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Efectos de la estimulación neonatal sobre alteraciones cognitivas, atencionales y de maduración cortical en un modelo animal genético de rasgos relacionados con la esquizofrenia

NATALIA PERALTA VALLEJO

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Efectos de la estimulación neonatal sobre alteraciones cognitivas, atencionales y de maduración cortical en un modelo animal genético de rasgos relacionados con la esquizofrenia

DISERTACIÓN DOCTORAL
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RESUMEN

Esta Tesis Doctoral da continuidad a la última década de trabajo realizado por el grupo de investigación, en la cual se han investigado los factores neurobiológicos, comportamentales y genéticos de las ratas "*Roman* de Alta Evitación" (RHA; vs. sus contrapartes, las "*Roman* de baja evitación", o RLA), con el fin de explorar su validez como modelo animal para la sintomatología positiva, negativa y cognitiva similar a la esquizofrenia. Además, se ha puesto particular interés en la investigación de los efectos positivos y duraderos de un tratamiento de estimulación neonatal (NH, del inglés *"Neonatal handling"*) sobre los aspectos conductuales, cognitivos y, por primera vez, genéticos, del modelo animal.

Para el desarrollo de los objetivos, se establecieron tres estudios. En el primer estudio se investigó la asociación entre el déficit en la prueba de inhibición de la respuesta de sobresalto por un prepulso (PPI) y el rendimiento deficiente en la conducta social, así como el deterioro en diversas tareas de memoria en las ratas genéticamente heterogéneas HS (*"Genetically Heterogeneous Stock rat"*). El propósito fue identificar comportamientos alterados en paradigmas atencionales que puedan predecir déficits en conducta social o cognitivos en modelos animales de rasgos relevantes para la esquizofrenia, como los síntomas negativos y cognitivos.

El segundo estudio se centró, por primera vez, en la evaluación conjunta de las diferencias en la exploración de objetos nuevos (NOE), la interacción social (IS), la inhibición prepulso de la respuesta de sobresalto (PPI), y diferentes tareas de memoria y aprendizaje en hembras de ambas cepas de ratas *Roman*. También se evaluaron los efectos duraderos del tratamiento con NH en los perfiles RHA frente a RLA en estas pruebas y tareas, y las asociaciones entre el rendimiento en las diferentes pruebas.

En el tercer estudio, se evaluaron las diferencias conductuales y cognitivas relacionadas con la esquizofrenia en ratas macho de ambas cepas *Roman* en dos periodos del desarrollo, adolescentes y adultos. Se analizó la expresión de ocho genes asociados con el neurodesarrollo y la plasticidad sináptica (*Cables1, Cdk5, Snap25, Homer1, Nrg1, Bdnf, Grin2b y Drd1*) en el córtex prefrontal (CPF). Además, se examinó el efecto de la

estimulación neonatal en los rasgos conductuales, cognitivos y moleculares en ambas cepas de ratas y en las dos edades.

Los resultados del primer estudio mostraron que, en la tarea de IS, las ratas HS de baja PPI mostraron un comportamiento social disminuido en comparación con las ratas HS de alta PPI. Además, en comparación con las ratas HS de alta PPI, el grupo de baja PPI mostró un rendimiento en la memoria espacial de trabajo más pobre, flexibilidad cognitiva deteriorada (en una tarea de "reversión" de aprendizaje espacial, o *"reversal task"*) y un déficit en la memoria espacial a largo plazo.

Los hallazgos del segundo estudio muestran comportamiento de exploración disminuido, y comportamiento de acicalamiento aumentado en las ratas hembras RLA (vs. las RHA), y que el NH normalizó ambos comportamientos a niveles similares a los de las ratas RHA. En la prueba IS, las ratas RLA mostraron una interacción social disminuida, que fue mejorada por el NH. Las hembras RHA mostraron un déficit de PPI, que no se vio afectado por el NH. Las tareas de aprendizaje/memoria espacial en el "laberinto acuático de Morris" (MWM, del inglés *"Morris water maze"*) mostraron alteraciones de la memoria de trabajo, el aprendizaje/memoria de referencia y el aprendizaje de "reversión" espacial (es decir, flexibilidad cognitiva) en las hembras RHA. El aprendizaje espacial de referencia y la flexibilidad cognitiva (es decir, la tarea de "reversión") mostraron cierta mejoría en las ratas (principalmente en las RHA) que habían recibido NH durante las tres primeras semanas de vida.

Los resultados del tercer estudio muestran diferencias conductuales y atencionales entre cepas incluso desde la adolescencia, y dichas diferencias son aún más marcadas en la adultez. Los resultados conductuales en adultos son consistentes con resultados previos, y por primera vez se evidenció, disminución de la actividad de acicalamiento, aumento de la actividad locomotora y un déficit de PPI en adolescentes de la cepa RHA. También, existen efectos en la expresión genética dependientes de la cepa y de la edad. Finalmente, se han observado importantes efectos de la estimulación neonatal (NH) sobre los rasgos conductuales, cognitivos y moleculares, predominantemente en la cepa RHA, que dependen de la edad. Es particularmente destacable que en las ratas RHA el tratamiento de NH incrementa, durante la adolescencia, la expresión génica en la fase postsináptica (genes *Ngr1, Homer1, Bdnf, Drd1, Grin2b*), así como, a nivel presináptico,

la expresión de *Cables1 y Cdk5*, mientras que disminuye drásticamente (hasta el nivel de las ratas RLA control) la expresión de *Snap25* tanto en las RHA adolescentes como en las adultas.

Los resultados de esta investigación han podido determinar las similitudes y diferencias en el perfil atencional, de un tipo de conducta social, y de la memoria y aprendizaje espacial entre ratas machos y hembras de las cepas *Roman*, las cuales aportan evidencia para establecer a la rata RHA como modelo animal de ciertos rasgos y síntomas asociados a la esquizofrenia. Por otro lado, se ha identificado que la estimulación neonatal (NH) tiene un efecto positivo perdurable sobre los rasgos conductuales y cognitivos de ratas machos y hembras de las cepas *Roman*. Además, el NH modula la expresión genética en el CPF en ratas macho RHA adolescentes y adultas.

ABSTRACT

This doctoral thesis builds upon the last decade of research conducted by the research group, in which the neurobiological, behavioral, and genetic profiles of RHA (Roman high-avoidance) rats, vs. their RLA ("Roman low-avoidance") counterparts, have been investigated, aiming to propose the former strain as an animal model for positive, negative and cognitive symptomatology similar to schizophrenia. Additionally, particular interest has been placed on exploring the positive and lasting effects of neonatal handling-stimulation (NH) treatment on the behavioral, cognitive, and, for the first time, genetic aspects of the animal model.

To achieve the objectives, three studies were established. The first study investigated the association between deficits in the prepulse inhibition of the startle response (PPI) and poor performance in social behavior, as well as impairment in various memory tasks in genetically heterogeneous HS rats. The purpose was to identify altered behaviors in attentional paradigms that may predict behavioral or cognitive deficits in animal models of traits relevant to schizophrenia, such as negative and cognitive symptoms.

The second study focused, for the first time, on the combined evaluation of differences in novel object exploration (NOE), social interaction (SI), prepulse inhibition of startle response (PPI), and various memory and learning tasks in females of both Roman rat strains. The lasting effects of NH treatment on RHA and RLA profiles in these tests and tasks were also evaluated.

In the third study, behavioral and cognitive differences related to schizophrenia were evaluated in males of both Roman rat strains at two developmental periods, adolescents and adults. The expression of 8 genes associated with neurodevelopment and synaptic plasticity (*Cables1, Cdk5, Snap25, Homer1, Nrg1, Bdnf, Grin2b, and Drd1*) in the prefrontal cortex was analyzed. Additionally, the effect of NH on behavioral, cognitive, and molecular (gene expression) traits in both strains of rats at the two ages was examined.

The results of the first study showed that in the SI task, low PPI-stratified rats (i.e., HS rats stratified for their low PPI scores) showed decreased social behavior compared to

high PPI rats. Additionally, compared to high PPI HS rats, the low PPI group showed poorer spatial working memory performance, impaired cognitive flexibility (in a reversal task) and worsened long-term spatial memory.

The findings of the second study show reduced novel object exploration and increased grooming behavior in female RLA rats (relative to RHAs), both of which were normalized by NH to levels similar to those shown by RHAs. In the SI test, RLA rats showed decreased social interaction, which was rescued by NH. Female RHA rats showed a deficit in PPI, which was not affected by NH. Spatial learning/memory tasks in the "Morris water maze" (MWM) showed alterations in working memory, reference learning/memory, and spatial reversal learning (i.e., cognitive flexibility) in female RHA rats. Spatial reference learning and cognitive flexibility (i.e., a spatial reversal task) showed some improvement in rats (mainly in RHA) that had received NH during the first three weeks of life.

The results of the third study show behavioral and attentional differences between the strains even from adolescence, which are still more marked in adulthood. Behavioral results in adults are consistent with previous findings, and for the first time, decreased grooming activity, increased locomotor activity, and a deficit in PPI were evidenced in adolescent RHA rats. In addition, there are effects on gene expression dependent on strain and age. Finally, we have observed important effects of neonatal stimulation (NH) on behavioral, cognitive, and molecular (gene expression) traits, predominantly in RHA rats, which depend on age. It is outstanding that in adolescent RHA rats NH treatment increases gene expression at the postsynaptic level (genes *Ngr1, Homer1, Bdnf, Drd1, Grin2b*), as well as, at the presynaptic level, the expression of *Cables1* and *Cdk5*, while the treatment dramatically decreases (to the level of control RLA rats) the expression of *Snap25* in both adolescent and adult RHAs.

The results of this research provide evidence on the similarities, and some differences, in the attentional, social behavior, learning and memory profiles between male and female rats of the Roman rat strains, which provide some support to propose RHA rats as an animal model of some traits (e.g., attentional and cognitive) and symptoms associated with schizophrenia. On the other hand, it has been shown that neonatal stimulation (NH) has positive and lasting effects on behavioral and attentional/cognitive

traits in male and female Roman rats. Additionally, NH modulates gene expression in prefrontal cortex in adolescent and adult male RHA rats.

ABREVIACIONES

5-CSRTT: Tarea de tiempo de reacción en serie de 5 elecciones

5-HT: Serotonina

5-HT1-7,1A,2A,2C,3: Subtipos del receptor de la serotonina

ADN: Ácido desoxirribonucleico

AMPA: Ácido α-amino-3-hidroxi-5-metilo-4-isoxazolpropiónico

APO-SUS: Ratas susceptibles a apomorfina

ATV: Área tegmental ventral

BDNF: Factor neurotrófico derivado del cerebro

BRAT: Ratas Brattleboro

C-ABL: Tirosina-proteína kinasa ABL1

CACNA1: Subunidad alfa 1A, de los canales de calcio dependiente de voltaje

CACNA1C: Subunidad alfa 1C, de los canales de calcio dependiente de voltaje

CACNB2: Subunidad auxiliar Beta 2 de los canales de calcio activados por voltaje

CDK5: Kinasa dependiente de ciclina 5

DPN: Días postnatales

COMT: Catechol-O-methyltransferasa

CPF: Córtex prefrontal

D2: Receptores dopaminérgicos D2

DA: Dopamina

Drd1: Receptor de dopamina D1

DSM: Manual Diagnóstico y Estadístico de Trastornos mentales

ErbB: Familia de proteínas de receptores dependientes de kinasa

ErbB4: Receptor Erb-B2 Tirosina Kinasa 4

GABA: Ácido gamma-aminobutírico

GRIA1: Subunidad 1 del receptor ionotrópico de glutamato tipo AMPA

GRIND2B: subunidad 2b del receptor ionotrópico de glutamato NMDA

GRM3: Receptor 3 metabotrópico del glutamato

HPC: Hipocampo

HS: Cepa de ratas genéticamente heterogéneas

IGF-1: Factor de crecimiento insulínico tipo 1

IS: Prueba de Interacción social

LSD: Ácido lisérgico

MK801: Dizocilpina

mRNA: Ácido ribonucleico mensajero

MRI: Imagen por Resonancia Magnética

MUNC18: Familia de proteínas de mamíferos no coordinados-18

NH: Estimulación neonatal

NIH: Instituto Nacional de Salud (USA)

NMDA: Ácido N-metil-D-aspártico

Nrg1: Neuregulina1

Nsf: Factor sensible a la N-etilmaleimida

P35: Proteína sustrato activador de CDK5

PCP: Fenciclidina

PET: Tomografía por emisión de positrones

PPI: Inhibición de la respuesta de sobresalto por un prepulso

PSD: Proteína de densidad sináptica

PT: Tarea de memoria espacial

RHA: Ratas *Roman* de alta evitación

RLA: Ratas *Roman* de baja evitación

RT: Tarea de flexibilidad cognitiva, o de "reversión" espacial

SGA: Antipsicóticos de segunda generación

SHR: Ratas espontáneamente hipertensas

SNAP 25: Proteína 25 asociada al sinaptosoma

SNARE: Receptores de proteínas de fijación soluble de N-etilmaleimida

SNC: Sistema nervioso central

SNP: Polimorfismos de un solo nucleótido

TT: Prueba de memoria de referencia espacial a largo plazo

SWM: Tarea de memoria de trabajo espacial

MWM: Laberinto acuático de Morris

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1. INTRODUCCIÓN

1.1. Antecedentes

Según el Instituto Nacional de la Salud Mental (National Institute of Mental Health NIMH, 2023) la esquizofrenia es un trastorno mental, grave, crónico e incapacitante. Afecta a casi el 1 % de la población mundial y se caracteriza porque quien lo padece tiene una percepción alterada de la realidad, cambios en la personalidad, razonamiento y comportamiento desordenado (Organización Mundial de la Salud., 2023). La edad de inicio de los síntomas difiere: en los hombres suele presentarse entre los 15 a 25 años y en las mujeres entre los 25 a 35 años (Li et al., 2022). Actualmente, se cree que la prevalencia entre los hombres es mayor (32,13%) que entre las mujeres (26,95%,) (Ferrara et al., 2024; Li et al., 2016). Existen diferencias con respecto al desarrollo de la enfermedad: las mujeres son más propensas a experimentar síntomas positivos y tienden a tener una mejor respuesta al tratamiento que los hombres. Por otro lado, los hombres tienden a presentar síntomas negativos, tienen un inicio más temprano del trastorno y suelen tener peores resultados a largo plazo (Leger and Neill, 2016).

1.2. Sintomatología

Aunque la esquizofrenia se considera un desorden mental complejo desde hace más de un siglo, sus definiciones y límites han cambiado a lo largo de este periodo (Tandon et al., 2013; Valle, 2020). Los profesionales clínicos han trabajado en el concepto y los criterios de diagnóstico, elaborando el "Manual Diagnóstico y Estadístico de Trastornos mentales" o DSM-V (por sus siglas en inglés, "*Diagnostic and Statistical Manual of Mental Disorders", Fifth Edition*), escrito por la Asociación Estadounidense de Psiquiatría en el 2013. Esta información ha promovido una mayor concordancia diagnóstica y, por lo tanto, una coherencia de los informes de estadísticas sanitarias en todo el mundo (American Psychiatric Association, 2013). Tomando en cuenta los criterios del DSM-V, actualmente se categorizan los principales síntomas de la esquizofrenia en tres grupos: síntomas positivos, síntomas negativos y síntomas cognitivos (Bowie and Harvey, 2006; Crawford and Go, 2022; Fletcher and Frith, 2009; Wu et al., 2021).

1.2.1. Síntomas positivos

Los síntomas positivos de la esquizofrenia están relacionados con la percepción que tiene el individuo sobre la realidad. Estas experiencias sensoriales pueden conducir a

cambios en el comportamiento de la persona. Los síntomas son: alucinaciones, delirios, pensamiento y discurso desorganizado (Crawford and Go, 2022; Fletcher and Frith, 2009).

1.2.2. Síntomas negativos

Los síntomas negativos se caracterizan por déficits motivacionales y conductuales. Dentro de la reducción motivacional/emocional se encuentran la abulia, la alogia y la anhedonia. Respecto a los rasgos conductuales, las personas con esquizofrenia suelen presentar asocialidad, siendo este un retraimiento de la conducta social. Este aspecto a menudo suele ser el más debilitante de la enfermedad. (Crawford and Go, 2022; Wu et al., 2021).

1.2.3. Síntomas cognitivos

Las personas con esquizofrenia suelen presentar dificultades en la atención, en la memoria de trabajo, en el aprendizaje, en la fluidez verbal y las funciones ejecutivas (Bowie and Harvey, 2006). Los tratamientos farmacológicos por sí solos no han demostrado tener un impacto claro sobre la naturaleza de las dificultades cognitivas. Sin embargo, combinados con un enfoque cognitivo-conductual podrían presentar una mejora de dichos déficits (Bowie and Harvey, 2006; Buchanan et al., 2007; Goff et al., 2011).

1.3. Neuroanatomía

En los estudios de Imagen por Resonancia Magnética (MRI, por sus siglas en inglés, *Magnetic Resonance Imaging*) en pacientes con esquizofrenia, los hallazgos más consistentes incluyen cambios estructurales y funcionales en la corteza cerebral. Con respecto a los primeros, se evidenció una reducción del volumen de materia gris de las áreas temporal medial, temporal superior y prefrontal (Kahn et al., 2015; Wright et al., 2000). Esta reducción cortical no refleja la pérdida de cuerpos celulares sino, una menor complejidad dendrítica y densidad sináptica, lo que podría afectar a la comunicación interneuronal (Glantz and Lewis, 2000). Dicha disminución en la comunicación neuronal, entre las regiones cerebrales, también conduce a cambios funcionales, debido a la pérdida de volumen de la sustancia blanca, responsable de las conexiones estructurales entre las regiones cerebrales. Es importante destacar que estas regiones están

involucradas directamente en la memoria, el procesamiento de la información auditiva y la toma de decisiones, funciones que a su vez están relacionadas con los síntomas de la enfermedad (Karlsgodt et al., 2010).

Este tipo de herramientas de neuroimagen también han permitido identificar alteraciones en regiones más específicas, que se han asociado con la esquizofrenia. Entre las principales estructuras se encuentra el córtex prefrontal (CPF), cuya función principal es integrar la información sensorial y la información motora procedente de regiones cerebrales subcorticales, junto con experiencias pasadas, para proporcionar respuestas adecuadas (Martucci et al., 2019; Siddiqui et al., 2008). Los estudios de neuroimagen molecular han informado de anormalidades estructurales y funcionales en el CPF de pacientes con esquizofrenia, lo que en parte podría estar relacionado con alteraciones en la neurotransmisión por dopamina (DA) (McCutcheon et al., 2020).

Por otro lado, la reducción del volumen del hipocampo (HPC) es uno de los hallazgos más consistentes encontrados por medio de MRI en pacientes esquizofrénicos (Anand and Dhikav, 2012; Dhikav and Anand, 2007). Actualmente hay datos convincentes que sugieren que existen aberraciones anatómicas y funcionales debidas a alteraciones neuronales en el HPC de dichos pacientes (Bast, 2011; Dhikav and Anand, 2007). Aunque la reducción del volumen hipocámpico en la esquizofrenia es modesta, y no tan marcada en comparación con la enfermedad de Alzheimer, por ejemplo, las alteraciones bioquímicas y funcionales proporcionan pruebas fiables de la implicación de esta región en la fisiopatología del trastorno (Dhikav and Anand, 2007). Esta área cerebral desempeña un papel relevante en la regulación de varias conductas y funciones cognitivas que están alteradas en la esquizofrenia, como el aprendizaje y la memoria (Lieberman et al., 2018). El HPC regula estas funciones cognitivas en conexión con el CPF. De hecho, la conectividad entre el CPF y el HPC se encuentra alterada en pacientes esquizofrénicos (Kahn et al., 2015; McCutcheon et al., 2020).

1.4. Etiología

Actualmente hay acuerdo en que la esquizofrenia tiene una etiología multifactorial que involucra componentes ambientales, genéticos y fisiológicos a lo largo del neurodesarrollo u ontogenia (p. ej. Millan et al., 2016; ver Figura 1). Aunque estos

factores no están del todo comprendidos, en los últimos años, la ciencia ha hecho importantes avances en identificar variables de riesgo para la esquizofrenia.

Hechos históricos como la hambruna de invierno holandesa de 1944 o la hambruna de China de 1959-1961 ya sugirieron una relación entre las condiciones ambientales (intrauterinas y extrauterinas) y la esquizofrenia (St Clair et al., 2005). Además, otros estudios sugieren que la vivencia de sucesos traumáticos, como violencia, aislamiento social o migraciones, especialmente en edades tempranas, podrían aumentar la susceptibilidad a presentar este padecimiento (Bentall et al., 2014; Bourque et al., 2011; Morgan et al., 2008). Otra variable de riesgo es el consumo de drogas, tales como la marihuana, los psicoestimulantes, alcohol o tabaco (Bourque et al., 2011; Susser, 1996)

Existe un factor hereditario que desempeña un papel fundamental**.** Por ejemplo, se evidenció que el 46% de gemelos monocigóticos tenían riesgo de desarrollar esquizofrenia, o hay un 40% de riesgo de presentar la enfermedad si ambos progenitores están afectados (Cardno and Gottesman, 2000; Mortensen et al., 1999). Además, estudios moleculares han asociado determinados genes con la susceptibilidad a la esquizofrenia. Entre ellos está el gen *Neuregulina-1* (*Nrg1*), que participa durante el neurodesarrollo e interviene en la señalización del glutamato. También se ha estudiado ampliamente al gen *Bdnf* (por sus siglas en inglés, *Brain Derived Neurotrophic Factor*), que contribuye a la neurogénesis del cerebro y la neuroplasticidad (Elfving et al., 2019; Roth et al., 2009; Sønderstrup et al., 2023; Stefansson et al., 2002).

Finalmente, las teorías más recientes postulan que la ocurrencia de la enfermedad puede ser el resultado de anomalías en la neurotransmisión central, como la hiperactividad dopaminérgica, serotoninérgica o alteraciones en la transmisión glutamatérgica y GABAérgica (Hu et al., 2015; Kapur and Remington, 1996; Matthysse, 1974; Ochoa-de la Paz et al., 2021; Stahl, 2018) (Figura 2).

