwent the following tests: open-field (exploratory activity), elevated zero-maze (anxiety-like behavior), marble burying (compulsive-like behavior), and PPI. Three groups of HS rats were formed according to their PPI scores, i.e. Low-PPI, Medium-PPI and High-PPI. The HS Low-PPI group displayed higher exploratory activity in the open-field than the HS Medium-PPI and HS High-PPI groups. Likewise, compared with their RLA counterparts, RHA rats exhibited lower PPI and more intense exploratory activity in the open-field test. Correlational and factorial analyses of the whole HS sample and the RHA/RLA data globally corroborated the results of the PPI-stratified HS subgroups. These data suggest that such a consistent association between impaired PPI and increased exploratory activity in outbred HS and inbred RHA/RLA rats is a relevant parameter that must be taken into account when modeling clusters of schizophrenia-relevant symptoms.

1. Introduction

Schizophrenia is mainly characterized by the presence of three groups of symptoms: positive (hallucinations, delusions and disorganized behavior), negative (anhedonia, apathy, reduced affect display) and cognitive (impaired sensorimotor gating, attention and executive functions). Moreover, schizophrenia also shows comorbidity with anxiety and obsessive-compulsive symptoms (Braga et al., 2013; Buckley et al., 2009). The complexity and diversity of schizophrenia hinders the full modelling of the entire constellation of symptoms in rodents, although it is desirable to evaluate all putative animal models with the broadest range possible of disease-related phenotypes.

Prepulse inhibition (PPI) of the startle response is a measure of sensorimotor gating, in which the magnitude of the startle is attenuated by the presence of a pre-stimulus of lower intensity (Graham, 1975). Impaired PPI has been proposed as an endophenotype for schizophrenia (Braff et al., 2008), although such an alteration is also present in other psychiatric disorders. Interestingly, the administration of amphetamine, which is known to cause psychotic-like symptoms in humans (Kokkinidis and Anisman, 1981; Snyder, 1973), disrupts PPI in humans (Hutchison and Swift, 1999; Kumari et al., 1998) and rodents (Kinney et al., 1999; Swerdlow et al., 2007). Apart from PPI deficits, amphetamine also induces hyperactivity in rodents, in line with the disorganized behavior of schizophrenia (Alsene et al., 2010; Blanc et al., 1994; Dickinson et al., 1988; Ott and Mandel, 1995; Powell and Miyakawa, 2006; Swerdlow et al., 2002). The association between increased activity and impaired PPI has also been found in other well-established animal models of schizophrenia, such as ventral

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hippocampal neonatal lesion (Tseng et al., 2009), NMDA infusion (Peleg-Raibstein and Feldon, 2006; Wang et al., 2015), NMDA-antagonist administration (Maple et al., 2017), genetic alterations in mice (Kulikov et al., 2016; Miyakawa et al., 2003; Munesue et al., 2010; Powell and Miyakawa, 2006; Takao et al., 2013; Young et al., 2014) and social isolation (Domeney and Feldon, 1998; Lukkes et al., 2009; Oliveras et al., 2016). Thus, as these studies suggest, impaired PPI and excessive motor/exploratory activity may constitute relevant parameters that must be taken into account when modeling clusters of schizophrenia-relevant symptoms. On the other hand, as mentioned above, compulsive and anxious behaviors have also been associated with schizophrenia, although research with rodent models has shown controversial evidence in that respect (Lindemann et al., 2008; McAuley et al., 2009).

This study was undertaken to evaluate the relationships among PPI and other schizophrenia-related symptoms, such as augmented exploratory activity, anxiety, and compulsivity in the genetically heterogeneous NIH-HS outbred rat stock (HS) and in the genetically-selected inbred Roman High-Avoidance (RHA) and Low avoidance (RLA) rats. The RHA and RLA rats were bidirectionally selected and bred for their very good (RHA) vs. extremely poor (RLA) ability to acquire the two-way active avoidance task (Driscoll et al., 1998; Escorihuela et al., 1999; Río-Álamos et al., 2017; Steimer and Driscoll, 2005). Compared with their RLA counterparts, RHA rats show deficient PPI, impaired working memory (Oliveras et al., 2015) and reduced latent inhibition (Esnal et al., 2016; Fernández-Teruel et al., 2006). These divergent behavioral profiles, together with the reported between-strain differences in dopaminergic (Giorgi et al., 2007; Guitart-Masip et al., 2008; Tournier et al., 2013), serotoninergic (Fomsgaard et al., 2017; Klein et al., 2014) and glutamatergic (Wood et al., 2017) systems, suggest that RHA rats may be a valid model for schizophrenia-related features. On the other hand, the HS rats were developed to obtain a stock of rats as genetically heterogeneous as possible (Hansen and Spuhler, 1984). To this aim, a crossing of eight parental inbred strains was carried out, generating a higher genetic recombination pattern and phenotypic variability than those shown by the most commonly used laboratory rat strains (Hansen and Spuhler, 1984). Since HS rats appear to show genetic variabilities more similar to human population, they constitute an excellent tool to study the neurobiological and genetic basis of both normal and abnormal (illness-related) complex traits (Baud et al., 2013; Díaz-Morán et al., 2013). Furthermore, similar to findings with the Roman rats, studies with the HS rats have shown tight associations among impaired PPI and other schizophrenia-related symptoms, such as deficient working memory (Oliveras et al., 2015) or reduced latent inhibition (Sánchez-González et al., 2016). These behavioral data suggest that HS rats stratified by low PPI may constitute a putative model of some schizophrenia-relevant features. It remains to be established whether exploratory activity in response to novelty, which is another schizophrenia-associated feature (Powell and Miyakawa, 2006), is also associated with PPI impairment in HS rats.

In this study, HS rats underwent the following tests: (i) open-field (to assess exploratory activity), (ii) elevated zero-maze (to evaluate anxiety-like behavior), (iii) marble burying (to examine compulsive-like behavior), and (iv) PPI. The data obtained from HS rats were analyzed both in the whole sample and in three groups of rats stratified by Low-, Medium- and High-PPI. Moreover, the same procedures were used with inbred RHA vs. RLA rats, so that we would be able to see the generalizability of the association among PPI and the other phenotypes from the Roman rat strains to the outbred HS stock.

2. Materials and methods

2.1. Subjects

Naïve male HS (n = 92), and inbred RHA (n = 12) and RLA (n = 12) rats, from the permanent colonies maintained at our laboratory (Medical Psychology Unit, Dept. Psychiatry and Forensic Medicine, School of Medicine, Autonomous University of Barcelona) since 1996 (RHA, RLA) and 2004 (HS), were used in this study. They were aged 3–4 months, having a weight range of 250-350 g. They were housed in pairs of the same strain in macrolon cages (50 × 25 x 14 cm) and maintained with food and water ad libitum (standard animal chow). These animals were bred and reared in our laboratory at Autonomous University of Barcelona. They were maintained under a 12:12 h light-dark cycle (lights on at 08:00 a.m.), with controlled temperature (22 ± 2 °C) and humidity (50–70%).

The HS rat stock was derived from eight inbred rat strains by Hansen and Spuhler (Hansen and Spuhler, 1984). These eight parental strains were the MR/N, WN/N and WKY/N (whose ancestors trace back to the original Wistar stock), the M520/N and F344/N (established in the 1920s with an unknown origin), the M520/N and the ACI/N (hybrids between the August and Copenhagen stocks), the BN/SsN (derived from a color mutant from a stock of wild rats kept at the Wistar Institute), and the BUF/N strain. To establish our colony, we received 40 pairs of NIH-HS rats from Dr. Eva Redei (Center for Comparative Medicine, Northwestern University, Chicago, USA) in 2004.

2.2. Experimental procedures

All behavioral testing was carried out during the light cycle between 09:00-14:00 h. Apart from the PPI test (see below), all the other test measures were taken by an expert observer, who was blind to group condition. After testing each rat, the corresponding apparatus was thoroughly wiped clean with 70% ethanol solution. In the marble burying test, new bedding was used for each animal and marbles were cleaned with a 70% ethanol solution between animals.

Experiments were performed in accordance with the Spanish legislation on “Protection of Animals Used for Experimental and Other Scientific Purposes” and the European Communities Council Directive (2010/63/EU) on this subject. Every effort was made to minimize any suffering of the animals used in this study.

Fig. 1 shows the experimental timeline.

2.2.1. Open-field

The apparatus was a circular arena (diameter, 83 cm) walled by white walls (height, 34 cm) and divided into 19 equal sectors by lines drawn on the floor. The test was carried out in a black-painted testing room, dimly illuminated with white fluorescent light (65 lx at the level of the apparatus). Each rat was individually placed in the periphery of the open-field, and behavior was videotaped and measured outside the

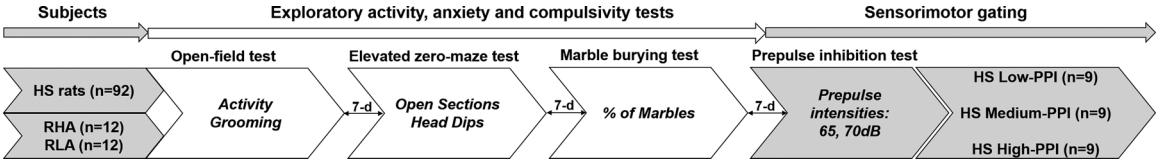

Fig. 1. Experimental timeline. HS, RHA and RLA rats underwent three consecutive tests (open-field, elevated zero-maze and marble burying test) before being tested for sensorimotor gating in the prepulse inhibition test (PPI). Each test was separated by a 7-day interval. The main variables of each test are indicated. As it is shown in the last arrows, HS rats were divided into Low-PPI, Medium-PPI and High-PPI to analyze their performance in the exploratory activity, anxiety and compulsivity tests.

testing room for 5 min. Total number of "exploratory activity" episodes (crossings + rearings), and "self-grooming" time were measured. The lower decile of the "grooming time" variable conformed the Low-Grooming (Low-Groom) group, the higher decile was for the H-Grooming (High-Groom) group, while a Medium-Grooming (Medium-Groom) group was randomly drawn from the intermediate deciles.

2.2.2. Elevated zero-maze

The maze comprised a circular corridor (105 cm diameter; 10 cm width) made of black plywood, elevated to 65 cm above the ground, having two open sections and two enclosed ones (walls 40 cm height). It was situated in a black-painted testing room, dimly illuminated with red fluorescent light (50 lx at the level of the apparatus). Each rat was placed in an enclosed section of the zero-maze facing the wall and behavior was videotaped and measured outside the testing room for 5 min. Measures taken were "time spent in open sections" and "number of head-dips" through the edge of the maze, as anxiety-related variables, as both parameters have previously been reported to be highly and positively correlated (r = 0.76; e.g. see (Martínez-Membrives et al., 2015)). Moreover, both measures have also been shown to be sensitive to anxiolytic and anxiogenic pharmacological and non-pharmacological treatments, in a manner that is independent of their effects on locomotor activity (Braun et al., 2011; Oliveras et al., 2016; Río-Alamos et al., 2015; Shepherd et al., 1994).

2.2.3. Marble burying test

Four white polyethylene box cages measuring 40 × 40 x 40, containing 5 cm depth bedding were used. Above the bedding were placed 16 translucent, light-green glass marbles, 20 mm in diameter arranged in 4 rows of 4 marbles each. The boxes were situated in a black-painted testing room, dimly illuminated with white fluorescent light (65 lx at the level of the apparatus). Each rat was placed in the center of the cage test for a 15-min period of observation. The "% of buried marbles" (i.e. marbles covered at least two-thirds by bedding) were counted.

2.2.4. Prepulse inhibition test

Four sound attenuated boxes (SR-Lab Startle Response System, San Diego Instruments, USA) were used. Each box consists of a Plexiglas cylinder situated on the top of a platform with a sensor that detects the strength made by the rat in each trial. Two speakers situated 15 cm from each side of the cylinder deliver the acoustic stimuli and a white noise generator provides the background noise. Each box was constantly lit by a 10 W lamp. The data were transduced by an accelerometer into a voltage which is amplified, digitized and saved into a computer for analysis. The session started with a 5 min habituation period in the startle chambers. Then, 10 "pulse-alone" trials (105 dB, 40 ms) were delivered in order to obtain a stable baseline of startle. After this, each one of the six different types of trials are randomly administered 10 times (60 trials in total):

1 Pulse-alone trials (105 dB 40 ms, "startle response", which was the variable used to calculate the percentage of prepulse inhibition (% PPI); see the formula below).
2 Prepulses of 65/70/75/80 dB (20 ms) followed by the startle stimulus (105 dB, 40 ms) with an inter-stimulus interval of 100 ms.
3 No stimulus trials (background noise at 55 dB).

The interval between trials was 10–20 s with a mean of 15 s. The startle magnitude was recorded during 200 ms after the onset of the pulse. The %PPI for each prepulse intensity was calculated by applying the following formula:

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

Subgroups of PPI were stratified according to their mean value between the two lower intensities of the PPI session (i.e. mean between the 65 dB and 70 dB). Lower pre-pulse intensities, which are closer to the lowest threshold, are known to elicit lower levels of PPI (Swerdlow et al., 2001). Therefore, these pre-pulse intensities may be more sensitive to detect differences in information-filtering. The lower PPI decile conformed the Low-PPI group, the higher decile was for the High-PPI group, while a Medium-PPI group was randomly drawn from the intermediate deciles.

2.3. Statistics

All the analyses were performed employing the "Statistics Package for Social Sciences" (SPSS, version 17).

For the analyses of the whole HS rat sample (n = 92), Pearson's correlation coefficients were performed among all the variables. Significance level was set at $p < 0.05$. Factorial analysis (direct oblimin; oblique rotation) was also performed on data from the whole HS rat sample.

For the assessment of PPI-stratified HS subgroups, One-Way ANOVAs were performed on all variables from the open-field, elevated zero-maze and marble burying tests, followed by Duncan's multiple range tests. Significance level was set at $p < 0.05$.

For the analyses of RHA vs. RLA rat groups, Student's t-tests were applied to the variables from the open-field, elevated zero-maze and marble burying tests. Significance level was set at $p < 0.05$.

3. Results

3.1. Deficient PPI is associated with increased exploratory activity in the open-field in HS and Roman rats

Pearson's correlations among variables from the 92 HS rats (Table 1) showed a significant negative moderate correlation between PPI and locomotor activity in the open-field test (r = −.32, p < .05). Accordingly, obliquely-rotated factor analysis (direct oblimin), which grouped the main behavioral variables of this study in 3 main factors, revealed a third factor comprising "OF_Activity" and "PPI" with loadings of −.76 and .77, respectively (Table 2). Otherwise, neither the anxiety- nor the compulsivity-like variables were associated with PPI in the correlational and factorial analyses. The first and the second factors will be dealt with in the following sections.

After correlational and factorial studies, comparisons among the three PPI subgroups of HS rats and between both Roman strains were performed to further investigate the relationships among deficient PPI and other behavioral responses. As expected, the division of HS rats in three subgroups according to PPI scores led to a significant *GROUP*

Table 1
Pearson's correlations among the main variables in the HS rats (n = 92).

Variables	1	2	3	4	5	6	7
OF_Activity	1						
OF_Grooming	−.13	1					
ZM_Osections	**.31****	.06	1				
ZM_Hdips	**.26***	.18	**.74****	1			
%MBT	.07	**.46****	−.07	−.03	1		
%PPI	−**.32****	.11	.09	−.12	.04	1	
StartleR	−.08	.08	−.11	−.17	.08	.09	1

"OF_Activity" refers to the number of activity episodes (crossings + rearings) in the open field. "OF_Grooming" corresponds to time spent self-grooming in the open field. "ZM_Osections", time spent in the open sections of the elevated zero maze. "ZM_Hdips", number of head dips in the elevated zero maze. "%MBT", percentage of buried marbles in the marble burying test. "%PPI", mean of the percentage of pre-pulse inhibition at pre-pulse intensities of 65 and 70 dB. "StartleR", baseline startle response.

* p < 0.05.

** p < 0.01 (2-tailed).

Table 2
Factorial analysis of the performance of HS rats (n = 92).

	Factors		
	1	2	3
OF_Activity	–	–	−.76
OF_Grooming	–	.83	–
ZM_Osections	.92	–	–
ZM_Hdips	.93	–	–
%MBT	–	.87	–
%PPI	–	–	.77
StartleR	–	–	–
% of cumulative variance	28.61%	50.35%	66.56%
Factor correlations	1		
	.05	1	
	.16	.08	1

Oblique three-factor solution (direct oblimin) with the main selected behavioral variables (2 from each test except for the marble burying test) and correlations between factors. Only factors with eigenvalues greater than 1 are considered. Loadings $\geq$.40 are shown. Symbols/abbreviations as in Table 1.

effect on PPI [One-Way ANOVA; $F_{(2,24)} = 66.021$, $p = < .001$; and Duncan's test confirmed the expected trend, High-PPI > Medium-PPI > Low-PPI; Fig. 2a]. Furthermore, RHA showed poorer PPI than RLA rats [Student's t-test; $t_{(1,22)} = 3.897$, $p = .001$] (Fig. 2b). With regard to exploratory activity in HS rats, one-way ANOVA revealed a significant *GROUP* effect on the "OF_Activity" variable [$F_{(2,24)} = 7.927$, $p = .002$]. Duncan's test confirmed that the Low-PPI group showed greater exploratory activity in the open-field test (Fig. 3a) than both the Medium-PPI and High-PPI groups, while no differences were observed between the Medium-PPI and the High-PPI group. Similarly, RHA rats displayed a higher number of exploratory activity episodes in the open–field test than the RLAs [$t_{(1,22)} = -5.138$, p = < .001] (Fig. 3c).

3.2. Reduced PPI is related to a higher number of head dips in the elevated zero-maze in HS and Roman rats

Correlational and factorial analyses showed no associations among PPI and anxiety-like behaviors measured in the elevated zero-maze, such as time spent in the open sections or number of head dips (Table 1 and Table 2). On the other hand, the three subgroups of PPI-stratified HS rats did not differ in time spent in open sections of the elevated zero-maze [one-way ANOVA, $F_{(2,24)} = 1.882$, $p = .174$] (Fig. 4a), but there was a significant difference in the number of head dips [one-way ANOVA, $F_{(2,24)} = 3.441$, $p = .049$]. Duncan's test confirmed that the Low-PPI group showed a higher number of head dips in the zero-maze than the High-PPI group, while no differences were observed with the Medium-PPI and between the Medium-PPI and the High-PPI groups (Fig. 4b). Likewise, and partly supporting these results, RHA rats (which are PPI-impaired) exhibited greater number of head dips [$t_{(1,22)} = -2.997$, p = .009] and spent more time in the open sections of the elevated zero-maze than their RLA counterparts [$t_{(1,22)} = -2.428$, p = .027] (Fig. 4c,d).

3.3. Impaired PPI is not linked to compulsive-like behaviors in HS and Roman rats

As shown in Table 1, neither of the behavioral traits supposed to measure compulsive-like behaviors, i.e. "Time Spent Grooming" and "% Marbles", were associated with PPI. Moreover, the PPI-stratified HS subgroups did not show significant differences either in the time spent self-grooming in the open-field [$F_{(2,24)} = 1.552$, $p = .232$] (Fig. 3b) or in the percentage of buried marbles in the marble burying test [$F_{(2,24)} = 2.000$, $p = .157$] (%Marbles, Low-PPI: 3.5 ± 2.4; Medium-PPI: 22.9 ± 10.7; High-PPI: 9.7 ± 5.2). Regarding the Roman rats, RLAs showed longer time spent grooming in the open-field than their RHA counterparts [$t_{(1,22)} = 7.089$, p = .010] (Fig. 3d), while no between-strain differences were observed in the percentage of buried marbles [$t_{(1,22)} = 1.433$, p = .171] (%Marbles, RHA: 11.5 ± 3.7; RLA: 7.3 ± 3.1).

3.4. Analysis of anxious-like and compulsive-like behaviors among HS rats

The main aim of this study was to investigate the association among PPI and some possibly co-selected behavioral features. However, additional analyses of the anxiety and compulsivity-relevant variables were needed to confirm their meaning in this study. As depicted in Table 1, positive high correlations were found between time in open sections and head-dips (r = .74) in the elevated zero-maze test (see Table 1). Accordingly, obliquely-rotated factor analysis (direct oblimin) grouped these two variables in a first factor of "anxiety-related behaviors" with loading of 0.92 and .93, respectively (Table 2). Regarding the compulsive-like behaviors, a significant positive mild correlation between the time spent grooming and the percentage of marbles buried was found (r = .46; Table 1). Additionally, the second component of the factorial analysis included these two variables related to "compulsivity-like responses" with loadings of .83 and .87, respectively (Table 2). The "baseline startle response" variable was not related to any of the three components of the factorial analysis (Table 2). The very low correlations observed among the three components of the factor analysis indicate that these three factors are essentially independent.

Interestingly, confirming the results from the factor analysis (see the second factor in Table 2), one-way ANOVA of Grooming-stratified HS subgroups yielded a significant *GROUP* effect in the percentage of

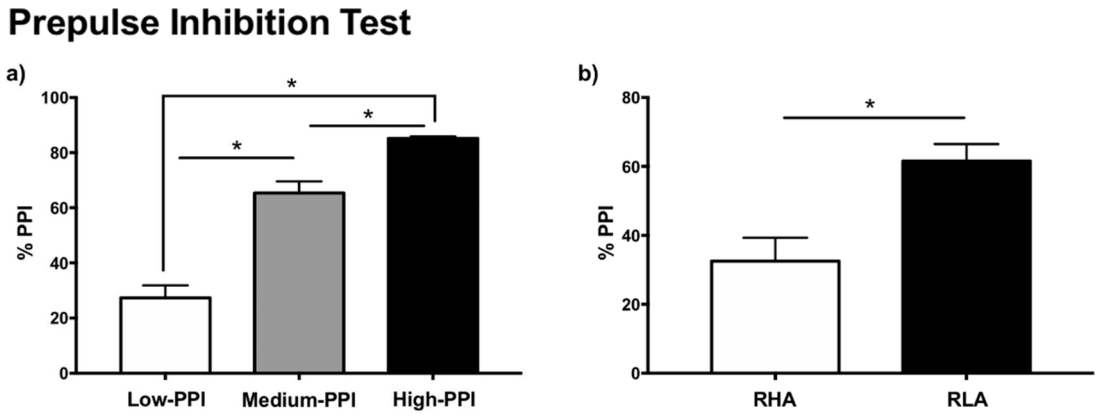

Fig. 2. Prepulse Inhibition Test. Mean ± SEM of the "% of PPI" averaged for the 65 and 70 dB prepulse intensities in **a)** HS rats with Low-PPI (n = 9), Medium-PPI (n = 9) and High-PPI (n = 9) and **b)** RHA (n = 12) and RLA (n = 12) rats. *: $p < 0.05$ between the indicated groups (Duncan's tests for "a" and Student's t-test for "b").

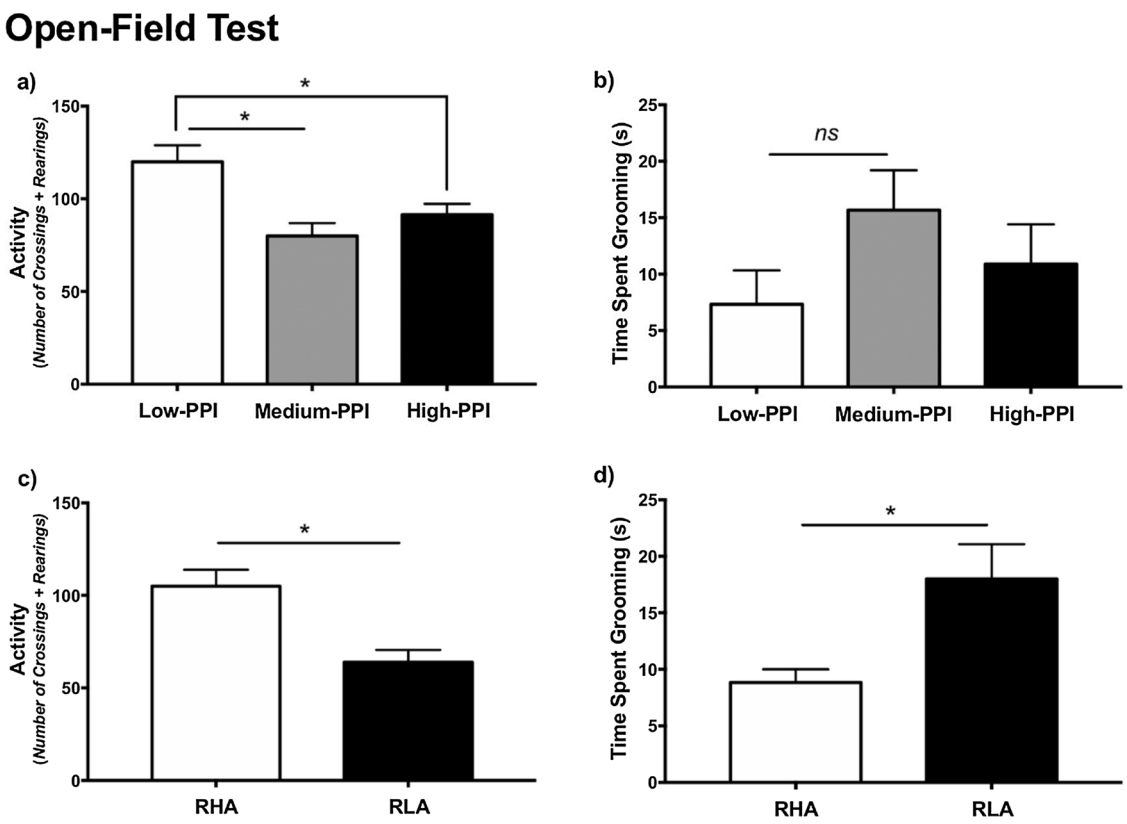

Fig. 3. Open-Field test. Mean ± SEM of **a)** number of exploratory activity episodes (crossings + rearings) and **b)** time spent self-grooming in HS rats with Low-PPI (n = 9), Medium-PPI (n = 9) and High-PPI (n = 9). Mean ± SEM of **c)** number of exploratory activity episodes (crossings + rearings) and **d)** time spent self-grooming in RHA (n = 12) and RLA (n = 12) rats. *: $p < 0.05$ between the indicated groups (Duncan's tests for "a" and Student's t-test for "c" and "d"), *ns*: not significant.

buried marbles [$F_{(2,24)} = 6.896$, $p = .004$]. Duncan's test revealed higher marble-burying behavior in the H-Groom rats than in the Medium-Groom and the Low-Groom groups, while no significant differences were found between the Medium-Groom and the Low-Groom groups (%Marbles, Low-Groom: 2.8 ± 1.8; Medium-Groom: 6.3 ± 2.9; High-Groom: 36.8 ± 11.9).