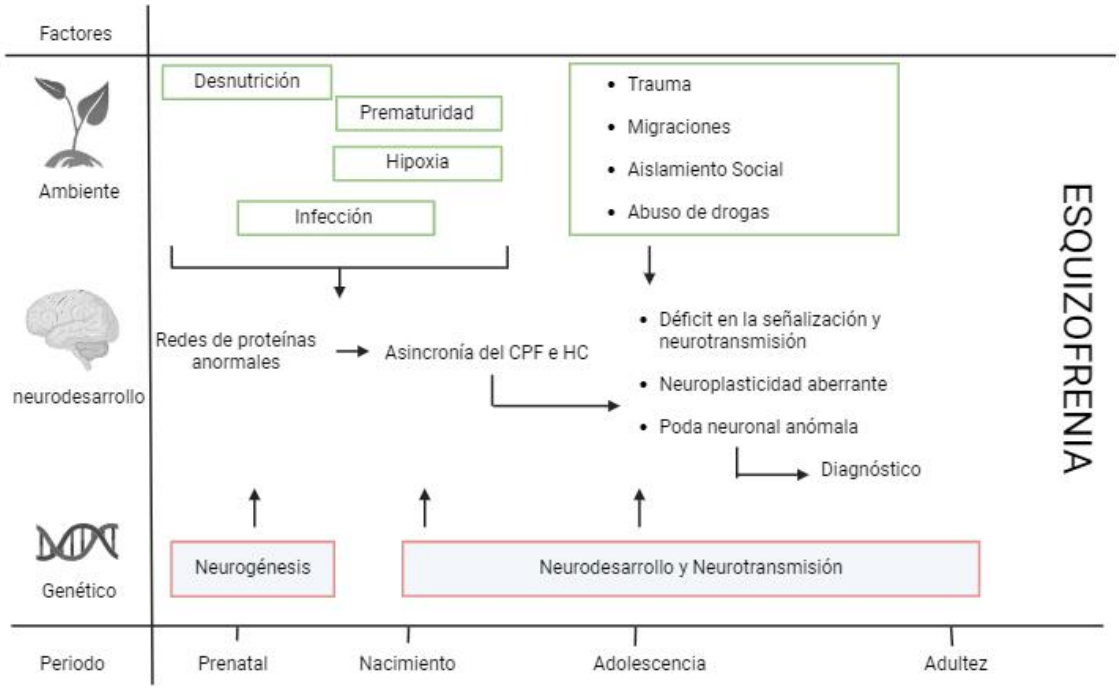


Figura 1. Asociación entre los factores genéticos y ambientales con el neurodesarrollo y su efecto en el cerebro en relación con la esquizofrenia. En las diferentes etapas de la ontogenia, la actividad de genes específicos puede modular o influir sobre el desarrollo de la enfermedad, y esto se suma a las condiciones ambientales principalmente desfavorables, como factores de riesgo para desarrollar el desorden. Adaptado de Millan et al. (2016).

1.4.1. Hipótesis Dopaminérgica

La dopamina (DA) es un neurotransmisor producido por las neuronas dopaminérgicas del área tegmental ventral (ATV) y en la sustancia negra. Estas neuronas proyectan hacia todo el cerebro por tres vías principales: nigroestriada, mesolímbica y mesocortical (Berke, 2018; Björklund and Dunnett, 2007; Lerner et al., 2021).

Esta hipótesis ha sido sustentada por la investigación fisiológica de las rutas dopaminérgicas a partir de tres observaciones principales: la primera, indica que los agentes liberadores o potenciadores de la DA, como las anfetaminas (o la cocaína), pueden producir síntomas psicóticos, similares a los de la esquizofrenia (Hermens et al., 2009). La segunda observación es referente a los antipsicóticos típicos, utilizados para tratar el trastorno, que bloquean los receptores dopaminérgicos postsinápticos del subtipo D2 (D2) en la vía mesolímbica, mecanismo que se considera responsable de la reducción de los síntomas positivos de la enfermedad (Seeman, 2021). La tercera observación es el hallazgo, mediante tomografía por emisión de positrones, de un mayor

número de receptores D2 en el estriado en cerebros postmortem de pacientes con esquizofrenia (Reynolds, 2022).

Se ha identificado una disminución de la actividad DAérgica de la vía mesocortical en la esquizofrenia. Esto podría deberse a que existe un déficit en la secreción de DA por las neuronas presinápticas. También puede ocurrir que los receptores dopaminérgicos en las neuronas postsinápticas se encuentren bloqueados, o que exista una inhibición de las neuronas presinápticas por un exceso de serotonina (Kapur and Remington, 1996; Stahl, 2018). La corteza prefrontal está relacionada con funciones de regulación emocional y social y, aunque no se conoce completamente el mecanismo, esto podría explicar parte de los síntomas negativos en la esquizofrenia (Brisch et al., 2014; Stahl, 2018). Finalmente, las proyecciones desde el ATV al córtex dorsolateral se relacionan con la regulación de funciones cognitivas, por lo que algunos de los síntomas negativos y cognitivos de la esquizofrenia pueden ser debidos a un déficit de actividad dopaminérgica a este nivel (Cropley et al., 2006).

La etiología de la esquizofrenia no puede explicarse teniendo en cuenta únicamente la neurotransmisión alterada de la DA. Con el descubrimiento de los antipsicóticos de segunda generación (SGA, por sus siglas en inglés, *Second-Generation Antipsychotics*), que actúan en otros receptores aparte del receptor de D2 (por ejemplo, sobre receptores serotoninérgicos), se pudo integrar la actividad de otros neurotransmisores como la serotonina, el glutamato y el GABA (ácido gamma-aminobutírico), como se ilustra el la Figura 2.

1.4.2. Hipótesis Serotoninérgica

La serotonina (5-HT) es un neurotransmisor perteneciente a la familia de las aminas biógenas, que regula las transmisiones excitatorias e inhibitorias mediante la modulación de la actividad de neurotransmisores como la DA, el GABA, el glutamato, entre otros (López-Giménez and González-Maeso, 2017). Los receptores de serotonina pueden ser de tipo metabotrópicos (5-HT1,2,4-7) e ionotrópicos (5-HT3) (Meltzer et al., 2003).

La hipótesis serotoninérgica de la esquizofrenia surge a partir de la observación de que la actividad de los agonistas de la serotonina como el ácido lisérgico (LSD) producen

síntomas positivos como alucinaciones o delirios, así como, la actividad de los antipsicóticos atípicos (o de segunda generación), los cuales se unen tanto a receptores de la DA como de la serotonina (Kapur and Remington, 1996; Stahl, 2018, 2016). Se ha observado que la hiperactivación de los receptores serotoninérgicos en las neuronas glutamatérgicas de la corteza prefrontal provoca una hipofunción dopaminérgica, debida al efecto inhibidor que tendría la serotonina a ese nivel, lo cual tendría relación con la sintomatología negativa de la esquizofrenia (Kapur and Remington, 1996) (Figura 2). El aumento en la activación de los receptores 5-HT2A de la serotonina en la psicosis podría deberse a un exceso de liberación de este neurotransmisor, al mayor número de receptores o al efecto de ambos (Ballanger et al., 2010; Huot et al., 2010). Existen estudios *postmortem* que han mostrado un incremento de la expresión de los receptores 5-HT1A y un aumento del número de receptores 5-HT2A en la corteza frontal, así como alteraciones en el transporte de la serotonina en pacientes con esquizofrenia (Stahl, 2018).

1.4.3. Hipótesis Glutamatérgica

El glutamato es un neurotransmisor excitatorio que cuenta con dos principales grupos de receptores, los receptores ionotrópicos y los receptores metabotrópicos. Dentro de los receptores ionotrópicos se encuentra el receptor NMDA (ácido N-metil-D-aspártico), AMPA (Ácido α-amino-3-hidroxi-5-metilo-4-isoxazolpropiónico) y kainato (Tsai and Coyle, 2002). La hipótesis glutamatérgica de la esquizofrenia tiene su fundamento en la hipoactividad de los receptores NMDA, principalmente en el CPF, y su efecto neurodegenerativo (Coyle, 2006; Hu et al., 2015).

Las neuronas corticales glutamatérgicas regulan la actividad de las interneuronas GABAérgicas, las cuales son responsables de la inhibición de otras neuronas glutamatérgicas (Figura 2). Este proceso funciona como frenado fisiológico para prevenir la hiperexcitación de las neuronas piramidales glutamatérgicas y el consiguiente daño neurotóxico (Blum and Mann, 2002). Cuando hay un agonismo de los receptores de NMDA, se altera el funcionamiento GABAérgico y por lo tanto se libera descontroladamente glutamato que se unirá a otros receptores ionotrópicos disponibles, pero que generan procesos neurotóxicos y la consiguiente apoptosis celular (Hu et al., 2015; Tsai and Coyle, 2002). Estudios en modelos animales han permitido

sustentar que a menor actividad glutamatérgica hay una menor actividad cortical, generando alteraciones cognitivas y de la conducta social, similares a los presentes en la esquizofrenia (Coyle, 2006; Lipska, 2000; Tsai and Coyle, 2002).

1.4.4. Hipótesis GABAérgica

El ácido gamma-aminobutírico o GABA es el principal neurotransmisor inhibitorio del sistema nervioso central (SNC), que tiene una función principal en las interneuronas GABAérgicas (Stępnicki et al., 2018).

La hipótesis GABAérgica de la esquizofrenia radica principalmente en la relación de GABA y la actividad glutamatérgica. Como se mencionó anteriormente, el GABA actúa en interneuronas inhibitorias de las neuronas glutamatérgicas. En pacientes con esquizofrenia se ha observado que los receptores NMDA glutamatérgicos en las neuronas GABAérgicas son anormales, evitando así la unión del neurotransmisor al receptor (Blum and Mann, 2002). Como consecuencia se genera un desequilibro entre la excitación e inhibición (balance excitatorio/inhibitorio) de las neuronas glutamatérgicas, y esta disfunción a nivel de la corteza prefrontal aumenta la actividad glutamatérgica del área tegmental ventral (Figura 2) (Elert, 2014). Este desbalance regulatorio en pacientes con esquizofrenia estaría influyendo en mecanismos cognitivos como la atención y la memoria de trabajo (Blum and Mann, 2002; Pratt et al., 2008; Stępnicki et al., 2018)

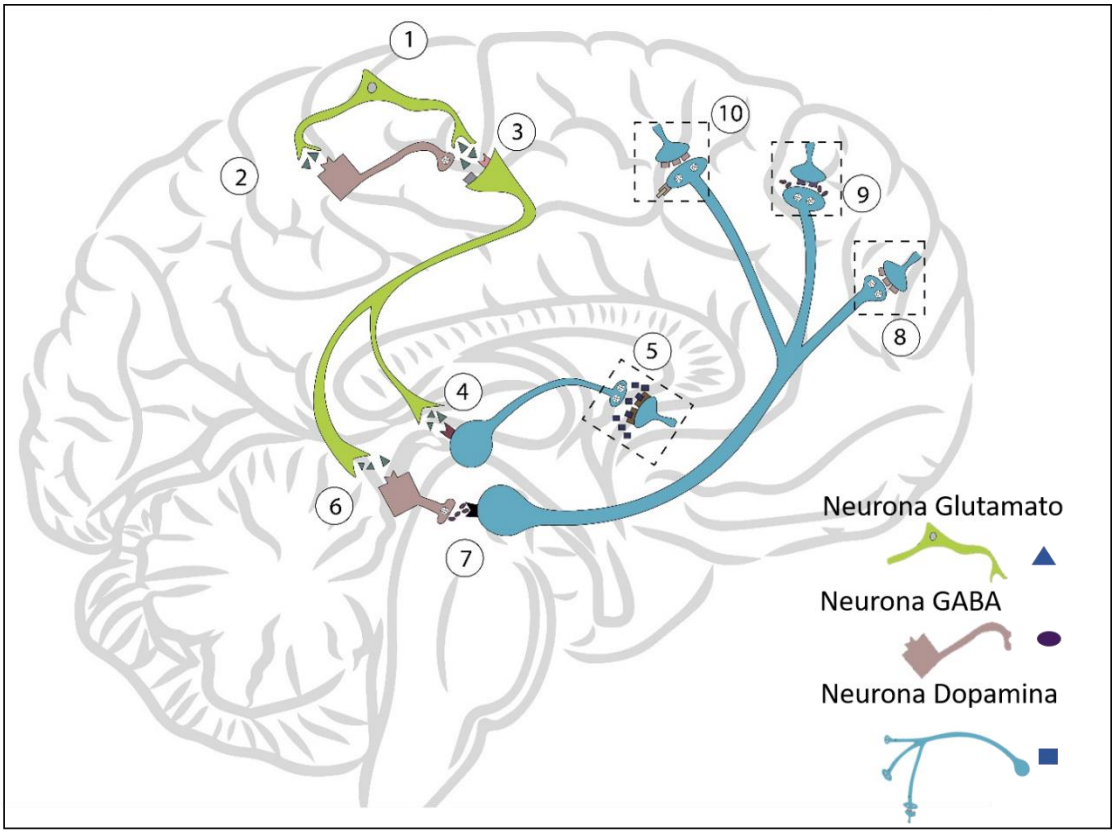


Figura 2. Esquema de la actividad de neurotransmisión en la hipótesis etiológica de la esquizofrenia (adaptado de Elert, 2014; Stahl, 2018). (1) Las neuronas glutamatérgicas corticales liberan glutamato, tanto hacia otras neuronas glutamatérgicas con proyección al área subcortical, como a interneuronas GABAérgicas. (2) Los receptores de las interneuronas GABAérgicas fallan en la unión con el glutamato, evitando así la salida de GABA y por lo tanto su acción inhibitoria sobre las neuronas glutamatérgicas. (3) El glutamato se une a los receptores de las neuronas glutamatérgicas sin inhibición de GABA, hiperactivando la vía hacia el área tegmental ventral. (4) El exceso de glutamato liberado hiperactiva la vía dopaminérgica mesolímbica, la cual proyecta hacia el núcleo accumbens. (5) El exceso de dopamina en esta región estaría relacionado con la aparición de alucinaciones y síntomas positivos de la enfermedad. (6) Por otro lado, en el área tegmental ventral, los receptores de las interneuronas GABAérgicas se unen al glutamato, (7) e inhiben la actividad de neuronas dopaminérgicas en la vía mesocortical que se proyecta hacia la corteza prefrontal. (8) En la corteza prefrontal se genera un déficit de función dopaminérgica. (9) Esto también puede deberse al bloqueo de los receptores D2 en las neuronas postsinápticas o (10) a una deficiencia debido a la inhibición por un exceso de serotonina en esta vía. En cualquier caso, se cree que el déficit de dopamina en el córtex prefrontal/frontal estaría relacionado con la llamada “hipofrontalidad” y la aparición de la sintomatología negativa (p. ej., aislamiento social, anhedonia) y cognitiva de la esquizofrenia.

1.5. Factores del desarrollo

Los primeros síntomas de la esquizofrenia suelen aparecer al final de la adolescencia o al principio de la edad adulta. Sin embargo, la investigación sugiere que alteraciones durante las diferentes fases del proceso madurativo del cerebro pueden desencadenar errores en las conexiones o en la transmisión nerviosas (Lieberman et al., 2001; Schmitt et al., 2023). También se ha planteado un vínculo entre errores a nivel genético y factores ambientales con la alteración en puntos críticos del desarrollo prenatal y/o postnatal, tales como la proliferación, migración, diferenciación celular, la sinaptogénesis y la poda neuronal (Feinberg, 1982; Gupta and Kulhara, 2010; Reckziegel et al., 2022; Schmitt et al., 2023; Van Os et al., 2008).

Los cambios normales que se observan en el cerebro durante la adolescencia, como la disminución del volumen de materia gris cortical y el metabolismo prefrontal, se muestran más pronunciados en cerebros de pacientes con esquizofrenia (Feinberg, 1982; Gupta and Kulhara, 2010). Esto podría deberse en parte a una alteración durante el proceso de poda sináptica que ocurre normalmente durante este periodo (Gupta and Kulhara, 2010; Sellgren et al., 2019). También, existe una reducción del neuropilo (reducción de la conectividad neuronal), lo que podría estar relacionado con los síntomas negativos de este trastorno. Finalmente, se ha demostrado que algunos de los genes que se han identificado como de susceptibilidad a la esquizofrenia están relacionados con el proceso de neurodesarrollo, tanto en la migración celular como en la formación de conexiones sinápticas y su regulación (Kowiański et al., 2018; Trubetskoy et al., 2022; Wolpowitz et al., 2000).

1.6. Factores genéticos

La esquizofrenia tiene un componente hereditario bien establecido, que puede estimar el riesgo genético a presentar la enfermedad. En un estudio pionero y extensísimo, de asociación entre marcadores genéticos en todo el genoma y la esquizofrenia, se identificaron 108 alelos de susceptibilidad (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Entre los genes involucrados se encuentran aquellos relacionados con la síntesis de la dopamina, como el gen *Catechol-O-methyltransferasa* (*Comt*), un grupo de genes que intervienen en la regulación de los

canales de calcio, (*Cacna1c, Cacnb2, Cacna1*), genes asociados con la neurotransmisión sináptica, como *Synapsin II* y el gen *Factor sensible a la N-etilmaleimida* (*Nsf*), y un grupo de genes de receptores del glutamato (*Grm3, Srr, Gria1*) y de la serotonina. Sin embargo, el componente genético sólo explicaría una parte de la variabilidad en la probabilidad de padecer esquizofrenia en la población general, lo que refleja el hecho de que una parte significativa puede deberse a influencias ambientales y/o interacciones gen-ambiente (Sawa and Snyder, 2002; Van Os et al., 2008)

Más recientemente, otra serie de genes han sido relacionados con el desarrollo del trastorno, algunos de los cuales, por su relevancia directa para la presente Tesis Doctoral, los describimos en los apartados siguientes y se resumen en la Tabla 1.

1.6.1. Genes de receptores de membrana

1.6.1.1. Gen receptor de la dopamina

El *gen receptor de la dopamina D1* (*Drd1*) codifica los receptores de dopamina subtipo D1. Este tipo de receptor es el principal para la vía dopaminérgica en el neocórtex (Lidow and Rakic, 1992), y desempeña un papel importante en la regulación del sistema de recompensa, y en los procesos cognitivos como la atención, el aprendizaje y la memoria (Beninger, 1998; Rinaldi et al., 2007).

Pruebas PET (por las siglas en inglés de*, Positron Emission Tomography*) en personas con esquizofrenia permitieron identificar una disminución de los receptores D1 en el CPF de los pacientes (Okubo et al., 1997), lo que concuerda con la expresión reducida del gen *Drd1*, encontrado en cerebros de pacientes esquizofrénicos (Kaalund et al., 2014). La disminución de receptores estaría relacionada con un déficit de la señalización de D1 en CPF y podría contribuir a los síntomas negativos y cognitivos asociados a esta enfermedad (Stahl, 2018). En contraste, en un modelo animal para la esquizofrenia, se encontró que la expresión de *Drd1* está aumenta en el CPF de ratas, lo que sugiere un endofenotipo neurobiológico más inmaduro (Elfving et al., 2019; Sønderstrup et al., 2023). Por otro lado, estudios poblacionales de asociación entre el gen y la esquizofrenia han revelado resultados negativos (Campion et al., 1994; Dolžan et al., 2007;

Hoogendoorn et al., 2005). Sin embargo, ensayos de polimorfismos de un solo nucleótido o SNP (por sus siglas en inglés, *Single-Nucleotide Polymorphism*), han identificado variantes de *Drd1* ligadas al riesgo de presentar este desorden cuyos efectos dependen del sexo (Hoenicka et al., 2010).

1.6.1.2. Gen de la Subunidad 2b del receptor ionotrópico de glutamato NMDA

El gen de la *subunidad 2b del receptor ionotrópico de glutamato NMDA* (*Grind2b*) codifica una de las siete subunidades que conforman al receptor de glutamato ionotrópico NMDA (Sabo et al., 2023). El receptor NMDA es importante durante la embriogénesis, en procesos como el correcto desarrollo del SNC y la formación de redes neuronales (Myers et al., 2019). Posteriormente, es responsable de mediar la neurotransmisión excitatoria del glutamato. La composición genética alterada podría contribuir a la variación de los receptores NMDA en las vías glutamatérgicas, dando como resultado una anormal diferenciación y migración neuronal y, por lo tanto, afectando a la plasticidad sináptica durante el desarrollo cortical (Myers et al., 2019; Sabo et al., 2023). Estos errores conllevarían más adelante a fenotipos negativos, déficit cognitivo y social, similares a los presentes en la esquizofrenia (Coyle, 2006; Tsai and Coyle, 2002).

Se ha observado un patrón de expresión diferencial del gen *Grin2b* en diferentes zonas cerebrales, asociado a desórdenes mentales. Por ejemplo, en pacientes con depresión psicótica está sobreexpresado en el córtex cingulado (Brown et al., 2023). Y, por otro lado, en cerebros *postmortem* de pacientes con esquizofrenia se ha visto subexpresado en el córtex temporal y frontal superior (Meador-Woodruff, 2000). Además, en estudios en un modelo animal de rasgos asociados a la esquizofrenia han encontrado que existe una expresión alterada de *Grind2b*, durante la infancia y la adolescencia en el CPF, lo que sugiere una alteración en el neurodesarrollo (Elfving et al., 2019). Actualmente más de 200 variantes en este gen se encuentran asociadas a diversos trastornos del neurodesarrollo (Hu et al., 2016). Y estudios poblacionales de asociación genética han identificado variantes que confieren susceptibilidad a presentar esquizofrenia o sus síntomas (Krzystanek et al., 2021).

1.6.2. Genes presinápticos

1.6.2.1. Gen Sinaptosoma asociado a la proteina25

El gen S*inaptosoma asociada a la proteína 25* (*Snap25*) codifica la proteína del mismo nombre. Es una proteína de la membrana plasmática presináptica que se expresa de forma elevada y específica en las células neuronales (Liu et al., 2017). SNAP25 forma parte del complejo de receptores de proteínas de fijación soluble de NSF o SNARE (por sus siglas en inglés SNAP, *Soluble NSF Attachment Protein Receptor*), que permiten la fusión de vesículas con la membrana neuronal para facilitar la exocitosis de neurotransmisores en el sistema nervioso. Además, interviene en la morfogénesis y densidad de las espinas dendríticas, en el tráfico de receptores postsinápticos y en la plasticidad neuronal (Houenou et al., 2017; Kádková et al., 2019).

Alteraciones en la estructura, expresión y/o función del gen parece que podrían estar relacionadas con varios trastornos neuropsiquiátricos y neurológicos. Por ejemplo, estudios en cerebros postmortem de pacientes con esquizofrenia mostraron expresión reducida de este gen y de su proteína en el HPC (Corradini et al., 2009; Thompson et al., 2003), así como en el lóbulo frontal. Por otro lado, se encontró una expresión aumentada tanto en el córtex cingulado como en el lóbulo prefrontal (Gabriel, 1997; Thompson et al., 1998). Estudios poblacionales han identificado que la variante rs6039769, en la secuencia del gen de *Snap25*, tiene un mayor nivel de expresión génica en el CPF y está asociada con la esquizofrenia (Houenou et al., 2017). Curiosamente, los polimorfismos en el gen, así como la expresión alterada de la proteína, se han asociado con un fenotipo conductual de hiperactividad-impulsividad y un déficit cognitivo en la inhibición prepulso de la respuesta de sobresalto (o PPI, por sus siglas en inglés, *Prepulse Inhibition*) en modelos animales (Bruno et al., 2007; Gunn et al., 2011; Jeans et al., 2007). Estos hallazgos podrían sugerir una asociación de *Snap 25* con la etiología de la esquizofrenia, así como con los síntomas cognitivos/atencionales de la enfermedad.

1.6.3. Genes Postsinápticos

1.6.3.1. Gen Neuregulin-1

Neuregulin-1 (*Nrg1*) es un gen de la familia del factor de crecimiento epidérmico, importante en la modulación de la migración neuronal, la sinaptogénesis, la comunicación neuronal y la mielinización (Wolpowitz et al., 2000). Tiene un rol importante en la regulación de la plasticidad sináptica, por ejemplo, en la ruta glutamatérgica dependiente de NMDA por medio de los receptores ErbB (Corfas et al., 2004; Stefansson et al., 2002).

Estudios previos han encontrado un aumento en los niveles de ARNm y de la proteína NRG1 en el lóbulo frontal de pacientes con esquizofrenia (Chong et al., 2008; Law et al., 2006), y, en un estudio con animales, se han observado resultados similares de expresión en el CPF de ratas (Elfving et al., 2019). Finalmente, grandes estudios poblacionales han demostrado asociaciones positivas entre el gen *Nrg1* y sus variantes con el riesgo de desarrollar esquizofrenia (Gurling et al., 2001; Li et al., 2006; Stefansson et al., 2002).

Nrg1 podría estar relacionado con la sintomatología esquizofrénica, como sugieren estudios en ratones transgénicos para el gen o su receptor *ErbB4*, en los cuales se muestran alteraciones de la sociabilidad, déficits en la memoria asociada al miedo y alteraciones en la sincronización sensoriomotora (Guerra et al., 2021; Olaya et al., 2018), además de una respuesta mejorada a la clozapina, un fármaco antipsicótico atípico (de segunda generación) utilizado para tratar la esquizofrenia (Corfas et al., 2004; Stefansson et al., 2002).

1.6.3.2. Gen Homer1

El gen *Homer1* codifica una familia de proteínas de densidad postsináptica con funciones clave en el control de la plasticidad sináptica, regulando los receptores glutamatérgicos, serotoninérgicos y dopaminérgicos (de Bartolomeis et al., 2013a, 2013b). *Homer1* tiene un rol activo en el CPF, en el mantenimiento del glutamato extracelular basal y en la regulación de la liberación de glutamato, serotonina y dopamina, mecanismos

relevantes para la esquizofrenia y la comorbilidad del abuso de estimulantes (Lominac et al., 2005).

Estudios de expresión genética o de los niveles de proteínas de densidad postsináptica en zonas cerebrales como el HPC o en el CPF apuntan a la etiología de la esquizofrenia (Elfving et al., 2019; Szumlinski and Kippin, 2008). Análisis *postmortem* de pacientes con esquizofrenia muestran que los niveles de proteína HOMER1 están alterados en el HPC (Matosin et al., 2016). También han demostrado que la sobreexpresión de *Homer1* en esta región deteriora la memoria espacial de trabajo y de referencia (Celikel, 2007), lo que se correlacionaría con los déficits cognitivos de esta patología.

En un estudio con ratas modelo de rasgos relevantes para la esquizofrenia se encontró que los niveles de proteína de HOMER1 en el CPF no se encontraban alterados significativamente, pero sí se observó una expresión aumentada de este gen (Elfving et al., 2019). Por otro lado, se encontró que ratones *Homer1-knockout* (*Homer1-KO*) presentan anomalías conductuales y neuroquímicas que concuerdan con los modelos animales de esquizofrenia, tales como déficits cognitivos en la ejecución del laberinto radial de ocho brazos, aumento de la ansiedad en una prueba de reconocimiento de objetos nuevos, incremento de la reactividad en entornos nuevos y de la conducta motora estimulada por MK-801 y metanfetamina (de Bartolomeis et al., 2013b; Szumlinski et al., 2005). Estos hallazgos podrían sugerir que una alteración, aumento o disminución, de la expresión de *Homer1* podría estar influyendo en la aparición de los rasgos asociados a la esquizofrenia.

1.6.4. Genes regulatorios del neurodesarrollo

1.6.4.1. Gen del Factor neurotrófico derivado del cerebro

El gen *Factor neurotrófico derivado del cerebro* (*Bdnf*) codifica la proteína del mismo nombre que actúa como factor de crecimiento de la familia de las neurotrofinas (Hohn et al., 1990; Kowiański et al., 2018). Durante el desarrollo cerebral, BDNF interviene en los procesos de neurogénesis, en la regulación de la muerte celular y la eliminación de conexiones mal formadas (Kowiański et al., 2018). Posteriormente, controla las interacciones sinápticas de corta y larga duración. Por ejemplo, mantiene la fuerza sináptica y la disminución de la excitabilidad de las interneuronas GABA del HPC. Estas

conexiones neuronales, a largo plazo, influyen en los mecanismos de la memoria y la cognición (Leal et al., 2015) como se mostró en un ensayo con animales. Los resultados mostraron un mejor rendimiento en tareas relacionadas con el aprendizaje/memoria (Vaynman et al., 2004).

Aunque el déficit de este gen no provoca una disminución significativa del número de neuronas, sí causa la inhibición de la arborización dendrítica y el deterioro de la plasticidad sináptica (Gao et al., 2009). Se ha demostrado que las experiencias sociales tempranas durante la primera semana postnatal podrían generar cambios permanentes en la metilación del ADN, como lo demostró el ensayo con ratas expuestas a condiciones de estrés durante la primera semana de vida: al evaluar patrones de metilación y expresión genética encontraron que el estrés temprano produjo cambios persistentes en la metilación del gen *Bdnf*, lo que causó una alteración en la expresión en la corteza prefrontal de las ratas adultas (Roth et al., 2009).

1.6.4.2. Gen de la Kinasa dependiente de ciclina 5

La Kinasa dependiente de ciclina 5 (CDK5) es una enzima kinasa de serina/treonina, que se ha revelado como un regulador crucial de la citoarquitectura del cerebro en desarrollo, interviene principalmente en la migración neuronal (Dhavan and Tsai, 2001; Shah and Rossie, 2018). A diferencia de las otras enzimas CDKs, CDK5 no es activada por ciclinas, sino que depende de activadores específicos del sistema nervioso, p35 y p39, para fosforilar sustratos (Dhavan and Tsai, 2001) .

CDK5 está implicada en la plasticidad funcional y estructural a través de su regulación de las vías de transducción de señales. Debido a esta asociación, se cree que CDK5 puede estar relacionada en los procesos de aprendizaje y formación de la memoria, así como en la adicción a las drogas (Bibb, 2003). También se ha demostrado que esta enzima ocupa un lugar importante en la regulación de la neurotransmisión dopaminérgica (Seiwell et al., 2007).

Estudios en roedores determinaron que al someterlos a condiciones de adicción a drogas la expresión de p*35* y de *Cdk5* estaba reducida en CPF, y mostraron fenotipos de déficits cognitivos (Lazenka et al., 2017). Esto se correlaciona con hallazgos en muestras de cerebro *postmortem* de pacientes esquizofrénicos, donde identificaron que los

niveles de expresión de *p35* están reducidos en el HPC y el CPF en humanos (Engmann et al., 2011).

1.6.4.3. Gen Cables1

El gen *Cables1* codifica para la proteína del mismo nombre, la cual sirve de sustrato para la CDK5, así como para la *tirosina kinasa* C-ABL (Groeneweg et al., 2011; Hernández-Ramírez et al., 2017; Zukerberg et al., 2000). *Cables1* se expresa en el cerebro, está presente en neuronas postmitóticas de la corteza cerebral y desempeña un papel importante en el desarrollo neuronal durante la embriogénesis, demostrado en ensayos in vivo con pez cebra (Groeneweg et al., 2011). Además, CABLES1 y CDK5 se localizan en las puntas de los axones en crecimiento, lo que sugiere que la asociación de CABLES1 y CDK5 forma parte de la vía de señalización que opera durante el desarrollo cerebral en el crecimiento de las neuritas (Zukerberg et al., 2000) y en el desarrollo del cuerpo calloso (Mizuno et al., 2014). Cambios en los patrones de expresión de *Cables1* podrían alterar los factores de transcripción neurales tempranos, lo que sugiere que CABLES1 es necesario para la diferenciación neural temprana (Groeneweg et al., 2011). La expresión reducida de *Cables1* podría, entonces, indicar un deterioro de la maduración sináptica en ratas adolescentes (Sønderstrup et al., 2023)

Tabla 1. Resumen de las categorías, función y expresión de los genes de susceptibilidad de la esquizofrenia analizados en esta Tesis.

Categoría	Nombre del gen	Funciones del gen	Expresión en la esquizofrenia	Expresión en modelos animales
Receptores de membrana	*Receptor de la dopamina* (*Drd1*)	▪ Regula el crecimiento y el desarrollo neuronal. ▪ Modulan los eventos mediados por el receptor dopaminérgico D2. ▪ Regula el sistema de recompensa, procesos cognitivos como la atención, el aprendizaje y la memoria.	▪ Disminución de los receptores D1 en el CPF de los pacientes (Stahl, 2018). ▪ Expresión reducida del gen *Drd1*, encontrado en cerebros de pacientes esquizofrénicos (Kaalund et al., 2014) .	▪ Expresión aumentada del gen en el CPF de ratas (Elfving et al., 2019).
	Subunidad 2b del receptor ionotrópico de glutamato NMDA (Grind2B)	▪ Relacionado con procesos del desarrollo del SNC y la formación de redes neuronales.	▪ Sobreexpresión en el córtex cingulado y subexpresado en el córtex temporal y frontal superior (Kaalund et al., 2014).	Expresión alterada en el CPF, durante el periodo juvenil en ratas (Elfving et al., 2019).

		▪ Codifica una de las siete subunidades que conforman al receptor de glutamato ionotrópico NMDA. ▪ Mediador de la neurotransmisión excitatoria del glutamato.		
Genes Presinápticos	*Sinaptosoma asociada a la proteína 25 (Snap25)*	▪ Interviene en la morfogénesis y densidad de las espinas dendríticas, en el tráfico de receptores postsinápticos y en la plasticidad neuronal. ▪ Forma parte del complejo SNARE, que facilita la exocitosis de neurotransmisores en el sistema nervioso.	▪ Expresión reducida del gen y de la proteína en el HPC, así como en el área del lóbulo frontal (Corradini et al., 2009; Thompson et al., 2003). ▪ Expresión aumentada tanto en el córtex cingulado como en el lóbulo prefrontal (Gabriel, 1997; Thompson et al., 1998).	▪ Polimorfismos y expresión alterada de la proteína (Bruno et al., 2007; Jeans et al., 2007).

Genes Postsinápticos	*Neuregulin-1 (Nrg1)*	▪ Modula la migración neuronal, la sinaptogénesis, la comunicación neuronal y la mielinización. ▪ Regula la plasticidad sináptica, en la ruta glutamatérgica dependiente de NMDA por medio de los receptores ErBb.	▪ Aumento de los niveles de ARNm y proteínas en el lóbulo frontal (Chong et al., 2008; Law et al., 2006).	▪ Aumento de la expresión en los niveles de ARNm y proteínas en el CPF (Elfving et al., 2019; Sønderstrup et al., 2023).
	Homer1	▪ Rol activo en la regulación de la liberación de glutamato y en el mantenimiento extracelular basal en el CPF ▪ Control de la plasticidad sináptica, regulando los receptores de neurotransmisores.	▪ Existe una sobreexpresión en el HPC (Matosin et al., 2016).	▪ No existen niveles proteicos alterados en el CPF, pero sí se observó una expresión aumentada de este gen (Elfving et al., 2019; Sønderstrup et al., 2023).

Genes regulatorios del neurodesarrollo	*Factor neurotrófico derivado del cerebro (Bdnf)*	▪ Interviene en los procesos de neurogénesis, en la regulación de la muerte celular y la eliminación de conexiones mal formadas. ▪ Controla las interacciones sinápticas de corta y larga duración.	▪ Se asocia con una disminución de la expresión en el CPF (Roth et al., 2009).	▪ Se asocia con disminución en la expresión en el CPF adulto (Elfving et al., 2019; Sønderstrup et al., 2023) .
	Kinasa dependiente de ciclina 5 (Cdk5)	▪ Regula la citoarquitectura del cerebro en desarrollo, específicamente en la migración neuronal.	▪ Reducción de los niveles de expresión de p35 en el HPC y el CPF en humanos (Engmann et al., 2011).	▪ La expresión de *p35* y de *Cdk5* estaba reducida en CPF, mostrando fenotipos de déficits cognitivos (Lazenka et al., 2017)
	Cables1	▪ Importante en el desarrollo neuronal durante la embriogénesis. ▪ Sustrato enzimático de Cdk5 y Ables 1 (C-Ables1).		▪ Expresión reducida de *Cables1*, en CPF de ratas adolescentes (Sønderstrup et al., 2023).

1.7. Factores ambientales e influencia en el neurodesarrollo

La manera en que se entiende la esquizofrenia hoy en día es diferente de como se la describió en el siglo XX. La etiología de la esquizofrenia es multifactorial y refleja una interacción entre la susceptibilidad genética y los factores ambientales que podrían alterar periodos críticos de la neurogénesis y del neurodesarrollo del cerebro (Stilo and Murray, 2019) (Figura1).

Las complicaciones obstétricas están documentadas como factor de riesgo de la esquizofrenia (Cannon et al., 2002). Estudios epidemiológicos han sugerido que mecanismos como la desnutrición fetal (Abel et al., 2010), la prematuridad, los episodios hipóxico-isquémicos (Rubio-Abadal et al., 2015) y las infecciones maternas durante el embarazo y el parto (Brown and Derkits, 2010; Buka et al., 2001), están asociados a un mayor riesgo de trastornos psicóticos.

Por otra parte, los estudios epidemiológicos muestran una asociación entre la susceptibilidad a desarrollar esquizofrenia y el consumo de drogas como el cannabis (Arseneault et al., 2002), y dicha relación tiende a ser más estrecha con respecto al inicio temprano (Van Os et al., 2002). También, el uso de psicoestimulantes (como las anfetaminas y la cocaína) (Sara et al., 2015), el alcohol (Koskinen et al., 2009) y el tabaco (Gurillo et al., 2015) podrían ser factores de riesgo para la psicosis.

Otros factores de riesgo son los asociados a las adversidades sociales o ambientales en diferentes formas, ya sea durante la infancia o la edad adulta. Se ha observado una relación entre el trauma infantil y los síntomas positivos de la esquizofrenia, especialmente alucinaciones (Bentall et al., 2014; Whitfield et al., 2005), así como con síntomas afectivos (Matheson et al., 2013). Además, comportamientos de aislamiento social han sido asociados a la psicosis (Morgan et al., 2008; Stilo et al., 2013). También, el trastorno estaría relacionado con el efecto de factores ambientales sociales adversos, como sugiere el hecho de que los grupos inmigrantes tienen un mayor riesgo de padecer esquizofrenia y otros trastornos psicóticos (Bourque et al., 2011; Morgan et al., 2008).

En general, se ha propuesto que diferentes tipos de experiencias o eventos ambientales adversos, en especial los experimentadas/os en fases tempranas del neurodesarrollo, pueden constituir factores de riesgo para la psicosis o/y la esquizofrenia. Así,

determinados tipos y niveles de estrés sufridos durante etapas tempranas de la vida (el denominado *"Early life stress"*) se han asociado con riesgo de esquizofrenia en humanos y con la aparición de fenotipos relacionados con el trastorno en diversos modelos animales (Hoffmann et al., 2017; McClelland et al., 2011; Millan et al., 2016; Richetto and Meyer, 2021). En cambio, la incidencia de experiencias tempranas enriquecidas (positivas) sobre la posterior vulnerabilidad a la esquizofrenia o a fenotipos relacionados con el trastorno no ha recibido apenas atención. Sí que existe evidencia, sin embargo, de que algunos tipos de experiencias infantiles-juveniles enriquecidas producen efectos positivos duraderos sobre procesos emocionales y cognitivos (aprendizaje, memoria), así como sobre el neurodesarrollo, la neuroplasticidad cerebral y otros procesos neurobiológicos. Aunque dichas evidencias provienen sobre todo de estudios con modelos animales, existen indicios claros en el mismo sentido también en humanos (Burrows and Hannan, 2016; Fernández-Teruel, 2021, 2022; Fernández-Teruel et al., 2002, 2021; McClelland et al., 2011; Raineki et al., 2014; Río-Álamos et al., 2017, 2019).

Uno de los tratamientos ambientales que implican estimulación infantil-juvenil enriquecida, utilizados a menudo en roedores (ratas o ratones, principalmente), y de larga tradición, es la estimulación neonatal (del inglés *"neonatal handling"*; a partir de ahora, "NH") (Fernández-Teruel et al., 2002; Levine, 1956; Meaney et al., 1988; Río-Álamos et al., 2017, 2019). Por su relevancia para la presente Tesis Doctoral, explicamos brevemente el tratamiento de la NH en la siguiente sección (véanse los métodos más detallados en la correspondiente sección, *"Materials and Methods"*, de los estudios 2 y 3 de esta Tesis).

1.8. Tratamiento de NH en roedores

El tratamiento de la NH en ratas (y ratones) es un procedimiento que, generalmente, se administra una o dos veces al día entre los días postnatales (DPN) 1- 21. Previamente se retira a la madre de la camada, para posteriormente colocar a las crías individualmente en cajas de plástico (35 × 15 × 25 cm) forradas con una toalla de papel. El tratamiento, comúnmente y a grandes rasgos, consiste en separar las crías (diariamente a la misma hora) de la madre durante unos 8-15 minutos (dependiendo del estudio concreto) y situarlas individualmente en esas cajas. En los minutos que las crías están separadas de la madre son masajeadas (acariciadas) suavemente durante un total de unos 10-12

segundos mientras se mantienen en la mano del experimentador. Tras esos minutos de separación son devueltas (una a una) a su caja-hogar, de nuevo con la madre y el resto de la camada.

Gran número de estudios realizados en ratas y ratones indican que, para rasgos comportamentales y cognitivos asociados a la esquizofrenia, la NH produce mejoras duraderas en el filtraje atencional (PPI), la inhibición latente y el rendimiento cognitivo en tareas muy diversas (Fernández-Teruel et al., 1997, 2002; Río-Álamos et al., 2015, 2017, 2019), junto con cambios en el volumen del HPC y la amígdala. También disminuye la ansiedad y aumenta la preferencia por la interacción social (IS) (Sampedro-Viana et al., 2021), y además tiene un impacto duradero en el neurodesarrollo y la neuroplasticidad (Fernandez-Teruel, 2021, 2022; Fernández-Teruel et al., 1997, 2002; Sampedro-Viana et al., 2023; Steimer et al., 1998).

En congruencia con el tratamiento de NH en roedores, la terapia de masaje en niños prematuros, un tratamiento inspirado en el NH en ratas, también produce efectos positivos en los seres humanos. En los bebés tratados con terapia de masaje, se produce una aceleración del incremento de peso, y un aumento del factor de crecimiento insulínico tipo 1 (IGF-1) en sangre, hormona que regula el crecimiento. También se evidenció un incremento del tono vagal y una disminución de cortisol, del que se sabe que su exceso crónico inhibe la secreción de IGF-1 (Field et al., 2008). Por otro lado, según los parámetros globales de la actividad electroencefalográfica y de potenciales evocados visuales y auditivos, se ha demostrado que dicha terapia de masaje infantil acelera la maduración cortical cerebral de los nacidos prematuros, de bajo riesgo y sin anomalías cerebrales (Guzzetta et al., 2009, 2011). Además, en ratas que recibieron un tipo de NH (similar al masaje infantil en niños neonatos), se observó que el antagonismo del IGF-1 revertía la aparición de tales efectos de maduración cerebral. Por lo tanto, estos hallazgos sugieren un papel causal de la función del IGF-1 en la mediación de los efectos madurativos cerebrales del NH en ratas y del tratamiento de masaje infantil en niños prematuros (Field et al., 2008; Guzzetta et al., 2009, 2011).

1.9. Modelos animales

Según el NIH (*National Institutes of Health, USA*) un modelo animal es una especie no humana, que se usa en estudios biomédicos porque puede replicar algunos aspectos de un proceso biológico o de una enfermedad, y que de otro modo serían impracticables en humanos o que estarían prohibidos por cuestiones éticas (National Human Genome Research Institute, 2024)

Para determinar si un modelo discrimina o refleja eficientemente los rasgos que quiere imitar, se necesita de un proceso de validación. En los trastornos psiquiátricos suelen discutirse en relación con tres criterios elaborados por primera vez por Willner: validez aparente, de constructo, y predictiva (Willner, 1984). La validez aparente corresponde a la semejanza entre los aspectos conductuales (validez etológica) o biológicos (validez de biomarcador) del modelo con respecto al síntoma o trastorno humano que se pretende imitar. La validez de constructo (mecanicista) concierne al hecho de que los mecanismos cognitivos o biológicos subyacentes al trastorno son similares tanto en humanos como en animales. Por último, la validez predictiva relaciona la semejanza entre el factor desencadenante y el resultado (validez de inducción) y entre los efectos de los tratamientos en los dos organismos (validez de remisión) (Belzung and Lemoine, 2011; Willner, 1984).