4. Discussion

The purpose of this study was to evaluate, in naïve outbred (HS) and inbred (Roman) rats, the relationships among PPI and other behavioral traits that are relevant in schizophrenia, such as altered exploratory/locomotor activity, anxiety and compulsive-like behavior. The present data suggest that augmented exploration is associated with deficient PPI in the genetically heterogeneous HS rats and the Roman rat strains.

The HS Low-PPI group displayed higher exploratory behavior in the open-field test than the Medium-PPI and High-PPI groups. The negative relationship between PPI and open-field exploratory activity was also supported by the following findings: (i) a significant negative correlation (-0.32) between both measures in the whole HS rat sample (n = 92); (ii) the fact that both variables grouped (with opposite sign) in the third factor of the factorial analysis; (iii) the fact that, compared with their RLA counterparts, RHA rats exhibited low PPI and increased exploratory behavior in the open-field test. The "low PPI – high exploratory activity" association has also been observed in rodent models of schizophrenia involving different manipulations or treatments. Examples of this are the social isolation syndrome (Domeney and Feldon, 1998; Lukkes et al., 2009; Oliveras et al., 2016), the ventral hippocampal syndrome (Peleg-Raibstein and Feldon, 2006; Tseng et al., 2009; Wang et al., 2015), genetic mouse models (Kulikov et al., 2016; Miyakawa et al., 2003; Munesue et al., 2010; Powell and Miyakawa, 2006; Takao et al., 2013; Young et al., 2014) or amphetamine administration (Alsene et al., 2010; Blanc et al., 1994; Dickinson et al., 1988; Ott and Mandel, 1995; Swerdlow et al., 2002). All of these models are characterized by an impairment of PPI and an increase of exploratory activity. However, to our knowledge, the present is the first study in which the "low PPI – high exploratory activity" association is reported in untreated rats (i.e. at baseline) derived from different sources and strains (i.e. HS rats derived from 8 inbred strains and inbred Roman rats, derived from Wistar rats). In this context, it is worth to mention that studies with HS rats stratified by their PPI levels have revealed positive associations among PPI and spatial working memory (Oliveras et al., 2015) and latent inhibition (Sánchez-González et al., 2016). Thus, these clusters among PPI and other attentional/cognitive and exploratory activity traits, and their consistency across different rat strains (i.e. HS and Roman rats), suggest that PPI may be a suitable predictor of other behavioral phenotypes related to schizophrenia.

No significant associations among PPI and anxiety-like behaviors were found in the correlational and factorial analyses. However, compared with the High-PPI group, the Low-PPI group showed an increased number of head dips in the elevated zero-maze, which may be indicative of lowered anxiety (Braun et al., 2011; Oliveras et al., 2016; Río-Alamos et al., 2015; Shepherd et al., 1994). Interestingly, similar to the Low-PPI group, RHA rats showed a greater number of head dips and spent more time in the open sections of the elevated zero-maze than RLA rats. These results agree with some previous studies reporting that deficient PPI is found in animals with low anxiety (Lindemann et al., 2008; McAuley et al., 2009).

Regarding measures of compulsive-like behavior, i.e. self-grooming (Kalueff et al., 2015) and marble burying (Andersen et al., 2010), they did not show significant associations with PPI. Furthermore, self-grooming was increased in RLA vs. RHA rats but did not differ between

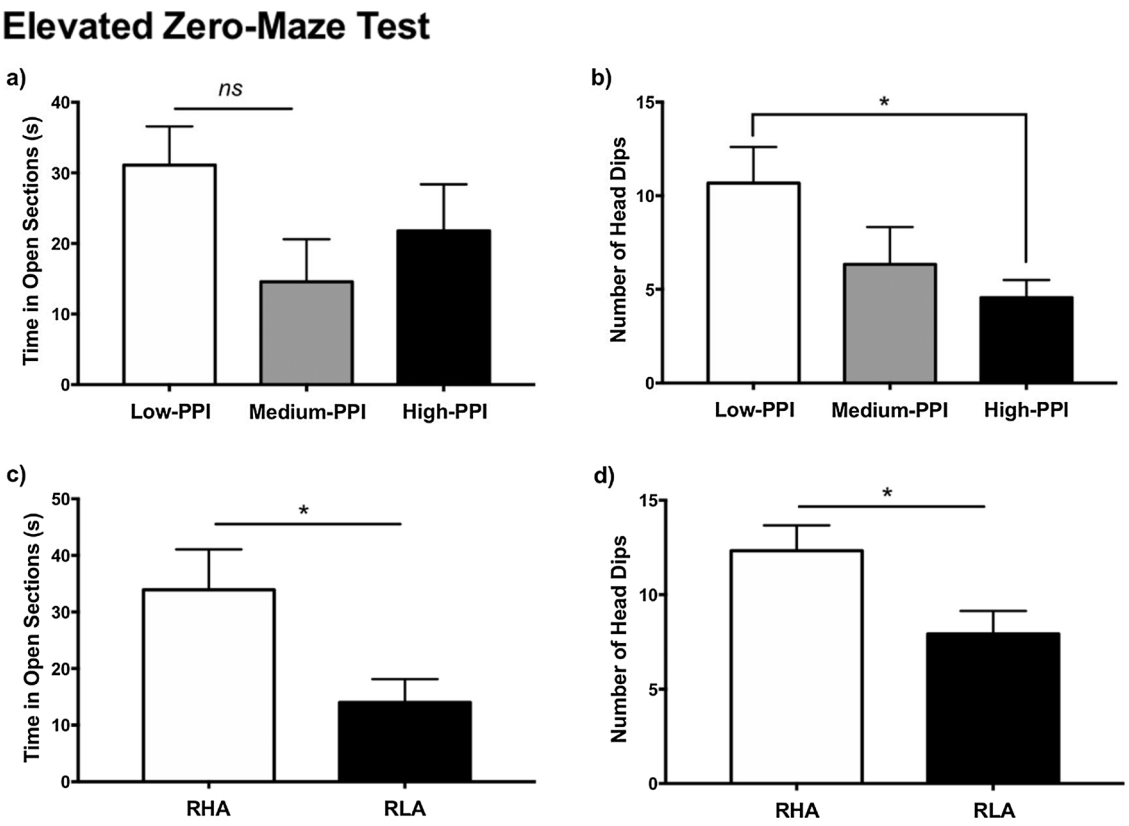

Fig. 4. Elevated Zero-Maze test. Mean ± SEM of **a)** time spent in the open sections, **b)** number of head dips in HS rats with Low-PPI (n = 9), Medium-PPI (n = 9) and High-PPI (n = 9). Mean ± SEM of **c)** time spent in the open sections, **d)** number of head dips in RHA (n = 12) and RLA (n = 12) rats. *: $p < 0.05$ between the indicated groups (Duncan's tests for "b" and "c", and Student's t-test for "d" and "e"), *ns*: not significant.

the PPI-stratified HS subgroups. Thus, contrary to the finding that schizophrenic patients –characterized by PPI deficits– often present obsessive-compulsive symptoms, in our HS rat sample compulsive-like behaviors were not associated with PPI levels (see Table 2). Remarkably, factor analysis of HS data revealed a strong positive relationship between self-grooming and marble burying, and this was confirmed by the comparison among grooming-stratified HS subgroups. Hence, these results suggest that both compulsive-like parameters are part of a common trait in the HS rats. This evidence agrees with previous studies using genetically-altered models of compulsivity in mice (Sungur et al., 2014) and rats (Bahi, 2016), which have also reported positive associations between both compulsive-like parameters.

As mentioned above, the evidence supporting the "low PPI – high exploratory activity" association is mostly derived from animals submitted to a variety of pharmacological and genetic manipulations, whereas there is a relative paucity of data derived from genetically heterogeneous (i.e. outbred) naïve animals, which have better translational value in view of the high genetic heterogeneity of the human population. Therefore, the present experiments performed in HS rats provide novel information that may be used to detect, in animal models, phenotypic traits (or clusters of them) that are reminiscent of schizophrenia-relevant symptoms.

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<u>Study 2</u>: "Schizophrenia-like reduced sensorimotor gating in intact inbred and outbred rats is associated with decreased medial prefrontal cortex activity and volume".

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ARTICLE

Schizophrenia-like reduced sensorimotor gating in intact inbred and outbred rats is associated with decreased medial prefrontal cortex activity and volume

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Prepulse inhibition (PPI) of startle response is a measure of sensorimotor gating that is impaired in schizophrenia and in many other clinical conditions. Rat models using pharmacological or surgical strategies reveal that PPI is modulated by the cortico-striatal-pallido-thalamic (CSPT) circuit. Here, we explore whether spontaneous variation in PPI in intact inbred and outbred rats is associated with functional and structural differences in the CSPT circuit. Inbred Roman High-(RHA) and Low-avoidance (RLA) and outbred heterogeneous stock (HS) rats were assessed for PPI, brain activity, and brain volume. Brain activity was assessed by c-Fos expression and brain volume by magnetic resonance imaging. Relevant structures of the CSPT circuit were evaluated, such as the medial prefrontal cortex (mPFC), cingulate cortex, hippocampus (HPC), amygdala, nucleus accumbens (NAc), and dorsal striatum. RHA showed lower PPI than RLA rats, while HS rats were stratified by their PPI levels in three groups. Reduced PPI was accompanied by decreased mPFC activity in Roman and HS rats and increased NAc shell activity in HS rats. Low PPI was also associated with decreased mPFC and HPC volumes in Roman and HS rats. This study reports a consistent relationship between decreased function and volume of the mPFC and spontaneous low-PPI levels in inbred and outbred intact rats. Moreover, our findings suggest that, apart from a hypoactive and smaller mPFC, a hyperactive NAc and smaller HPC may underlie reduced PPI levels. Our results support the notion that sensorimotor gating is modulated by forebrain structures and highlight the importance of the mPFC in its regulation.

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INTRODUCTION

Prepulse inhibition (PPI) of the startle response is a measure of sensorimotor gating, in which the magnitude of a startle stimulus is attenuated by the presence of a pre-stimulus of lower intensity [1]. PPI impairments are present in several neuropsychiatric disorders [2], including schizophrenia [3, 4]. In this sense, studying the neural mechanisms involved in PPI is relevant for progress in our knowledge of the neurobiological basis of schizophrenia-related features [5].

Rodent studies reveal that PPI is modulated by the cortico-striatal-pallido-thalamic (CSPT) circuit involving the prefrontal cortex, thalamus, hippocampus (HPC), amygdala, nucleus accumbens (NAc, via ventral pallidum), and dorsal striatum (via ventral pallidum) efferents to the pedunculopontine nucleus [6–8]. In particular, rat models involving brain alterations have mostly focused on the medial prefrontal cortex (mPFC), HPC and NAc. As shown in these studies, PPI is reduced by treatments that either decrease or increase activity in the mPFC [9–12], HPC [5, 13–18], and NAc [19–23]. Moreover, several studies using neurodevelopmental models of schizophrenia report PPI deficits paralleled by mPFC and HPC abnormalities, such as models of prenatal administration of phencyclidine [24], prenatal LPS [25], or isolation rearing [26, 27]. Additionally, electrical stimulation of the NAc alleviates PPI deficits in poly I:C offspring [28, 29], while it disrupts PPI in control-saline offspring. This evidence, derived from rat models involving neural manipulations, is generally consistent with the idea that alterations in the "mPFC-HPC-NAc" circuit [13] play a role in PPI deficits and other cognitive impairments found in schizophrenic patients [8, 30–33].

Here, we explore whether spontaneous variation in PPI in intact inbred and outbred rats is associated with functional and structural differences in the CSPT circuit. Thus, we evaluated neuronal activity (through c-Fos expression) and volume (through structural magnetic resonance imaging—MRI) of relevant regions of the CSPT circuit, such as the mPFC, cingulate cortex (Cg), HPC, amygdala, NAc, and dorsal striatum, in intact rats displaying spontaneous differences in PPI, i.e., the Roman rats and the outbred heterogeneous rat stock.

The inbred Roman high- (RHA) and low-avoidance (RLA) rats were bidirectionally selected for rapid vs. non-acquisition of the two-way active avoidance task, respectively [34, 35]. Interestingly, compared with their RLA counterparts, RHA rats show spontaneous reductions in PPI, working memory [36] and latent inhibition [37]. These divergent behavioral profiles, together with differences in dopaminergic [38, 39], serotoninergic, and glutamatergic systems [40–42], suggest that RHA rats may be a valid

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model of schizophrenia-related features. On the other hand, the outbred rat heterogeneous stock (hereafter, HS rats) was developed from a crossing of 8 inbred strains to obtain a stock of rats as heterogeneous as possible [43]. Their genetic heterogeneity makes them a unique tool to study the neurobiological/genetic basis of normal and abnormal complex traits [44, 45]. Previous studies suggest that HS rats stratified by low-PPI may constitute a putative model of some schizophrenia-relevant traits [36, 46, 47].

As reviewed above, PPI is impaired after several alterations that can either increase or decrease activity in the mPFC, HPC and NAc. Thus, we hypothesized that differences in PPI would be paralleled by differences in activity and/or volume within these regions, although the direction of these differences was difficult to predict.

MATERIALS AND METHODS

Subjects

We used male Roman (RHA = 28; RLA = 28) and HS rats ($n = 100$) from our permanent colony (Dept. Psychiatry and Forensic Medicine, Universitat Autònoma de Barcelona). They were aged 3–4 months, weighing 250–350 g. They were housed in pairs in macrolon cages (50 × 25 × 14 cm) and maintained with food and water ad libitum, maintained under a 12:12 h light–dark cycle (lights on at 08:00 a.m.) and with controlled temperature (22 ± 2 °C) and humidity (50–70%).

Experimental procedures

All behavioral and MRI testing was carried out during the light cycle between 09:00–14:00 h. Experiments were performed in accordance with the Spanish legislation on "Protection of Animals Used for Experimental and Other Scientific Purposes" and the European Communities Council Directive (2010/63/EU) on this subject. Every effort was made to minimize any suffering of the animals used in this study.

See experimental overview in Fig. 1.

Prepulse inhibition (PPI)

PPI was conducted in four sound attenuated boxes (SR-Lab Startle Response System, San Diego Instruments, US), as previously described [47]. Briefly, animals were individually located in an acrylic cylinder, which was situated in a dimly illuminated box and on the top of a platform with a sensor that detects the strength made by the rat in each trial. Noise bursts were presented via a speaker mounted 15 cm above the cylinder. After 5 min of habituation, 10 "pulse-alone" trials (105dB(A), SPL, 40 ms) were delivered in order to obtain a stable baseline of startle (BL1_Startle). Next, each one of the six types of trials were randomly administered ten times (60 trials in total): (i) Pulse-alone trials (105dB(A), SPL, 40 ms; BL2_Startle; used to calculate the percentage of PPI); (ii) prepulses of 65/70/75/80dB(A), SPL (20 ms) followed by the pulse stimulus (105dB(A), SPL, 40 ms) with an inter-stimulus interval of 100 ms; or (iii) no-stimulus trials (background noise of 55dB). The interval between trials was 15 s (range 10–20 s). The %PPI for each prepulse intensity was calculated by applying the following formula: %PPI = [100−(startle amplitude on prepulse trials / startle amplitude on "BL2_Startle" pulse-alone trials × 100)].

HS rats were stratified by their average %PPI at prepulse intensities 65 and 70dB(A). Lower prepulse intensities, which are closer to the lowest threshold, are known to elicit lower levels of PPI [5]. Therefore, these prepulse intensities may be more sensitive

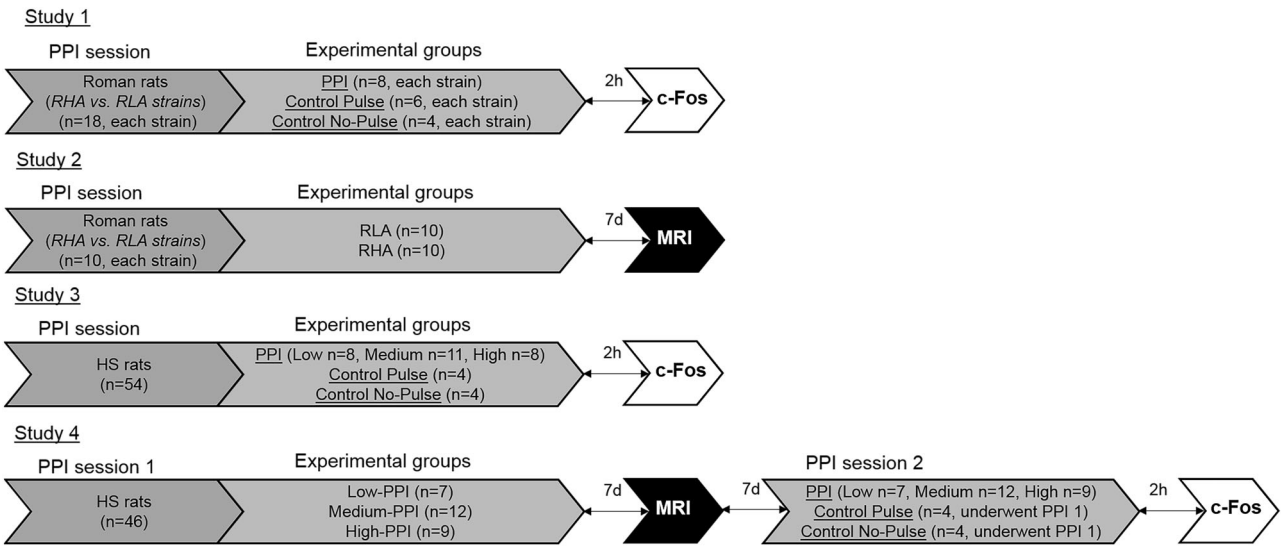

Fig. 1 Experimental overview. **Study 1:** PPI and c-Fos expression in the RHA and RLA rats. RHA ($n = 8$) and RLA ($n = 8$; from six different litters in both cases) underwent a PPI test. RHA ($n = 6$) and RLA ($n = 6$; from six different litters in both cases) underwent a pulse-alone session (Pulse). RHA ($n = 4$) and RLA ($n = 4$; from four different litters in both cases) underwent a background noise (No-Pulse) session. They were euthanized 2 h later to obtain samples for the c-Fos expression study. **Study 2:** PPI and MRI in RHA and RLA rats. RHA ($n = 10$) and RLA ($n = 10$) underwent a PPI test and a single MRI session with a 7-day interval. Rats from both groups were obtained from at least eight different litters. **Study 3:** PPI and c-Fos expression in the outbred heterogeneous rat stock (HS rats). HS rats ($n = 54$, from 40 different litters) underwent a PPI session and they were stratified as Low-PPI ($n = 8$), Medium-PPI ($n = 11$) and High-PPI ($n = 8$) groups by their PPI levels (see "Materials and Methods"). Randomly selected animals underwent control sessions of pulse-alone (Pulse, $n = 4$) or background noise (No-Pulse, $n = 4$). All rats were euthanized 2 h after the respective experimental sessions to obtain samples for the c-Fos expression study. **Study 4:** PPI, MRI and c-Fos expression in HS rats. HS rats ($n = 46$, from 40 different litters) underwent a PPI session ("PPI session 1") and they were stratified as Low-PPI ($n = 7$), Medium-PPI ($n = 12$), High-PPI ($n = 9$) groups by their PPI levels. Two additional control groups, Pulse ($n = 4$) and No-Pulse ($n = 4$), randomly selected from the whole sample ($n = 46$), also underwent a PPI session ("PPI session 1"). After a 7-day interval, the Low-PPI, Medium-PPI and High-PPI groups underwent a single MRI session, while rats from the Pulse and No-Pulse control groups were transported and kept in the MRI testing room for an equivalent period of time as for the PPI-stratified groups. After another 7-day interval, HS rats from the PPI-stratified groups underwent a second PPI session ("PPI session 2"), while the Pulse and No-Pulse control groups were submitted to a "pulse" or a "background noise" session, respectively. All rats were euthanized 2 h after the respective second PPI session ("PPI session 2") to obtain samples for the c-Fos expression study. See variable definitions and further details in "Materials and Methods" section

to detect differences in PPI. For both experiments with HS rats (studies 3 and 4, see Fig. 1), subjects that were one standard deviation below or above the group mean %PPI conformed the Low-PPI and High-PPI groups, while a Medium-PPI group was randomly drawn from the intermediate values.

The "Pulse" and "No-Pulse" control groups for each strain of the Roman and HS rats underwent sessions of pulse-alone trials (i) or non-stimulus trials (iii), respectively, of the same duration as the PPI session.

Assignment of rats to testing box and time of testing was counterbalanced across groups to avoid order effects. Sample size of each experimental group (Fig. 1), including $n = 4$–6 animals in control groups, was determined based on previous c-Fos studies suggesting relatively high homogeneity of c-Fos measures in control groups of similar size (e.g., [48, 49]).

Tissue processing, c-Fos immunohistochemistry, and microscopy

c-Fos immunostaining was performed with modifications as described earlier [50]. Animals were euthanatized 120 min following the PPI, Pulse and No-Pulse sessions. Brains were removed, immersed in isopentane (SIGMA 320404) and stored at 80 °C until sectioned. Coronal 20-µm thick sections were cut in cryostat (Leica CM3050S) and thaw-mounted onto slides (Thermo scientific). Slides were stored at −80°. Prior to staining, the slides were allowed to reach RT, then they were immersed in Formaldehyde 3.7–4% for 12 min at RT. After fixation, the slices were washed several times in TBS (0.05, 0.15 M, pH 7.4) and TBS-T (0.05%). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (SIGMA 216763) in 70% methanol (SIGMA 179337) in 27% TBS, followed by several washes in TBS-T. Afterwards, endogenous protein was blocked incubating the slices with 10% normal horse serum (Sigma, H0146) in "antibody diluent" (TBS-T with 1% (w/v) BSA (SIGMA A9647). Slices were then incubated overnight at 4 °C with the primary antibody (goat anti-c-Fos IgG, Santa Cruz, SC52G; 1:250). On the next day, slices were washed in TBS-T, and were then incubated with the secondary antibody (biotinylated horse anti-goat IgG, Vector laboratories, BA-9500; 1:200) for 30 min. After washes in TBS-T, the samples were incubated with Peroxidase Streptavidin (HRP conjugated, Jackson immunoresearch, 016-030-084; 1:250) for 30 min. Afterwards, slices were washed in TBS-T, TBS and TB. Then, HRP activity was demonstrated with DAB (50 mg DAB (SIGMA, D5637) + 100 ml of TB + 33 microliters of H_2O_2 (SIGMA 216763)) for 10 min. Afterwards, slices were washed in TB, dehydrated, cleared, and cover-slipped. Microphotographs were captured with an Eclipse 80i Nikon microscope attached to a Nikon DXM1200F70 digital camera at ×10 magnifications. The regions of study were the mPFC (bregma: from 3.72 to 2.52 mm); Cg (bregma: from 3.72 to −1.56 mm); dorsal HPC: CA1 of the dorsal HPC (Dorsal CA1), dentate gyrus of the dorsal HPC (Dorsal DG) (bregma: from −2.40 to −3.84 mm); ventral HPC: CA1 of the ventral HPC (Ventral CA1), dentate gyrus of the ventral HPC (Ventral DG) (bregma: from −4.80 to −5.52 mm); NAc: shell and core (bregma: from 2.28 to 1.56 mm); dorsal striatum: caudate-putamen (Striatum CP) and globus pallidus (Striatum GP) (bregma: from −1.80 to −3.12 mm); amygdala: basolateral amygdala (BLA) and central amygdala (CeA) (bregma: from −1.92 to −3.36 mm). The borders of each area were identified with the help of a rat brain atlas [51]. See further details of the regions of interest in Fig. S1e–k. The ImageJ software ("analyze particles" function) was employed to automatically identify and count the number of c-Fos immunostained nuclei in three histological sections of each brain region/mm^2 and averaged for each animal. Particle size and appropriate grey threshold were set for each region and maintained for all subjects.