1.10. Síntomas relevantes para (o similares a) la esquizofrenia en roedores

La principal dificultad de modelar la esquizofrenia en roedores es que no pueden manifestar (al menos de forma evidente para los observadores humanos) alucinaciones, delirios, o alteraciones en el lenguaje, por ejemplo. Por lo cual, el campo de la neurociencia ha debido buscar otros fenotipos y comportamientos de los animales que puedan modelar y/o aportar información sobre los procesos vinculados al trastorno. Hay una diversidad de comportamientos y procesos en los roedores de laboratorio que son relevantes para el estudio de los síntomas positivos, negativos y cognitivos de la esquizofrenia (Jones et al., 2011; Powell and Miyakawa, 2006).

Los recientes avances en genómica humana y en la manipulación del genoma del ratón han permitido avanzar en la modelización de ciertos síntomas de la esquizofrenia en roedores. Sin embargo, estos síntomas (validez aparente) deben ir acompañados de

alteraciones en los circuitos cerebrales relacionados con la esquizofrenia (validez de constructo), así como de respuestas significativas a tratamientos eficaces relacionados con la esquizofrenia (validez predictiva).

1.10.1. Síntomas positivos en roedores

Dentro de los comportamientos de posible relevancia para los síntomas positivos de la esquizofrenia en roedores se encuentra la agitación psicomotriz (en relación con el comportamiento gravemente desorganizado en la esquizofrenia). Esta hiperactividad psicomotora se mide como un aumento espontáneo de la actividad locomotora dentro de la jaula doméstica, en respuesta a la novedad o en respuesta a drogas estimulantes (p. ej., anfetamina, cocaína) (Powell and Miyakawa, 2006).

1.10.2. Síntomas negativos en roedores

Para modelar los síntomas negativos de este desorden en roedores se toman en cuenta comportamientos indicadores de anhedonia (p. ej., diminución de la preferencia por sustancias agradables o reforzantes) o de retraimiento social (Powell and Miyakawa, 2006). Ejemplos de síntomas de alteraciones del comportamiento social son:

a. Menor interacción con un congénere juvenil

b. Disminución de la preferencia de lugar por un congénere enjaulado

c. Menor preferencia por la novedad social

d. Dominancia social alterada en la prueba del tubo

e. Alteración del comportamiento agresivo en el ensayo del “residente-intruso”

f. Disminución del comportamiento de nidificación

g. conducta social en la jaula-hogar

1.10.3. Síntomas cognitivos en roedores

Los síntomas cognitivos se modelan en roedores a través de comportamientos en relación a tres aspectos principales (Powell and Miyakawa, 2006), los cuales son:

a) Memoria de trabajo (por ejemplo, alteración del rendimiento de la memoria de trabajo en el laberinto en T, el laberinto radial de 8 brazos o el "laberinto acuático de Morris" –MWM-).
b) Atención, sincronización sensoriomotora y funciones ejecutivas (p. ej., disminución de la PPI, disminución de la inhibición latente, empeoramiento en la tarea de "tiempo de reacción en serie de 5 elecciones" -*5-CSRTT, del inglés "5-choice serial reaction time task"*-)
c) Aprendizaje espacial (por ejemplo, disminución del aprendizaje espacial en el MWM o en el laberinto espacial de 8 brazos).

1.11. Modelos animales para la esquizofrenia

Los modelos animales existentes pueden clasificarse en cuatro categorías: modelos del neurodesarrollo, modelos farmacológicos, modelos de lesiones y modelos genéticos (Jones et al., 2011; Vales and Holubova, 2021; Wang et al., 2021).

Los modelos del neurodesarrollo pueden implicar manipulaciones ambientales durante los periodos críticos del desarrollo, como la etapa perinatal, posnatal o juvenil. Como ejemplo de estos modelos, roedores sometidos a experiencias negativas como el aislamiento social durante los primeros días (o semanas) de vida, generan cambios conductuales en la adultez, provocando aumento en la ansiedad, la agresión y en la actividad locomotora, así como un deterioro cognitivo y déficits en el filtraje atencional (Vales and Holubova, 2021). También, dentro de estos puntos críticos del neurodesarrollo, la administración de ciertos fármacos o sustancias (p. ej., sustancias que provocan reacciones inmunológicas con efectos cerebrales) permite investigar procesos neurobiológicos que conducen al desarrollo de fenotipos similares a la esquizofrenia y el potencial tratamiento en la progresión a la psicosis (Wang et al., 2021).

Los modelos animales farmacológicos más utilizados siguen la hipótesis glutamatérgica o dopaminérgica de la esquizofrenia. Se trata de la administración de drogas que generan síntomas relevantes para la esquizofrenia, entre las que se encuentran los potenciadores de la DA (p. ej., anfetamina, cocaína y apomorfina), y los antagonistas no competitivos del receptor del glutamato NMDA (p. ej., ketamina, fenciclidina –PCP-, o el MK801) (Jones et al., 2011). Estos modelos permiten estudiar comportamientos como

la conducta social, déficits en la PPI, deterioros cognitivos e hiperactividad, similares a la esquizofrenia (Wang et al., 2021; Winship et al., 2019).

Con respecto a los modelos por lesión, aunque existen varios, el más destacado es el de lesión a nivel del HPC ventral bilateral en ratas entre el DPN 7-14, el cual ha permitido obtener un modelo que presenta anormalidades en la memoria espacial y aprendizaje, déficits sensoriomotores (p. ej., en PPI), déficits en conducta social y un aumento de la agresividad, entre otros (Jones et al., 2011). Además, la lesión compromete la integridad de la corteza prefrontal medial y del núcleo accumbens (aparte del HPC), produce alteraciones en la función dopaminérgica mesolímbica y mesocortical, y genera una serie de alteraciones neurobiológicas características de la esquizofrenia (Tseng et al., 2009).

Finalmente, en los últimos años se ha producido un auge en la utilización de modelos genéticos. El uso de la ingeniería genética ha permitido, por ejemplo, la eliminación (*knock-out* en inglés) o la reducción de la expresión de un solo gen (*knock-down* en inglés), así como la inserción o sustitución de secuencias de ADN específicas (*knock-in* en inglés) (Powell et al., 2011; Vales and Holubova, 2021; Winship et al., 2019). El beneficio de estos modelos animales genéticos de ratón radica en la especificidad en la función que se pretende estudiar, seguimiento de rutas fisiológicas de un conjunto de genes y la asociación de marcadores moleculares con el riesgo a presentar síntomas/fenotipos relacionados con la enfermedad (Jones et al., 2011; Powell and Miyakawa, 2006).

Los avances en tecnología de manipulación genética en ratas de laboratorio han progresado mucho más lentamente que en ratones. Por ello, se han desarrollado históricamente otras estrategias y modelos con el objetivo de estudiar características relacionadas con la esquizofrenia en ratas.

Una estrategia clásicamente utilizada ha sido la de generar modelos de rata (o ratón) a través de la crianza selectiva bidireccional a partir de ciertos fenotipos comportamentales y/o farmacológicos, la denominada selección psico-genética o fármaco-genética, respectivamente. Esta estrategia a dado lugar, por ejemplo, a varios modelos genéticos de esquizofrenia (o, más bien, de procesos asociados al trastorno),

tales como: las ratas susceptibles a la apomorfina (APO-SUS), las ratas seleccionadas por baja PPI, las ratas de la cepa Brattleboro (BRAT), y las ratas espontáneamente hipertensas (SHR), entre otras (Oliveras et al., 2023).

Un modelo animal genético derivado por cruce selectivo bidireccional que usamos desde hace años en el grupo de investigación son las cepas *Roman*, seleccionadas *por su* gran facilidad (RHA¸ por sus siglas en inglés, *Roman High-Avoidance*) vs su extrema dificultad (RLA, por sus siglas en inglés, *Roman Low-Avoidance*) para aprender la tarea de evitación activa en dos sentidos (Fernández-Teruel et al., 2023; Oliveras et al., 2023). Como constituyen el modelo central de la presente Disertación Doctoral, ampliaremos su descripción en la siguiente sección.

1.12. Ratas *Roman*

Las líneas de ratas romanas (del inglés, *"Roman"*) se establecieron en Roma a principios de los años 60 mediante la selección bidireccional por la adquisición de la respuesta de evitación activa en dos sentidos. Con este test se establecieron dos perfiles muy separados de ratas Wistar, las que mostraban alta respuesta de evitación, que recibieron el nombre de *"Roman High-Avoidance"* o RHA, frente a las ratas con extremadamente bajas respuesta de evitación, *"Roman Low avoidance"* o RLA (Bignami, 1965). Desde la generación de ambas líneas/cepas de ratas *Roman* hasta la actualidad se han publicado más de 270 artículos internacionales sobre estas cepas o como parte de estudios más amplios en los que han sido incluidas (Fernández-Teruel et al., 2021, 2023). En 1996 se estableció una colonia permanente de ratas *Roman* RHA y RLA consanguíneas en el laboratorio de la Unidad de Psicología Médica, Departamento de Psiquiatría y Medicina Legal (Facultad de Medicina, Universidad Autónoma de Barcelona, España), donde se han llevado a cabo multitud de estudios sobre aspectos conductuales, cognitivos, farmacológicos, neurobiológicos y genéticos con las cepas RHA y RLA (p. ej., Fernández-Teruel et al., 2021, 2023; Giorgi et al., 2019; Oliveras et al., 2023).

1.12.1. Roman Low Avoidance (RLA)

Las ratas RLA, en comparación con las RHA y otras cepas de ratas, muestran un perfil ansioso ante situaciones amenazantes condicionadas o no condicionadas (aprendidas o espontáneas, respectivamente), así como mayores signos de frustración en tareas de

devaluación de la recompensa (Fernández-Teruel et al., 2021, 2023; Ferre et al., 1995; Giorgi et al., 2019; López-Aumatell et al., 2009b; Steimer and Driscoll, 2003). Además, las ratas de esta cepa muestran una elevada respuesta hormonal al estrés (Díaz-Morán et al., 2012; Steimer and Driscoll 2003; revisado por Fernández-Teruel et al., 2023). En cuanto a otros rasgos cognitivos y comportamentales, se ha visto que las RLA presentan mejor respuesta atencional, mejor capacidad de aprendizaje y memoria y mayor conducta social que las RHA (Oliveras et al., 2015; Río-Álamos et al., 2019; Sampedro-Viana et al., 2021, 2023a).

1.12.2. Roman High avoidance (RHA)

En esta sección se presentan los hallazgos y perfiles cognitivos/conductuales, neurobiológicos y relacionados con el neurodesarrollo de las ratas RHA comparadas con las ratas RLA y otras cepas o modelos. Los resultados han permitido considerar a RHA como un modelo para algunos rasgos característicos de la esquizofrenia, resumidos en la Figura 3.

1.12.2.1. Rasgos del neurodesarrollo, neurotransmisión y neuroplasticidad

Con respecto a los rasgos característicos de la cepa RHA en su neurodesarrollo, se han encontrado diferencias significativas en regiones cerebrales altamente ligadas a la etiología de la esquizofrenia. En el HPC, la amígdala y el CPF se observa una disminución del volumen y de la actividad neuronal. En el CPF también se ha observado un mayor número de espinas dendríticas “inmaduras” en las ratas RHA (Meyza et al., 2009; Río-Álamos et al., 2019; Sánchez-González et al., 2020, 2021; Tapias-Espinosa et al., 2019, 2023). Por otro lado, no se encontraron diferencias de volumen en regiones cerebrales como el núcleo accumbens, el estriado y el córtex cingulado anterior entre RHA y RLA (Río-Álamos et al., 2017; Tapias-Espinosa et al., 2019). Existen diferencias de expresión del gen *Bdnf* entre cepas de ratas Roman en el CPF y en HPC. Además, en esta última región, la diferencia también se observa en los niveles de la proteína BDNF entre cepas (Elfving et al., 2019; Sanna et al., 2019). La evidencia nos ha permitido concluir que las ratas RHA parecen presentar déficits de maduración cortical en comparación con las ratas RLA (Elfving et al., 2019; Sønderstrup et al., 2023).

Con respecto a la actividad de los neurotransmisores, se halló una activación diferencial de las interneuronas GABA en el CPF presentando las ratas RHA una activación reducida en comparación con las ratas RLA (Tapias-Espinosa et al., 2023). Además, existe un aumento del tono dopaminérgico funcional mesolímbico (detectado mediante micro diálisis en el núcleo accumbens), mayor densidad del receptor D1 en áreas límbicas y mayor cantidad de mRNA de dicho receptor en el CPF. Se observó una disminución de la densidad de receptores D2 en áreas límbicas en ratas RHA (Elfving et al., 2019; Giorgi et al., 2019; Guitart-Masip et al., 2006; Tournier et al., 2013). Finalmente, las RHA tienen mayor densidad de receptores 5-HT2A en el CPF. Esta diferencia en la densidad podría estar relacionada con diferencias funcionales en la transmisión de glutamato, dado que las ratas RHA presentan un dramático déficit de receptores mGlu2 (metabotrópicos de glutamato subtipo 2) en córtex frontal, HPC y estriado (Elfving et al., 2019; Fomsgaard et al., 2018; Klein et al., 2014).

1.12.2.2. Rasgos conductuales

Multitud de estudios han comparado ratas RHA con ratas RLA (u otro tipo de ratas de estudio) respecto a diferentes rasgos conductuales y hormonales. Entre los resultados se muestra que las ratas RHA presentan ansiedad y respuestas de frustración reducidas, respuestas hormonales al estrés disminuidas, un aumento en la búsqueda de sensaciones novedosas y recompensas, además de una mayor actividad locomotora (p. ej., Escorihuela et al., 1999; Fernández-Teruel et al., 1997; López-Aumatell et al., 2009a; Moreno et al., 2010; revisado en Fernández-Teruel et al., 2021, 2023; y Giorgi et al., 2019). Además, este perfil comportamental se ve reforzado por una mayor vulnerabilidad a la autoadministración de drogas (p. ej., alcohol, cocaína) y a la adicción, sumado al hecho de que las ratas RHA muestran un perfil impulsivo en varias tareas (Giorgi et al., 2019; Moreno et al., 2010). Estudios de la conducta social han manifestado que las ratas macho RHA presentan un déficit en la preferencia social (Oliveras et al., 2022b; Sampedro-Viana et al., 2021, 2023a). Además, las ratas madres RHA muestran menor conducta protectora de sus crías que las ratas madres RLA (Driscoll et al., 1979; Fernández-Teruel et al., 2021; Río-Álamos et al., 2014).

1.12.2.3. Rasgos cognitivos en RHA

Las ratas RHA muestran una reducción de la inhibición latente en los paradigmas de evitación activa bidireccional y de sobresalto potenciado por el miedo, además de una disminución en el tiempo de "congelación" y de la respuesta de sobresalto condicionadas a una señal discreta o a un contexto en comparación con las ratas RLA y con ratas de otras cepas (Esnal et al., 2016; Fernández-Teruel et al., 2006; López-Aumatell et al., 2009a, 2009b). Con respecto al filtraje atencional, ensayos de PPI, muestran consistentemente que las ratas RHA presentan un deterioro de la PPI en comparación con las ratas RLA y ratas genéticamente heterogéneas (Oliveras et al., 2015, 2022a; Río-Álamos et al., 2019; Tapias-Espinosa et al., 2018, 2019). Finalmente, estudios para evaluar diferentes tipos de aprendizaje y memoria en el laberinto acuático de Morris (MWM, *"Morris Water Maze"*) y otras tareas, muestran que las ratas RHA presentan un deterioro de la memoria de trabajo espacial, de la memoria espacial de referencia y de la flexibilidad cognitiva en relación con las RLA y ratas genéticamente heterogéneas (Escorihuela et al., 1995; Martínez-Membrives et al., 2015; Oliveras et al., 2015; Río-Álamos et al., 2019).

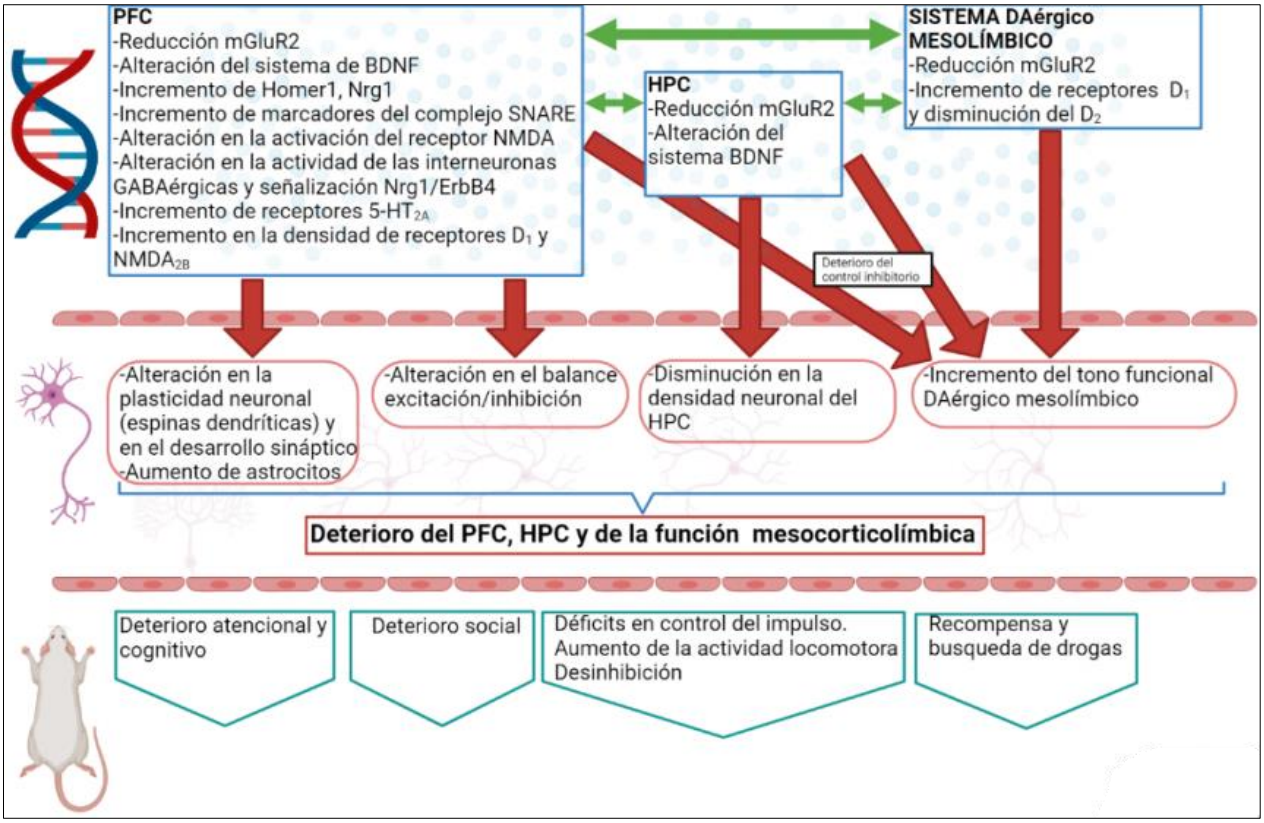


Figura 4. Esquema de las principales alteraciones moleculares, celulares y conductuales del modelo de rata RHA para el estudio de la esquizofrenia. Adaptado de Fernández-Teruel et al., (2021). Las flechas verdes indican "interacción" mientras que las flechas rojas muestran "alteración" o "deterioro". PFC=Corteza prefrontal; HPC=HPC; DA=Dopamina; mGluR2=Receptor metabotrópico de glutamato 2; BDNF= Factor neurotrófico derivado del cerebro; Nrg1=Neuregulin 1; SNARE= Receptores de proteínas de fijación soluble del factor sensible a la N-etilmaleimida; NMDA= N-metil-D-aspartato; ErbB4= Proteína tirosina kinasa receptora; 5-HT= Serotonina.

2. OBJETIVOS

2.1. Objetivos generales

Explorar las asociaciones entre la inhibición prepulso de la respuesta de sobresalto (PPI), interacción social (IS), y algunas funciones cognitivas (aprendizaje y memoria espacial), en las ratas heterogéneas HS y las cepas Roman (RHA y RLA), así como evaluar en estas últimas los efectos de la estimulación neonatal (NH) sobre aquellos procesos y sobre la expresión de genes relacionados con función sináptica y neuroplasticidad en dos edades diferentes.

2.2. Objetivos específicos

ESTUDIO 1

1. Evaluar las asociaciones entre la inhibición prepulso (PPI), interacción social (IS) y procesos cognitivos en ratas HS.

ESTUDIO 2

2. Evaluar las asociaciones entre la inhibición prepulso (PPI), interacción social (IS) y procesos cognitivos en ratas hembra RHA vs. RLA.
3. Estudiar, en ratas hembra RHA vs. RLA, las diferencias en fenotipos de conducta exploratoria y social, así como en procesos atencionales (PPI) y cognitivos (aprendizaje y memoria espacial).

4. Evaluar, en hembras RHA vs. RLA, si el tratamiento de estimulación neonatal (NH) produce efectos sobre dichos comportamientos y procesos atencionales/cognitivos similares a los ya observados en machos de ambas cepas.

ESTUDIO 3

5. Estudiar, en machos de ambas cepas, y en dos fases del neurodesarrollo (adolescencia y adultez), las diferencias en conducta exploratoria, social y en la PPI, así como en la expresión de genes relacionados con la neuroplasticidad, la maduración cerebral y función sináptica en la corteza prefrontal (CPF).

6. Evaluar los efectos de la estimulación neonatal (NH) sobre la conducta exploratoria, social y la PPI, así como sobre la expresión génica en CPF, en ratas machos de ambas cepas y a las dos edades.

7. Evaluar, en machos de ambas cepas y ambas edades, si los efectos de la estimulación neonatal (NH) sobre la conducta exploratoria, social y la PPI se relacionan con los efectos del tratamiento sobre la expresión génica en la CPF.

3. RESULTADOS

ESTUDIO 1

Low prepulse inhibition predicts lower social interaction, impaired spatial working memory, reference memory and cognitive flexibility in genetically heterogeneous rats

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ABSTRACT

The “Genetically Heterogeneous National Institutes of Health (NIH–HS)” stock rat (hereafter HS) shows a wide phenotypic variation, as a result of having been derived from eight inbred rat strains. Thus, these rats may be a conceivable parallel model of a healthy human sample. In order to evaluate whether HS rats have face validity as an animal model of schizophrenia-relevant features, it should be demonstrated that they present behavioural traits that may model negative and cognitive symptoms of the disorder. Previous studies on HS rats have shown that prepulse inhibition (PPI, a measure of sensorimotor gating processes), which is impaired in schizophrenic patients, is correlated with their working memory performance. In this study, we evaluated whether low PPI in the HS stock rat predicts impairments of spatial working memory (SWM), spatial reference memory and cognitive flexibility in the Morris water maze (MWM) test, and we evaluated HS rats for social interaction (SI) in a social investigation task. HS rats were stratified into 2 different groups according to their PPI scores, i.e., low- and high-PPI. In the SI task, low-PPI rats showed decreased social behaviour compared to high-PPI rats. In addition, relative to high-PPI HS rats, the low-PPI group displayed poorer SWM performance, impaired cognitive flexibility (in a reversal task) and worsened long-term spatial memory. Such differential behaviours in social and cognitive paradigms provide evidence on the face validity of low-PPI HS rats as a model of negative-like and cognitive schizophrenia-relevant traits.