Structural magnetic resonance imaging (MRI)

Settings, parameters and brain region analyses were conducted as previously described [52]. Briefly, we obtained 35 coronal T2-weighted fast spin-echo images from the olfactory bulb to the cerebellum, which allowed us to manually count pixels of the whole brain, mPFC (bregma: from 3.72 to 2.52 mm), Cg (bregma: from 3.72 to −1.56 mm), NAc (including shell and core sub-regions, bregma: from 2.52 to 0.84 mm), HPC (including dorsal and ventral parts, bregma: from −2.40 to −5.52 mm), dorsal striatum (bregma: from 2.28 to 0.96 mm), and amygdala (bregma: from −1.20 to −5.04 mm). The borders of each area were identified with the help of a rat brain atlas [51]. See further details of the regions of interest in Fig. S1a–d. Counting of pixels was performed using ImageJ software outlined by an experimenter blinded to group status. Volumes of each delimitated area were calculated using the following formula: [(Field of view (3,5 × 3,5 cm^2)/Matrix size (256 × 256)) × Slice thickness (0.5 mm)] × number of pixels included in delimitated area. The percentage of relative volume (% volume) was obtained by using the following formula: [(volume of a delimitated region)/(total brain volume)] × 100. "% volume" variable was used for analyses in the Roman rat strains, because we found between-strain differences in "total brain volume" measured in mm^3. Sample size of each experimental MRI group from studies 2 and 4 (Fig. 1) was determined based on previous MRI studies in rats (e.g., [27, 53]).

Statistics

All the analyses were performed employing the "Statistics Package for Social Sciences" (SPSS). Significance level was set at $p < 0.05$.

A 2 × 3 ('2 strains × 3 conditions') ANOVA followed by post hoc Duncan's test were used to determine differences between the RHA and RLA rats in the different conditions (i.e., No-Pulse, Pulse, PPI) of the c-Fos study 1.

Student's *t*-test was used to test significant differences between RHA and RLA rats from study 2.

One-way ANOVA followed by post hoc Duncan's test were used to determine differences among the HS rats with Low-PPI, Medium-PPI and High-PPI, as well as the No-Pulse and Pulse control groups in studies 3 and 4.

To study associations among variables, multiple linear regression (forward stepwise method) and factorial (direct oblimin; oblique rotation) analyses were applied to c-Fos, MRI and PPI data from studies 1, 3, and 4.

RESULTS

PPI and c-Fos in RHA and RLA rats (study 1)

RHA rats had lower PPI than their RLA counterparts (Table S1). No differences were found in "BL1_Startle" between the RHA-Pulse and RLA-Pulse groups, while the RLA-PPI group showed higher baseline startle response (both in BL1 and BL2) than the RHA-PPI group (Table S1). However, analyzing groups of RHA and RLA rats previously matched for their baseline startle response, we found that the between-strain differences in %PPI were maintained (Fig. S2a, b).

2 × 3 ANOVA revealed a "Strain" effect on c-Fos in the "mPFC" [$F_{(1,30)} = 6.628$; $p = 0.015$] and the BLA [$F_{(1,30)} = 7.573$; $p = 0.010$; Table 1], as both regions were more activated in RLAs than in their RHA counterparts, and a "Condition" effect in the "mPFC" [$F_{(2,30)} = 4.488$; $p = 0.020$], the Cg [$F_{(2,30)} = 3.488$; $p = 0.043$; Table 1] and the CeA [$F_{(2,30)} = 4.048$; $p = 0.028$; Table 1], indicating that these regions had higher activation in the PPI and Pulse conditions than in the No-Pulse condition in both strains. Importantly, there was a "Strain × Condition" effect in the mPFC [$F_{(2,30)} = 8.243$; $p = 0.001$]. RLA-PPI rats showed higher c-Fos expression than the RLA-Pulse, RLA-No-Pulse and all RHA groups (Duncan's post hoc test, $p < 0.05$ (Fig. 2a); representative microphotographs in Fig. 2b). No significant "Strain" (all $Fs_{(1,30)} \leq 4.112$; all $ps \geq 0.052$), "Condition" ($Fs_{(2,30)} \leq 2.358$; all $ps \geq 0.112$) or "Strain × Condition" ($Fs_{(2,30)} \leq 1.737$; $ps \geq 0.193$) effects were found in other brain regions (Table 1).

Table 1. Study 1: c-Fos activation in various brain regions of Roman rats after a single PPI session

	Number of c-Fos labeled neurons/mm^2						
	RHA			RLA			
	No-Pulse *(n = 4)*	Pulse *(n = 6)*	PPI *(n = 8)*	No-Pulse *(n = 4)*	Pulse *(n = 6)*	PPI *(n = 8)*	Effects
Cg	31.4 ± 1.6	68.6 ± 10.5*	61.5 ± 11.2*	33.8 ± 2.8	52.9 ± 9.6*	51.6 ± 9.5*	Condition
Dorsal CA1	3.0 ± 0.7	3.4 ± 1.7	3.0 ± 0.6	3.0 ± 0.8	4.7 ± 0.9	5.9 ± 0.9	
Dorsal DG	9.3 ± 3.0	8.4 ± 1.2	10.1 ± 1.3	8.2 ± 2.8	16.5 ± 2.9	9.0 ± 1.3	
Ventral CA1	7.9 ± 1.3	11.0 ± 1.8	10.6 ± 1.6	9.1 ± 1.4	8.2 ± 1.0	11.3 ± 1.1	
Ventral DG	4.2 ± 1.7	7.9 ± 3.5	11.4 ± 2.9	6.8 ± 1.7	11.2 ± 1.9	7.0 ± 1.0	
NAc shell	23.3 ± 2.4	28.3 ± 5.9	35.0 ± 5.4	21.7 ± 7.6	38.8 ± 7.9	24.5 ± 5.1	
NAc core	18.4 ± 4.3	21.7 ± 4.6	25.7 ± 4.6	15.6 ± 5.8	17.1 ± 4.6	13.5 ± 3.0	
Striatum CP	3.7 ± 0.7	10.1 ± 2.1	7.3 ± 1.7	7.5 ± 2.4	9.3 ± 2.5	11.3 ± 1.1	
Striatum GP	3.3 ± 1.2	5.4 ± 0.8	6.7 ± 1.9	4.9 ± 3.1	6.4 ± 2.0	5.5 ± 1.3	
BLA	11.2 ± 1.4	15.7 ± 2.9	15.0 ± 3.2	20.5 ± 2.0	20.3 ± 2.1	19.2 ± 1.9	Strain
CeA	6.5 ± 1.7	12.3 ± 2.1*	11.1 ± 2.1*	1.7 ± 2.0	18.5 ± 3.9*	15.1 ± 1.6*	Condition

Three groups of RHA and RLA rats were randomly distributed in the background noise control group (No-Pulse), in the pulse-alone control group (Pulse) or the PPI group. Effects of "Condition" (with the Pulse and PPI groups showing higher Cg and CeA activation than the NP group) and "Strain" (with RLA rats showing higher BLA activation than RHAs) are indicated in the table (ANOVA). Values are mean ± SEM

Cg cingulate cortex, *Dorsal CA1* CA1 of the dorsal hippocampus, *Dorsal DG* dentate gyrus of the dorsal hippocampus, *Ventral CA1* CA1 of the ventral hippocampus, *Ventral DG* dentate gyrus of the ventral hippocampus, *NAc shell* nucleus accumbens shell, *NAc core* nucleus accumbens core, *Striatum CP* striatum caudate-putamen, *Striatum GP* striatum globus pallidus, *BLA* basolateral amygdala, *CeA* central amygdala

*$p < 0.05$ vs No-Pulse group of the same strain (Duncan's multiple range test)

PPI and MRI in RHA and RLA rats (study 2)

RHA rats had lower PPI than their RLA counterparts (Table S2). No differences were found in "BL1_Startle" and "BL2_Startle" between the RHA and RLA strains (Table S2).

ANOVA revealed a Strain effect in volume (%) in the mPFC [$t_{(1,18)} = 2.189$; $p = 0.042$], HPC [$t_{(1,18)} = 8.886$; $p = <0.001$] and amygdala [$t_{(1,18)} = 5.370$; $p = <0.001$] (Student's *t*-test; Fig. 3a). RHA strain showed lower relative volume of the mPFC, HPC, and amygdala than the RLAs. No statistical differences were found between groups in "% volume" in other brain regions (all $ts \leq {}_{(1,18)}1.737$; $ps \geq 0.193$; Table S3).

PPI and c-Fos in HS rats (study 3)

The expected differences in PPI were found after stratification in the three rat groups, HS Low-PPI < Medium-PPI < High-PPI, while there were no significant differences in "BL1_Startle" and "BL2_Startle" among the three PPI-stratified groups (Table S4). Albeit non-significant, a numerical trend towards reduced baseline startle in Low-PPI rats was observed. For this reason, we reanalyzed the data from the HS Low-PPI and High-PPI rats after matching them for their baseline startle response amplitude and found that the between-group differences in %PPI were maintained (Fig. S2c–f).

ANOVA revealed a Group effect in c-Fos expression in the "mPFC" [$F_{(4,30)} = 7.971$; $p = <0.001$] and "NAc shell" [$F_{(4,30)} = 2.896$; $p = 0.039$]. The number of c-Fos positive cells in the "mPFC" was higher in the High-PPI rats than in the Medium-PPI and Low-PPI groups (Duncan's post hoc test, $p < 0.05$; Fig. 2c). Moreover, the High-PPI group was the only one that statistically differed from both the No-Pulse and Pulse control groups. Conversely, in the "NAc shell", the number of c-Fos labeled neurons was greater in the Low-PPI group than in the High-PPI group (Duncan's post hoc test, $p < 0.05$; Fig. 2d). In addition, the Low-PPI group was the only group that statistically differed from the No-Pulse control group. No differences were found among groups in other brain regions (all $Fs_{(4,30)} \leq 2.524$; all $ps \geq 0.062$; Table 2a). Multiple regression revealed that c-Fos expression in the mPFC and the NAc shell predicted PPI performance ($R^2 = 0.593$; $p = <0.001$; Table S5). In line with that, factor analysis of PPI, startle and c-Fos variables, pooling HS (study 3) and Roman rats (study 1), revealed a 6-factor solution, which grouped PPI and mPFC activation (both with positive sign) in the second factor (Table S6).

MRI and c-Fos in HS rats (study 4)

We found the expected differences in %PPI variables ("PPI 65_70 pre", "PPI total pre", and "PPI 65 post") among the three PPI-stratified HS groups: Low-PPI < Medium-PPI < High-PPI. No differences were found in "BL1_Startle pre", "BL2_Startle pre", "BL1_Startle post", and "BL2_Startle post" among the three PPI-stratified groups (Table S7).

ANOVA revealed a Group effect in c-Fos expression in the "mPFC" [$F_{(4,31)} = 4.053$; $p = 0.009$], "dorsal CA1" [$F_{(4,31)} = 2.797$; $p = 0.043$] and "CeA" [$F_{(4,31)} = 3.624$; $p = 0.016$]. The Low-PPI group showed lower number of c-Fos positive neurons in the "mPFC" than the Pulse, Medium-PPI and High-PPI groups (Duncan's post hoc test, $p < 0.05$; Fig. 2e). In the "dorsal CA1", the High-PPI group also showed higher c-Fos expression than the Low-PPI and No-Pulse groups (Duncan's post hoc test, $p < 0.05$, Fig. 2f). In the "CeA", the Pulse control group had greater activation than all the PPI groups and the No-Pulse group, while the High-PPI group revealed higher c-Fos expression than the No-Pulse group (Duncan's post hoc test, $p < 0.05$, Table 2b). No differences were found among groups in other brain regions (all $Fs_{(2,25)} \leq 1.117$; all $ps \geq 0.343$; Table 2b).

Regarding volumetric measures, ANOVA revealed a Group effect in the mPFC [$F_{(2,25)} = 4.750$; $p = 0.018$] and the HPC [$F_{(2,25)} = 4.734$; $p = 0.018$]. The HS Low-PPI group showed lower mPFC and HPC volumes than the Medium-PPI and High-PPI groups (Duncan's post hoc test, $p < 0.05$, Fig. 3b; see representative MRI images in Fig. 3c, d). No group-related statistical differences were found in other brain regions (all $Fs_{(4,31)} \leq 2.552$; all $ps \geq 0.059$; Table S8A). Multiple regression revealed that volumes of the HPC and mPFC predicted PPI levels ($R^2 = 0.513$; $p = 0.005$; Table S8b). In relation to this, factor analysis of study 4 (including PPI, startle, MRI and c-Fos) showed a 4-factor solution, in which the first factor grouped PPI (pre and post-MRI), mPFC activation and mPFC volume with positive sign (Table S9).

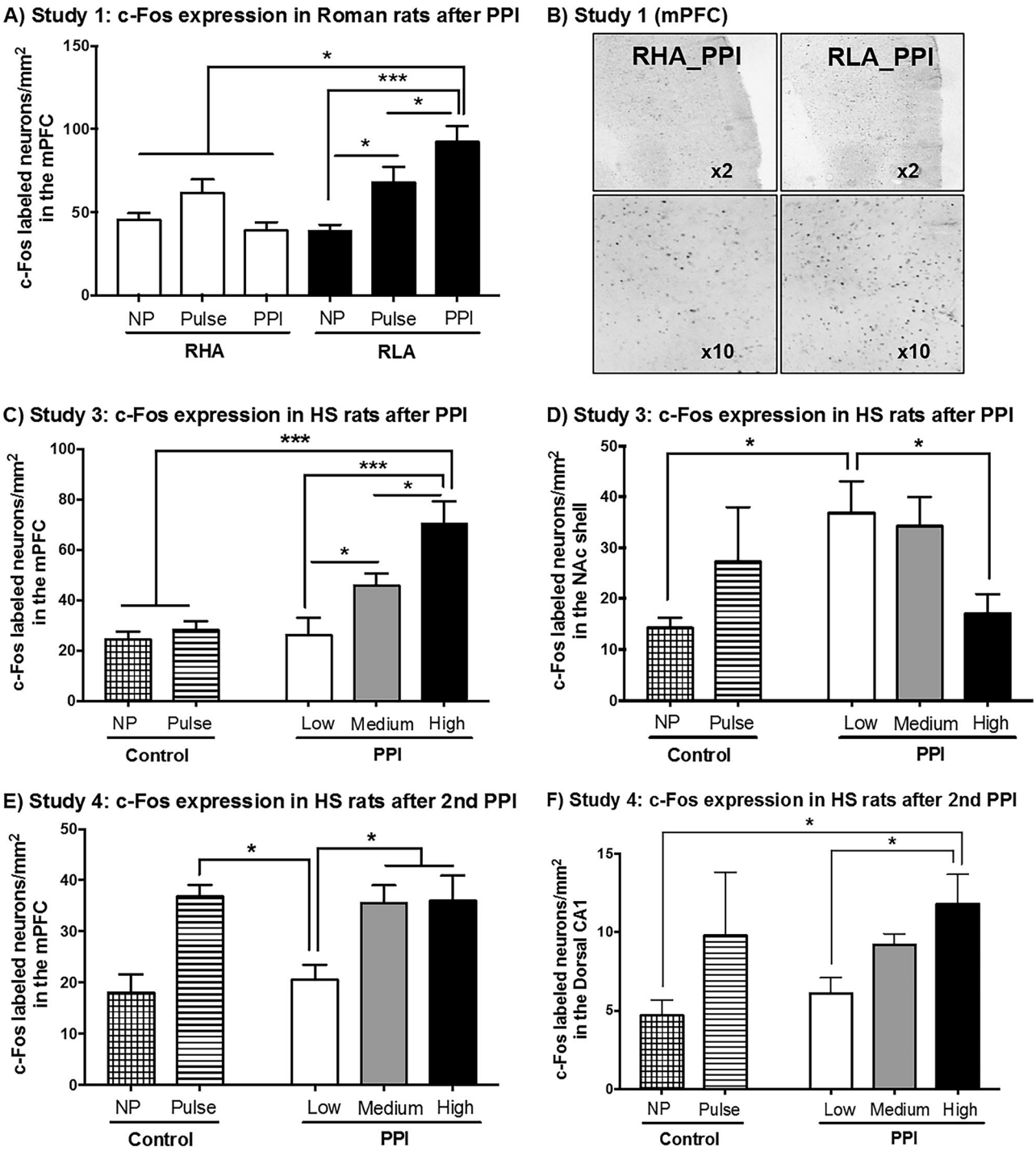

Fig. 2 Reduced PPI is associated with decreased medial prefrontal cortex (mPFC) activation in Roman and HS rats. **a** mPFC activation in RLA rats was significantly higher in the PPI condition than in the NP (No-Pulse) and Pulse conditions and was also higher than all conditions in the RHA rats. Conversely, RHA rats did not show differences in mPFC activation among the three conditions. **b** Representative photomicrographs of c-Fos differences in the mPFC (c-Fos expression) between the Roman rats. RHA rats exposed to a PPI session (RHA_PPI) had lower mPFC activation than their RLA (RLA_PPI) counterparts, as observed both at x2 and x10 magnifications. **c** HS rats stratified for high-PPI scores (High-PPI) showed increased neuronal activity in the mPFC compared to HS rats stratified by medium-PPI scores (Medium-PPI) and HS rats stratified by low-PPI scores (Low-PPI). The Medium-PPI had higher mPFC activation than the Low-PPI. Additionally, the High-PPI group showed higher mPFC activation than the No-pulse control group (NP, control) and the Pulse control group (Pulse, control). **d** Nucleus accumbens shell (NAc shell) activation in the Low-PPI group was greater than in the High-PPI and NP groups. **e** Following a second PPI session (study 4), the HS Low-PPI group showed lower c-Fos expression in the mPFC than Medium-PPI, High-PPI and Pulse groups. **f** The dorsal CA1 of the hippocampus (Dorsal CA1) in the the High-PPI group had higher c-Fos expression than the Low-PPI and NP groups. Values are mean ± SEM. See "n"/group in Fig. 1. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Duncan's multiple range test)

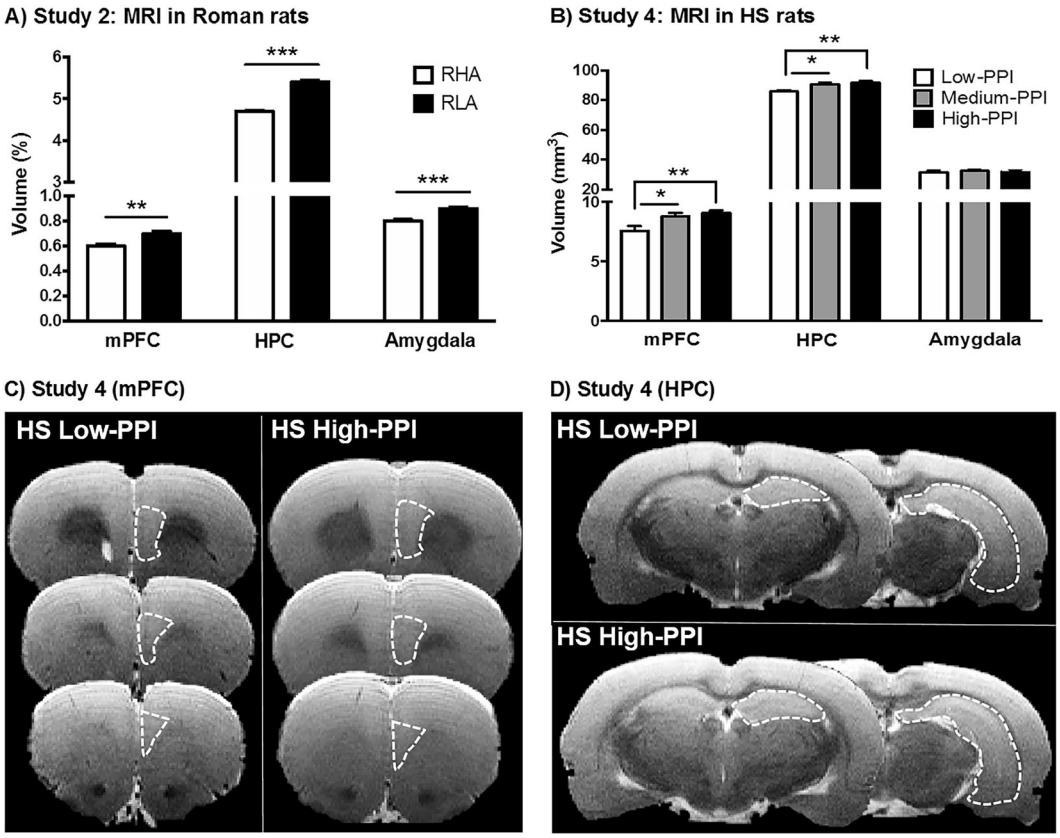

Fig. 3 Lower PPI is associated with decreased MRI volumes in the medial prefrontal cortex (mPFC) and hippocampus (HPC) in Roman and HS rats. **a** RHA rats showed significant reductions in the "% volume" of the mPFC, HPC and amygdala compared to their RLA counterparts. "% volume" variable was used for analyses in the Roman rat strains, because we found between-strain differences in "total brain volume" measured in mm^3 (see Table S3). **b** The HS rats stratified by low PPI (Low-PPI) had lower mPFC and HPC volumes than HS rats stratified by medium (Medium-PPI) or high (High-PPI) PPI. No statistical differences were found in the amygdala. Values are mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Student's *t*-test in "**a**"; Duncan's multiple range test in "**b**"). **c**, **d** Representative coronal MRI images showing volumetric differences in the mPFC (**c**) and HPC (**d**) between the HS Low-PPI and High-PPI rats. The HS Low-PPI showed a thinner mPFC and HPC (white dashed lines) than HS High-PPI rats. The borders of each area were identified with the help of a rat brain atlas [51]

DISCUSSION

This work was aimed to investigate the neurobiological mechanisms associated with reduced sensorimotor gating in the Roman and in the outbred heterogeneous HS rats. Our results showed that both neuronal activity and volume of the mPFC, as well as HPC volume, were highly and consistently associated with spontaneous differences in PPI in intact rats.

PPI is related to mPFC activation and mPFC and HPC volumes in Roman and HS rats

Results of c-Fos expression in the Roman rats (study 1) indicated that the RLA rats showed higher activation of the mPFC than the RHAs during the PPI test. Interestingly, the different conditions markedly affected RLA rats, i.e., much higher c-Fos activation in the PPI condition than the No-Pulse and Pulse conditions, while did not significantly influence c-Fos expression in RHA rats. Moreover, c-Fos results from study 3 revealed that the HS High-PPI group had higher neuronal activity in the mPFC than all the other four groups, thus suggesting specificity of the mPFC-PPI association. Similarly, c-Fos results in HS rats from study 4 showed that the Low-PPI group displayed lower mPFC activation than the High-PPI, Medium-PPI and Pulse groups. The differences in c-Fos expression between both studies using HS rats (Fig. 2c, e) may be due to habituation, since all animals from study 4 were tested twice in the PPI apparatus. Importantly, it is noteworthy that differences in c-Fos activation between the Pulse and No-Pulse control groups and the PPI groups in study 4 may reflect PPI-related mechanism, although it cannot be excluded that these differences reflect divergences in the experimental history between control and PPI groups, including that PPI groups had undergone MRI measurements under anesthesia.

The main conclusion arising from studies 1, 3, and 4 is that there is a strong and consistent positive relationship between activation of the mPFC and PPI response, which is generalizable through different genetic backgrounds (i.e., different rat strains). Remarkably, this is globally supported by regression analysis revealing that activation of the mPFC (together with NAc shell) predicts PPI levels, and by factor analysis (pool of studies 1 and 3, Table S6). In contrast, the Cg, which is a region adjacent to the mPFC and that has been previously related to schizophrenia ([54] but see also [55]), was equally activated by PPI and Pulse conditions in study 1 in both RHA and RLA and there were no differences among HS rats (study 3). This result reinforces the specificity of the association between mPFC activation and PPI. Importantly, our findings agree with previous studies showing that PPI is: (i) positively associated with mPFC activation in rats selectively bred for low or high PPI [56]; (ii) impaired after several pharmacological and neurodevelopmental treatments in the mPFC [9–11, 24, 25]; or (iii) improved in the poly I:C rat model treated with electrical stimulation of the mPFC [28, 29]. However, in the only previous study using untreated and unselected animals, there was no difference in c-Fos expression between PPI-exposed and Pulse-exposed mice in the mPFC [48]. One possible explanation for the

Table 2. c-Fos activation in various brain regions in HS rats from studies 3 and 4

(A) Study 3: c-Fos activation in various brain regions of HS rats after PPI

	Number of c-Fos labeled neurons/mm^2				
	No-Pulse (n = 4)	*Pulse (n = 4)*	*Low-PPI (n = 8)*	*Medium-PPI (n = 11)*	*High-PPI (n = 8)*
Cg	53.2 ± 5.4	58.6 ± 18.2	66.9 ± 9.8	75.2 ± 14.3	84.8 ± 13.0
Dorsal CA1	4.4 ± 0.8	5.1 ± 2.7	4.9 ± 1.0	5.9 ± 1.5	2.3 ± 0.7
Dorsal DG	11.4 ± 2.3	10.0 ± 3.1	11.6 ± 2.6	10.2 ± 1.0	5.0 ± 0.7
Ventral CA1	11.2 ± 0.8	11.6 ± 2.1	12.2 ± 1.6	14.2 ± 1.3	12.6 ± 2.6
Ventral DG	7.9 ± 1.8	19.5 ± 7.8	12.8 ± 2.0	18.5 ± 3.7	17.2 ± 3.1
NAc core	5.8 ± 2.2	10.2 ± 2.8	10.9 ± 1.6	17.1 ± 3.4	9.7 ± 1.7
Striatum CP	9.5 ± 3.5	13.7 ± 4.5	13.7 ± 3.3	12.4 ± 2.4	15.6 ± 2.5
Striatum GP	5.1 ± 1.5	8.1 ± 3.7	8.0 ± 1.3	6.3 ± 1.5	6.3 ± 1.2
BLA	10.5 ± 4.1	8.4 ± 2.6	13.7 ± 2.3	19.7 ± 2.0	15.8 ± 3.0
CeA	7.9 ± 1.8	12.3 ± 4.3	15.4 ± 5.5	10.1 ± 1.4	8.0 ± 1.5

(B) Study 4: c-Fos activation in various brain regions of HS rats after second PPI

	(n = 4)	*(n = 4)*	*(n = 7)*	*(n = 12)*	*(n = 9)*
Cg	15.0 ± 6.9	12.9 ± 0.5	18.3 ± 2.1	23.1 ± 4.2	16.3 ± 2.2
Dorsal DG	25.1 ± 7.1	22.6 ± 2.8	25.4 ± 4.0	24.5 ± 3.0	24.8 ± 3.3
Ventral CA1	9.3 ± 1.1	13.0 ± 0.8	14.9 ± 1.3	13.8 ± 1.0	15.4 ± 2.6
Ventral DG	9.3 ± 1.0	8.6 ± 2.1	16.0 ± 1.7	16.4 ± 2.1	12.3 ± 1.6
NAc shell	13.5 ± 2.3	17.9 ± 9.7	21.3 ± 5.4	15.0 ± 3.0	20.8 ± 3.3
NAc core	9.8 ± 2.7	11.9 ± 4.2	11.2 ± 1.8	14.9 ± 1.6	12.6 ± 1.9
Striatum CP	3.9 ± 2.0	13.7 ± 4.5	11.8 ± 1.3	10.9 ± 1.5	12.3 ± 2.7
Striatum GP	6.3 ± 1.6	8.1 ± 1.7	11.6 ± 1.8	9.0 ± 1.6	10.9 ± 1.6
BLA	17.5 ± 7.2	26.8 ± 3.6	20.7 ± 3.4	20.1 ± 2.3	23.9 ± 3.1
CeA	8.4 ± 3.2*	26.8 ± 3.2	17.2 ± 2.2*	14.7 ± 1.6*	18.0 ± 3.4$^{*\#}$

(A) In study 3, HS rats were stratified by their PPI scores in rats with low-PPI scores (Low-PPI), medium-PPI scores (Medium-PPI) and high-PPI scores (High-PPI). Moreover, control groups for background noise (No-Pulse) and pulse-alone (Pulse) were used. **(B)** In study 4, one week after undergoing an MRI session, HS rats were submitted to another PPI session to obtain samples to conduct c-Fos analyses. See further procedural details in "Materials and Methods" and Fig. 1. Values are mean ± SEM. Abbreviations as in Table 1.
$^*p < 0.05$ vs. Pulse group, $^\#p < 0.05$ vs. No-Pulse group in the CeA (Duncan's multiple range test following significant ANOVA effect)

discrepancy with our findings is that in the mice study, there was just a PPI-exposed group, and animals were not stratified by differential PPI. In fact, the average percentage of PPI in that study was around 50%, which roughly corresponds to Low-PPI rats in the present study 3. Moreover, it is not yet clear that the same neural mechanisms are involved in the regulation of PPI in different species (e.g., mice and rats) [13, 48].

Conversely, we found no group-related differences in c-Fos expression in the different HPC sub-regions in studies 1 and 3. These results may seem surprising in light of the literature relating HPC function and sensorimotor gating [5, 13, 32], although there have also been some controversial findings [48, 57]. However, in study 4 the Dorsal CA1 had higher activation in the HS High-PPI group than in the Low-PPI and No-Pulse groups. In this regard, it is assumed that the Dorsal CA1 is critically involved in several types of memory [58]. As animals were tested twice for PPI in study 4, this may suggest that such an increase of c-Fos activation in the Dorsal CA1 of High-PPI rats is due to a memory process (i.e., habituation of PPI).

In contrast to our findings, previous studies show that PPI is also impaired by manipulations that increase activity in the mPFC [12] or the HPC [14–18]. However, recent evidence suggests that the hippocampal and prefrontal modulation of PPI may be strain-dependent as, for instance, prefrontal disinhibition in Sprague–Dawley rats [12] and ventral hippocampal disinhibition in Wistar rats [16] disrupt PPI, but none of these effects are observed in Lister hooded rats [59, 60].

Regarding the MRI results, study 2 revealed that RHA rats had lower "% volume" of the mPFC and the HPC than the RLAs. These findings agree with previous evidence showing decreased volume of both areas and reduced PPI in RHA compared with RLA rats, as well as a significant positive correlation between HPC volume and PPI [52, 61]. In keeping with that, in the present study 4, the HS Low-PPI group had lower mPFC and HPC volumes than the Medium-PPI and High-PPI groups. Regression analysis supports these findings, as mPFC and HPC volumes positively predict PPI levels (see Table S8b). In this regard, it is known that variations in the volume of particular brain regions may reflect microscopic changes within these regions, including changes in synaptogenesis, dendritic arborization, number of neurites, and neuronal and glial genesis, that can, in turn, influence behavioral responses [62–64]. For instance, a study using a "double hit" rat model of schizophrenia reports volume reductions in the mPFC accompanied by several neurochemical alterations in cortical inhibitory circuits within this region, such as a reduction in parvalbumin expressing interneurons, in mRNA levels of calbindin and ERbB4, and in expression of PSA-NCAM and GAD67 [55]. To the extent that macroscopic variations in the volume of a specific brain region are accompanied by microscopic changes that can influence behavioral processes, our MRI results suggest that the mPFC and HPC may regulate PPI. Moreover, our

experimental strategy (study 4) enabled us to study PPI, MRI and c-Fos in the same subjects. Thus, factor analysis of the most relevant parameters (explaining 64.1% variance) revealed a 4-factor solution, with a first factor grouping PPI variables with mPFC volume and activation (see Table S9), which is globally consistent with the results of group comparisons and regression analyses (studies 3–4). Our findings are in line with previous reports showing both impaired PPI and reduced mPFC volume in several neurodevelopmental rat models [10, 26, 27, 53]. In addition, MRI studies in schizophrenic patients have shown significant correlations between PPI and gray matter volume in the dorsolateral prefrontal, middle frontal and orbital/medial prefrontal cortices [8], whereas HPC volume correlated with PPI in healthy subjects [33]. Moreover, patients with first-episode schizophrenia show significant volumetric reductions in the HPC [65].

The present strategy, which considers the spontaneous variation of PPI in intact rats, may be a parallel of the study of brain function/structure and PPI in humans. Nevertheless, the rat model approach has the obvious advantage of enabling application of invasive procedures and enhanced control of variables. For example, our c-Fos results are in line with a previous functional MRI (fMRI) study in humans that demonstrated that patients with schizophrenia had lower activation of the frontal and parietal cortical regions than healthy subjects during a PPI test [32]. Importantly, however, the higher spatial resolution of c-Fos over fMRI improves detection of very small nuclei and single neurons involved, which is not applicable to humans [66, 67].

NAc and amygdala activation during PPI in the Roman and HS rats

The HS Low-PPI group showed higher NAc shell activity than both the High-PPI group and the No-Pulse control group (study 3), revealing an opposite relationship with PPI compared to the findings from the mPFC (see also regression analysis in Table S5). A similar, albeit non-significant, trend was observed in study 1 between the Roman strains, as the mean c-Fos value of the PPI-deficient RHA rats was 35.0(±5.4) vs. 24.5(±5.1) of their RLA counterparts (see Table 1). These findings are consistent with evidence of reduced neuronal activity in the mPFC and increased activity in the NAc of rats selectively bred for deficient sensorimotor gating [56]. Nevertheless, it is worth to point out that the relationship between NAc function and PPI is still a matter of controversy. In fact, PPI is impaired after (i) ablative lesion of the NAc shell [19]; (ii) functional inhibition of the NAc core, but not shell [23]; (iii) amphetamine infusion into the NAc core, but not the NAc shell [68]; or (iv) high-frequency electrical stimulation of the NAc shell in control-saline offspring, while somewhat paradoxically it alleviates PPI deficits in the poly I:C offspring [29]. On the other hand, it has been reported that direct stimulation of alfa-1 and beta adrenoceptors in the NAc shell, but not core, disrupts PPI, and that blocking alfa-1 receptors within NAc shell reverses amphetamine-induced PPI impairments [69]. In relation to this, the acute administration of methylphenidate, which stimulates dopamine transmission in the NAc, elicits an increase in c-Fos expression in both the NAc shell and core, but more markedly in the former, in parallel to PPI impairments in mice [70]. In addition, the disruptive effects of PPI by dopamine agonists seem to be strain-dependent, as Sprague–Dawley are more sensitive to PPI-disruptive effects of apomorphine and amphetamine than Lister Hooded rats [5, 68]. To sum up, studies investigating NAc function and PPI have provided mixed and even paradoxical results, which indicates the need for further research. Our results, however, would be in line with those findings suggesting that a hyperactive NAc shell is associated with reduced PPI. Nevertheless, in study 4 no trend for a negative association between NAc shell activation and PPI was observed, which is likely because c-Fos expression was measured after repeated PPI exposure. In this context, there is wide evidence that repeated exposure to a novel environment or stress leads to decreased c-Fos response to those stimuli [71]. Therefore, c-Fos results from study 4 may be to some extent influenced by a process of habituation [72].

On the other hand, previous studies suggest that both the amygdala and NAc may interact to modulate PPI [2]. However, we did not find a specific relationship between PPI and activation in the CeA or BLA. Instead, there was (i) an unspecific "pulse" effect in the CeA, as reflected by higher c-Fos activation in the PPI and Pulse conditions than in the No-Pulse condition (studies 1 and 4); and (ii) a "strain" effect in the BLA in study 1, as the BLA was globally more activated in RLAs than in their RHA counterparts. This finding, together with the increased amygdala volume in RLA rats (see Fig. 3a), is consistent with previous results of strain-related differential stress sensitivity and amygdala volume and function [49, 52].

CONCLUSIONS

PPI is a translational measure of sensorimotor gating that is impaired in several human diseases, such as schizophrenia, and is used in many rodent animal models to investigate brain mechanisms underlying these diseases. A common approach in rodent studies has been to assess the impact of specific brain manipulations on PPI, while neural correlates of PPI in humans have preferentially been studied using brain imaging. In this regard, our present work might contribute to bridge the gap between rodent and human studies by investigating structural and functional brain correlates in intact inbred and outbred rats with spontaneous differences in PPI. We report a consistent positive association between PPI and mPFC activity and volume. Moreover, our results suggest that, apart from a hypoactive and smaller mPFC, a hyperactive NAc shell and a smaller HPC may underlie reduced PPI levels. Overall, our findings support the notion that sensorimotor gating is modulated by forebrain structures and highlight the importance of the mPFC in its regulation.

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ADDITIONAL INFORMATION

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Supplementary Figures and Tables

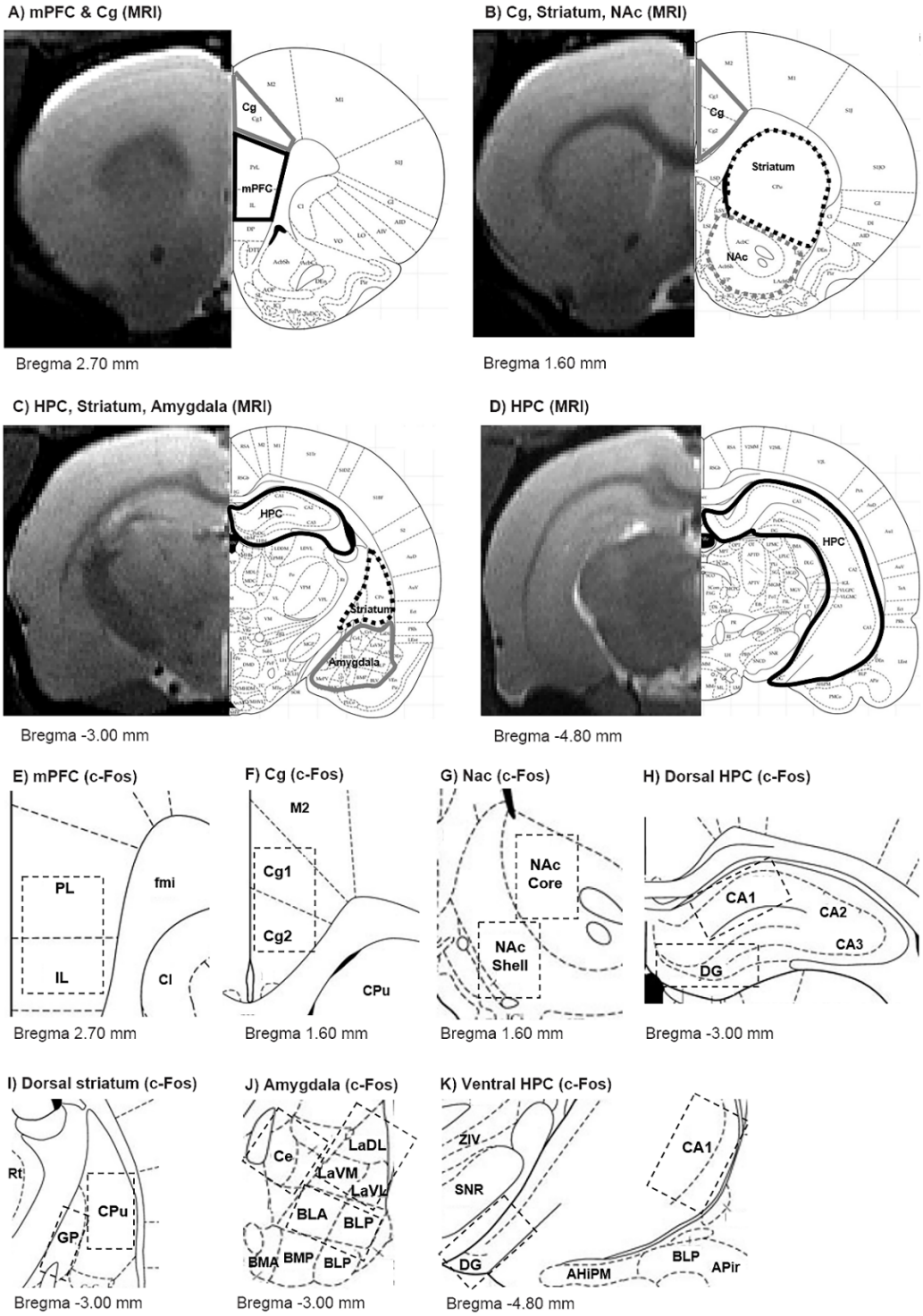

Figure S1. Regions of interest for c-Fos and MRI studies. a-d Schematic diagrams adapted from the Paxinos and Watson atlas [51] showing the regions of interest for the MRI studies 2 and 4, which include the mPFC (a; black lines; bregma: from 3.72 to 2.52mm), Cg (a and b; grey lines; bregma: from 3.72 to -1.56mm), NAc (b; dashed grey lines; shell and core sub-regions, bregma: from 2.52 to 0.84mm), HPC (c and d; black lines; dorsal and ventral parts, bregma: from -2.40 to -5.52mm), dorsal striatum (b and c; black dashed lines; bregma: from 2.28 to 0.96mm) and amygdala (c; gray lines; bregma: from -1.20 to -5.04mm). **e-k** Schematic diagrams adapted from the Paxinos and Watson atlas [51] showing the regions of interest (delineated with dashed black lines) for the c-Fos studies 1, 3 and 4, including mPFC (e; bregma: from 3.72 to 2.52mm); Cg (f; bregma: from 3.72 to -1.56mm); NAc: shell and core (g; bregma: from 2.28 to 1.56mm); dorsal HPC: CA1 of the dorsal HPC (Dorsal CA1), dentate gyrus of the dorsal HPC (Dorsal DG) (h; bregma: from -2.40 to -3.84mm); dorsal striatum: caudate-putamen (Striatum CP) and globus pallidus (Striatum GP) (i; bregma: from -1.80 to -3.12mm); amygdala: basolateral amygdala (BLA) and central amygdala (CeA) (j; bregma: from -1.92 to -3.36mm); and ventral HPC: CA1 of the ventral HPC (Ventral CA1), dentate gyrus of the ventral HPC (Ventral DG) (k; bregma: from -4.80 to -5.52mm). The figure includes a representative schematic diagram for each region.

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Supplementary Figures and Tables

	Startle amplitude and %PPI			
	RHA groups		RLA groups	
	Pulse (n=6)	PPI (n=8)	Pulse (n=6)	PPI (n=8)
BL1_Startle	2389.1 ±259.3	1569.9 ±274.5	2790.8 ±668.5	3022.2 ±560.4*
BL2_Startle	*See legend*	745.2 ±122.9	*See legend*	1995 ±377.7*
PPI65	-	5.3 ±14.1	-	57.2 ±7.5*
PPI70	-	37.0 ±7.1	-	69.4 ±6.0*
PPI 65_70	-	21.1 ±9.6	-	63.3 ±6.1*
PPI_75	-	44.2 ±8.6	-	71.5 ±5.0*
PPI_80	-	72.5 ±4.3	-	77.0 ±3.9
PPI_75_80	-	58.4 ±5.5	-	74.3 ±4.4*
PPI Total	-	39.7 ±7.3	-	68.8 ± 5.3*

Table S1. Startle amplitude and %PPI in the Roman rats from Study 1. Mean±SEM of startle baseline (BL1_Startle) amplitude in Roman high-(RHA) and low-avoidance (RLA) rats of the pulse-alone control (Pulse) and PPI groups, as well as %PPI (PPI groups) averaged for pre-pulses 65dB and 70dB (PPI 65_70) and 65dB, 70dB, 75dB and 80dB (PPI Total). Student’s t-tests revealed significant Strain effects in “PPI65” [$t_{(1,14)}$=3.242; p=0.008], “PPI70” [$t_{(1,14)}$=3.481; p=0.004], “PPI_65_70” [$t_{(1,14)}$=3.624; p=0.003], “PPI75” [$t_{(1,14)}$=2.516; p=0.018], “PPI_75_80” [$t_{(1,14)}$=2.009; p=0.041] and “PPI total” [$t_{(1,14)}$=3.210; p=0.007], indicating that RHA rats showed lower PPI than the RLAs. However, no differences were found in “PPI80” [$t_{(1,14)}$=0.838; p=0.451]. On the other hand, no differences were found in “BL startle” either between the RHA and RLA Pulse control groups [$t_{(1,10)}$=0.560; p=0.594], while the RLA group showed higher startle response than the RHA-PPI group in “BL1_Startle” [$t_{(1,14)}$=2.327; p=0.042] and “BL2_Startle” [$t_{(1,14)}$=3.149; p=0.013]. The value of “BL2_Startle” for the “Pulse” group is not shown, as this group underwent 50 pulse-alone trials after the 10 first pulse trials (BL1_Startle), which is not comparable to the condition of PPI-stratified rats that underwent just 10 pulse-alone trials. Values are mean±SEM. *, significant difference (see above) vs corresponding RHA group.

Supplementary Figures and Tables

	Startle amplitude and %PPI	
	RHA (n=10)	RLA (n=10)
BL1 Startle	1107.3 ±157.5	1908.7 ±384.7
BL2_Startle	872.2 ±157.4	1313 ±377.7
PPI_65	19.9 ±8.2	47.3 ±5.9*
PPI_70	30.2 ±9.4	59.7 ±3.4*
PPI 65_70	25.1 ±8.3	53.5 ±4.1*
PPI_75	42.3 ±7.6	62.9 ±5.1*
PPI_80	62.6 ±5.2	72.9 ±2.3
PPI Total	38.8 ±7.1	60.7 ±3.1*

Table S2. Startle amplitude and %PPI in the Roman rats from Study 2. Mean±SEM of startle baseline (BL1_Startle and BL2_Startle) amplitude in Roman high-(RHA) and low-avoidance (RLA) rats of the pulse-alone control (Pulse) and PPI groups, as well as %PPI (PPI groups) averaged for each pre-pulse. Student’s t-tests showed Strain effects on “PPI65” [$t_{(1,18)}$=2.706; p=0.015], “PPI70” [$t_{(1,18)}$=2.951; p=0.013], “PPI_65_70” [$t_{(1,18)}$=3.079; p=0.009], “PPI75” [$t_{(1,18)}$=2.249; p=0.039] and “PPI total” [$t_{(1,18)}$=2.833; p=0.011], indicating that RHA rats showed lower PPI than the RLAs. However, no differences were found in “PPI80” [$t_{(1,18)}$=1.810; p=0.095]. On the other hand, no strain differences were found in “BL1_Startle” [$t_{(1,18)}$=1.884; p=0.082] and “BL2_Startle” [$t_{(1,18)}$=1.078; p=0.302]. Values are mean±SEM. *, significant difference (see above) vs corresponding RHA group.

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	Volume (mm^3)		Volume (%)	
	RHA (n=10)	RLA (n=10)	RHA (n=10)	RLA (n=10)
mPFC	10.4 ±0.5	12.6 ±0.5**	0.6 ±0.02	0.7 ±0.02*
Cg	10.3 ±0.1	11.2 ±0.2**	0.3 ±0.01	0.3 ±0.01
NAc	8.4 ±0.3	9.2 ±0.3	0.5 ±0.02	0.5 ±0.01
HPC	80.1 ±1.6	100.2 ±1.3***	4.7 ±0.04	5.4 ±0.07***
Striatum	42.8 ±0.7	46.4 ±1.1*	2.5 ±0.02	2.5 ±0.05
Amygdala	26.4 ±0.6	33.7 ±0.9***	0.8 ±0.02	0.9 ±0.02***
Whole brain	1722.1 ±22.1	1861.6 ±36.2**	100	100

Table S3. Study 2: Volume of the various brain regions in the Roman rats. mPFC, medial prefrontal cortex; Cg, cingulate cortex; NAc, nucleus accumbens; HPC, hippocampus; Striatum, dorsal striatum. Values are mean±SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs. RHA group (Student’s t-test).

Supplementary Figures and Tables

	Startle amplitude and % of PPI			
	Pulse (n=4)	Low-PPI (n=8)	Medium-PPI (n=11)	High-PPI (n=8)
BL1_Startle	935.9 ±223.5	1074 ±260.7	1649.2 ±476.1	2235.5 ±594.5
BL2_Startle	*See legend*	526.3 ±212.8	802.6 ±333.7	1445.3 ±346.7
PPI_65	-	31.7 ±4.6	63.0 ±2.5*****	81.1 ±2.1******
PPI_70	-	43.2 ±5.0	70.4 ±2.3*****	88.2 ±1.0******
PPI 65_70	-	37.2 ±2.8	66.7 ±2.0*****	84.6 ±1.1******
PPI_75	-	56.3 ±2.6	74.4 ±1.7*****	85.7 ±2.2******
PPI_80	-	67.2 ±3.8	79.2 ±2.7*****	91.2 ±1.2******
PPI Total	-	49.5 ±2.6	72.8 ±1.8*****	86.5 ±.8******

Table S4. Startle amplitude and %PPI in HS rats from Study 3. Mean±SEM of baseline startle (BL Startle) amplitude in HS rats of the pulse-alone control (Pulse) group and the PPI groups (HS rats stratified by their PPI scores in Low-PPI, Medium-PPI and High-PPI), as well as percentage %PPI averaged for pre-pulses 65dB and 70dB (PPI 65_70) and 65dB, 70dB, 75dB and 80dB (PPI Total). ANOVA analyses showed there was a Group effect among the three PPI-stratified subgroups in “PPI_65” [$F_{(2,24)}$=56.559; p=<0.001], “PPI_70” [$F_{(2,24)}$=47.359; p=<0.001], “PPI_65_70” [$F_{(2,24)}$=113.400; p=<0.001], “PPI_75” [$F_{(2,24)}$=42.280; p=<0.001], “PPI_80” [$F_{(2,24)}$=16.406; p=<0.001] and “PPI_total” [$F_{(2,24)}$=90.222; p=<0.001]. The post-hoc Duncan test confirmed the expected differences: Low-PPI < Medium-PPI < High-PPI. One-way ANOVA (with the 4 groups) showed no significant group differences in “BL1_Startle” [$F_{(3,24)}$=1.212; p=0.315] and in “BL2_Startle” [$F_{(2,24)}$=1.983; p=<0.160] among the PPI-stratified groups. The value of “BL2_Startle” for the “Pulse” group is not shown, as this group underwent 50 pulse-alone trials after the 10 first pulse trials (BL1_Startle), which is not comparable to the condition of PPI-stratified rats that underwent just 10 pulse-alone trials. Values are mean±SEM. ** p < 0.05 vs Medium-PPI and Low-PPI; * p< 0.05 vs Low-PPI (Duncan’s multiple range test).