Keywords

Schizophrenia-linked traits, outbred HS rats, prepulse inhibition, social interaction, working memory, reference memory, cognitive flexibility, long-term memory.

1. Introduction

Impairments of attention-related processes, such as sensorimotor gating, are considered to be a common psychophysiological feature of schizophrenia [1–3]. Sensorimotor gating relates to information processing, and filtering out unnecessary sensory information, which may have downstream effects on core symptoms of the disease. One way to measure sensorimotor gating, which refers to the ability of filtering out irrelevant inputs from significant ones, is prepulse inhibition (PPI), which measures the ability of a rather weak acoustic stimulus to reduce the acoustic startle response (ASR) triggered by a subsequent acoustic pulse of higher intensity. PPI is impaired in patients with schizophrenia, amongst other neuro-psychiatric disorders, such as obsessive-compulsive disorder, Tourette syndrome, and Huntington's disease, amongst others [3]. PPI appears to be a rather simple quantifiable measure in both rodents and humans, therefore providing great value for translational research on the neurobiological mechanisms underlying neuropsychiatric disorders such as schizophrenia [4, 5]. Several studies have shown PPI to be disrupted in patients with schizophrenia, as well as in unaffected relatives of patients with schizophrenia and in other mental disorders, and thus it is widely considered an endophenotype of the disorder (e.g., reviews by [3,4, 6–9]).

Although its main regulatory circuit involves the midbrain inferior colliculus, the superior colliculus, the pedunculopontine tegmental nucleus, and the caudal pontine reticular nucleus, PPI is also known to be modulated by several cortical and subcortical areas, including the orbitofrontal cortex, anterior cingulate, medial prefrontal cortex, nucleus accumbens, basolateral amygdala, and the hippocampus [3,8]. The fact that these high-order-processing brain regions modulate PPI suggests that this process might be related to other schizophrenia-linked symptoms.

PPI in rodent models can be impaired by several manipulations, including brain lesions, social isolation rearing and pharmacologically- induced PPI deficits (used mainly to test antipsychotic drugs), such as administration of dopaminergic psychostimulants or glutamate NMDA-receptor antagonists amongst others. Still, non-pharmacological models of reduced PPI remain key models in revealing information about the neurobiological bases of PPI impairments (e.g. [10]).

An important issue in schizophrenia research is to explore whether PPI performance goes along with other schizophrenia-relevant (positive, negative and cognitive) symptoms, as such clusters of symptoms may facilitate progress in neurobiological (and treatment) research on the disorder. In this context, for example, Braff et al. [11] reported that PPI levels negatively correlated with scores on both the Scale for the Assessment of Positive Symptoms (SAPS) and the Scale for the Assessment of Negative Symptoms (SANS), which measure severity of positive and negative symptom in patients with schizophrenia. In addition, low PPI levels characterized on healthy human subjects were found to be correlated with poor strategy scores on executive functions [12]. These findings raise the question of whether (or to what extent) sensorimotor filtering deficits could be involved in (or could precede) the appearance of (some) positive, negative and cognitive symptoms related to schizophrenia.

In the present study we asked whether PPI might predict (or be associated with) negative-like and/or cognitive schizophrenia-relevant symptoms (as measured in rodents). Social withdrawal is one of the main negative symptoms of schizophrenia, and it may originate from general anhedonia, anxiety and/or social cognitive deficits. There are many ways of measuring sociality vs. asociality in rodents. In this regard, Deak et al. [13] and Gururajan et al. [14] have proposed an interesting social interaction (SI) paradigm in rats that separates anti- psychotic/propsychotic drug effects on social motivational processes from their effects on non-social exploration, social dominance and locomotor activity. Using this procedure, we have reported that the bidirectionally and selectively-bred Roman high- (RHA) vs. low-avoidance (RLA) strains, which exhibit low vs. high PPI scores, respectively [15–19], also show differences in social interaction preference (RHA<RLA) that are independent of their levels of non-social exploration, locomotor activity and anxiety [20–22].

The aim of this study was to characterize a cohort of genetically heterogeneous (outbred) rats by their PPI performance, and to explore the possible associations of PPI with social behaviour and spatial cognition. The “National Institutes of Health Genetically Heterogeneous stock” (HS) rat [23] constitutes the most genetically heterogeneous laboratory rat in existence, and thus they might function as a conceivable analogous model of a healthy human sample which could provide more

generalizable results than the ones achieved with common laboratory rodent lines/strains (e.g. [17,24–27] and references therein).

Thus, we intended to evaluate, in a group of adult male HS rats, whether sensorimotor gating (i.e., PPI) profiles might predict performance in tests of social interaction (SI), learning and memory, including spatial reference and working memory, and cognitive flexibility (i.e., spatial reversal learning). The central hypothesis was that, if PPI shares some underlying mechanism with sociability and/or spatial learning- memory and cognitive flexibility processes, then we would expect that low-scored PPI rats would show deficits in sociability (i.e., time spent in social interaction), as well as in cognitive performance in spatial working memory, reference learning/memory, reversal spatial learning and/or long-term spatial reference memory.

2. Material and methods

2.1. Animals

The subjects used were 44 males of the genetically heterogeneous stock (HS) rat from the permanent colony bred and maintained at our laboratory (Medical Psychology Unit, Dept. Psychiatry & Forensic Medicine, School of Medicine, Autonomous University of Barcelona) since 2004. The HS stock was established in the 1970s at the NIH [23], derived from an eight-way cross of the following 8 independent inbred rat strains: Maudsley Reactive (MR/N), Wistar-Nettleship (WN/N) and Wistar-Kyoto (WKY/N) from the original Wistar stock; M520/N and Fischer 344 (F344/N), that were established in 1920 and have an un- known origin; Brown Norway (BN/SsN), from a colour mutant stock of wild rats; the Agouti (ACI/N), from hybrids between two stocks; and the Buffalo (BUF/N). This eight-way cross led to a particular genetic mosaic of parental haplotypes and high genetic heterogeneity in the HS rats ([24,28–30]).

The rats were approximately 3–4-month-old when the experiments began, and had a weight range of 384 ± 47.6 g (mean ±SD). It is important to highlight that these 44 rats were taken from 40 different families/litters, thus representing the genetic variability of the whole HS rat colony. They were kept in same-sexed pairs in standard macrolon cages, under a 12:12 h light-dark cycle (lights on at 8:00 a.m.), with temperature and

humidity set at 22 ± 2 ◦C and 50 ± 20% (respectively) and with ad libitum access to food and water.

After approval by the Committee of Ethics of the Autonomous University of Barcelona in accordance with the European Communities Council Directive (86/609/EEC) regarding the care and use of animals for experimental procedures, the experiments were performed from 9:00 to 14:00 h. and the whole study was performed within the frame of rules specified by the National Institute for animal experiments.

2.2. Experimental procedures

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

For testing the acoustic startle response (ASR), acoustic stimuli were delivered using two identical startle response systems (SR-Lab Startle Response System, San Diego Instruments, San Diego, USA). Each one consisted of a non-restrictive tubular enclosure, located on the top of a Plexiglas platform, in a ventilated and sound-attenuated 90 × 55 × 60 cm chamber lit by a 10 W lamp. The rat was placed in the cylinder, which had a sensor that detects the movements made by the rat. Acoustic stimuli were delivered through 2 high frequency speakers situated 15 cm from each side of the cylinder, and a white noise generator provided background noise. The recorded data was transduced by an accelerometer into a voltage which was amplified, digitalized and saved into a computer in a single-file for further exportation and analysis.

The experiment was conducted in an isolated laboratory room. Each startle session started with a 5 min habituation period, where rats were exposed only to continuous background noise. Next, 10 pulse-alone trials (40 ms pulses of 105 dB) were delivered in order to obtain a basal measure of the ASR (“baseline-1” trials). Afterwards, in a randomized order and with a randomized intertrial interval of 10 to 20 s (with an average of 15 s), 60 more trials were delivered, consisting of 10 repetitions of each of the following 6 trial types: startle-alone trials (40 ms pulses of 105 dB; “baseline-2” trials); PPI trials (prepulses of 65/70/ 75/80 dB, 20 ms each, precede the 40 ms startle pulse of 105 dB, with an interval between prepulse and pulse of 100 ms); control trials (no stimulus delivered, rats were merely exposed to background noise of 55 dB). The session ended with five pulse-alone trials, which were not considered for the present study. The

startle amplitude magnitude was measured by an accelerometer and calculated as an integrated response of the enclosure vibrations caused by the whole rat's body startle re-action after startle stimulus onset (recording range: 200 ms). Startle response amplitude was defined as the maximum accelerometer voltage during the first 200 ms following the startle stimulus onset. Data was obtained in an Excel file. PPI values obtained at each prepulse intensity were indexed by percent PPI and calculated as follows:

% PPI = 100 - [(startle amplitude on prepulse trials / startle amplitude on pulse "baseline-2" trials) * 100]

Eight rats were excluded from the rest of studies due to technical problems with the startle system. Taking as a reference the median value (median = 52.72) of "Total%PPI" (averaged from the 4 prepulse intensities) of the remaining 36 rats, they were divided in two groups, either showing scores above (high-PPI group) or below (low-PPI group) the median. These selected 36 rats remained in their home cages (in pairs) until the end of the experiment. Three weeks after forming the two PPI groups, rats were evaluated in a social interaction (SI) test and, 72 h after the SI test they underwent several spatial tasks in the Morris water maze (MWM) following the timeline depicted in Fig. 1.

The observers were always blind to the group condition (high- or low-PPI) to which rats belonged.

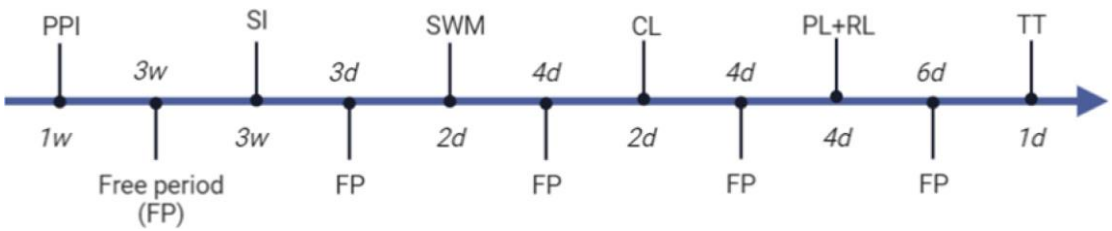


Fig. 1. General overview of the experimental procedure, timeline of behavioural tasks and free periods. Abbreviations: PPI, prepulse inhibition test; SI, social interaction test; SWM, spatial working memory task; CL, cued learning task; PL, place learning task; RL, reversal learning task; TT, transfer test. "2d-6d", "1w-3w", days or weeks elapsed, respectively.

2.2.2. Social interaction (SI)

The social investigation apparatus and social interaction (SI) testing procedure have been previously described ([13,14]; see Drawing 1 in [21]). The apparatus consisted of two adjacent solid semi-transparent dark methacrylate boxes measuring 65 cm long x 20 cm wide x 23 cm high. Each box had 2 holes measuring 3 cm in diameter, a diameter that allows an adult rat to put its head through but not the rest of the body (they could not escape, only explore). The hole that faced the contiguous box was labelled as the social hole, and the hole that faced the opposite wall was the non-social one. The distance between both social holes was 12 cm, a distance that allowed the rats to perceive each other, but prevented physical interaction or contact between them. There was a digital video camera located on middle-top of the boxes 120 cm above from the floor of the arena. SI testing took place in a dimly red-lighted room.

Unrelated and unfamiliar rats were matched according to their weight (with a maximum body weight difference of ± 20 g) and paired with a rat from the same (high- or low-) PPI group (that is, 9 rat pairs per group) for SI testing. None of the pairs were housed together.

In days 1–3 the rats underwent three habituation sessions (one per day) in which they were placed for 30 min/day in the testing room remaining in their home cage (without food or water during these 30 min). In the 4th day each rat underwent an individual 30 min habituation session to the experimental SI cage within the testing room. In the SI testing day (day 5), one rat of each pair to be tested for SI was placed in the middle of each SI testing box. Experimenters immediately left the test room and the rats' behaviour was recorded with a video camera for 15 min. The protocol was randomized in every possible way in order to avoid any external effect on the rats' behaviour: rat pairs on the habituation procedures were different from rat pairs on the SI test day, and the testing order was randomized in both habituation and SI testing procedures. Each rat was tested only once. Between tests, the boxes were cleaned with a 70% ethanol solution and dried with a paper towel to remove defecations and eliminate olfactory signals. Both habituation and experimental SI sessions were performed from 9:00 to 14:00 h.

Social and non-social investigative behaviour was defined based on the total amount of time that rats spent at both holes over the 15 min session. Locomotor activity (crossings across the 3 squares –marked on the floor- that divided the SI boxes) and self-grooming were also scored (see procedural details in [21]). Datasets for the total time spent in each session were grouped into 5 min time slots.

2.2.3. Morris water maze tasks (MWM)

Tasks in the Morris water maze (MWM) were carried out in a circular water plastic tank (140 cm diameter, 30 cm deep) filled with water (maintained at 24±1 ◦C) made opaque with nontoxic white tempera paint. The escape platform was a circular plastic cylinder (16 cm diameter, 28 cm height) fixed and submerged 2 cm below the water surface (except otherwise indicated), therefore invisible to the rats. The pool was surrounded by visual cues present at the laboratory that remained unchanged during the whole experiment. An imaginary cross formed by two axes (each one perpendicular to the other) established 4 cardinal points: North (N), South (S), East (E) and West (W). This maze division created 4 quadrants. Rats began each trial facing the wall of the tank (see detailed procedure for each task below).

Swimming behaviour was recorded with a video camera placed above the water maze. The videos were further analyzed with a tracking system (Smart v.2.5.14, PANLAB, Barcelona, Spain). This system pro- vided the latency to find the platform (time elapsed until a rat reached the platform) and path length (distance travelled) from each rat and trial.

Five different tasks were conducted in the MWM. Latencies and distances travelled were measured (except otherwise specified). Detailed procedures and schedule are schematized in Fig. 2 and explained below.

i Spatial working memory (SWM)

Rats were tested on 2 consecutive days, and they went through 2 trials per day. Both the starting point and the platform location were constant within a day, and changed from day 1 to day 2 (see Fig. 2).

In each trial rats were allowed to swim for 90 s or until the platform was located. If the subject failed to find the platform within 90 s, the rat was gently guided (not placed) to the platform. After the platform was reached, the rat remained at the platform for 15 s, after which it was placed in its cage for another 15 s before the second trial began. After the second trial, which was equal to trial 1, the rat was put back in its home cage.

The improvement of latencies/distances in trial 2 vs. trial 1 represents the level of spatial working memory (SWM).

The second SWM session (day 2) was identical to the first one except for changing the platform position and the starting point (see Fig. 2).

ii Cued learning task (CL)

The "Cued learning task" (CL) was carried out as a way to assess the validity of the spatial tasks. The platform was 1 cm above the water surface and was cued with a 15 cm striped black and white flag. This design ensures that the rat swims directly to the platform (guided by the proximal cue/s, i.e., the black and white flag and the visible platform), unless it has impaired perceptual, visual or motor deficits, lack of motivation, etc. (which could interfere with spatial learning in the other tasks or even influence PPI performance).

For the CL task 9 rats/group were randomly selected from the respective high-PPI (n = 18) and low-PPI (n = 18) groups. This was done because our previous observations (unpublished) suggested that testing in the cue task before performing other tasks could sometimes lead to carryover effects that might alter performance in successive spatial tasks. To prevent this (and to test it) we decided to test only half of the rats. The task was performed during 2 consecutive days, 4 trials/day, in which the platform remained in the same position. Starting points were N-S-E-W in both days (see Fig. 2). Duration and procedure for each trial were as in the SWM task. After each trial the rat remained in their home cage for 15–20 min until the next trial started.

iii Spatial place/reference learning (PL)

During this task the platform was hidden (and non-cued) 2 cm below the water surface (as in the SWM task) and remained always in the same position (see Fig. 2). Rats

underwent 4 trials/day for 2 consecutive days. Duration and procedure for each trial were as in the SWM task. Starting points were N-S-E-W in day 1, and E-N-S-W in day 2 (Fig. 2). After each trial the rat remained in their home cage for 15–20 min until the next trial started.

iv Reversal spatial learning task (RL) – cognitive flexibility

During this task the hidden platform was placed in the quadrant opposite to that of the PL task and remained always in the same position (see Fig. 2). Rats underwent 4 trials/day for 2 consecutive days. Duration and procedure for each trial were as in the SWM and PL tasks. Starting points were W-S-N-E in day 1, and S-E-W-N in day 2 (Fig. 2). After each trial the rat remained in their home cage for 15–20 min until the next trial started.

v Transfer test/trial (TT)

One week after the RL task, a transfer trial (TT) was conducted to assess long-term reference memory. The platform was removed from the pool, and rats' platform-searching swimming behaviour was tracked for 60 s. The starting point was S (see Fig. 2). The time spent in the last targeted quadrant (where the platform was placed during the RL task) and number of crossings over the last platform location (annulus crossings) were measured.

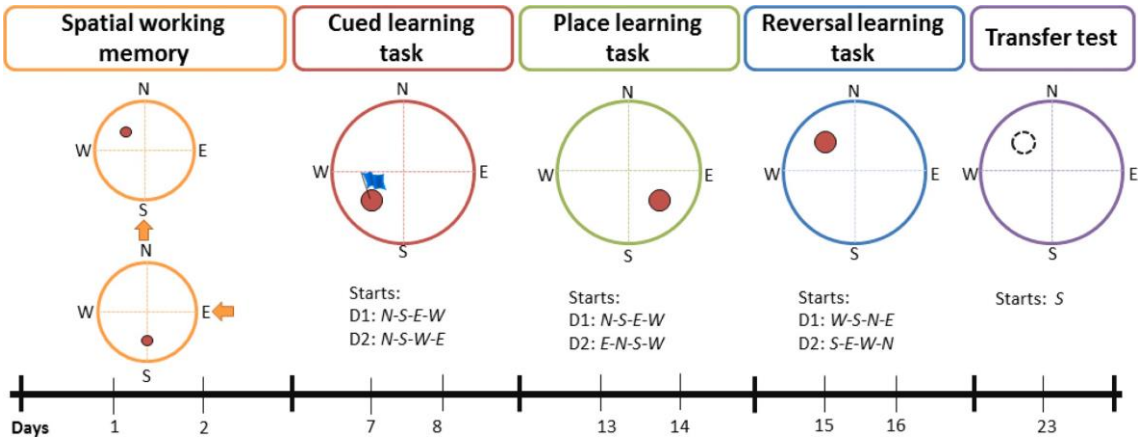


Fig. 2. Sequence, timing, order and details of the learning tasks performed in the MWM. Abbreviations: N, S, E, W; north, south, east, west, respectively (starting points). D1, D2; day 1, day 2, respectively.

2.3. Statistical analysis

Statistical analysis was performed using the “Statistical Package for Social Science” (SPSS, version 17).

Repeated measures (2 groups x 4 prepulse intensities) ANOVA was applied to %PPI scores of the two stratified PPI groups (between-subject factor) across the 4 prepulse intensities (within-subject factor). Stu- dent’s t-tests for independent samples were applied to baseline startle 1 and 2, as well as to total %PPI (averaged for the 4 prepulse intensities). For the SI, repeated measures ANOVAs (“high vs. low-PPI” as between-subject factor, and the three 5-min intervals as within-subject factor) were conducted with the amount of time spent on social and non-social holes.

In the MWM tasks, latency and/or distance travelled to reach the platform were taken as main dependent variables for the SWM, PL, RL and CL tasks. For SWM measures, repeated measures ANOVAs were performed with latencies and distances from the 2 trials from the 2 days as within-subject factors (“high vs. low-PPI” as between-subject factor). Student’s t-tests (for independent samples) were applied to the per- centage latency or distance of trial 1 vs. trial 2.

Analyses of the 8 trials of the CL task were carried out with repeated measures ANOVAs taking the 8 distance measures as within-subject factors (and “high vs. low-PPI” as between-subject factor). Analyses of the 8 trials of the PL and RL tasks were carried out with repeated measures ANOVAs taking the 8 distance measures as within-subject factors, and “high vs. low-PPI” and “cued vs. non-cued” as between- subject factors. Regarding the TT, the recall of the platform location was measured with a two-way ANOVA on annulus crossings and on time spent in the last targeted quadrant (with “high vs. low-PPI” and “cued vs. non-cued” as between-subject factors). Student’s t-test (for independent samples) between both PPI-stratified groups was then applied to both variables.

To explore whether PPI levels and scores in the behavioural and cognitive tests/tasks may be related to (or may share association with) some common underlying trait a principal component factor analysis (PCA) was carried out with the most relevant variables of the study.

The level of significance of all comparison analyses was set at $p < 0.05$, two-tailed, except otherwise indicated (since in some particular cases one-tailed significance was used because we had an a priori hypothesis on the direction of effects).

3. Results

3.1. Prepulse inhibition

Table 1 shows the mean ±S.E.M of the different variables from the PPI session from the two subgroups of HS rats stratified on the basis of their %PPI performance. Results show that the 2 groups do not diverge in the magnitude of the baseline startle response (baseline-1, baseline-2; both $t(1,34) < 0.59$, $p > 0.55$). Repeated measures ANOVA between the two groups (between-subjects factor) and the net startle response under the 5 stimulus conditions of the PPI phase (i.e., within-subjects factor "stimulus type": baseline-2, prepulse 65, prepulse 70, prepulse 75, prepulse 80) revealed significant effects of "stimulus type" (Huynh-Feldt (df 4, 40.90) = 23.83, $p < 0.001$) but no significant group effect ($F(1, 34) = 0.80$, $p = 0.38$) (Table 1).