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Dependent variable	Method	Model	Predictor variables	*r*	*R* (Model)	R^2 (Model)	p
%PPI	Forward stepwise	2	mPFC_cFos NAcShell_cFos	0.639 -0.418	0.770	0.593	<0.001

Table S5. Study 3: Multiple stepwise regressions between PPI session and c-Fos activation in various brain regions in HS rats. Forward stepwise multiple regressions between “%PPI”, as a dependent variable, and all the c-Fos analyzed regions, as independent variables, showed that the medial prefrontal cortex c-Fos activation (mPFC_cFos) and the nucleus accumbens shell c-Fos activation (NAcShell_cFos) predicted PPI performance. r, bivariate Pearson’s correlation.

Supplementary Figures and Tables

	Factors					
	1	2 “Frontocortical Inhibition” factor	3	4	5	6
BL1_Startle	-	-	-	-	0.88	-
PPI	-	0.77	-	-	-	-
mPFC_cFos	-	0.81	-	-	-	-
Cg_cFos	-	-	-	-	-	-
NAc shell_cFos	0.86	-	-	-	-	-
NAc core_cFos	0.62	-	-	-	-	-
BLA_cFos	-	-	-	-	-0.51	-
CeA_cFos	-	-	-	-0.86	-	-
Dorsal CA1_cFos	0.78	-	-	-	-	-
Dorsal DG_cFos	0.74	-	-	-	-	-
Striatum CP_cFos	-	-	-	-	-	0.76
Striatum GP_cFos	-	-	-	-	-	0.92
Ventral CA1_cFos	-	-	-0.87	-	-	-
Ventral DG_cFos	-	-	-0.65	0.50	-	-
% of cumulative variance	24.3%	40.1%	51.4%	61.9%	70.2%	77.5%
Factor correlations	1					
	-0.07	1				
	0.02	-0.16	1			
	-0.107	-0.01	-0.07	1		
	-0.25	-0.04	-0.01	-0.04	1	
	0.168	0.01	-0.12	-0.14	-0.12	1

Table S6. Factorial analysis of PPI, Startle and c-Fos parameters in HS and Roman rats (studies 1 and 3). Six-fold solution of factor analysis (oblique rotation) including prepulse inhibition (PPI; 65_70dB), Startle and c-Fos parameters and pooling Roman (study 1) and HS (study 3) rats. Kolmogorov-Smirnov normality analyses confirmed that all included variables fit with a normal distribution. Importantly, the 6-factor solution showed a 2nd factor (tentatively named “frontocortical inhibition”) that grouped PPI and medial prefrontal cortex (mPFC) c-Fos activation with the same sign (loadings of 0.77 and 0.81, respectively), which goes along with group comparisons of both parameters between the Roman strains and among the 3 HS sub-groups stratified by PPI (see main text). It collectively indicates a strong positive association between mPFC activation and PPI. Factors 1,3,5,6 showed associations among neural regions, such as positive associations (i.e. high positive loadings) among nucleus accumbens shell (NAc shell) and NAc core and dorsal hippocampus regions (i.e. dorsal CA1 and dorsal DG) in factor 1, between both ventral HPC regions (ventral CA1 and ventral DG) in factor 3, between amygdala (CeA) and ventral hippocampus (ventral DG) and between both striatal regions (i.e. striatum caudate-putamen (CP) and globus pallidus (GP)) in factor 6. Factor 5 showed associations between basolateral amygdala (BLA) and Startle response. The 6 factors had very low inter-correlations, so that they can be considered as practically independent from each other. Only loadings > 0.50 are shown.

Supplementary Figures and Tables

	Startle amplitude and % of PPI				
	No-Pulse (n=4)	Pulse (n=4)	Low-PPI (n=7)	Medium-PPI (n=12)	High-PPI (n=9)
BL1_Startle *pre*	527.2 ±110.7	971.5 ±454.7	1290.2 ±459.2	1474.9 ±558.3	1415.9 ±387.1
BL2_Startle *pre*	461.0 ±168.4	494.0 ±117.5	352.5 ±99.4	808.5 ±287.2	1158.3 ±417.3
PPI_65 *pre*	67.2 ±6.2	59.0 ±7.3	26.9 ±10.0	66.3 ±2.9*	80.7 ±1.0**
PPI_70 *pre*	73.1 ±4.4	72.9 ±4.0	52.5 ±5.0	76.1 ±2.2*	83.9 ±0.9*
PPI 65_70 *pre*	70.1 ±5.2	66.0 ±5.6	39.7 ±3.4	71.2 ±2.2*	82.3 ±0.4**
PPI_75 pre	76.8 ±5.3	76.1 ±1.8	66.3 ±3.4	79.7 ±2.0*	86.1 ±1.6*
PPI_80 pre	79.3 ±5.1	77.9 ±5.2	75.4 ±2.6	86.2 ±1.3*	89.0 ±1.1*
PPI Total *pre*	74.1 ±5.0	71.5 ±4.0	54.8 ±2.7	77.1 ±1.6*	84.9 ±.6**
BL1_Startle *post*	-	1444.1 ±890.7	1071.2 ±258.5	1602.1 ±343.6	1194.9 ±315.4
BL2_Startle post	-	*See legend*	525.3 ±170.8	971.0 ±267.0	854.9 ±213.6
PPI_65 *post*	-	-	38.5 ±9.9	62.4 ±2.8*	65.4 ±4.1*
PPI_70 *post*	-	-	63.9 ±9.9	72.6 ±4.8	69.0 ±5.3
PPI_65_70 *post*	-	-	50.6 ±9.2	67.5 ±3.6	67.2 ±4.4
PPI_75 *post*	-	-	74.0 ±5.2	77.2 ±2.9	78.5 ±3.5
PPI_80 *post*	-	-	83.5 ±3.7	80.1 ±4.5	82.4 ±3.2
PPI Total *post*	-	-	66.6 ±6.2	73.1 ±3.3	73.8 ±3.6

Table S7. Startle amplitude and %PPI in HS rats from Study 4. HS rats underwent two PPI sessions, one before (*“pre”* in the Table) and another after (*”post”* in the Table) a single magnetic resonance imaging (MRI) session. See further procedural details in “Methods” and Figure 1. The table shows mean±SEM of baseline startle (BL Startle, *pre* and *post* MRI measurement) amplitude in RHA and RLA rats, as well as %PPI averaged for prepulses 65dB and 70dB (PPI 65_70 *pre* and *post*) and prepulses 65dB, 70dB, 75dB and 80dB (PPI Total, *pre* and *post*). ANOVA analyses showed a Group effect among the three PPI-stratified groups in “PPI_65 pre” [$F_{(2,25)}$=27.824; p=<0.001], “PPI_70 pre” [$F_{(2,25)}$=29.200; p=<0.001], “PPI 65_70 pre” [$F_{(2,25)}$=76.359; p=<0.001], “PPI_75 pre” [$F_{(2,25)}$=16.501; p=<0.001], “PPI_80 pre” [$F_{(2,25)}$=16.389; p=<0.001], “PPI total pre” [$F_{(2,25)}$=60.772; p=<0.001] and “PPI 65 post” [$F_{(2,25)}$=6.657; p=0.005]. The post-hoc Duncan test confirmed the expected differences in “PPI 60_70 pre” and “PPI total pre”: Low-PPI < Medium-PPI < High-PPI, and in “PPI 65 post”: Low-PPI < Medium-PPI and High-PPI. No differences were found in “PPI_70 post” [$F_{(2,25)}$=0.462; p=0.635], “PPI 65_70 *post*” [$F_{(2,25)}$=2.760; p=0.083], “PPI_75 post” [$F_{(2,25)}$=0.333; p=0.720], “PPI_80 post” [$F_{(2,25)}$=0.184; p=0.833] and “PPI total *post*” [$F_{(2,25)}$=0.770; p=0.473] among the three PPI-selected groups and “BL1_Startle *pre*” [$F_{(2,25)}$=1.340; p=0.947], “BL2_Startle pre” [$F_{(3,25)}$=0.031; p=0.280], “BL1_Startle *post*” [$F_{(2,25)}$=0.743; p=0.486] and “BL2_Startle post” [$F_{(2,25)}$=0.801; p=0.460] among the three PPI-stratified groups. The value of “BL2_Startle” for the “Pulse” group is not shown, as this group underwent 50 pulse-alone trials after the 10 first pulse trials (BL1_Startle), which is not comparable to the condition of PPI-stratified rats that underwent just 10 pulse-alone trials. Values are mean±SEM. **, p< 0.05 vs Medium-PPI and Low-PPI; *, p< 0.05 vs Low-PPI (Duncan’s multiple range test).

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A) ANOVA

	Volume (mm^3)			%Volume		
	Low-PPI (n=7)	Medium-PPI (n=12)	High-PPI (n=9)	Low-PPI (n=7)	Medium-PPI (n=12)	High-PPI (n=9)
mPFC	7.6 ±0.4	8.8 ±0.3*	9.1 ±0.2**	0.4 ±0.02	0.5 ±0.02*	0.5 ±0.01**
Cg	14.7 ±0.7	16.2 ±0.8	15.3 ±0.4	0.8 ±0.04	0.9 ±0.04	0.8 ±0.03
NAc	16.8 ±0.4	16.9 ±0.4	16.6 ±0.4	1.0 ±0.03	1.0 ±0.02	1.0 ±0.02
HPC	85.7 ±1.1	90.6 ±1.5*	91.8 ±1.2**	4.9 ±0.07	5.1 ±0.08	5.1 ±0.05
Striatum	73.0 ±0.7	74.0 ±1.6	73.6 ±0.9	4.2 ±0.05	4.2 ±0.08	4.1 ±0.05
Amygdala	31.5 ±1.3	32.4 ±1.0	31.7 ±1.4	1.8 ±0.07	1.8 ±0.06	1.8 ±0.09
Whole brain	1750.7 ±11.5	1766.1 ±20.0	1806.1 ±18.1	100	100	100

B) Multiple stepwise regressions

Dependent variable	Method	Model	Predictor variables	*r*	*R* (Model)	R^2 (Model)	p
%PPI	Forward stepwise	1	mPFC_MRI	0.565	0.650	0.422	0.001
			HPC_MRI	0.554			

Table S8. Study 4: Volume of various brain regions in the HS rats as a function of different PPI levels. a HS rats were stratified by their PPI scores in rats with low PPI scores (Low-PPI), medium PPI scores (Medium-PPI) and high PPI scores (High-PPI). Values are mean±SEM. *p < 0.05 **p < 0.01 vs. Low-PPI. Abbreviations as in Table 2. **b** Forward stepwise multiple regressions between “%PPI”, as a dependent variable, and all the c-Fos analyzed regions, as independent variables, showed that the hippocampal volume (HPC_MRI) and the medial prefrontal cortex volume (mPFC_MRI) predicted PPI performance. r, bivariate (Pearson’s) correlation.

Supplementary Figures and Tables

	Factors			
	1 “Frontocortical Inhibition” factor	2	3	4
BL1_Startle *pre*	-	-	0.92	-
BL1_Startle *post*	-		0.84	-
PPI *pre*	0.90	-	-	-
PPI *post*	0.58	-0.50	-	-
mPFC_cFos	0.56	-	-	-
NAc shell_cFos	-	-	-	-0.72
Striatum CP_cFos	-	0.60	-	-
Ventral DG_cFos	-	-	-	0.79
CeA_cFos	-	0.64	-	-
mPFC_mm^3	0.78	-	-	-
NAc_mm^3	-	0.71	-	-
% of cumulative variance	21.7%	38.7%	53.3%	64.1%
Factor correlations	1			
	-0.01	1		
	0.08	-0.02	1	
	-0.11	-0.07	0.01	1

Table S9. Factorial analysis of PPI, Startle, MRI and c-Fos parameters in HS rats (study 4). Four-factor solution (oblique rotation) including prepulse inhibition (PPI; 65_70dB), Startle, c-Fos and magnetic resonance imaging (MRI) variables from outbred HS rats (study 4). To avoid redundancy among variables (i.e. highly correlated variables) and to reduce them to the minimum meaningful ones, varimax factor analyses were applied to c-Fos and MRI data separately, and one variable (the one showing the highest loading) from each of the resulting factors was selected for the final combined (obliquely-rotated) factor analysis (which combined behavioral, C-Fos and MRI data) shown in the table. Varimax analysis of c-Fos data led to 5 components from which mPFC_cFos, NAc shell_cFos, Striatum CP_cFos, Ventral DG_cFos and CeA_cFos were chosen, whereas varimax factor analysis of MRI data led to a 2-factor solution, from which mPFC_mm^3 and NAc_ mm^3 were selected. These c-Fos and MRI selected variables, together with Startle-Pre, Startle-Post, PPI-Pre and PPI-Post, were submitted to an obliquely-rotated (oblimin direct) factor analysis. Direct oblimin rotation grouped the main variables in four main components that explained 64.1% of the variance. The first component (tentatively named “frontocortical inhibition”, as in the previous table) collected with positive sign “PPI_pre” (0.90), PPI_post (0.58), “mPFC_cFos” (0.56) and “mPFC_MRI” (0.78) variables, revealing tight associations between the mPFC, both activity and structure, and PPI scores. The second component grouped with negative sign PPI_post (-0.50) and with positive sign Striatum CP c-Fos (0.60), CeA_cFos (0.64) and NAc_mm^3 (0.71). The third component clustered with positive sign Startle_pre (0.92) and Startle_post (0.84). The last component of the factorial analysis collected with negative sign Striatum CP_cFos (-0.72) and with positive sign Ventral DG_cFos (0.79). The 4 factors had very low inter-correlations, so that they can be considered as practically independent from each other. Only loadings > 0.50 are shown. See variable definitions in “Materials and Methods” section.

Supplementary Figures and Tables

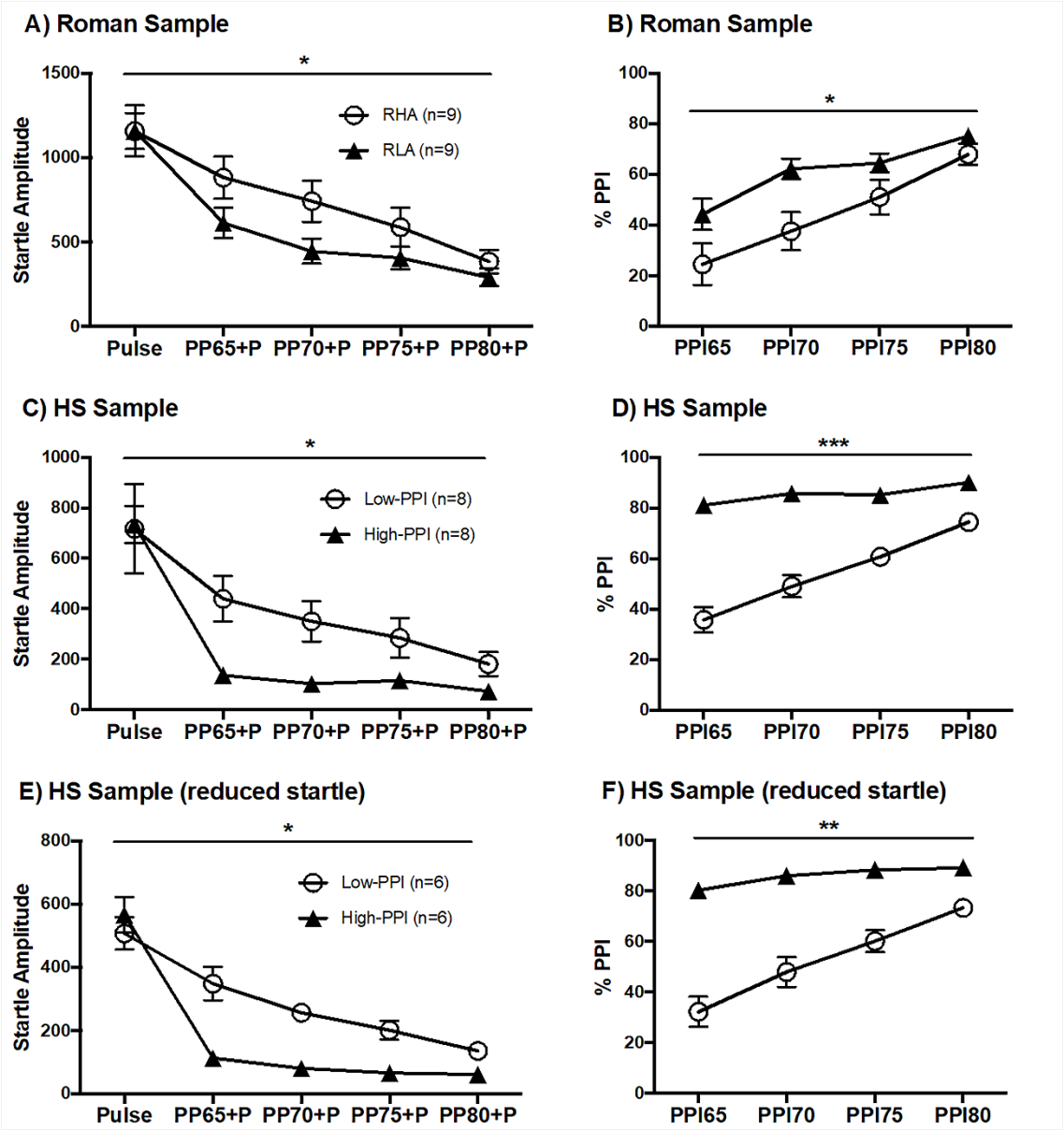

Figure S2. Influence of matching Roman (a, b) or HS (c-f) rats for their baseline startle response on startle amplitude on “prepulse + pulse” trials (a, c, e) and on the percentage of prepulse inhibition (%PPI; b, d, f). We conducted additional analyses to test whether there was a possible floor effect due to reduced baseline startle in low-PPI rats (i.e. RHAs or HS Low-PPI). Thus, we first matched baseline startle response (from pulse-alone trials) in the different groups of Roman and HS rats and then we applied repeated measures ANOVA. We pooled rats from studies 1 and 2 (total “Roman sample” of RHA and RLA rats, n=18 each strain) and studies 3 and 4 (total “HS sample” of HS rats, with Low-PPI, n=15, and High-PPI, n=17) to match them by their startle response to pulse-alone trials. For the “Roman sample” analysis, half of the RHA rats showing the greater startle amplitude to “pulse-alone” trials (“BL2_Startle” variable, from Tables S1-2) were selected (n=9), while half of the RLA rats showing the lower startle amplitude were also selected (n=9). As shown in “**a**”, even though RHA and RLA rats started from the same level of “Startle amplitude” in pulse-alone (“Pulse”) trials, RLA rats showed a higher reduction in their startle amplitude across the different prepulse+pulse (i.e. PP65+P to PP80+P) trials (significant interaction “group x trial type”, $F_{(4,64)}$=2.875, p=0.030, repeated-measures ANOVA). Moreover, and most importantly, we conducted a repeated-measures ANOVA analysis on %PPI (taking the different prepulse intensities –PPI65 to PPI80- as the “within-subjects” factor; see “**b**”), which showed significant “group x trial type” ($F_{(3,48)}$=3.088, p=0.036) and “group” ($F_{(1,16)}$=4.996, p=0.041) effects, revealing an apparently genuine deficit in PPI in the RHA rats. As done for the Roman rats, in the “total HS sample” analysis, half of HS Low-PPI rats showing the greater startle amplitude to “Pulse” trials (“BL2_Startle” variable from Supplemental tables 4 and 7) were selected (n=8), while half of the HS High-PPI rats showing the lower startle amplitude were selected (n=8). As shown in “**c**”, even though HS Low-PPI and High-PPI rats started from the same level of “Startle amplitude” in “Pulse” trials, HS High-PPI rats showed a higher reduction in their startle amplitude in the different prepulse+pulse trials (significant interaction “group x trial type”,

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$F_{(4,56)}$=4.280, p=0.004, repeated-measures ANOVA). Moreover, we conducted a repeated-measures ANOVA analysis on %PPI (taking the different prepulse intensities as the “within-subjects” factor). As shown in “**d**”, there were highly significant “group x trial type” ($F_{(3,42)}$=12.654, p=<0.001) and “group” ($F_{(1,14)}$=124.204, p=<0.001) effects, showing an apparently genuine deficit in PPI in the HS Low-PPI rats. Finally, with the idea to test whether a further reduced baseline startle amplitude leads to reduced %PPI, we conducted the same previous analysis with the HS rats but removing the two animals with higher baseline startle response from each group. We obtained the same differences as in “**c”** and “**d”**: “group x trial type” interaction on “startle amplitude” ($F_{(4,40)}$=11.376, p=<0.001; **“e”**) and “group x trial type” ($F_{(3,30)}$=9.157, p=<0.001; “**f**”) and “group” ($F_{(1,10)}$=93.714, p=<0.001; “**f**”) effects on %PPI. In conclusion, since reduced %PPI in HS Low-PPI and RHA rats (vs. HS High-PPI and RLA rats, respectively) occur with or without parallel reductions in the startle amplitude to pulse-alone (“Pulse”) trials, this indicates that the reduction observed in %PPI reflects a “real” impairment in sensorimotor gating.

Pulse: Mean ± SEM of startle amplitude in “Pulse-alone” trials. PP65+P to PP80+P: Mean ± SEM of startle amplitude in “Prepulse + Pulse” trials at different prepulse intensities. PPI65 to PPI80: Mean ± SEM of percentage of PPI in “Prepulse + Pulse” trials at different prepulse intensities. *p < 0.05, **p<0.01, ***p<0.001, “group x trial type” effect (repeated measures ANOVA).

Discussion

The main aim of this Doctoral Dissertation was to study the behaviors and neurobiological mechanisms associated with spontaneous low PPI in intact inbred and outbred rats (i.e. RHA and HS Low-PPI), as well as testing a treatment to increase PPI in these animals. Thus, this Doctoral Dissertation provides evidence for the validity of RHA and HS Low-PPI rats that goes from behavior to brain mechanisms, as well as from brain mechanisms to behavior. Fig. 7 illustrates a summary of our main results.

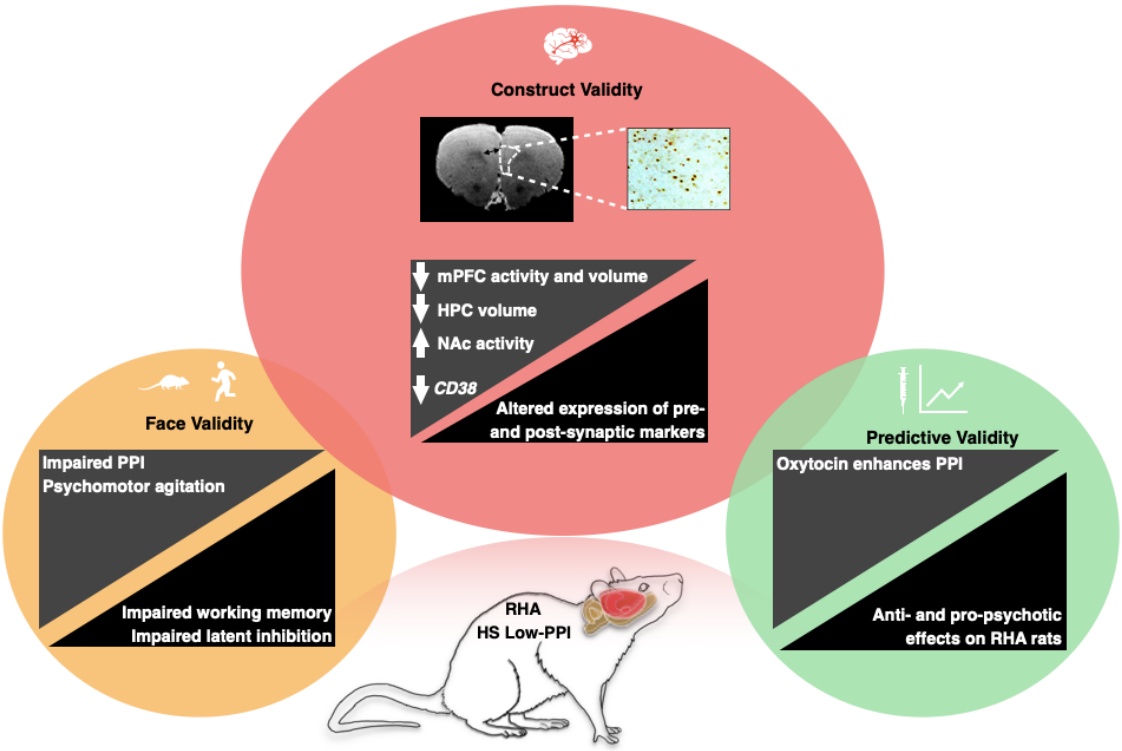

Figure 7. Summary of results from this Doctoral Dissertation and previous findings in the HS Low-PPI and RHA rats. To show a more comprehensive representation, findings are grouped in three clusters according to the three validity criteria of animal models (face, construct, and predictive validity). In gray triangles, results derived from this Doctoral Dissertation. Note: *CD38* findings only refer to the RHA rats. In black, previous findings in the HS Low-PPI and RHA rats.

1. Relationship among impaired PPI and other schizophrenia-like behavioral features in Roman and HS rats.

Schizophrenia is an exclusively human condition, and it is impossible to develop a model expressing all schizophrenia symptoms. However, as face validity is fundamental for a rodent model of a mental disorder, it is important to evaluate all putative models with the widest range conceivable of schizophrenia-like symptoms. In this sense, in Study 1, we aimed to explore whether spontaneous low PPI in intact inbred Roman and outbred HS rats was associated with increased exploratory activity, anxiety-like, or compulsive-like behaviors.

As described earlier, increased exploratory activity in response to novelty is a key feature to model positive-like symptoms in rodents [103], while anxiety and obsessive-compulsive symptoms are highly comorbid in schizophrenic patients [13,14]. We used different behavioral tasks in order to test these behavioral phenotypes, such as the open-field (exploration), the elevated zero-maze (anxiety), and the marble burying test (compulsivity). Our subjects of study were the outbred HS rats stratified by Low-, Medium-, and High-PPI, and the inbred Roman rats that show spontaneous differences in PPI (RLA > RHA). This study allowed us to explore (i) whether the stratification for low PPI also co-stratifies other schizophrenia-like symptoms in HS rats and (ii) whether the RHA rats show other schizophrenia-relevant symptoms.

Our results revealed that the HS rats stratified by Low-PPI and the RHA rats showed increased exploratory activity in response to novelty. In this regard, previous findings from our laboratory showed that reduced PPI was associated with other schizophrenia-like cognitive symptoms in both HS Low-PPI and RHA rats, such as impaired latent inhibition and working memory [164,165,179]. Thus, our results showing a cluster of positive schizophrenia-like (psychomotor agitation) and cognitive schizophrenia-like symptoms in HS Low-PPI and RHA rats provide substantial face validity for the human condition. Of note, the evidence supporting the association between “low PPI” and “increased exploratory activity” is typically derived from animals submitted to a variety of manipulations or treatments, including social isolation [181,182], ventral hippocampal lesion [183–185], genetic models [103,186–190], NMDA infusion [183,185], NMDA antagonists administration [191] or amphetamine administration [137,192–195]. However, there is a relative paucity of data derived from outbred naïve animals. The use

of HS rats may have better translational value in view of the high genetic heterogeneity of the human population.

On the other hand, contrary to what is reported in some human studies [14,196], there were no associations between low PPI and the presence of anxious-like or compulsive-like behaviors. In fact, RHA and HS rats with reduced PPI showed low anxiety-like behavior, which is in line with previous reports in rodents [197,198]. Moreover, the association between “high PPI” and “high anxiety-like behavior” seen in the RLA and HS High-PPI rats agreement with the profile of hypervigilance previously observed in the high-PPI Wistar-Kyoto rats [198].

In summary, this study suggests that reduced PPI and increased exploratory activity, as well as impaired latent inhibition and working memory [164,165,179], are part of a cluster of symptoms that must be taken into consideration when modeling schizophrenia-like features. Moreover, it provides higher face validity to our two rat models of schizophrenia-related features, i.e. RHA and HS Low-PPI rats.

2. Association between structural and functional brain differences and deficient PPI in the Roman and HS rats.

The underlying mechanisms of PPI have been extensively studied in rodent models of schizophrenia evaluating the impact of specific brain manipulations on PPI. These studies suggest that PPI is modulated by the CSPT circuit [39,123,128]. On the other hand, PPI mechanisms have also been investigated in humans by exploring the neural correlates of PPI using non-invasive brain imaging. In this regard, in Study 2, we aimed to explore neural correlates of PPI using non-invasive and invasive procedures in the inbred Roman and outbred HS rats. Specifically, we aimed to evaluate whether spontaneous PPI variation in intact inbred Roman and outbred HS rats was associated with functional and structural differences in the CSPT circuit. Thus, we explored brain volume (by structural MRI) and neuronal activity (by c-Fos expression) of relevant regions of the CSPT circuit, such as the mPFC, cingulate cortex, HPC, amygdala, NAc and dorsal striatum, in intact rats with spontaneous differences in PPI. Moreover, this study allowed us to provide evidence for the construct validity of our two rat models of schizophrenia, i.e. RHA and HS Low-PPI rats.

First, our data revealed that differences in sensorimotor gating in intact inbred Roman and outbred HS rats were associated with divergences in mPFC activity and volume. Specifically, low PPI was linked to decreased mPFC activity and volume. This finding agrees with a previous report showing reduced mPFC *in vivo* neuronal activity in rats selectively bred for low PPI [116]. Moreover, several reports reveal that PPI is modified by treatments that alter the normal functioning of the mPFC, such as pharmacological treatments [199–201], neurodevelopmental approaches [125,126,202–204] or deep brain stimulation [205,206]. In agreement with rodent research, human studies using functional and structural MRI have revealed reduced PFC activity and volume in relation to PPI performance in healthy and schizophrenic subjects [38,39,154]. Importantly, it must be taken into consideration the higher spatial resolution of c-Fos over functional MRI, as c-Fos allows the identification of very small nuclei and single neurons [207,208]. In keeping with that, in Study 3, we aimed to elucidate what kind of neurons are involved in the association between low neuronal activity and reduced PPI. In order to do so, in line with the GABA hypothesis (see *1.5.3. Glutamate hypothesis* and *1.5.4. GABA hypothesis*), we conducted a fluorescent immunostaining with c-Fos, PV, and PSD-95 (a marker for excitatory inputs) in the mPFC of Roman rats. Our data revealed higher

activity of PV interneurons (double staining PV and c-Fos) in the RLA group that underwent the PPI session than the RLA control groups and all the RHA groups (including RHA under the PPI condition). In agreement with our result, previous reports have revealed that prenatal administration of PCP or lipopolysaccharide reduces the density of PV-positive cells and the c-Fos activity in the mPFC in parallel to PPI impairments [209,210]. Similarly, several treatments used to model schizophenia-like symptoms have also reported a reduction in PV cell density in the mPFC [74,108,145,211–215]. Likewise, in another study using genetically modified mice, it was demonstrated that the PV-deficient (PV–/–) mice show lower PPI than the PV+/+ control group [216]. On the other hand, we found "Strain" differences in the mean density of PV interneurons (RLA>RHA, which could be indicative of reduced PV expression and activity [209,215,217]) and the PSD-95 puncta (a marker for excitatory synapses) on active PV interneurons. The RHA rats showed globally lower PSD-95 density on active PV cells than the RLA, which agrees with the idea that excitatory drive plays a role in the activity of PV interneurons in schizophrenia [72] (see Fig. 3). This study, using fluorescent immunostaining, adds further knowledge to our findings indicating reduced general volume and activity of the mPFC in the RHA rats and identifies inhibitory PV interneurons as part of the circuit that modulates PPI.

Second, in Study 2, we found that the HPC volume was positively associated with PPI performance in both HS and Roman rats. In line with the PFC results, the RHA rats, compared to the RLAs, and the HS Low-PPI rats, compared to the HS Medium-PPI and High-PPI groups, showed reduced volume of the HPC. This finding agrees with previous reports showing that the HPC volume is positively associated with the magnitude of PPI response in both control rats [125] and healthy human subjects [154].

Third, our data revealed increased neuronal activity in the NAc shell in the HS Low-PPI rats compared to the HS High-PPI group. Regarding the Roman rats, a similar but non-significant trend was observed, as the RHA showed higher c-Fos mean value in the NAc shell than the RLAs. This result, together with increased mPFC activity and volume, replicates what is observed in rats selectively bred for low PPI [116], since they also show reduced mPFC activity and increased NAc activity. However, research studying the role of the NAc activity in PPI has provided diverse and paradoxical findings [129,136,192,206,218–220], which suggest the need for further investigation.

On the other hand, in this study, we conducted additional analyses of PPI measures to see whether reduced PPI in both RHA and HS Low-PPI meant actual deficits in PPI, rather than simple baseline startle differences (see Study 2, Figure S2). Specifically, we proved that matching Roman and HS rats by their baseline startle levels (from pulse-alone trials) maintained PPI differences between the RHA and RLA rats and between the HS Low-PPI and High PPI. This evidence points out, for the first time, genuine deficits in PPI in both the RHA and the HS Low-PPI rats that reinforce the validity of both rat models of schizophrenia-like features.

In summary, the structural and functional findings, along with behavioral results from Study 1, indicate that deficient PPI is associated with several neurobiological and behavioral traits that resemble schizophrenia features in both RHA and HS Low-PPI. Thus, our results underline the usefulness of PPI to model schizophrenia-related features and, in turn, provide more validity to our two rat models of schizophrenia.

3. Effects of peripheral oxytocin administration on PPI in the Roman and HS rats.

Oxytocin has been proposed as an alternative natural drug with potential antipsychotic-like effects for schizophrenia [84,85,92]. In this regard, previous research suggests that oxytocin would have PPI-enhancing effects in rats [85,96], which lead us to test oxytocin in our two rats of study, i.e. HS and Roman rats. First, we conducted a pilot dose-response study in the HS rats. We matched HS rats in three groups according to similar low PPI levels to have room for PPI improvement. A week later, they were randomly distributed to either saline, 0.04, or 0.2mg/kg group conditions, and received a dose of saline or oxytocin 30min before the PPI test. Second, derived from the pilot study, we tested the same doses in the RLA and RHA rats. Finally, as the Roman rats showed divergent oxytocin effects on PPI and they differ in several oxytocin-related behaviors, such as maternal nurturing, social interaction, and stress responses [107,158,165], we studied differences in gene expression in the oxytocin pathway.

Our data from the pilot dose-response study in the HS rats revealed that oxytocin increased PPI at dose of 0.04 compared to vehicle. Importantly, the dose of 0.04 did not differ from the dose of 0.2. For this reason, we decided to test both doses in the Roman rats. In this regard, as expected, oxytocin had a more powerful PPI-enhancing effect on the schizophrenia-like RHA rats than in RLAs. Specifically, oxytocin at 0.2mg/kg improved PPI at the PPI intensity of 75dB, while it had no significant effects on the RLAs. These results are in line with previous findings indicating that oxytocin improves PPI or attenuates PPI deficits in rodents [85,95,96,221], as well as other schizophrenia-related behaviors and cognitive functions [84,94,222–224].

Interestingly, the fact that we found divergent effects in the RHA and RLA may suggest baseline differences in the oxytocin pathway between the strains, which might be relevant for PPI modulation. In order to test this hypothesis, we conducted a gene expression study in the mPFC of representative samples of RHA and RLA rats from the saline groups and the 0.2mg/kg effective oxytocin dose. We reported a significant “Strain” effect in the regulator of oxytocin release *CD38*, as we found higher expression in the RLA rats than in the RHAs. This gene encodes the protein CD38 that is involved in the secretion of oxytocin and shows a positive association with oxytocin release [88,225,226]. In this sense, our data indicate that the RHA rats could have lower baseline oxytocin levels and this may explain why they had a greater benefit from the oxytocin

administration to improve PPI [225,227]. Our findings on *CD38* are consistent with several studies reporting alterations in the oxytocin pathway both in animal models of schizophrenia-relevant phenotypes [85,228–233] and human schizophrenia patients [89,101,226,234]. Particularly, a previous mice study reported that animals lacking *CD38* show deficiencies in several PFC-dependent behaviors [232]. On the other hand, oxytocin administration increased oxytocin receptor (*OXTR*) expression on both Roman rat strains. This effect is in agreement with previous findings indicating that oxytocin administration restores the reduction of *OXTR* expression in the mPFC triggered by prolonged stress in rats [235]. Importantly, it is noteworthy that *OXTR* plays a relevant role on the regulation of excitatory-inhibitory balance in the mPFC that might be relevant for schizophrenia-related disorders [236]. In this regard, it has been reported reduced *OXTR* expression in the PFC in both the schizophrenia-like Wisket rats [233] and schizophrenic patients [234].

Consistent with the GABA hypothesis of schizophrenia (see *1.5.4. GABA hypothesis* and *1.6.3. Oxytocin*), we speculate that oxytocin increased PPI in the RHA rats by regulating dopamine release through the modulation of the excitatory-inhibitory cortical circuit. Previous findings indicate that oxytocin improves PPI by regulating dopamine transmission, as oxytocin attenuates the PPI disrupting effects of amphetamine [95] and the cocaine-induced mesolimbic dopamine release and hyperactivity [222]. Even though altered dopamine transmission underlies psychotic symptoms of schizophrenia, recent data indicate that this alteration might be caused by an imbalance between neocortical excitatory glutamatergic and inhibitory GABAergic neurons [31,48]. In this regard, previous studies showing that the RHA rats could have an excessive glutamatergic and dopaminergic tone in the PFC and striatum [172,174], as well as reduced activity of PV interneurons (study 3), suggest that the behavioral deficits observed in the RHA might be related to an excitation-inhibition imbalance in the PFC. Accordingly, as oxytocin administration has been linked to a decrease in glutamate and an increase in GABA release [97,99,237,238], oxytocin might have improved PPI in the RHA by increasing activation of OXTR in inhibitory interneurons in the mPFC and, in turn, reducing mesolimbic dopamine transmission. Further studies are needed to confirm this hypothesis.

4. Reduced PPI in the RHA and HS Low-PPI rats: Are they valid and useful rat models for schizophrenia?

PPI is a translational measure of sensorimotor gating that is used in several human clinical conditions and in relevant rodent models to probe brain mechanism underlying these conditions. After the observation in 1978 that PPI is deficient in schizophrenia [117], as well as in many other clinical conditions [113], the use of rodent models of impaired sensorimotor gating has increased in the last 40 years [119].

The development of animal models to study the impact of brain-site specific or environmental manipulations on PPI is relevant to investigate schizophrenia-related disorders, as most of them show face, predictive, and construct validity [78]. However, the use of rodents that spontaneously differ in PPI or other schizophrenia-related phenotypes allows studying illness-related features in a way that is similar to human research (see *2.4. Experimental strategies*). Moreover, these "intact" animal models may offer the chance to understand the mechanisms involved in schizophrenia-like symptoms without confounding effects of a given manipulation or treatment. In this regard, the RHA and HS Low-PPI rats, which are both characterized by spontaneous low PPI, have shown some behavioral (see Study 1) and neurobiological features that are relevant for schizophrenia-related symptoms and have contributed to investigate its mechanisms (see [138,164,165,172,179,180] and Fig. 7). For example, in Studies 2 and 3, by studying associations between spontaneous differences in PPI and brain activity and volume, we contribute to bridge the gap between rodent studies that examined the impact of specific brain manipulations on PPI and human imaging or postmortem studies that examined brain correlates of PPI.

On the other hand, taking into account the anatomical and genetic heterogeneity of schizophrenia, as well as the severe side effects of the current antipsychotic medications (see *1.6. Pharmacological treatments*), the potential usefulness of PPI to provide deeper knowledge for schizophrenia is a matter of controversy [78]. Alternatively, PPI has been proposed as a helpful biomarker for schizophrenia-related disorders in humans and rodents. Following this point of view, PPI would be a suitable candidate to reduce the heterogeneity and multiple interaction in the pathogenesis of schizophrenia by stratifying individuals according to differences in this simple measure [239]. In this sense, as the pathogenesis of schizophrenia occurs during the early brain maturation (see *1.3. Risk*

factors), PPI would be helpful in the early life to detect individuals that are at risk of suffering from schizophrenia. Thus, using rodents that spontaneously differ in PPI (e.g. HS Low-PPI and RHA) allows reducing the entire constellation of schizophrenia symptoms and focusing on the study of individuals that are "at risk" of suffering from schizophrenia-like disorders or symptoms. Furthermore, as previous evidence indicates that high PPI levels are associated with a higher benefit from learning-based therapies in schizophrenic patients, it would be important to develop animal models that allow testing the potential PPI-enhancing effects of drugs, rather than studying the impact of different treatments that disrupt PPI or attenuate the effects of a brain manipulation [78,239]. With the idea to test the potential PPI-enhancing effects of drugs, some intact animals selectively bred or stratified by low basal PPI have shown to be more sensitive to the PPI-enhancing effects of various treatments [240–242]. Accordingly, subjects with low basal PPI might allow to identify appropriate candidates for enhancing the efficacy of cognitive-behavioral therapy. In this regard, the RHA and HS Low-PPI rats that show spontaneous relatively lower PPI than the RLA and HS High-PPI, respectively, might be useful rat models to identify drugs (i.e. oxytocin) suitable for increasing the efficacy of cognitive-behavioral therapy. These behaviorally stratified or selectively bred animal models are less specific than animal models based on hypothesis-driven approaches, but they do not have the limitation of assuming a particular neurobiological mechanism.

Overall, despite the fact that animal models with brain-site specific or environmental manipulations are valid and important for the study of the mechanisms underlying schizophrenia and related disorders, the HS Low-PPI and the RHA rats are also valid models that can contribute to provide relevant knowledge about the disorder. Moreover, as the HS Low-PPI and the RHA are more sensitive to the PPI-enhancing effects of drugs, they would be useful to find treatments to improve the efficacy of cognitive therapies.

Conclusions

The data obtained in the present Doctoral Dissertation identifies schizophrenia-like reduced sensorimotor gating in intact inbred RHA and outbred HS rats as a valid and relevant tool to explore the behavioral and neural mechanisms underlying schizophrenia. Specifically, the main conclusions we have drawn are the following:

1. Increased exploratory activity in response to novelty is related to PPI deficits in RHA and HS Low-PPI rats, showing a link between positive-like and cognitive-like symptoms.

2. Reduced PPI is not associated with anxious-like or compulsive-like responses in RHA and HS Low-PPI rats.

3. Increased anxiety is associated with higher PPI in HS and Roman rats, which is in agreement with a behavioral profile of hypervigilance.

4. PPI deficits in intact RHA and HS Low-PPI rats are associated with different brain abnormalities observed in schizophrenic patients, such as decreased mPFC activity and volume, as well as reduced HPC volume.

5. As described in schizophrenia and in contrast to the mPFC findings, HS Low-PPI rats show increased NAc shell activity.

6. The PPI-dependent activity of GABAergic PV interneurons is lower in RHA rats than in their RLA counterparts, suggesting that reduced cortical inhibition could underlie the behavioral differences between the Roman rat strains.

7. Exogenous oxytocin administration, which shows an inhibitory profile on GABA/Glutamate function, attenuates natural PPI deficits in HS Low-PPI and RHA rats, while it does not affect RLAs.

8. Oxytocin increases *OXTR* expression in the mPFC of both Roman rat strains.

9. RHA rats show lower constitutive expression of the regulator of oxytocin release *CD38* in the mPFC than RLAs, which is in agreement with the differential PPI effects of oxytocin.

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Annex

Annex 1

Study 3: "*Decreased activity of parvalbumin interneurons in the medial prefrontal cortex in intact inbred Roman rats with reduced sensorimotor gating*".

In preparation.

SHORT COMMUNICATION

Decreased activity of parvalbumin interneurons in the medial prefrontal cortex in intact inbred Roman rats with reduced sensorimotor gating

Carles Tapias-Espinosa, …, Alberto Fernández-Teruel

ABSTRACT

Prepulse inhibition (PPI) of the startle response is a wide-spread cognitive paradigm to assess schizophrenia-like sensorimotor gating deficits in rodents. Preclinical and clinical studies indicate that PPI is modulated by the medial prefrontal cortex (mPFC), which is in line with previous findings indicating that natural PPI differences in the Roman rats (RLA>RHA) are associated with differences in mPFC activity (RLA>RHA). In this context, a dysfunctional cortical excitatory-inhibitory balance is proposed as the main neural substrate for cognitive dysfunction in schizophrenia. Here, we explore whether PPI differences in the Roman rats could be associated with differences in the activity of inhibitory neurons (parvalbumin-containing GABAergic (PV) interneurons) and/or reduced excitatory drive to PV interneurons (PSD-95 puncta on PV interneurons). Our data showed that reduced PPI in the RHA rats was associated with reduced activity of PV interneurons, while the RHA and RLA rats globally differed in the density of both PV interneurons and PSD-95 puncta on active PV interneurons. These findings point to reduced cortical inhibition as a candidate to explain the schizophrenia-like features observed in the RHA rats and supports the role of impaired cortical inhibition in schizophrenia.

INTRODUCTION

Sensorimotor gating is the process to filter out relevant from irrelevant information [1], which is deficient in several neuropsychiatric conditions, including schizophrenia [2]. Sensorimotor gating can be operationally measured by prepulse inhibition (PPI) of the startle response [3]. Impaired PPI in rodents is used as a common endophenotype to model this basic attentional schizophrenia-like deficiency and to try to elucidate the mechanisms underlying schizophrenia. In this regard, findings from clinical and preclinical studies indicate that PPI would be modulated by the cortico-striato-pallido-thalamic (CSPT) circuit [2,4–8]. In agreement with the role of the CSPT circuit in PPI, we found that PPI differences were associated with differences in volume and activity of the medial prefrontal cortex (mPFC) in both intact inbred Roman and outbred NIH-HS rats [9]. Interestingly, our data revealed fewer c-Fos-labelled neurons in the mPFC of rats with reduced PPI, which raised the question of what kind of neurons in the mPFC may be involved in the modulation of PPI. In this regard, several hypotheses have been proposed to try to explain the etiology of schizophrenic symptoms based on different neurotransmission systems, such as the dopaminergic, serotoninergic, glutamatergic, GABAergic systems.

The GABA hypothesis postulates that GABA-mediated cortical inhibition is dysfunctional in schizophrenia, leading to excessive excitatory neural transmission to subcortical brain regions [10–12]. In this regard, it has been suggested that dysfunctional NMDA receptors in GABAergic interneurons do not activate these inhibitory neurons, and this leads to overactivation of glutamatergic projections [10,13]. In turn, this higher glutamatergic transmission would over-activate (i) the mesolimbic dopaminergic neurons, responsible for positive symptoms of schizophrenia; and (ii) the GABAergic neurons that project to the PFC, causing negative and cognitive symptoms [10]. This hypothesis is based on the evidence that schizophrenic patients show poor gamma oscillation frequency in the PFC, a pattern of neuronal firing critical for cognitive abilities, such as attention and working memory [13–16]. Gamma oscillations depend on cortical inhibitory circuitry and it seems that dysfunctional cortical GABA-mediated synaptic inhibition would explain cognitive impairment in schizophrenia [16,17]. Particularly, a subpopulation of GABA interneurons that expresses the calcium-binding protein parvalbumin (PV) seems to be essential to provide inhibitory inputs and to drive cortical gamma oscillations [15,16]. Importantly, consistent with the GABAergic hypothesis, it

has been recently demonstrated that excitatory synapses (measured by PSD-95) are selectively decreased on PV interneurons in the PFC of schizophrenic patients and this predicts the activity-dependent downregulation of PV [18]. In this regard, studies have repeatedly shown reduced number and/or expression of PV interneurons in both schizophrenic patients [19–23] and animal models of schizophrenia [3,16,20,24–28].

Regarding PPI in rats, previous findings indicate that prenatal phencyclidine (PCP) or lipopolysaccharide reduce the density of PV-positive cells and the c-Fos activity in the mPFC in parallel to PPI impairments [29,30]. Similarly, the PV knockout (PV–/–) mice show lower PPI than the PV+/+ control group [31]. Regarding the Roman rats, we found that the spontaneous PPI-deficient Roman high-avoidance (RHA) rats showed lower mPFC activity related to PPI performance than the Roman low-avoidance (RLA) rats. In this context, we aimed to explore whether the differences observed in the mPFC activity associated with PPI differences in the Roman rats might be explained by differences in PV interneuron activity and/or differences in excitatory synaptic density in PV interneurons. The strength of the excitatory synapses can be measured by PSD-95 puncta, which is the major scaffolding protein in the excitatory postsynaptic density [32].

The inbred RHA and RLA rats were bidirectionally selected for rapid vs. non-acquisition of the two-way active avoidance task, respectively. Interestingly, compared with their RLA counterparts, RHA rats show spontaneous reductions in PPI, working memory, and latent inhibition, among other schizophrenia-relevant features [9,33]. These behavioral differences in parallel to between-strain divergences in the dopaminergic, serotoninergic, and glutamatergic systems, indicate that the RHA rats might be a valid animal model to study the underlying mechanisms of schizophrenia-related symptoms [34].

In this study, we investigated the colocalization of c-Fos with PV interneurons and PSD-95 puncta on PV cell bodies in the mPFC of the Roman rats after a PPI session. We hypothesize that RHAs, compared to the RLA strain, would show fewer number of active PV interneurons and lower excitatory synaptic inputs (PSD-95) to PV interneurons following PPI testing.

METHODS

Subjects

We used 42 male Roman rats (RHA = 21; RLA = 21) from our permanent colony at the Department of psychiatry and Forensic Medicine, Universitat Autònoma de Barcelona. They were aged 2.5-3 months old, weighing 220-290 g. They were housed in pairs in macrolon cages (50 x 25 x 14 cm) and maintained with food and water ad libitum, under a 12:12 light-dark cycle (lights on at 08:00am) and controlled temperature (22±2 C) and humidity (50-60%).