Regarding %PPI scores, repeated measures ANOVA with the 2 groups as between-subject factor and the 4 prepulse intensities as within-subject factor, revealed a significant effect of prepulse intensity (Huynh-Feldt (df 3, 78.1) = 43.39, $p < 0.001$), indicating that %PPI increased in parallel to the increase of prepulse intensity. ANOVA also yielded a very significant group effect ($F(1, 34) = 87.21$, $p < 0.001$), confirmed also by a very significant group difference in total PPI (averaged for the 4 pre pulse intensities; $t(34) = 9.34$, $p < 0.001$; Table 1). Likewise, the ANOVA on square root-transformed %PPI data (Table 1) yielded significant effects of prepulse intensity (Huynh-Feldt (df 2.33, 79.29) = 40.33, $p < 0.001$) and group ($F(1,34) = 85.87$, $p < 0.001$) (Table 1).

Table 1. Startle response in different trial types and percent prepulse inhibition in high- and low-PPI stratified HS rats

Groups Variables	*High*-PPI (n = 18)	*Low*-PPI (n = 18)
(A) STARTLE RESPONSE		
Baseline-1	1723.90 ± 293.90	1998.40 ± 355.60
Baseline-2 (#)	945.00 ± 315.80	906.80 ± 139.80
Prepulse 65	535.12 ± 175.17	721.73 ± 124.35
Prepulse 70	334.60 ± 99.46	560.44 ± 70.98
Prepulse 75	286.85 ± 100.84	465.71 ± 65.35
Prepulse 80	180.74 ± 60.99	338.26 ± 44.96
Average (65 to 80)	334.32 ± 107.75	521.53 ± 72.78
(B)% PPI		
%PPI65	43.6 ± 3.5	19.7 ± 6.3 **
%PPI70	61.9 ± 2.6	32.9 ± 4.5 **
%PPI75	71.2 ± 2.2	46.3 ± 3.3 **
%PPI80	80.3 ± 1.9	60.1 ± 2.2 **
Total%PPI	64.3 ± 1.4	39.8 ± 2.3 ***
(C) Sqrt%PPI		
Sqrt-%PPI65	6.48 ± 0.29	4.57 ± 0.43 **
Sqrt-%PPI70	7.83 ± 0.17	5.71 ± 0.34 **
Sqrt-%PPI75	8.41 ± 0.13	6.69 ± 0.30 **
Sqrt-%PPI80	8.95 ± 0.09	7.73 ± 0.14 **
Sqrt-Total%PPI	8.00 ± 0.85	6.25 ± 0.19 *

Mean ± S.E.M of startle response in (A) baseline-1, baseline-2 and "prepulse (65 to 80 dB) + pulse" trials, and (B)%PPI and (C) square-root-transformed %PPI levels. (#) Baseline-2; averaged startle response for the 10 trials that was used for %PPI calculation (see formula in "Material and methods"). **p < 0.001, "group" effect from repeated measures ANOVA including the 4 prepulse intensities (%PPI65 to %PPI80, or Sqrt-%65 to Sqrt-%80) as within-subject factor (see text for details). *p < 0.05, *** p < 0.001 between both groups (Student's t- test).

3.2. Social interaction test

Fig. 3A shows the "social time" (time spent exploring the social holes) every 5 min. Repeated measures ANOVA showed a significant "group x time" effect on social activity at the social hole ($F(1,34) = 6.45$, $p < 0.016$) (Fig. 3A). To better explore the source of this "group x time" interaction we applied one-way ANOVA to social time 5–10 min and to the sum of social time 5–10 min plus social time 10–15 min.

These analyses revealed group effects on both measures ($F(1,34) = 4.12$, $p = 0.025$; and $F(1,34) = 3.10$, $p = 0.044$, respectively; both one-tailed, as we had specific hypothesis on the direction of the effects, i.e., we expected that the high-PPI group would exhibit higher social interaction) (see "Group effect" indicated in Fig. 3A).

No significant differences were found between both groups in "non- social time", locomotor activity or grooming (all Fs $(1,34) < 1.80$, all $p > 0.19$); the only effects on these variables were of "time interval" on grooming (Huynh-Feldt (df 2, 51.77) = 7.34, $p = 0.004$) and locomotor activity (Huynh-Feldt (df 2, 58.17) = 29.67, $p < 0.001$) (Fig. 3B-D).

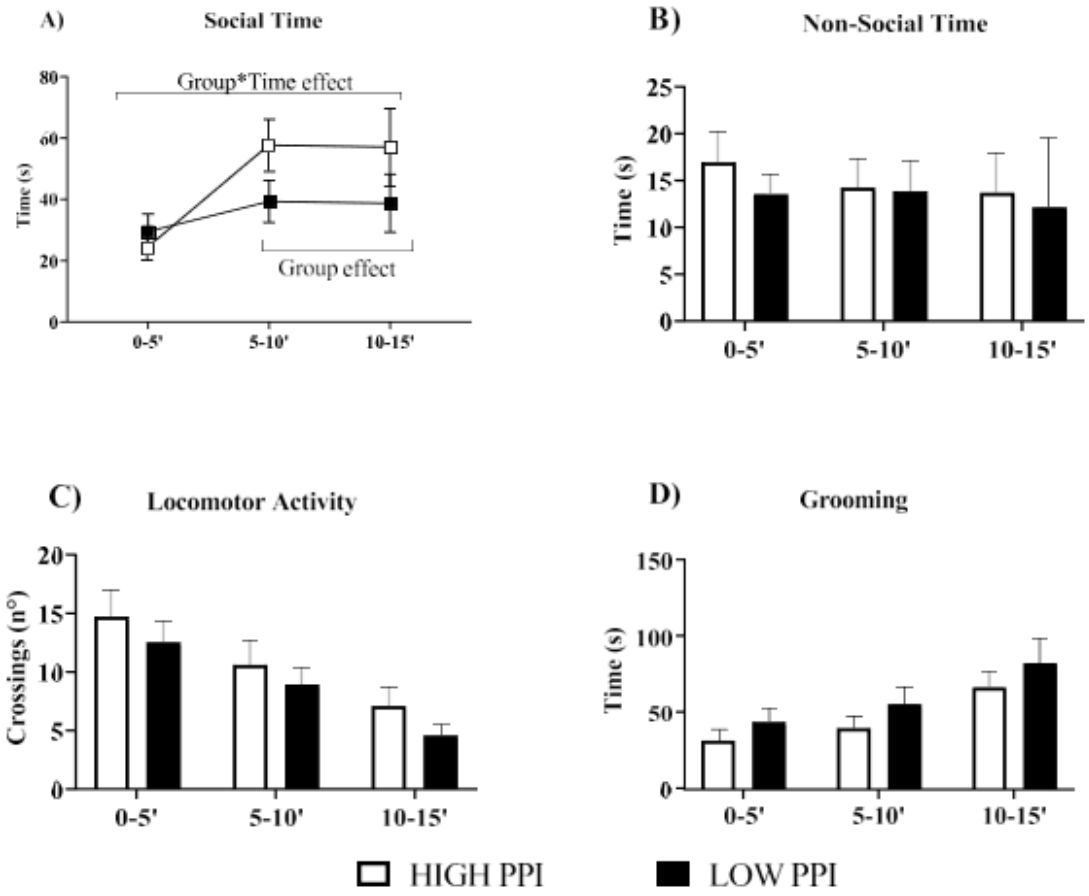


Fig. 3. Behaviour of high- and low-PPI rats in the social interaction test. Time spent investigating at the social (A) or non-social (B) holes divided in three 5-min intervals. (C) Locomotor activity (number of crossings) in 5-min intervals. (D) Time spent self-grooming in 5-min intervals. "Group x time" significant effect, repeated measures ANOVA (see Results for details). "Group" effect, indicates between-group significant differences (one-way ANOVA) during the 5–10 min and 5–15 min intervals (see Results for details). All data are expressed as mean ± S.E.M

3.3. Morris water maze

3.3.1. Spatial working memory (SWM)

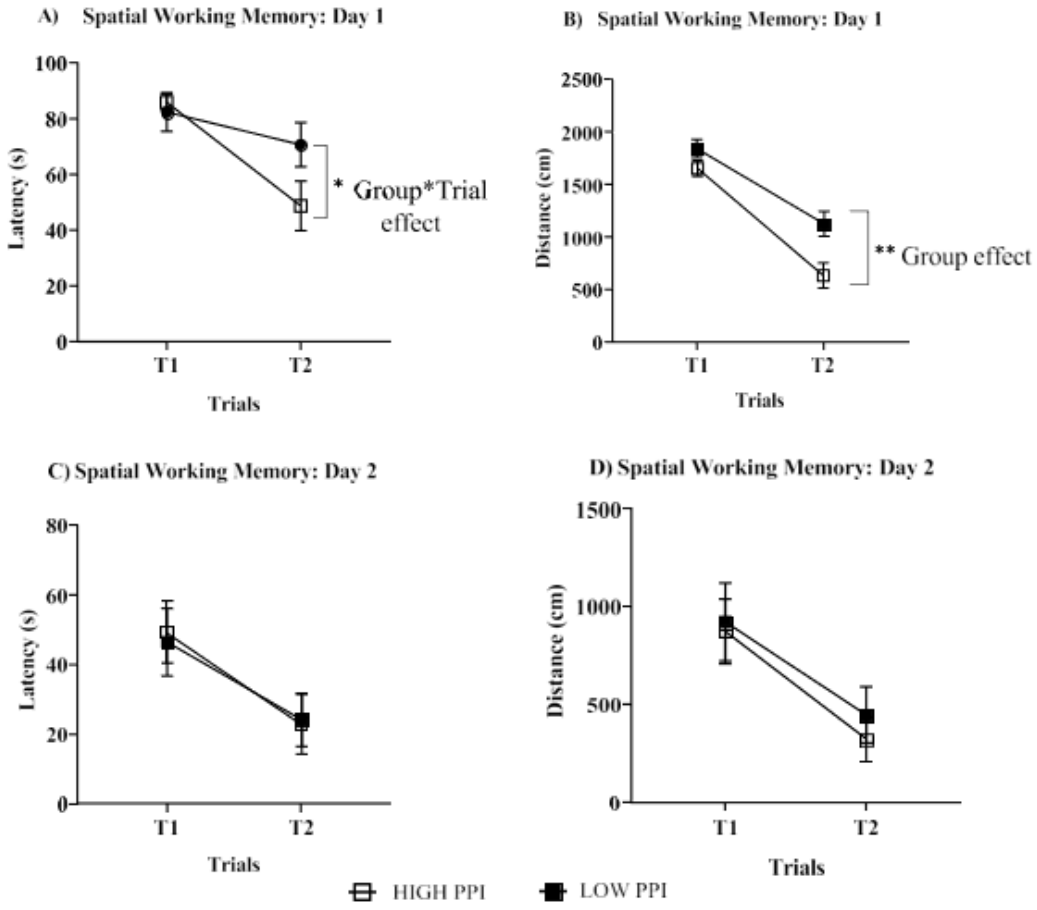
Data from both latencies (to find the platform) and distance travelled were analyzed in the SWM task because they provided complementary results (Fig. 4A-D). In the remaining tasks only distances are presented, since latencies are usually more dependent on swimming speed and because latencies did not contribute anything to the results other than the distances in the rest of the tasks.

For the SWM analyses, rats with latency values below 10 s in the first trial were removed from the analysis (i.e., not included in the analysis). This was done to ensure that the experience of the first (acquisition) trial must be sufficient (and comparable between subjects), since rats that in the first trial find the platform in e.g., 5–10 s, (i) do not have a true acquisition experience (of spatial navigation) as have those rats that take, e.g., 30, 40, 60 or 80 s swimming in the tank to find the platform, and (ii) when a rat takes so short time (i.e., < 10 s) to find the platform it is rather unlikely that it can show any working memory in trial 2, because of a floor effect. Thus, the initial "n" (18 subjects/group) was reduced to n = 12 (high-PPI) and n = 11 (low-PPI) as a consequence of removing rats with latency values below 10 s on the first trial.

Thus, in the SWM task, repeated measures ANOVA showed a nearly significant effect of "group x trial" on latency values from the first day ($F(1,21) = 4.18$, $p = 0.054$) of the SWM task (Fig. 4A). Student's t-test for latency in trial 2 was $t(21)=1.81$, $p = 0.042$ (one-tailed, given that we had a directed hypothesis in the sense of the difference in trial 2, i.e. latency in trial 2 should be lower in the high-PPI group) (Fig. 4A). Repeated measures ANOVA showed a significant global group effect on the distance travelled in the first and second trials from the first day ($F(1,21) = 8.20$, $p < 0.01$) (Fig. 4B). No significant effects were observed in the second day of the SWM task (Fig. 4C-D).

We calculated the percentage of latency and distance of trial 1 with respect to trial 2 for each day (so, higher values indicate greater per- centage of trial 1 relative to trial 2, i.e., better working memory), and after a ln(sqrt) transformation of the percentages we applied Student's t- test for independent samples to compare both groups. The results, as shown in Fig. 4E, revealed significant differences between both groups for day 1

latency (t(21) = 2.08, p = 0.05) and distance (t(21) = 2.53, p = 0.02), whereas there were no effects on any variable in day 2 (Fig. 4E).



E) Percent differences of latency and distance between trials 1 and 2 in the SWM task

	DAY 1		DAY 2	
	Latency	Distance	Latency	Distance
High-PPI	2.69 ± 0.10 **a**	2.87 ± 0.09 **b**	2.98 ± 0.19	2.97 ± 0.20
Low-PPI	2.38 ± 0.11	2.58 ± 0.06	2.73 ± 0.18	2.72 ± 0.19

Fig. 4. Performance of high- and low-PPI rats in the spatial working memory task(A) Latency to reach the platform in the first (T1) and second (T2) trial of day 1 in the spatial working memory (SWM) task. (B) Distance (cm) travelled by the rats in the first (T1) and second (T2) trial of day 1 in the SWM task. (C) Latency to reach the platform in the first (T1) and second (T2) trial of day 2 in the SWM task. (D) Distance (cm) travelled by the rats in the first (T1) and second (T2) trial of day 2 in the SWM task. (E) Percent latency and distance [both ln(square root)-transformed] of trial 1 vs. trial 2 of day 1 and day 2 in the SWM task. All values are means ± SEM. "Group x trial" and "Group" effects are from repeated measures ANOVA (see Results for details). a, $p \leq 0.05$; b $p \leq 0.02$ between high-PPI and low-PPI groups (Student's t-test).

3.3.2. Cued learning task (CL)

Fig. 5A shows the distance travelled in the 8 trials of the CL task. The results of the repeated measures ANOVA did not reveal significant differences between the two groups in acquisition ($F(1,16) = 0.89$, $p = 0.35$), nor significant "group x trial" interaction ($F(1,16) = 3.07$, $p = 0.053$).

3.3.3. Spatial acquisition–place learning task (PL)

A repeated measures ANOVA on the distance across the 8 trials was performed, with "group" ("high vs. low-PPI") and "cued" ("cued vs. non- cued") as between-subject factors. There were no "Cued" effects or interactions (both $F(1,34) < 3.2$, $p > 0.08$), so results in Fig. 5B are pooled by "group". There was an overall trial effect (Huynh-Feldt (df 3.59, 118.56) = 6.47, $p < 0.001$), but neither group effect nor its interaction with trials revealed statistical significance, indicating that the two groups did not differ in performance in the PL task ($F(1, 34) = 1.77$, $p > 0.19$) (Fig. 5B).

3.3.4. Spatial reversal–reversal learning task (RL)

A repeated measures ANOVA on the distance across the 8 trials was performed with "group" and "cued" as between-subject factors. "Cued" effects or interactions with "group" or with trials were not significant (both $F(1,34) < 1.7$, $p > 0.22$), so results in Fig. 5C were pooled by "group" ("high vs. low-PPI"). ANOVA (group x trials) showed a significant trial effect (Huynh-Feldt (4.72, 160.53) = 18.21, $p < 0.001$), as well as a group effect on distances travelled in the 8 trials ($F(1,34) = 5.27$, $p < 0.028$) (Fig. 5C), as low-PPI rats exhibited worse ability to learn the new platform position (Fig. 5C).

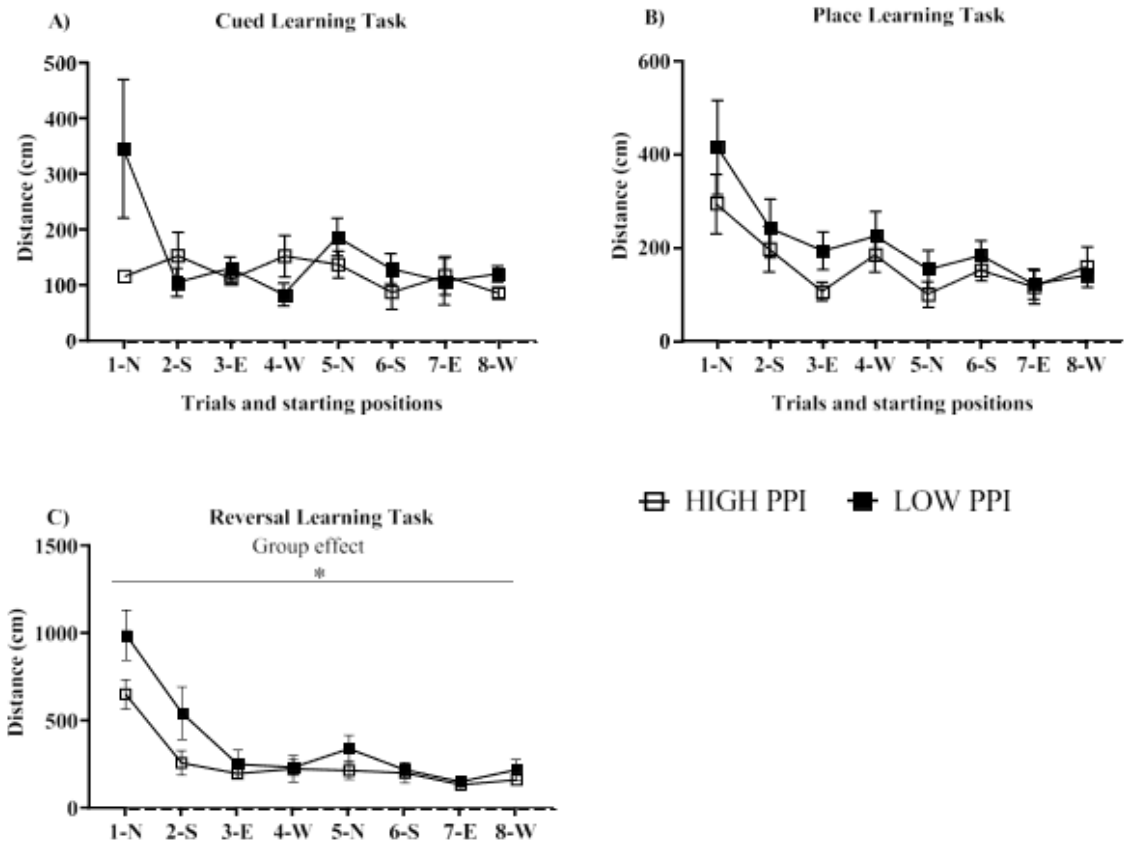


Fig. 5. Performance of high- and low-PPI rats in cued learning, spatial place learning and spatial reversal learning tasks. (A) Mean ±S.E.M. of the distance travelled by the rats in each of the 8 trials of the cued learning (CL) task. (B) Mean ± S.E.M. of the distance travelled by the rats in each of the 8 trials of the place learning (PL) task. (C) Mean ± S.E.M. of the distance travelled by the rats in each of the 8 trials of the reversal learning (RL) task. *, $p < 0.029$ between high- and low- PPI groups ("group" effect from repeated measures ANOVA; see text).

3.3.5. Transfer test (TT)

"Cued" effects or interaction with "group" were not significant ($Fs(1,34) < 0.23$, $p > 0.6$), so results from the TT task were pooled by "group" ("high vs. low-PPI"; Fig. 6A-B). Student's t-tests on the "total time spent in the last target quadrant" (i.e. platform position during the RL task) and "annulus crossings" yielded significant differences between both groups ($t(34) = 2.52$, $p = 0.017$, Fig. 6A; and $t(34) = 2.31$, $p = 0.027$, Fig. 6B, respectively), indicating that low-PPI rats spent less time searching the platform in the target quadrant and, accordingly, made lesser annulus crossings than high-PPI rats.

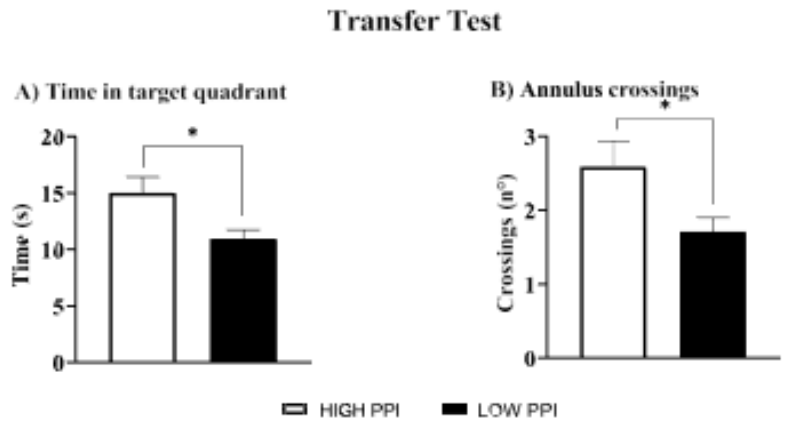


Fig. 6. Performance of high- and low-PPI rats in the transfer test of long-term spatial memory. (A) Time spent (s) in the target quadrant, i.e., the quadrant where the platform was located in the transfer test (TT) task. (B) Number of crossings through the annulus where the platform was located during the TT *p ≤ 0.027 between high- and low- PPI groups (Student's t-test for independent samples). Data expressed as mean ± S.E.M.

3.3.6. Principal components analysis

To explore further the associations between PPI and the most meaningful variables from the SI and cognitive tasks, we selected the measures that showed sensitivity to group differences and included them in a principal component analysis. To reduce the number of variables to the minimum we selected the Total PPI scores and the "sensitive" measures selected from the other tests/tasks. These were the "social time" from the SI test, "distances travelled in trials 1–2" from the first day of the SWM task, the first four trials (averaged) of the RL task and the "time spent in the target quadrant" from the TT task.