Prepulse inhibition of the startle response (PPI)

PPI was performed as previously described in [9]. In short, rats were individually located in an acrillic cylinder within an attenuated box (SR-Lab Startle Response System, San Diego Instruments, US). Noise bursts were presented through a speaker located 15cm above the cylinder. The PPI session consisted of: (i) 5-min acclimation period; (ii) 10 pulse-alone trials (105dB(A), SPL, 40ms); (iii) 60 random trials of: 10 pulse-alone (used to calculate %PPI), 10 prepulses of each intensity 65/70/75/80dB(A, SLP, 20ms) followed by pulse stimulus with an inter-stimulus interval of 100ms, or non-stimulus trials (background noise of 55dB). The %PPI for each prepulse intensity was obtained by applying the following formula: %PPI = [100 – (startle amplitude on prepulse trials/startle amplitude on pulse-alone trials x 100). The No-Pulse (NP), Prepulse-alone (Pre), and the Pulse-alone (Pulse) control groups for each strain underwent sessions of non-stimulus trials, prepulse-alone trials, and pulse-alone trials of the same duration as the PPI test. Animals were randomly assigned to time and box of testing to avoid order effects. Group n's were as follows: RHA-NP = 4; RHA-Pre = 4; RHA-Pulse = 5; RHA-PPI = 8; RLA-NP = 4; RLA-Pre = 4; RLA-Pulse = 6; RLA-PPI = 7.

Tissue processing and florescent Immunohistochemistry

Two hours after the PPI, Prepulse-alone, Pulse-alone, or No-Pulse sessions, rats were anaesthetized with a lethal dose of pentobarbital and perfused transcardically with a solution of 0.1M PBS (pH 7.6), followed by 3.7-4% paraformaldehyde in PBS (Casa Álvarez). Once the brains were removed, they were immersed in 4% paraformaldehyde for 2h and then in 30% sucrose in PBS for 2 days. Coronal 30-µm thick sections of the mPFC (bregma: from 3.72 to 2.52mm) were cut in cryostat (Leica CM3050S), cryopreserved, and stored at 80°C until free-floating florescent immunohistochemistry

staining. Prior to stanning, sections were treated with antigen retrieval (citrate buffer pH 6). After several PBS and PBS-T washes, endogenous peroxidase was blocked incubating the slices with 10% normal horse serum (Sigma H0146) in “antibody diluent” (PBS-T with 1% (w/v) BSA). Slices were then incubated overnight at 4°C with the primary antibodies (goat anti-c-Fos IgG, Santa Cruz, SC52G, 1:500; mouse anti-PV, 1:2000, sigma; Rabbit anti-PSD-95, 1:1000, Abcam). On the next day, free-floating samples were washed in PBS-T and then incubated with the secondary antibody for c-Fos (biotinylated horse anti-goat IgG, Vector Laboratories, 1:400) for 40 min. After washes in PBS-T, the slices were incubated for 45 min with Alexa Flour 555 conjugated with streptavidin, Alexa Flour 488 conjugated with anti-mouse, and Alexa Flour 647 conjugated with anti-rabbit (all of them from ThermoFisher Sciencific, diluted 1:750). Then, sections were washed in PBS-T and PBS, incubated for 5 min with DAPI (Hoechst, 1:1000), and washed in PBS and PB. Finally, samples were mounted, and cover slipped using mounting solution.

Image Acquisition, Post-Image Processing and Object Sampling

Images were taken on a confocal microscope (Leica SP5) using a 63x/1.50 NA SC oil immersion objective. For each animal, fifteen image stacks (512x512; 0.7µm) of the mPFC were randomly selected. The IMARIS 9.5 software was used to automatically identify and count the number of c-Fos and PV nuclei and PSD-95 puncta in three sections of the mPFC/mm^2 and averaged for each animal. Based on the ability of antibodies to penetrate tissue, we obtained a representative image of objects that were in the middle of the z-planes. No differences were observed in the mean volume of tissue sampled among groups.

Statistics

Co-localization of c-Fos and PV cell bodies was calculated in order to determine the density of active PV interneurons in the total image area. The percentage of PV interneurons colocalization with c-Fos (%PV+/c-Fos) was determined by the following formula: PV/c-Fos density = [number of “PV+c-Fos+” cells / “PV+ total cells” x 100]. The number of PSD-95 puncta per surface area of PV+ or “PV+c-Fos+” cells was calculated in order to determine the density of excitatory synapses on PV interneurons or active PV interneurons, respectively.

All the analyses were performed using the SPSS statistical software. Data are expressed as Mean ± SEM. Significance level was set at $p<0.05$.

Student's *t*-test was used to test for significant PPI differences between RHA and RLA rats.

A 2 x 4 ('2 strains x 4 conditions') ANOVA was used to explore differences in the different neural markers (PV, c-Fos, and PSD95, and their combination) between the RHA and RLA rats in the different experimental conditions (i.e. No-Pulse, Prepulse-alone, Pulse-alone, and PPI).

RESULTS

As expected, the RHA rats showed lower PPI than their RLA counterparts and 2x4 ANOVA revealed a "Strain x Condition" effect in c-Fos activity in the mPFC ($F_{(3,34)}$=2.985; p=0.045). Importantly, Duncan's post hoc test confirmed that the RLA rats under the PPI conditions showed higher c-Fos activity than the NP, Pre, and Pulse RLA groups and all the RHA groups, while there were no differences among RHA groups (Fig. 1b).

Regarding PV interneurons, 2x4 ANOVA revealed a significant "Strain" effect ($F_{(1,34)}$=15.891; p=<0.001; Fig. 1a), as the RLA rats showed a higher number of PV+ cells in the mPFC than the RHA rats, both in cell bodies and surface area. There were no "Condition" ($F_{(3,34)}$=0.256; p=0.857) or "Strain x Condition" ($F_{(3,34)}$=1.172; p=0.335) effects.

On the other hand, concerning the percentage of active PV interneurons, 2x4 ANOVA showed a significant "Strain effect" ($F_{(1,34)}$=4.864; p=0.034), as it was globally higher in the RLA than in the RHA rats, and a "Strain x Condition" effect ($F_{(3,34)}$=2.894; p=0.049). Post-hoc Duncan's test confirmed that the RLA under the PPI condition showed higher percentage of active PV interneurons than the RLA control groups and the RHA groups ($p<0.05$; Fig. 2a). In fact, the RHA-PPI rats, compared to their RLA-PPI counterparts, exhibit an approximately 60% reduced activation of PV interneurons.

Finally, regarding PSD-95, no significant effects were observed in either total number of PSD-95 puncta or PSD-95 puncta on PV+ cells (Fig. 1c and Fig. 2b, respectively; all $p \geq 0.498$). However, we found a "Strain" effect in the density of PSD-95 puncta on active PV+ cells (PSD-95 puncta on "PV+c-Fos+" cells; $F_{(1,34)}$=6.447; p=0.016), as it was globally higher in RLA rats than in RHAs (Duncan's post hoc test, $p<0.05$; Fig. 2c). No "Condition" ($F_{(3,34)}$=0.227; p=0.841) or "Strain x Condition" ($F_{(3,34)}$=0.159; p=0.923) effects were found in the density of PSD-95 puncta on active PV+ cells.

DISCUSSION

This study aimed to elucidate the specific mechanisms associated with reduced neuronal activity in the mPFC during PPI in the RHA rats compared to the RLAs. Our results indicated that the differences in neuronal activity might be partly explained by differences in activity of PV interneurons. Specifically, the RLA rats showed both higher PPI and activity of PV interneurons than the RHAs. Additionally, our data revealed two relevant strain differences between the Roman rats, i.e. the density of PV interneurons and the density of PSD-95 puncta on active PV+ cells.

Our result showing reduced activity of PV interneurons agrees with several studies reporting reduced PV interneurons in schizophrenia [19–23] and animal models of schizophrenia-related features [3,16,20,24–28]. In this regard, prenatal and postnatal neurodevelopmental models of schizophrenia have shown impaired PPI associated to reduced density of PV interneurons and c-Fos activity after PPI [29,30], while loss of PV gene involves impaired PPI [31]. In this study, we have also shown that the schizophrenia-like RHA rats show lower density of PV interneurons in the mPFC than the RLAs. This evidence is supported by previous findings indicating that prenatal exposure to MK-801 or PCP (NMDA antagonists) decreases PV interneuron density in the PFC of rats, enhances locomotion, and reduces PPI [29,30]. However, as noted by [27,29], it is noteworthy that using florescent immunohistochemistry we could not detect whether the RHA rats had lower PV expression below detection limit rather than loss of PV interneurons, as it happens in the NR1-deficient mice model of schizophrenia [35]. In this regard, the possible differences in PV expression between the Roman rats are in line with differences observed in PV activity, as lower PV expression would also indicate lower activity [18]. In fact, in a previous study with the Roman rats using stereology, there were no differences in PV density between the strains in the PFC, even though there were some important methodological differences between the studies, such as region delineation, immunohistochemical procedures and antibody dilution, effects of testing, or animal age [36]. Our results regarding the activity of PV interneurons are consistent with previous reports in schizophrenic patients showing that PV interneurons express lower GAD67 [37,38], as well as reduced PV density [19–23,39]. In this sense, the reduction in the activity of PV interneurons could drive to the disinhibition of pyramidal neurons that causes increased mesolimbic dopamine and schizophrenia-like symptoms in the RHA rats [34].

On the other hand, according to the GABA hypothesis of schizophrenia, it has been suggested that dysfunctional excitatory drive to cortical PV interneurons causes hypoactivation of these interneurons and it leads to overactivation of glutamatergic projections to the mesolimbic system [10,13]. To explore this issue, we analyzed the colocalization of PSD-95 puncta (a marker for excitatory projections) on PV interneurons. We did not find differences in the RHA and RLA rats either between strains or among the PPI subgroups, which contrasts with a previous study in humans reporting an 18% reduction in mean density of PSD-95+ puncta on PV+ interneurons in schizophrenic patients [18]. However, interestingly, we found strain differences in the mean density of PSD95+ puncta on “PV+c-Fos” interneurons, as the schizophrenia-like RHA rats showed globally lower PSD-95 density on active PV+ cells than the RLA strain. This finding agrees with the idea that the excitatory drive play a role in the activity of PV interneurons, even though PSD-95 seems to be related to PPI performance.

CONCLUSIONS

Our data indicate that the higher neuronal activity in the mPFC during PPI, showed by the RLA rats compared to the RHAs, is partly explained by differences in PV interneurons activity. This result points to reduced cortical inhibition as a likely candidate to explain the schizophrenia-like features observed in the RHA rats and supports the role of impaired cortical inhibition in schizophrenia.

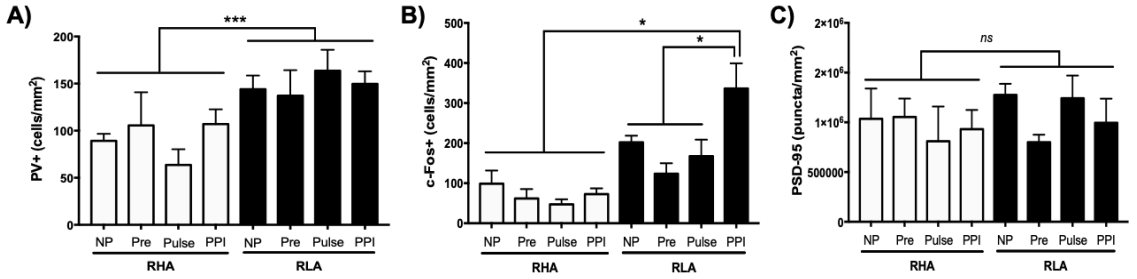

Figure 1. Low PPI is related to reduced medial prefrontal (mPFC) activity (RLA>RHA), while the Roman rats differ in the density of parvalbumin-positive (PV+) cells. **A)** The RHA rats show globally lower PV+ density than the RLAs. **B)** The RLA rats in the PPI condition showed higher c-Fos activity in the mPFC than the RLA control groups (NP, no-pulse; Pre, prepulse-alone; Pulse, pulse-alone) and all the RHA groups. **C)** No differences in the density of PSD-95 puncta were observed. Values are mean±SEM. See "n"/groups in Methods. **p*<0.05; ****p*<0.001; *ns*, non-significant (Duncan's multiple range test).

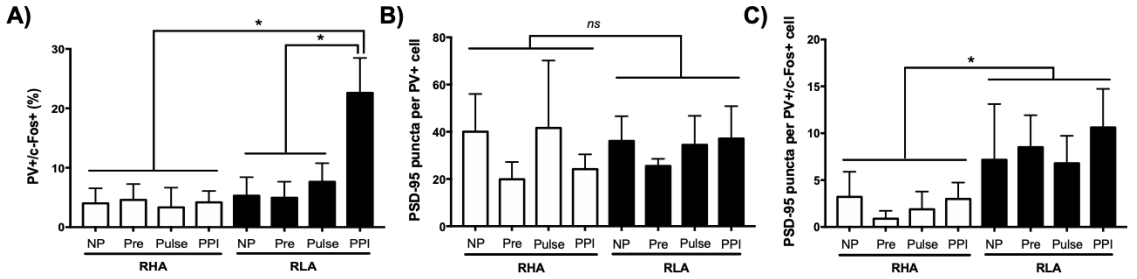

Figure 2. Reduced PPI is related to decreased activity of parvalbumin-positive (PV+) cells in the medial prefrontal of Roman rats. **A)** The RLA rats in the PPI condition show a higher percentage of active PV+ interneurons than the RLA control groups (NP, no-pulse; Pre, prepulse-alone; Pulse, pulse-alone) and all the RHA groups. **B)** No differences in the density of PSD-95 puncta on PV+ interneurons were observed. **C)** The RHA rats show globally lower density of PSD-95 puncta on PV+ interneurons than the RLAs. Values are mean±SEM. See "n"/groups in Methods. **p*<0.05; *ns*, non-significant (Duncan's multiple range test).

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Annex 2

Study 4: "*Oxytocin attenuates sensorimotor gating impairments in inbred Roman rats in line with strain differences in CD38 gene expression*".

Submitted.

Oxytocin attenuates schizophrenia-like sensorimotor gating deficits in inbred Roman rats in line with strain differences in CD38 gene expression.

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ABSTRACT

Prepulse inhibition (PPI) of the startle response is a measure of sensorimotor gating that is impaired in many clinical conditions, including schizophrenia. The inbred Roman high-avoidance (RHA) rats, compared to their low-avoidance (RLA) counterparts, show distinct schizophrenia-like phenotypes, such as spontaneous deficits in PPI accompanied by decreased medial prefrontal cortex (mPFC) activity and volume. Schizophrenia-like deficits are usually attenuated by antipsychotic drugs, but these drugs often produce severe side effects. In order to reduce these side effects, the neuropeptide oxytocin has been proposed as an alternative natural antipsychotic for schizophrenia. Here, we examined the effects of peripheral oxytocin administration (saline, 0.04, and 0.2mg/kg) on PPI in the RHA vs. RLA rats, as well as in the outbred heterogeneous stock (HS) rats. Our results showed that oxytocin increased PPI in the HS rats and attenuated PPI deficits in the RHA rats, but it did not affect PPI in the RLAs. To explore whether these divergent effects were due to differences in the oxytocin pathway, we analyzed gene expression of the oxytocin receptor (*OXTR*) and the regulator of oxytocin release (*CD38*) in the mPFC of the Roman rats. Consistent with the differential oxytocin effects on PPI (RHA>RLA), constitutive *CD38* expression was reduced in the RHA rats compared to the RLAs, while oxytocin administration increased *OXTR* expression in both strains. Overall, the present work reveals that oxytocin administration shows antipsychotic-like effects on PPI in outbred and inbred rats, and it suggests that these effects may depend on basal differences in the oxytocin pathway.

Keywords: schizophrenia, oxytocin, sensorimotor gating, gene expression, qPCR, CD38, OXTR, Roman rats

INTRODUCTION

Prepulse inhibition (PPI) of the startle response is a measure of sensorimotor gating that is impaired in many neuropsychiatric disorders, including schizophrenia. PPI shows the ability of a sensory pre-stimulus to reduce a startle motor response [1] and is modulated by the medial prefrontal cortex (mPFC) in rats [2–5] and the PFC in humans [6–8].

The inbred Roman high-avoidance (RHA) rats display, compared to their low-avoidance counterparts (RLA), several schizophrenia-like behavioral phenotypes, such as (i) impaired PPI, working memory, and latent inhibition [9,10]; (ii) increased exploratory activity [11]; (iii) poorer maternal behavior [12]; and (iv) decreased social behavior (unpublished results). Moreover, molecular, neuroanatomical, and neurochemical analyses have shown that the RHA rats exhibit several brain abnormalities that resemble schizophrenia [13–16]. For instance, the RHA rats show low PPI accompanied by reduced mPFC activity and volume [14]. Furthermore, pharmacological studies have shown that the administration of several antipsychotic drugs in the RHA rats can attenuate schizophrenia-like phenotypes, such as hyperactivity and PPI deficits [17]. In this regard, even though antipsychotic drugs usually attenuate schizophrenia-like impairments in rodents and humans [5,18,19], they produce severe side effects [20]. Importantly, the neuropeptide oxytocin has been proposed as an alternative natural antipsychotic for schizophrenia, which would have the advantage of presenting less side effects than antipsychotics [21–24]. Thus, oxytocin can become a candidate of interest to replace or be adjuvant of the current schizophrenia medication.

Oxytocin is a neuropeptide synthesized in the paraventricular and supraoptic nuclei of the hypothalamus [25,26]. It is known to promote uterine contractions and breastfeeding, but recent evidence has shown that oxytocin might be also critical for social attachments and cognitive-relevant behaviors [23,27]. Specifically, high oxytocin levels in blood plasma are associated with several positive events, such as trust, physical contact with a partner, and reduced hormonal response to stressors or reduced anxiety. In contrast, low levels of oxytocin have been related to several psychiatric conditions, such as autism spectrum disorders, depression, and schizophrenia [21]. In this sense, recent findings have pinpointed that oxytocin administration induces several

antipsychotic-like effects, as it increases trustworthiness in healthy subjects [28], reduces positive and negative symptoms in schizophrenia [29], increases eye gaze in schizophrenia [30], and improves several schizophrenia-like behaviors in rodents, such as social interaction [31] or PPI [32,33].

Oxytocin acts on oxytocin receptors (OXTR) and its secretion is regulated by the CD38 protein [34,35]. In this regard, studies in rodents lacking *OXTR* or *CD38* show impairments in oxytocin-related behaviors, such as maternal nurturing [34,36] and social behavior [34,36]. Interestingly, it has been recently found that mice lacking *CD38* show PFC abnormalities accompanied by alterations in several behaviors that depend on this region [37]. Of note, the *OXTR* and *CD38* genes have been implicated in complex human behaviors, such as the processing of anticipatory, appetitive, and aversive cognitive states [38]. Regarding the role of oxytocin in the brain, findings are generally consistent with the idea that oxytocin has an inhibitory profile, as it reduces glutamate and increases GABA release [39–41].

In order to test the oxytocin effects on PPI, based on previous works by [32,33,42], first we conducted a dose-effect pilot study in the outbred heterogeneous stock (HS) rats. Derived from this study, we examined the effects of oxytocin on PPI in the RHA and RLA rat strains. Then, given the behavioral differences between the Roman rats, we explored differences in the oxytocin pathway (*OXTR* and *CD38*) by real-time quantitative polymerase chain reaction (qPCR). Our major hypotheses were that oxytocin would cause a more marked PPI improvement in the RHA than in the RLA rats and there would be between-strain differences in *OXTR* and *CD38* expression in the mPFC.

MATERIAL AND METHODS

Subjects

We used naïve male HS (n= 46), and inbred RHA (n=54) and RLA (n=45) rats from our breeding colonies (Dept. Psychiatry and Forensic Medicine, Universitat Autònoma de Barcelona). They were aged 3-4 months, weighting 320-390 g. They were housed in pairs in macrolon cages (50 x 25 x 14) and kept with food and water *ad libitum*, maintained under a 12:12h light-dark cycle (lights on at 08:00 a.m.) and with controlled temperature (22 ± 2°C) and humidity (50-70%).

Experimental procedures

Oxytocin administration and behavioral testing were conducted during the light cycle (9:00–14:00). Experiments were carried out in accordance with the Spanish legislation on "Protection of Animals Used for Experimental and Other Scientific Purposes" and the European Communities Directive (2010/63/EU) on this subject. Every effort was made to minimize any suffering of animals used in this study.

First, we carried out a pilot study using the genetically heterogeneous (outbred) HS rats (the "National Institutes of Health genetically heterogeneous" rat stock; see [43,44]), which served as a pilot study designed to select effective doses of oxytocin. Second, we conducted the Roman rat study, in which we aimed to test the hypothesis that oxytocin (the dose/s selected from the pilot HS study) would had improving effects on PPI particularly in the RHA rats.

As illustrated in Fig. 1, before the PPI test, the HS rats underwent a "screening" PPI test to select them according to relatively low PPI levels compared to those found in the HS colony, while the Roman rats did not undergo it.

Oxytocin administration

Following random assignment to group condition, HS groups and RHA and RLA rat strains received subcutaneous injections 30-min before the PPI test of either sterile 0.9% saline vehicle, 0.04mg/kg oxytocin or 0.2mg/kg oxytocin (VWR International Eurolab, S.L., Barcelona, Spain) dissolved in sterile saline, based on previous studies [32,33,42]. Pair-housed rats were injected and behaviorally tested at the same time.

Prepulse inhibition

PPI was conducted in four sound attenuated boxes (SR-Lab Startle Response System, San Diego Instruments, USA), as previously described by our laboratory with minor modifications [11]. Briefly, rats were located in a cylinder, which was situated in a dimly illuminated box and on the top of a platform with a sensor that detects the strength made by the rat in each trial. Noise bursts were presented via a speaker mounted 15cm above the cylinder. After 5 min of acclimation period, 10 "pulse-alone" trials (105dB(A), SPL, 40ms; Startle_Block1) were delivered in order to obtain a stable baseline of startle. After this pulse-alone period, six types of trials were randomly administered ten times (60 trials in total): (i) Pulse alone trials (105dB(A), SPL, 40ms; used to calculate the percentage of PPI; Startle_Block2); (ii) prepulses of 60/65/70/75dB(A), SPL, 20ms, followed by the pulse stimulus with an inter-stimulus interval of 100ms; or (iii) no-stimulus trials (background noise of 55dB). The interval between trials was 15 s (range 10-20 s). The percentage of PPI for each prepulse intensity was calculated by applying the following formula: %PPI = [100-(startle amplitude on prepulse trials/startle amplitude on pulse trials)x100].

For selection of HS rats with relatively low PPI levels, based on a previous work [45], we used a shorter version of the PPI test. This session consisted of (i) a 2-min acclimation period; (ii) 5 pulse-alone trials; and (iii) randomly administered trials of 12 pulse-alone trials and 3 pre-pulse 65dB + pulse trials. The percentage of PPI for selection of HS rats was calculated by applying the following formula: baseline %PPI = [100-(startle amplitude on the 3 prepulse trials/startle amplitude on 12 pulse trials)x100]. This baseline PPI ("PPI_screening" session) allowed us to select a sub-sample of HS rats by their similar and relatively low PPI levels to have room for improvement in the "final" PPI session upon oxytocin administration.

RNA extraction, Reverse Transcription, and qPCR

Immediately after the PPI test, a random and representative sample of RHA and RLA rats belonging to the saline or 0.2mg/kg oxytocin groups were euthanized to conduct qPCR analyses (≥8 rats per group). Their mPFC was dissected out, immediately frozen in liquid nitrogen, and stored at -80°C.

RNA was extracted from the mPFC tissue as described earlier in [46] with minor modifications. Briefly, RNA from tissue samples (around 30µg) was extracted using the

miRNeasy mini kit (Qiagen; cat. no. 217004) in a RNAase-free environment. RNA samples were subjected to DNAse treatment using the Turbo DNA-free kit (Ambion; cat. no. AM1907). RNA concentration was determined using Thermo ScientificTM NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, USA). RNA integrity number (RIN) was determined using the 2100 Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and only samples with RIN value ≥8 were and A260/A280 ratio ≥ 1.8 were included in the analyses. The purified RNA (200ng) was transcribed into complementary DNA (cDNA) using qScript cDNA SuperMix kit (Quanta Biosciences, cat. no. 95048), according to the manufacturer instructions. The cDNA products were diluted 1:5 with RNase/DNase-free water.

Each sample was run on a 96-well plate in duplicates and one reaction contained 3µL diluted cDNA, 0.6µL of each primer (final concentration 300nM), 0.8µL of RNAse-free water, and 5µL of Fast SYBR Green Master Mix (Applied Biosystems, cat. no. 438512). The following primers were used: *GAPDH* (*F*: CATCAAGAAGGTGGTGAAGCA, *R*: CTGTTGAAGTCACAGGAGACA); *RPL13A* (*F*: AGCAGCTCTTGAGGCTAAGG, *R:* GGGTTCACACCAAGAGTCCA); *CD38* (*F*: GAAAGGGAAGCCTACCACGAA, *R*: GCCGGAGGATTTGAGTATAGATCA); *OXTR* (F: TCGTACTGGCCTTCATCGTG, R: TGAAGGCAGAAGCTTCCTTGG). To compare the multiple samples between the assays, a positive control (a pool of cDNA from all samples used as a calibrator) and a negative control (RNase/DNase-free water) were included in each run. To check for gDNA contaminations, a negative control of RNA from which the reverse transcription had been omitted was included for each sample analysis.