The principal component analysis is shown in Table 2. It shows a first factor explaining the highest percentage (38%) of variance, grouping with high loadings the distances in the SWM task (loading 0.79 and 0.71) and the distance travelled during trials 1–4 of the reversal task (loading 0.63). Total PPI (loading -0.58), social time (loading -0.55) and time spent in the target quadrant during the TT (-0.34) also load in this first factor with opposite sign (Table 2). This factor literally indicates that worse PPI relates to both lesser social interaction and time spent in the target quadrant in the TT, as well as with longer distances in the SWM and reversal learning tasks.

The second factor mainly shows that PPI (loading 0.69) and the time spent in the target quadrant during the TT (loading 0.64) retain a strong direct association, whereas social

time is negatively associated to them (loading -0.64). Thus, one part of the variance of social time is directly related to PPI and TT performance (indicated by the loadings of both variables with the same sign in the first factor), whereas another part of social time variance relates negatively to PPI and TT (indicated by the opposite sign of loadings in the second factor) (Table 2).

Table 2. Principal components analysis including the most important variables from the PPI test, the SI test, SWM (working memory), RL (reversal learning) and TT (long-term memory) tasks.

Factors:	Factor 1	Factor 2
Variables:	*"Attention – Cognition - Social motivation"*	
Total PPI	**-0.58**	0.69
Social time	**-0.55**	-0.64
Dist-SWM-1	0.79	0.31
Dist-SWM-2	**0.71**	—
Dist-Reversal learning 1–4	**0.63**	—
TT-Time in Quadrant	**-0.34**	0.64
Variance explained	38.0%	23.4%
Total variance explained (the 2 factors)	61.4%	

Only factors with eigenvalues greater than 1 are considered. Only variables with absolute loadings > 0.30 are presented. Highlighted in bold and underlined are the variable loadings that are most relevant for the hypotheses of the present study. The variables included in the analysis are those that showed sensitivity to group (i.e., high-PPI vs. low-PPI) differences in the study. Abbreviations. -" Total PPI"; %PPI score in "pre-pulse + pulse" trials averaged for the 4 prepulse intensities (i.e., 65, 70, 75, 80 dB). "Social time"; total time spent exploring the social hole in the social interaction (SI) test. "Dist-SWM-1, Dist-SWM-2"; distance travelled during trial 1 and trial 2, respectively, in day 1 of the spatial working memory (SWM) task. "Dist-Reversal learning 1–4"; average distance travelled in trials 1–4 (first training day) of the reversal learning (RL) task. "TT-Time in Quadrant"; time spent in the target quadrant (i.e., the quadrant where the platform was placed during the RL task) in the transfer test (TT). N = 23 (the reduction in the "N" being due to the rats excluded from the SWM task analyses; see explanation in "Results" section).

4. Discussion

The present study shows the first combined characterization of PPI, social interaction, spatial working memory, place learning, spatial reversal learning (cognitive flexibility)

and long-term reference memory in the genetically heterogeneous HS stock rat. In keeping with our hypothesis that HS rats could be a suitable model to discern cognitive and negative-like traits connected to some schizophrenia-related symptoms, we have found that the subgroup of HS rats exhibiting lower PPI levels also displays worse spatial working memory (SWM), impaired cognitive flexibility and long-term reference memory, as well as lowered social interaction (or social investigation). The principal components analysis (PCA) applied to the main variables (those variables that were sensitive to group differences) of the study also yielded results that cohere with the observed differences between both PPI- stratified groups: PCA shows a first factor in which all variables (from PPI, SI, SWM, RL and TT tests) are grouped with moderate-to-high loadings and with the sign expected according to the hypotheses we had. Thus, we provide evidence in support of a positive association between individual differences in PPI and some negative and cognitive schizophrenia-relevant traits.

Identifying symptom clusters may lead to disclosing possible com- mon mechanisms that might to some extent underlie the neurobiological basis of different schizophrenia symptoms. Generally, PPI deficits in patients with schizophrenia are found to correlate with some cognitive symptom, but possible connections to positive or negative symptoms have been far less investigated [11]. Former studies in animal models have demonstrated that rats selectively bred for low PPI are useful models to study the biological basis of negative symptoms of schizophrenia as these animals show decreased motivation and social withdrawal [31]. However, since similar studies in unselected outbred rats are scarce, our first purpose was to test whether an intact (untreated, unselected) population of genetically heterogeneous (outbred) rats stratified by PPI scores performed differently in a behavioural paradigm used to model social interaction and asociality as a function of drug treatments or genetic factors (e.g. [14,21,32]; see also [15]). This hypothesis was already explored in a previous study [33], where the possible link between sensorimotor gating deficits and negative symptoms was examined by stratifying a sample of Wistar rats according to their extreme PPI scores and by evaluating the subjects in a social investigation task. Nonetheless, that study was performed with a notably reduced “n” (n = 5/group) and used a SI model in which confusion elements between social/non-social behaviour, social dominance and

locomotor activity may coexist. Actually, the authors found that rats with increased social interaction also exhibited higher locomotor activity in their SI test [33]. Instead, our results were obtained with a substantially greater “n” per group (n = 18) and with genetically heterogeneous HS rats. Our data show that high-PPI rats display higher social interaction without any parallel increase of locomotor activity. Thus, our results confirm and extend those previous findings, suggesting that the positive association between PPI and SI might be a generalizable phenomenon which, at least with the present procedure, is independent of locomotor activity at a group level. This conclusion is in accordance with previous findings on the absence of association between social interaction and activity in the present SI procedure [21]. In future studies it would be interesting, in order to better discriminate between the rats’ interest for “social interaction” vs. pure “object/novelty interaction”, to include some object in the non-social hole, or even to have three types of holes, one social, one non-social including an object and one non-social including no object.

Braff et al. [11] were first to report a significant correlation between PPI and both positive and negative symptoms assessed through rating scales that measure symptom severity of patients with schizophrenia, although the level of correlation was not as robust as those found with cognitive functions [11]. Our present findings tend to support the notion that PPI deficits are associated with diminished interest for social investigation/interaction, in line with the findings by Braff et al. It would be interesting to further investigate this association in healthy human individuals at risk for schizophrenia.

Some studies have suggested that deficits in PPI are correlated with specific impairments in cognitive performance in patients with schizophrenia ([34–36]; but see also [17] and references therein). In healthy humans, high and low PPI scores may reveal high and low levels of attention, respectively [31], and several studies have associated high PPI performance with superior executive functions such as strategy formation [37,38], planning efficiency [39], working memory performance as measured by the Letter-Number Span Task [40] and strategy score on the WM test from the Cambridge Neuropsychological Test Automated Battery [12].

Regarding rodent models, some manipulations are known to affect rats' PPI and cognitive performance, such as the administration of various glutamate NMDA-receptor antagonists (e.g., [14,22,41]). Still, there is a paucity of data on similar associations in untreated rodents differing in PPI levels. In an untreated cohort of inbred mice, Singer et al. [42] reported that %PPI obtained with the 69 dB prepulse correlated with saving scores obtained in a spatial WM task. In a sample of HS rats, Oliveras et al. reported positive association between %PPI and WM in a similar task [17]. To our knowledge, only these two studies performed in untreated rodents precede our present results. We report here for the first time that low-PPI-stratified rats underperformed their high-PPI counterparts in the spatial WM task, in the spatial RL task (cognitive flexibility) and in the TT test (long-term reference memory), thus extending to cognitive flexibility and long-term reference memory the PPI-cognition associations observed in the two above mentioned reports. There were no differences in the standard place learning (PL) task. The results observed in the SWM task deserve some comment. Our results, in line with Oliveras et al. [17], indicate that low-PPI rats displayed lesser latency (and a clear trend for lesser distance) savings between both trials of the SWM task in day 1, but no between-group differences were observed in day 2 of the task. This is likely due to the fact that rats learn non-spatial strategies that likely account for the markedly reduced latencies and distances in day 2 of the WM task compared with day 1. Finally, no group differences were found in the CL task, which ruled out any possible gross motivational, motor or perceptual (visual) impairment that could bias the results of the spatial learning/memory tasks or be involved in PPI performance.

The present findings suggest that, besides their association with SWM, PPI (or sensorimotor processes) might be associated with (or have an influence on) long-term spatial memory (TT test) and the cognitive ability to switch to new strategies when the requirements of the task have changed (RL task). Notably, however, the results reflect that impairments in both the SWM and RL tasks in the low-PPI group appeared only in the initial performance (i.e., day 1 of the SWM task, and also in the first day of the RL task). In fact, both experimental groups reached equal asymptotic levels of acquisition from the third trial of the first day in the RL task (as also occurred in the PL task). This could be related to the fact that the tank we used was smaller (140 cm diameter) than

those commonly used in the literature (which usually have diameters of 160–200 cm), because of space limitations of our laboratory. Moreover, the platform (16 cm diameter) used in the MWM tasks was also wider than the usual ones (which commonly are around 10 cm diameter). Both aspects, tank and platform sizes, have likely contributed to reduce the difficulty to learn the MWM tasks in the present study, and may have prevented us from getting even better results between the two PPI groups. On the other hand, in the transfer test (TT) a possibility would have been that rats that were very good performers in the RL task could have spent less (rather than more) time searching the platform where it was located in the RL task. However, this doesn't seem to be the case since, together with its superiority in the RL task, the high-PPI group spent longer time in the target quadrant and made more annulus crossings than the low-PPI group, which is compatible with the idea of better long-term reference memory in high-PPI rats.

Even keeping the above nuances in mind, the present findings are overall congruent with previous studies dealing with the association of PPI levels and cognitive/behavioural flexibility. Thus, Freudenberg et al., [43] reported that selectively-bred low-PPI rats were less capable to switch their behaviour upon changing requirements in an operant conditioning paradigm [43]. Likewise, Río-Álamos et al. [44] reported that the genetically-selected RHA rats, which are characterized by impaired PPI, exhibit a deficit of spatial-cognitive flexibility (in a spatial reversal learning task) relative to their RLA counterparts. Taken together, the present findings and these two studies with selectively-bred rats suggest that the association "weak PPI – worse cognitive flexibility" may be a generalizable finding in rats. It would be interesting to explore whether these findings extend to humans, in patients with schizophrenia and/or in at-risk individuals.

Understanding the neurobiology behind naturally-occurring PPI variations might facilitate studying shared neurobiological bases behind this endophenotype and related traits. Notably, we have reported that prefrontal cortical or striatal expression of dopamine D2 and mGlu2 (metabotropic glutamate type 2) receptors, and to a lesser extent expression of 5HT1a and 5HT2a receptors, are negatively associated to PPI levels in HS rats (stratified for low or high PPI levels; [45]). All these systems/receptors have been directly or indirectly related to PPI (reviewed by [45]) and, in particular, mGlu2 receptors (which primarily function as presynaptic autoreceptors to regulate glutamate

release) affect the functionality of striatal dopamine release as well as serotonin activity in the prefrontal cortex (reviewed by [45]; and [15]), both of which have been related with schizophrenia-relevant features (e.g. [45]; review by [15]). In fact, Gonzalez-Maeso et al. [46] and Delille et al. [47], amongst others, have proposed that a receptor complex containing 5-HT2A and mGlu2 receptors integrates serotoninergic and glutamatergic signaling, preferentially in the prefrontal cortex (PFC), in order to convey sensorimotor information in a proper manner. In this context, impaired PFC function and hippocampal deficits have been associated to schizophrenia symptoms. Accordingly, low-PPI-stratified HS rats have been reported to show lowered PFC and hippocampal activation (as well as decreased PFC and hippocampal volume) following a PPI session (see [48], and references therein). The present behavioural associations, between PPI and both SI and cognitive tasks, may suggest that some of the above neurobiological changes associated with variation in PPI levels might also be related with these other behavioural/cognitive functions.

Colony breeding constraints (i.e., limited husbandry space and the need to keep the females for breeding), and limited capacity of our animal facility (taking into account that we also maintain other rat colonies at our laboratory), precluded the use of female rats in the present study, which constitutes a limitation of it. Given our previous findings of consistent sex-related differences in PPI and social behaviour in other rat strains and the known sex differences observed in schizophrenia ([18],b; and references therein), it would be desirable that future studies address whether the association between PPI and social behaviour and cognitive processes holds also in female HS rats. On the other hand, studies exploring if the observed associations are also present in rat strains modelling differential schizophrenia-related phenotypes (e.g., [16]) would be of great value in establishing the generalizability of the present findings. As initial steps in that direction, our previous findings have shown a positive PPI-spatial working memory association in HS and RHA and RLA rats ([17]; see also [44]), but it would be necessary to explore whether using the present experimental procedures the relations amongst the processes studied here hold also in RHA/RLAs as well as other rat strains.

The present study was carried out in a sample of HS rats, the most genetically heterogeneous laboratory rat at present. Therefore, we expect that their greater genetic

variability may bring more generalizable results than the ones obtained with common laboratory rodent strains. Our results suggest that, by stratifying genetically heterogeneous rats by their PPI scores, it is conceivable to identify a suitable at-risk phenotype to study cognitive and negative-like traits/symptoms that might be relevant for several (neuro-) psychiatric disorders, including schizophrenia.

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ESTUDIO 2

Schizophrenia-relevant social, attentional and cognitive traits in female RHA vs. RLA rats: effects of neonatal handling

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Abstract

The Roman high- (RHA) and low-avoidance (RLA) rats were bidirectionally selected and bred for, respectively, their rapid vs. extremely poor acquisition in the two-way active avoidance task. Consistent between-strain neurobehavioural differences have been found in anxiety- and stress-linked traits, as well as in schizophrenia-related phenotypes. RLAs display enhanced anxious- and stress-related phenotypes, whereas RHA rats show impulsivity, hyperactivity and attention/cognition-related impairments. Many of these typical behavioral phenotypes have been reported to be positively modulated by environmental treatments such as neonatal handling (NH). However, most studies on the Roman rat strains have been carried out in males. Thus, the present study for the first time focused on the joint evaluation of differences in novel object exploration (NOE), social interaction (SI), prepulse inhibition of the startle response (PPI), and cognitive performance and flexibility in various spatial tasks (using the Morris water maze, MWM) in females of both Roman rat strains. We also aimed at evaluating the long-lasting effects of NH treatment on the RHA vs. RLA profiles in these tests/tasks. Results show that anxiety-related behavior, as measured by the NOE test and self-grooming in the SI test, was increased in RLA rats, and dramatically reduced by NH. In the SI test RLA rats displayed diminished social interaction, which was rescued by NH. RHA females exhibited a deficit of PPI, which was not affected by NH. Spatial tasks in the MWM showed impairments of working memory, reference learning/memory and spatial reversal learning (i.e., cognitive flexibility) in RHA females. Spatial reference learning and cognitive flexibility (i.e., reversal task) showed some improvement in rats (mainly in RHAs) that had received NH during the first three weeks of life. With the exception of the SI test, the pattern of differences between female RHA vs. RLA profiles was overall consistent with what has previously been found in males of both strains, and NH treatment was able to enduringly improve some emotion-related and (spatial) cognitive outcomes in both strains.

Key words. - Schizophrenia; Social behavior; Cognition; Neonatal handling; Females; Roman rats

1. Introduction

For the research on the neurobiological basis of schizophrenia (and other psychopathological conditions), as well as to evaluate and propose new potentially effective treatments (pharmacological or not), it is essential to have valid animal models that mimic as much as possible the symptoms and neurobiological mechanisms of the disorder (Jones et al., 2011; Powell and Miyakawa, 2006).

In the present work we focus on a genetically-based rat model of schizophrenia-relevant features, the Roman high-avoidance (RHA) and Roman low-avoidance (RLA) rat strains, which were derived from Wistar rats through bidirectional selective breeding for high (RHA) or extremely low ability (RLA) to acquire two-way active avoidance learning (Bignami 1965; Driscoll and Bättig 1982). Over 50 years of research have revealed important phenotypic differences between RHA and RLA rats in anxiety/fear-related traits, coping styles, and stress sensitivity. RLA rats show higher levels of anxiety in both unconditioned and conditioned tests compared to RHA, and also present enhanced stress-induced hormone responses (Carrasco et al., 2008; Díaz-Morán et al., 2012; Fernández-Teruel et al., 2021; Giorgi et al., 2019; Río-Álamos et al., 2015, 2017).

Most importantly, in relation to the present work, the two Roman strains diverge in other phenotypic aspects that are thought to be relevant for schizophrenia. Thus, relative to RLA and outbred rats from other strains/stocks, RHA rats are characterized by increased impulsive behaviour, and enhanced behavioural (e.g., hyperactivity) and neural responses (e.g., increased function of the nucleus accumbens) to dopaminergic psychostimulants. (e.g., amphetamine, cocaine) (Giorgi et al., 2019). RHA rats also exhibit impaired working memory and cognitive flexibility, attentional deficits and elevated sensation/novelty seeking, enhanced vulnerability to drug abuse, relative decreased preference for social interaction and signs of impaired function of the prefrontal cortex and hippocampus (Elfving et al., 2019; Fernández-Teruel et al., 2021; Giorgi et al., 2019; Meyza et al., 2009; Oliveras et al., 2015; Sampedro-Viana et al., 2021, 2023; Tapias-Espinosa et al., 2023, 2019). These phenotypes might be related to impaired neurodevelopment in the RHA strain, including alterations in the mesolimbic dopaminergic system, as well as in cortical (and likely, hippocampal) serotonergic, GABAergic and glutamatergic function (Elfving et al., 2019; Fernández-Teruel et al.,

2021; Sánchez-González et al., 2021; Sønderstrup et al., 2023). Based on the aforementioned phenotypic/neurobiological characteristics (among many others) RHA rats have been proposed as a reasonably valid animal model of relevant behavioural, neural and neurodevelopmental traits/phenotypes linked to schizophrenia (reviewed by Fernández-Teruel et al., 2021; Giorgi et al., 2019).

The so-called neonatal handling (NH) treatment of rat (or mice) pups, which includes mild stimulation and brief daily separations from the mother and litter during the first 21 days of life, produces enduring positive neurobehavioural effects in rats and mice (Levine 1956; reviewed by Fernández-Teruel et al., 2002, and Raineki et al., 2014). Thus, among others, NH leads to life-long reductions of anxiety and stress-hormone responses (Meaney et al., 1988; Núñez et al., 1996; Raineki et al., 2014; Viau et al., 1993) and to increases of novelty/reward seeking and social play (Aguilar, 2010; Fu and Depue, 2019). NH treatment also produces long-lasting improvements in attentional/cognitive processes as well as in hippocampal plasticity and function (Fernandez-Teruel, 2022; Meaney et al., 1988; Taschetto Vey et al., 2020). Many of the typical behavioural, emotional and cognitive phenotypes of male RHA and RLA rats have been found to be sensitive to the long-term positive influence of NH (Aguilar et al., 2002; Fernández-Teruel et al., 1997, 2002; Río-Álamos et al., 2015, 2017; Steimer et al., 1998)

There are sex differences in schizophrenia incidence, age of onset and symptoms. Men develop schizophrenia around five years before than women on average (Li et al., 2016), the disorder appears to be more frequent in men than women (1.4/1 risk ratio), and men appear to have more negative symptoms and more severe clinical features (including social withdrawal, substance abuse and alterations of affect) than women (Li et al., 2016; Millan et al., 2016; Räsänen Sami, 2000). Most of the preclinical research with animal models relevant to schizophrenia (or schizophrenia-related phenotypes or symptoms) has been carried out in males, including studies on the Roman rats. To date no study has explored the cognitive profiles of females of both Roman rat strains, nor the effects of NH treatment on their social behaviour and cognitive profiles. Therefore, we aimed at exploring differences in relevant traits for this disorder and the possible long-term effects of NH treatment in females of the RHA and RLA rat strains. Thus, the present study evaluated the differences between female RHA and RLA rats and the

effects of NH on phenotypes related to exploratory behaviour and emotionality/anxiety (exploration of a novel object, grooming behaviour), as well as traits that are thought to be related to positive symptoms (locomotor activity), negative symptoms (social interaction vs. asociality), and cognitive/attentional symptoms (PPI, spatial learning/memory, working memory, cognitive flexibility) of schizophrenia. It was expected that RHA females would exhibit worsened PPI and working memory, as well as impaired place task learning, reference memory and impaired reversal learning. We also expected that NH treatment would reduce signs of anxiety (novelty exploration, grooming) and increase social interaction in both rat strains, and that NH would improve PPI, spatial learning/memory performance and cognitive flexibility (in a reversal learning task), particularly in female RHA rats.

2. Materials and methods

2.1 Animals

The animals used in this study were females of the inbred Roman high- (RHA, n = 32) and low-avoidance (RLA n = 30) rat strains. All rats came from the permanent colonies maintained at the laboratory of the Medical Psychology Unit, Dept. Psychiatry and Forensic Medicine (School of Medicine, Autonomous University of Barcelona, Spain), since 1996. Each experimental group consisted of rats from 9 different litters (1-2 rats per litter). Litters were culled to 10 pups (approximately half of each sex) on the second postnatal day (PND 2). Rats were approximately 2.5 months old at the beginning of the experiments (except otherwise indicated) with an average weight of 191 ± 16 g (mean + SD). They were maintained under a 12:12 h light-dark cycle (lights on at 08:00 a.m.), with controlled temperature (22 + 2º C) and humidity (50-70%). They had food and water available ad libitum.

All testing was carried out between 09:00 and 14:00h. All procedures were carried out in accordance with the Spanish Legislation (Royal Decree 53/2013, 1st February 2013) and the current regulation related to “Protection of Animals used for Scientific Purposes” established by the European Union (2010/63/UE, 22 September 2010).

2.2 Neonatal Handling (NH) Treatment

Thirty female RHA (n = 16) and RLA (n = 14) rats received NH treatment. NH was administered twice daily (at 9:30 and 17:00 h) between PND1-21, in a room with a temperature of 22 ± 2 ◦C. Each handling session consisted of first removing the mother from the litter and then placing the pups gently and individually in plastic cages (35 x 15 x 25 cm) lined with paper towel for a total period of 8 min. Each pup was gently stroked for 3–4 s at 0, 4 and 8 min with bare hands, after which they were returned to their home-cage with their mother and litter. Non-handled (Control) rats (n = 32, 16/strain) were left undisturbed except for the weekly cleaning of their home cages. Rats were weaned at PND21, and were housed in pairs of the same experimental group in regular (50 x 25 x 14 cm) macrolon cages.