All qPCR reactions were run on a QuantStudio3 qPCR system (Applied Biosystems) using a standardized 40-cycle Fast SYBR Green program with annealing/acquisition segments adjusted for each primer sets: *GAPDH*, *RPL13A*, and *CD38* at 61°C; *OXTR* at 58°C. Expression levels of housekeeping genes did not differ across groups. A comparative cycle of threshold fluorescence (Ct) method was used and the relative transcription level of the target gene was normalized to that of average for housekeeping genes (*GAPDH* and *RPL13A*) and expressed as relative quantity to the calibrator sample using the Pfaffl method [47].

Statistics

All the analyses were performed using the "Statistics Package for Social Sciences" (SPSS). Significance level at $p < 0.05$.

3 x 4 ("3 treatments x 4 prepulse intensities") repeated measures ANOVA was applied followed by post hoc Duncan's test to determine differences among the three HS groups, as we had the *a priori* hypothesis that the drug treatment was expected to increase PPI levels [32,33]. 3 x 2 ("3 treatments x 2 startle Blocks – Startle_Block1 and Startle_Block2") repeated measures ANOVA was applied to determine differences among groups in baseline startle response (to pulse-alone trials).

2 x 3 x 4 ("2 strains x 3 treatments x 4 prepulse intensities") repeated measures ANOVA was applied to data from the Roman rat study, followed by post hoc Duncan's test, as we also had the *a priori* hypothesis that the drug treatment would increase PPI levels particularly in the PPI-impaired RHA strain. 2 x 3 x 2 ("2 strains x 3 treatments x 2 startle Blocks – Startle_Block1 and Startle_Block2") repeated measures ANOVA was applied to determine differences among groups.

2 x 2 ("2 strains x 2 treatments") ANOVAs were applied to data from the gene expression study, followed by post hoc Duncan's test to determine differences in gene expression between the RHA and RLA that underwent the two treatment conditions (saline vs. 0.2mg/kg oxytocin). Before the gene expression analysis, the Grubbs outlier test was run, and significant outliers removed (final sample was of 5-8 rats per group).

Within-strain Spearman's rank correlations were performed between PPI scores and gene expression values, with Bonferroni's correction for multiple coefficients.

RESULTS

In the HS rats, 3 x 4 repeated measures ANOVA revealed a “Prepulse” effect [$F(3,45)=24.52$; $p=<0.001$], as PPI was greater at higher prepulse intensities; while there was no “Treatment x Prepulse” effect [$F(6,45)=0.82$; $p=<0.560$]. However, there was a “Treatment” effect on PPI [$F(2,15)=4.20$; $p=0.036$]. Particularly, the HS group that received a dose of 0.04mg/kg showed higher PPI than the saline control group in “PPI_75dB” and “PPI_Total” measures, and did not differ from the dose of 0.2mg/kg (Duncan’s post hoc test, $p<0.05$; Fig. 2). Notably, there were no differences among the three groups in their levels of PPI in the “PPI_Screening” session [$F(2,15)=0.001$; $p=0.999$], indicating that the groups were matched correctly. With regard to baseline startle response, there was a “Block” effect [$F(1,15)=24.778$; $p=<0.001$], indicating startle habituation, while there was no significant “Block x Treatment” [$F(2,15)=0.035$; $p=0.966$] or “Treatment” [$F(1,15)=0.059$; $p=0.943$] effects (see Fig. 2). Thus, oxytocin had a specific effect on PPI in HS rats previously matched for their PPI levels in the “PPI_screening” session.

In the Roman rats, 2 x 3 x 4 repeated measures ANOVA showed a “Prepulse” effect on PPI [$F(3,279)=110.80$; $p=<0.001$], as PPI was higher at higher prepulse intensities; and a “Strain x Prepulse” effect on PPI [$F(3,279)=4.91$; $p=0.009$], as PPI was higher in the RLA rats than their RHA counterparts. No significant “Strain x Treatment x Prepulse” effect on PPI was found [$F(6,279)=1.28$; $p=0.265$]. Importantly, however, there was a “Treatment x Prepulse” effect on PPI [$F(6,279)=2.98$; $p=0.008$], as RHA rats treated with the dose of 0.2mg/kg significantly improved PPI compared to the RHA control saline, and did not statistically differ from the RLA rats at the “PPI_75dB” (Duncan’s post hoc test, $p<0.05$; Fig. 3). Regarding baseline startle, there was a “Block” effect [$F(1,93)=67.77$; $p=<0.001$], indicating startle habituation from Startle_Block1 to Startle_Block2, and a “Strain” effect [$F(1,93)=21.10$; $p=<0.001$], as startle was overall higher in the RLA rats than the RHA rats. However, there was no “Block x Treatment” [$F(2,93)=0.842$; $p=0.434$] or “Block x Strain x Treatment” [$F(2,93)=0.347$; $p=0.708$] effects (see Fig. 3). Thus, oxytocin had a specific improving effect on PPI in RHA rats that was absent in RLA rats.

In line with the behavioral data, as shown in Fig. 4, ANOVA revealed a “Strain” effect on *CD38* gene expression in the mPFC [$F(1,21)=11.61$; $p=0.003$], which was

higher in the RLA rats than the RHA rats. This finding coheres with the fact that oxytocin was effective to improve PPI in the RHAs, while being devoid of effects in the RLA rats. Moreover, there was a “Treatment” effect on *OXTR* expression [$F(1,21)=5.50$; $p=0.029$], as it was globally higher in subjects treated with oxytocin than in saline groups (Fig. 4).

Within-strain Spearman's rank correlations among the two genes and PPI scores (“PPI_75dB” and “PPI_TOTAL”) yielded non-significant results for RHAs (coefficient range 0.03 to – 0.28, all non-significant after Bonferroni correction; n=13) as well as for RLA rats (coefficient range 0.03 to -0.58, all non-significant after Bonferroni correction; n=12). This indicates that there are no systematic associations among individual PPI levels and gene expression, thus allowing to consider the observed (group) gene effects as genuine and not influenced by the PPI session itself.

DISCUSSION

The main aim of the current study was to test the potential antipsychotic-like effects of oxytocin on PPI in our rat model of schizophrenia-relevant symptoms, i.e. the RHA rats. Our results showed that oxytocin was effective to improve PPI performance in inbred RHA rats compared to the saline group, while oxytocin had no effect on the RLAs. Moreover, a novel outstanding finding is that gene expression analysis revealed differences in the oxytocin pathway between the Roman rats, as RHA rats showed lowered expression of *CD38* than their RLA counterparts.

Together with results from the pilot study in the HS rats, PPI findings in the RHA rats are in line with previous studies showing that oxytocin administration reverses the PPI-disrupting effects of amphetamine and the NMDA antagonist MK-801 [32], attenuates the natural PPI deficits in Brown-Norway rats [33] and increases PPI in C57BL/6N mice [48]. In this sense, it has been reported that the sensitivity to the PPI disrupting effects of the NMDA antagonist PCP are increased in oxytocin knock-out mice [22]. Apart from PPI, oxytocin has shown a potential antipsychotic profile in other behaviors and cognitive functions, such as hyperactivity [49], social withdrawal [31,50], aggressive behavior [51], and impaired latent inhibition [42].

The present work adds further evidence on the potential antipsychotic value of oxytocin for two main reasons. First, we show that oxytocin improved PPI in a sub-sample of genetically heterogeneous rats, selected for their relatively low PPI scores in relation to the HS population. The HS rats are known to have higher genetic heterogeneity than other laboratory rats, which confers these results an enhanced translational value in view of the heterogeneity of the human population [11,43]. Second, we report divergent strain-related effects of oxytocin in the Roman rats, as the treatment improved PPI in the PPI-deficient RHA strain but not in the RLA strain, suggesting possible endogenous differences in oxytocin related to PPI.

To see whether these divergent effects in the Roman rats were related to differences in the oxytocin pathway, we analyzed gene expression of the oxytocin receptor (*OXTR*) and the oxytocin regulator (*CD38*) in the mPFC. Consistent with the differential oxytocin effects on PPI (RHA>RLA), our data revealed that *CD38* expression in the mPFC was reduced in RHA rats compared to their RLA counterparts. Interestingly,

loss of *CD38*, which is associated with low oxytocin levels [34], causes abnormalities in PFC-dependent behaviors [37]. Together with the fact that RHA rats display attentional and sensorimotor gating deficits, reduced social behavior and impaired working memory, the reduced *CD38* expression in the PFC of RHA rats may be consistent with alterations in the oxytocin pathway reported from animal models of schizophrenia-relevant features [22,37,52–54] and human schizophrenia studies [21,35,55,56]. For example, the vasopressin-deficient Brattleboro rats show abnormal oxytocin release in response to stress [57], as well as several natural schizophrenia-like deficits, including impairments in PPI, social discrimination, and memory [58]. Thus, it seems reasonable that the RHA rats would benefit more from oxytocin administration than the RLAs. On the other hand, *OXTR* expression was increased in both RHA and RLA rats that underwent oxytocin administration. In agreement with our data, a recent finding indicates that oxytocin administration reverses reduced *OXTR* expression in the mPFC caused by prolonged stress [59]. In addition, the schizophrenia-like Wisket rats show reduced *OXTR* expression related to decreased acute pain sensitivity [54]. Moreover, it has been reported that *OXTR* expression is reduced in post-mortem brains of schizophrenic patients [56]. Regarding oxytocin administration, the entry of exogenously administered oxytocin into the brain is still a matter of controversy [48,60]. However, our data suggest that oxytocin administration increased *OXTR* in the mPFC of the Roman rats. Thus, oxytocin increased *OXTR* in the mPFC in both rat strains, but not *CD38*. Since the Roman rats were euthanized around 1 hour after the administration of oxytocin (when they finished the PPI test), the possibility remains that the *CD38* gene in the mPFC, if altered by oxytocin administration, had already returned to its basal levels (RHA < RLA), while oxytocin had more long-lasting effects on the *OXTR*. Also, even though we did not observe “treatment” effect in *CD38* levels in the PFC, we cannot rule out that exogenous oxytocin administration increases *CD38* in the hypothalamus [26]. On the other hand, the absence of significant gene-PPI correlations indicates that there are no systematic associations among individual expression of both genes and “PPI_75dB” and “PPI_Total” levels. This suggests that the gene expression effects observed here are more likely due to the “strain” or “treatment” effects rather than influences from the PPI experience or PPI differences.

Here, we focused on the mPFC, as previous studies have indicated differences in volume, activity, and gene expression between the Roman rats in this region [13,14,16,61]. However, focusing only on the mPFC is a limitation of the present study,

as for instance the hippocampus or the nucleus accumbens have also been involved in PPI modulation [4,14,62] and the hypothalamus is critical for oxytocin synthesis [25,26]. Future studies will be addressed to a wider range of brain areas.

Regarding the neural mechanisms through which oxytocin improves PPI, one could speculate that it does it through the inhibition of dopamine transmission, as oxytocin attenuates the PPI disrupting effects of amphetamine [32] and the cocaine-induced mesolimbic dopamine release and hyperactivity [49]. Accordingly, impaired dopamine transmission has been described as one of the fundamental factors in the etiopathology of psychotic symptoms of schizophrenia [63]. However, recent findings have highlighted that dopamine transmission is regulated by the balance between neocortical excitatory glutamatergic and inhibitory GABAergic neurons, and this balance is primarily affected in schizophrenia [63,64]. Specifically, schizophrenic patients would have excessive glutamate release and reduced GABA inhibition. In this sense, previous findings indicate that, compared to RLAs, the RHA rats could have an excessive glutamatergic and dopaminergic tone in the PFC and striatum that could drive an imbalance between excitation and inhibition [16,65]. Consequently, as oxytocin administration has been associated with a reduction in glutamate and an increase in GABA release [39–41,48], and *OXTR* is expressed in GABAergic interneurons [66], it is possible that oxytocin reduces dopamine transmission by increasing inhibition in mPFC neurons.

CONCLUSIONS

Overall, the present work reveals that oxytocin administration shows antipsychotic-like effects on PPI in both outbred HS and inbred RHA rats, and it suggests that these effects may depend on basal differences in the oxytocin pathway. To the best of our knowledge, this is the first study that combines the oxytocin effects on PPI and gene expression of oxytocin-related genes in the mPFC in a genetically based rat model of schizophrenia-like features. Our present results support the notion that oxytocin administration regulates sensorimotor gating in a strain-dependent manner, while also highlighting basal differences in *CD38* expression in the mPFC between the RHA and RLA rat strains that may be relevant for neurobiological research on schizophrenia.

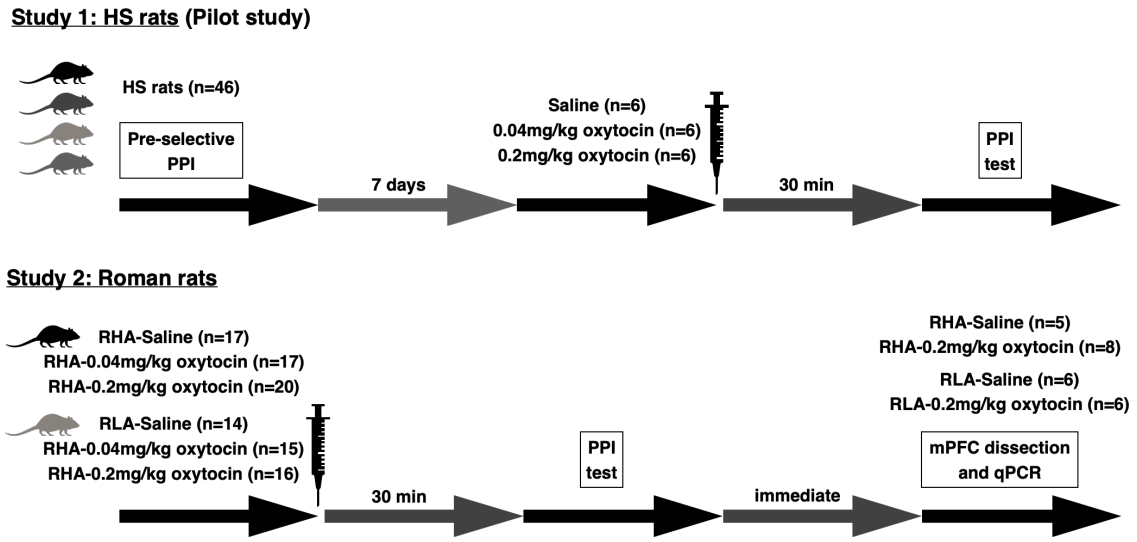

Figure 1. Experimental timeline. Study 1: HS rats underwent a short pre-selective version of the PPI test that was used to conform three similar random groups with relative low PPI. After a 7-day rest period, animals were injected with saline solution, 0.04mg/kg oxytocin, or 0.2mg/kg oxytocin 30-min before a PPI test. Study 2: RHA and RLA rats were randomly distributed in three groups that received saline solution, 0.04mg/kg oxytocin, or 0.2mg/kg oxytocin 30-min before a PPI test. Immediately after ending the PPI session, random and representative samples of the saline and 0.2mg/kg oxytocin from both strains were euthanized and the mPFC was dissected out to conduct gene expression analyses by quantitative polymerase chain reaction (qPCR).

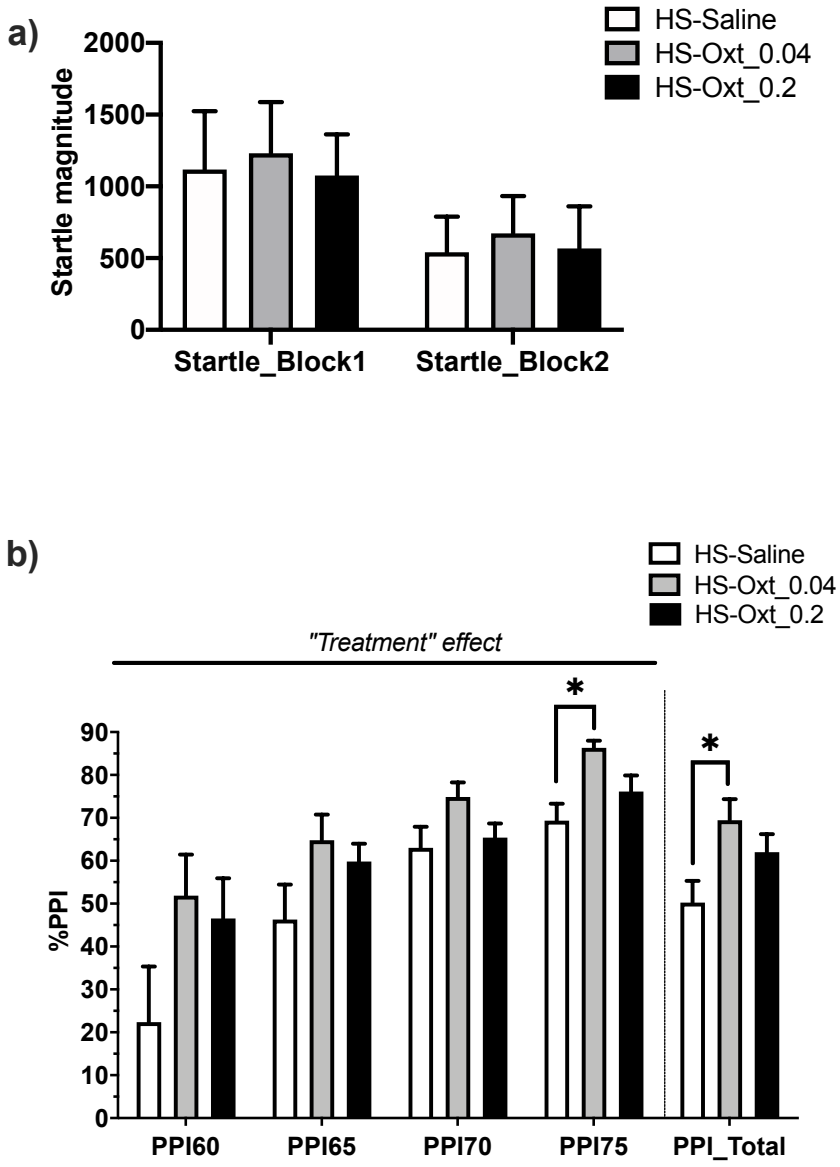

Figure 2. Oxytocin improves PPI in the HS rats treated with oxytocin compared to saline group and has no effects on startle response. **a)** No "treatment" effects were observed among the three groups of HS rats treated with saline solution, oxytocin 0.04mg/kg, or 0.2mg/kg in the baseline startle blocks (1 and 2). **b)** A significant "treatment" effect was observed among the three groups of HS rats across the different prepulse intensities (PPI60, 65, 70, and 75dB), as PPI was higher in the HS rat group treated with oxytocin 0.04mg/kg compared to the saline group. *, $p<0.05$ (Duncan's multiple range test).

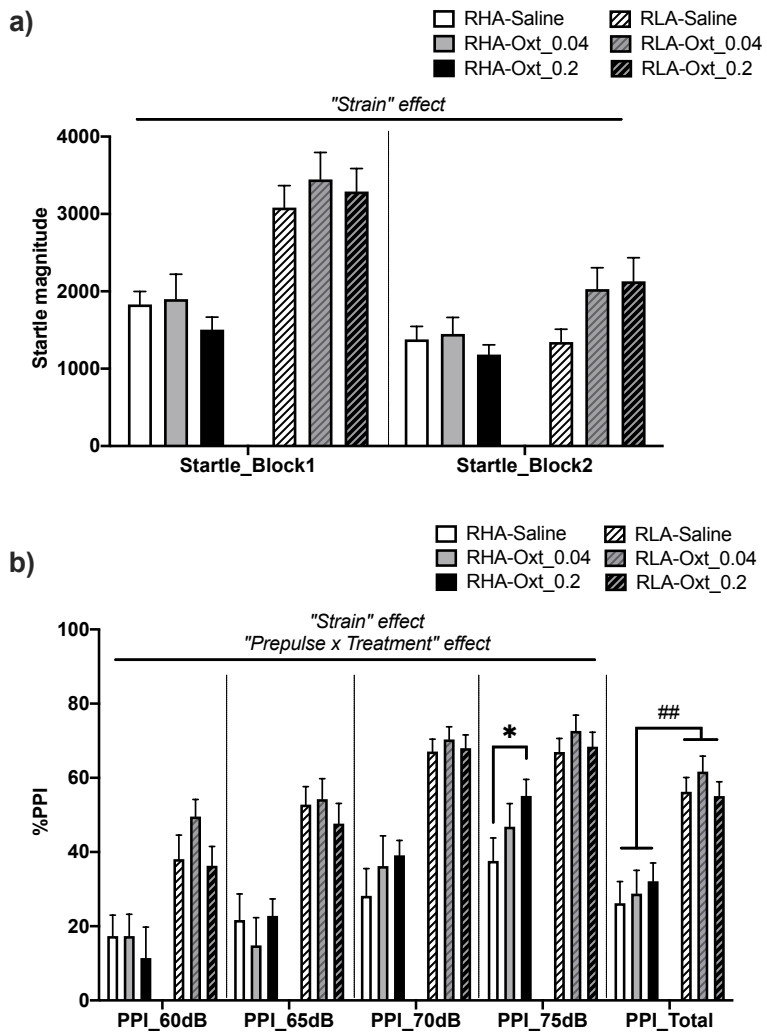

Figure 3. Oxytocin attenuates PPI deficits in the RHA rats at the 75dB prepulse intensity and has no effects on startle response. **a)** No "treatment" or "strain x treatment" effects were observed among the six groups of RHA and RLA rats treated with saline solution, oxytocin 0.04mg/kg, or 0.2mg/kg in the baseline startle blocks (1 and 2). However, there was a "strain" effect, as startle was globally higher in the RLA rats than in the RHAs. **b)** A significant "prepulse x treatment" effect was observed among groups across the different prepulse intensities (PPI_60, 65, 70, and 75dB), as oxytocin improved PPI in the RHA rats treated with oxytocin 0.2mg/kg compared to saline group at the 75dB prepulse intensity. *, $p<0.05$; ##, $p<0.01$ (Duncan's multiple range test).

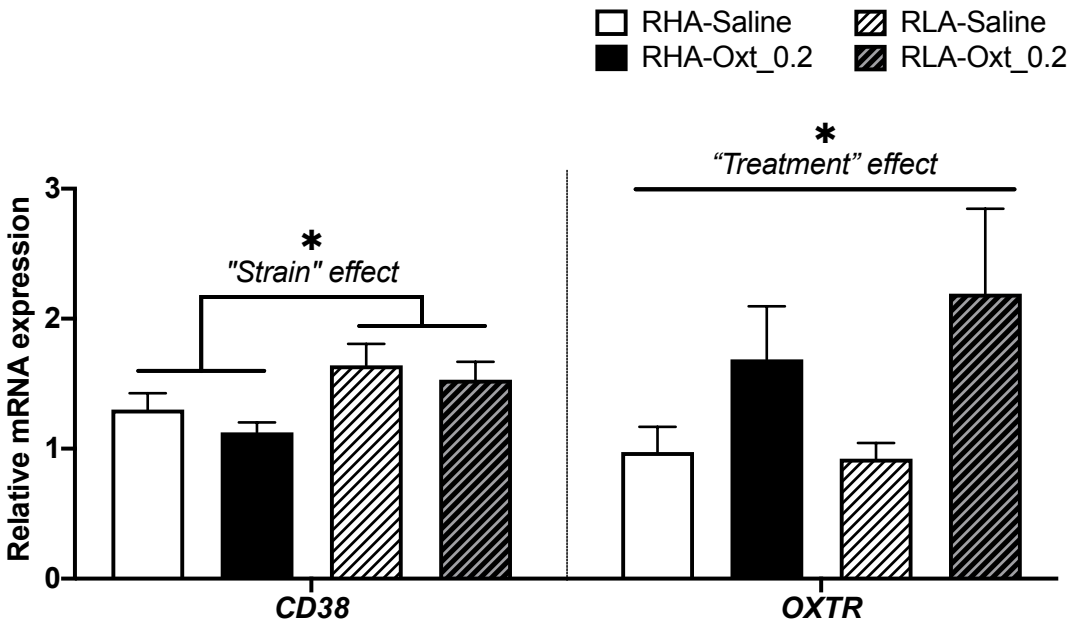

Figure 4. The RHA rats show lower *CD38* expression than the RLA rats, while oxytocin increases *OXTR* expression in the medial prefrontal cortex (mPFC). A significant "strain" effect was observed in *CD38* relative mRNA expression in the mPFC, as the RLA rats globally showed higher expression than the RHAs. On the other hand, there was a significant "treatment" effect in *OXTR* expression in the mPFC, as both RHA and RLA rats treated with oxytocin 0.2mg/kg showed higher expression than RHA and RLA rats treated with saline solution. *, $p<0.05$.

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