2.3 Novel Object Exploration (NOE) test

At PND52 the Novel Object Exploration (NOE) test was performed (Río-Álamos et al., 2015). This test was used to test anxiety-related exploratory behaviour in response to novelty. Food was removed from the home cage (except 4 food pellets left in each cage) and the cage was pulled away from the rack 20 cm to allow direct observation of the rats' behaviour. One hour later, the novel object (graphite pencil Staedtler Noris, HB nº2) was introduced vertically in the cage through the grid cover until it reached the cage bedding, and then the "Latency to the first exploration" (NOE latency time) and the "total time of exploration" (NOE exploration time) were measured for 3 minutes by a trained researcher standing approximately 40-50 cm away from the cage front. Exploration of the novel object was defined as approaching the nose to it at 1 cm distance (or less) or touching/manipulating the object.

2.4 Social Interaction (SI) test

For the social interaction (SI) test, two acrylic boxes (65 x 23 x 20 cm) were placed in front of each other at 12 cm, to prevent physical contact between the animals, in a red-lit room with a camera on the roof connected to a monitor outside the room to record the test. Each box had two 3 cm diameter holes at its ends. The hole facing the room wall (distal hole) was named "Non-social hole", while the one facing the other box was named "Social hole" (Sampedro-Viana et al., 2021, 2023).

The SI test was carried out at PND74. The day before the test, animals were habituated to the room and the box in a 30-min habituation session. During this time, the two holes of each box were covered with white tape and a cardboard barrier was placed between the boxes to prevent exploration of the other box. A pair of non-familiar weight-matched animals of the same strain were placed into the testing boxes (one rat in each box) for the 30-min habituation period.

In the test day, for SI assessment, the holes were opened and two weight-matched non-familiar rats (maximum weight difference 20 g) of the same strain were placed (one rat in each box) into the set-up for a 15-min test. The time spent exploring the social hole (Social Time) and the non-social hole (Non-Social Time), time spent self-grooming (Grooming Time) and total number of crossings (Crossings) were recorded (each box was divided into three equal squares by lines painted on the floor to measure horizontal activity –crossings-) by two well-trained observers (between-observer reliability r = 0.95) watching the TV monitor outside the testing room. The SI boxes were cleaned with 70% ethanol solution after each test and dried with paper towel.

2.5 Prepulse inhibition of the acoustic startle response (PPI)

Two days after finishing the SI test (PND76) the PPI test was conducted in two sound-attenuated startle boxes (SR-Lab Startle Response System, San Diego Instruments, US). Animals were individually placed 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 delivered via a speaker mounted 15 cm above the cylinder. The data were transduced by an accelerometer into a voltage which is amplified, digitized, and saved into a computer for further analysis.

The session consisted of 5 minutes of habituation, after which 10 “pulse-alone” trials (105 dB(A), SPL, 40 ms) were delivered in order to obtain a stable baseline of startle responses (BL1). Next, six types of trials were randomly administered 10 times (60 trials): (i) Pulse-alone trials (105 dB(A), SPL, 40 ms; BL2; used to calculate the percentage of PPI); (ii) prepulses of 65/70/75/80 dB (A), SPL (20 ms) followed by the pulse stimulus (105 dB (A), SPL, 40 ms) with an inter-stimulus interval of 100 ms; or (iii) no-stimulus trials (background noise of 55 dB). Finally, 5 “pulse-alone” trials were administered

(BL3). The inter-trial interval was 15 s (range 10–20 s). The %PPI for each prepulse intensity was calculated with the following formula: %PPI = [100−(startle amplitude on prepulse trials/startle amplitude on “BL2” pulse-alone trials × 100)].

For each trial, the startle amplitude magnitude was measured by an accelerometer and calculated as an integrated response of the enclosure vibrations caused by the whole rat’s body startle reaction after startle stimulus onset (recording range: 200 ms). Startle response amplitude was defined as the maximum accelerometer voltage during the first 200 ms following the startle stimulus onset.

2.6 Morris water maze (MWM) tasks

The Morris water maze (MWM) tasks were performed in a circular pool (diameter: 140 cm, heigh 60 cm), filled with water mixed with nontoxic white tempera paint (to make it opaque) to 30 cm deep and maintained at 24 ± 1 ºC. The escape platform was a circular plastic cylinder (16 cm diameter) that was fixed in a determined position and hidden 2 cm below the water surface (except otherwise indicated), in order to be invisible to the rats.

Some distal visual cues surrounded the pool during the experiments (i.e., in the laboratory walls, windows, etc.) that were unchanged during the whole experimental procedures, including the seating position of the researcher/observer. There were no local cues inside the pool. Four points equally spaced around the perimeter of the tank were designed, establishing the 4 cardinal points: North (N), South (S), East (E), and West (W). Therefore, the pool was divided in 4 quadrants.

The parameters measured in all tasks were the latency (time elapsed until the rat found the hidden platform), distance travelled to reach the platform and swimming speed. Animal behaviour was monitored by a video camera placed on the ceiling above the center of the pool, and using a computerized tracking system (ANY-maze Video Tracking System, v.7.16. Stoelting.Co).

2.6.1 Delayed-matching-to-place (DMTP): working memory (WM) task

At PND 120, animals performed the DMPT task, which consisted of 2 trials per day during 3 consecutive days. The platform was located at NW the first day, the second day at the

center of the pool and the last day at S (Figure 1A). The platform was moved daily to a different position within the pool in order to measure only working memory and avoid (as much as possible) other types of memory from the previous days. The starting positions of the animals were also defined pseudorandomly, being S, N and W, in days 1, 2 and 3 respectively, and each rat was placed into the pool always facing its wall (Figure 1A).

In the first trial the rat was allowed to swim for 90 s to locate the platform. If during this time the subject wasn't able to find the platform, the animal was gently placed on it. Once in the platform, animals remained 15 s on it in order to orientate themselves with the external cues. After this time, the animal was placed into a cage containing sawdust for 15 s before the next trial started. In this second trial the subject was placed in the same starting position and had 90 s to reach the platform. As in the first trial, if the animal didn't reach the platform in time, it was gently placed there and left 15 s. After that, the rat was returned to its home cage. The same procedure, but changing platform position and starting position, was followed the two following days.

The first daily trial is thought to be the sample trial, in which the animals find the location of the platform by trial and error. If the animal remembers where the platform was in the first trial, then he swims a shorter path to it in the second trial, and the difference of latency and distance in the second trial with respect to the first trial represents a measure of working memory.

2.6.2 Place learning task (PT): spatial reference learning/memory

Four days after the DMTP task, the place task (PT) was performed, consisting of 3 trials per day during 4 consecutive days. The escape platform remained always in the same position from trial to trial and across days, and the position of the platform was chosen randomly to the NW. The starting position for each trial was chosen randomly between W, S and E (Figure 1B). The animals performed 3 trials each day, in which the order of the starting position was also randomly changed from day to day. In each trial, the animal had 90 s to locate the escape platform and then 15 s on it, before it was returned to the home cage. In this task, spatial reference learning/memory are tested, as the rats

learn to orientate themselves by the fixed distal cues present in the room. The latency and distance travelled to reach the platform were measured.

2.6.3 Transfer test (TT) task: spatial long-term reference memory

The next day after finishing the PT task, the transfer test (TT) was performed. The platform was removed from the pool. The distance and latency to reach the position where the platform was located in the PT (i.e., target position, or "annulus"), the number of annulus crossings and mean swimming speed, were scored in a 60-s trial test in which the animal's starting position was S. This task provides a measure of long-term reference memory (Figure 1C).

2.6.4 Reversal learning task (RT): spatial cognitive flexibility

Two days after the transfer test (TT), the subjects underwent the reversal task (RT). The platform was located in the opposite quadrant that was used in the place task (PT) and stayed fixed for the whole procedure (Figure 1D). It consisted of 3 trials/day on 3 consecutive days, in which the starting position was also randomly assigned each day and trial, varying from W, N and E. The rat had to reach the platform within 90 s, if not, it was gently placed on it and allowed to stay there for 15 s before it was returned to the home cage. The time elapsed between consecutive trials was approximately 20 min, as in the PT. The measures taken were as for the PT phase.

2.6.5 Cue task (CT)

Four days after the Reversal task (RT), animals were tested in a cue task (CT). It consisted of 8 trials during 2 consecutive days (4 trials/day). Approximately 15 min elapsed between consecutive trials. For this test, the platform was 1 cm above the water surface, and it was cued with a small black and white flag. The platform was fixed at position NE during the two-day test and the order of the starting positions was pseudorandomly established and was the same for the two training days (N, S, E and W) (Figure 1E).

This test is performed as a control condition in which the ability to swim to the visible and cued platform goal is measured, that's why the location of the escape platform is clearly indicated. The subjects are expected to swim directly to the platform unless they have gross visual, motor or motivational impairments that might interfere with the

spatial learning. If any subjects have bad performance in this test, e.g., rats that predominantly remained passively floating (i.e., they did not search the platform), they were excluded from analyses of this task and the previous spatial tasks.

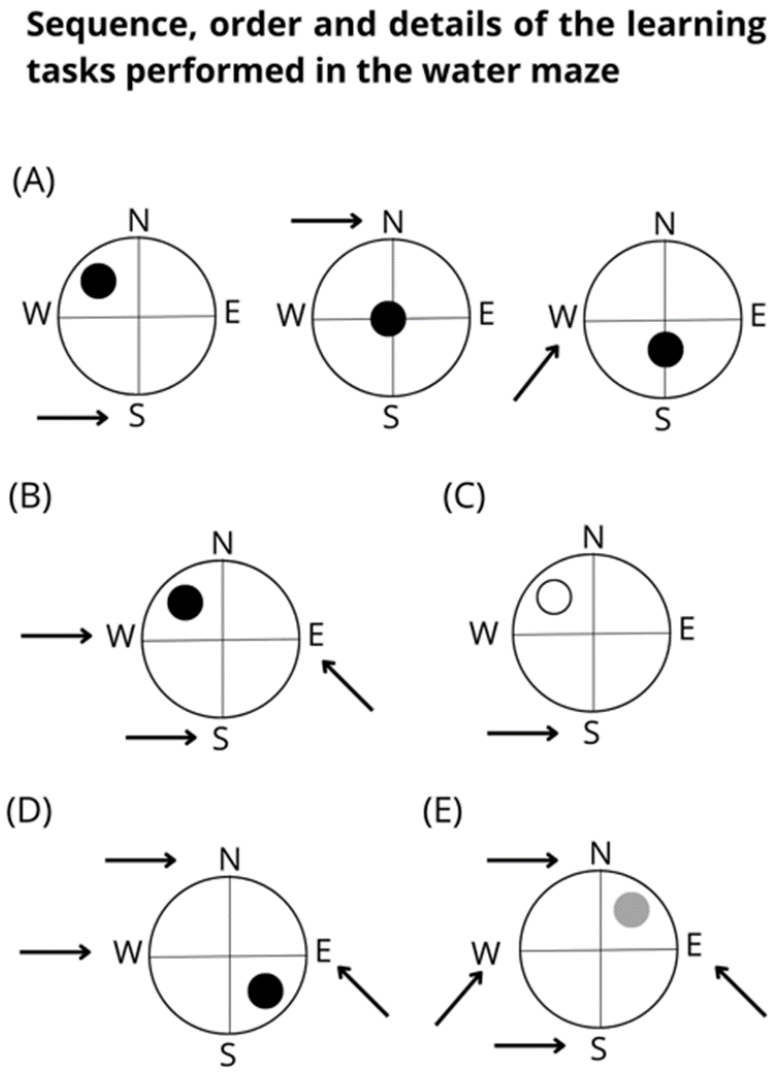


Figure 1. The arrows indicate the starting positions, black circles indicate the platform location, open circle indicates that the platform had been removed, grey circle indicate that the platform was above the surface of the water and marked by a black and white flag. A) MWM-Delayed Matching to Place; position of the submerged escape platform represented by the black circles. The starting points of each day are represented by the black arrows. N, E, S, W are the cardinal points. B) MWM-Place Task; position of the hidden escape platform represented by the black circle and the starting positions of the trials each day are represented by the arrows. C) Transfer test; platform removed. The open circle represents the position where the escape platform was located in the previous place task. The arrow indicates the starting position. D) MWM-Reversal Task; position of the submerged escape platform represented by the black circle and starting points of each trial represented by the arrows. E) MWM-Cue Task; position of the visible escape platform represented by the grey circle and starting positions represented by the arrows.

2.7 Statistical analyses

Factorial ANOVA (2 strain x 2 treatments), in some cases with repeated measures (different prepulse intensities in the PPI test, trials or sessions in MWM tasks), were carried out. In some cases, factorial ANOVAS were performed with covariates to control for the influence of some variables.

From our previous studies, mostly carried out with males, but some of them including females (Oliveras et al., 2022b; Río-Álamos et al., 2015) we had specific hypotheses on the direction of results. Thus, we expected that, relative to RLAs, RHA rats would show shorter latency to explore the novel object and longer time (exploring the novel object) in the NOE test, higher social and non-social time in the SI test, poorer PPI, as well as impaired performance in the cognitive tasks (WM, PT, TT and RT) in the MWM. On the other hand, we also hypothesized that NH would shorten NOE latency, increase NOE time and reduce grooming in the RLA strain, and NH would increase social interaction in both strains and improve performance in the different learning/memory tasks in the MWM (except in the cue task) in RHA rats. That is why following any significant ANOVA main effect or interaction, posthoc Duncan's multiple range tests were performed to explore differences between groups. We also performed Pearson's correlations to study association among variables. Factor analysis is a statistical data reduction and analysis technique that aims at explaining associations among multiple observed variables in terms of a potentially lower number of unobserved (underlying) factors (or constructs). Thus, we applied factor analysis (obliquely-rotated) to the main variables of the study in order to see whether they cluster within a lower number of underlying and meaningful factors.

3 Results

3.1 NOE test

The factorial ANOVA showed a "Strain" ($F(1,61) = 38.9$, $p < 0.001$), "Treatment" ($F(1,61) = 23.0$ $p < 0.001$) and "Strain * treatment" effects ($F(1,61) = 11.2$, $p < 0.001$) on "Latency". The posthoc Duncan test showed that RLA-Control group was different from both RLA-NH and RHA-Control (Figure 2A). "Strain" ($F(1,61) = 36.9$, $p < 0.001$) and

"Treatment" (F (1,61) = 43.6, p < 0.001) effects were found on "Exploration Time", showing that both NH groups markedly increased their scores in this variable (. Figure 2B).

The factorial ANOVA (2 strain x 2 NH x 2 "NOE-Duration" conditions) applied to both NOE "latency" and "exploration time" parameters showed the expected "strain" (both Fs > 73.1 p < 0.001), NH (both Fs > 43.3 p< 0.001) and "NOE-Duration" (both Fs > 22.3 p< 0.001) effects, as well as "strain x NH x "NOE-Duration" interactions (both Fs > 5.9 p< 0.02).

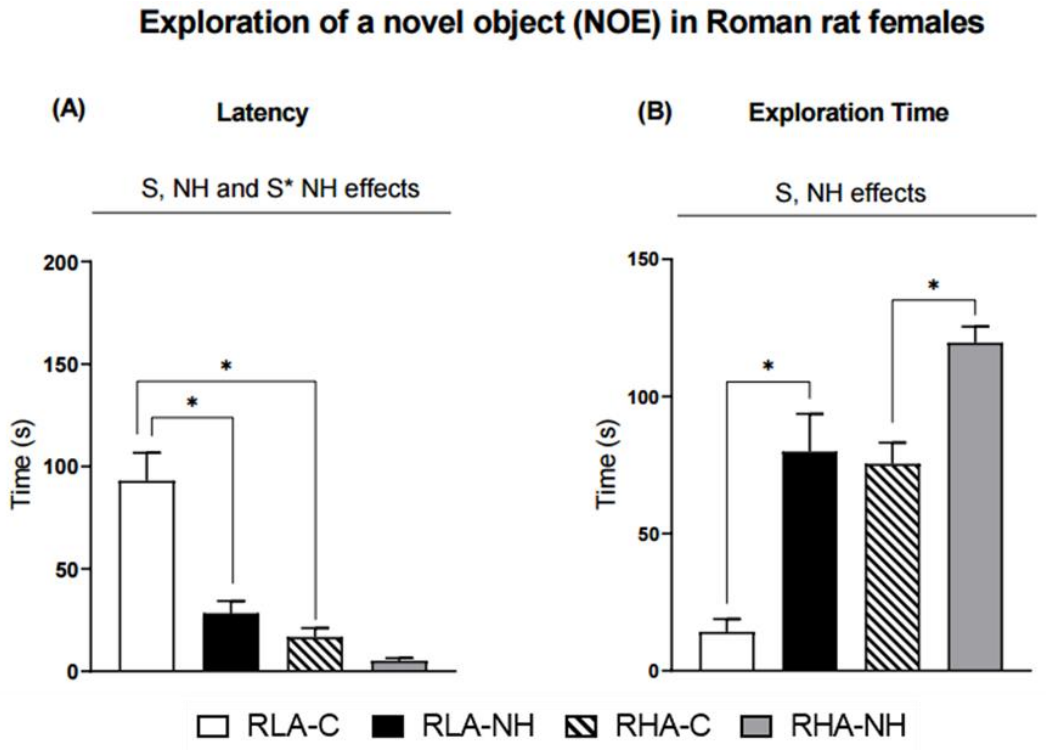


Figure 2. Means (± SEM) for the novel object exploration (NOE) test in female RLA and RHA rats and effects of the NH treatment on: A) "Latency"; time elapsed until the first exploration of the novel object. B) "Exploration Time"; time spent exploring the novel object. Abbreviations: RLA-C, control RLA rats; RLA-NH, neonatally-handled RLA rats; RHA-C, control RHA rats; RHA-NH, neonatally-handled RHA rats. n = 16/group, except RLA-NH group that had n= 14. * p<0.05, Duncan's multiple range test. S, strain effect (ANOVA); NH, treatment effect (ANOVA).

3.2 Social Interaction (SI) test

The factorial ANOVA for the variables for the SI test revealed a "Strain" effect on Social Time (F (1,58) = 11.53, p < 0.001, figure 3A), in Grooming (F (1,58) = 25.84, p < 0.001,

Figure 3D) and on Locomotor activity (F (1,58) =63.1, p < 0.001, figure 3E). "Strain * Treatment" approached significance in Social Time (F (1,58) =3.6, p < 0.06, Figure 3A) as well as "Treatment" for the variable Grooming (F (1,58) =3.22, p = 0.077, Figure 3D). Since we had hypotheses on the positive effects of NH on social interaction and grooming, Duncan's tests were performed, showing that RLA-NH group differed from its respective RLA control group in both "social time" (Figure 3A) and grooming (Figure 3C).

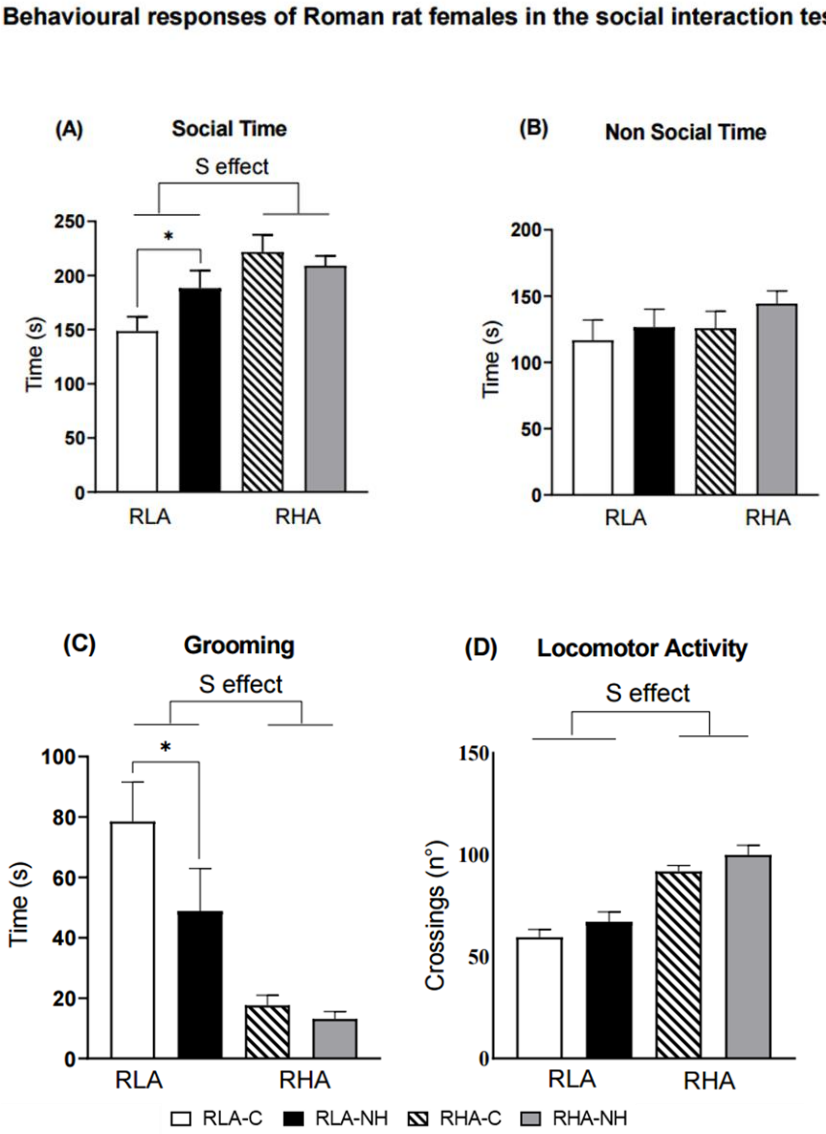


Figure 3. Means (± SEM) for the social interaction (SI) test in female Roman rats and effects of the NH treatment on: A) Total Social Time; B) Non-Social Time; C) Grooming; D) Locomotor Activity. Group abbreviations as in Figure 2. n = 16/group, except RLA-NH n= 14. * p<0.05, Duncan's multiple range test. S, strain effect (ANOVA).

3.3 Prepulse inhibition (PPI)

A repeated measures two-factor ANOVA on the 4 prepulse intensities (Figure 4A) and the ANOVA for the total PPI (averaged for the 4 intensities; Figure 4B) revealed a "Strain" effect (both $F(1,58) > 15.4$, $p < 0.001$, Figure 4B), but no interaction effects were observed. ANOVA detected no effects on baseline startle measures ($F(1,58) = 1.63$, $p < 0.21$, Figure 4C).

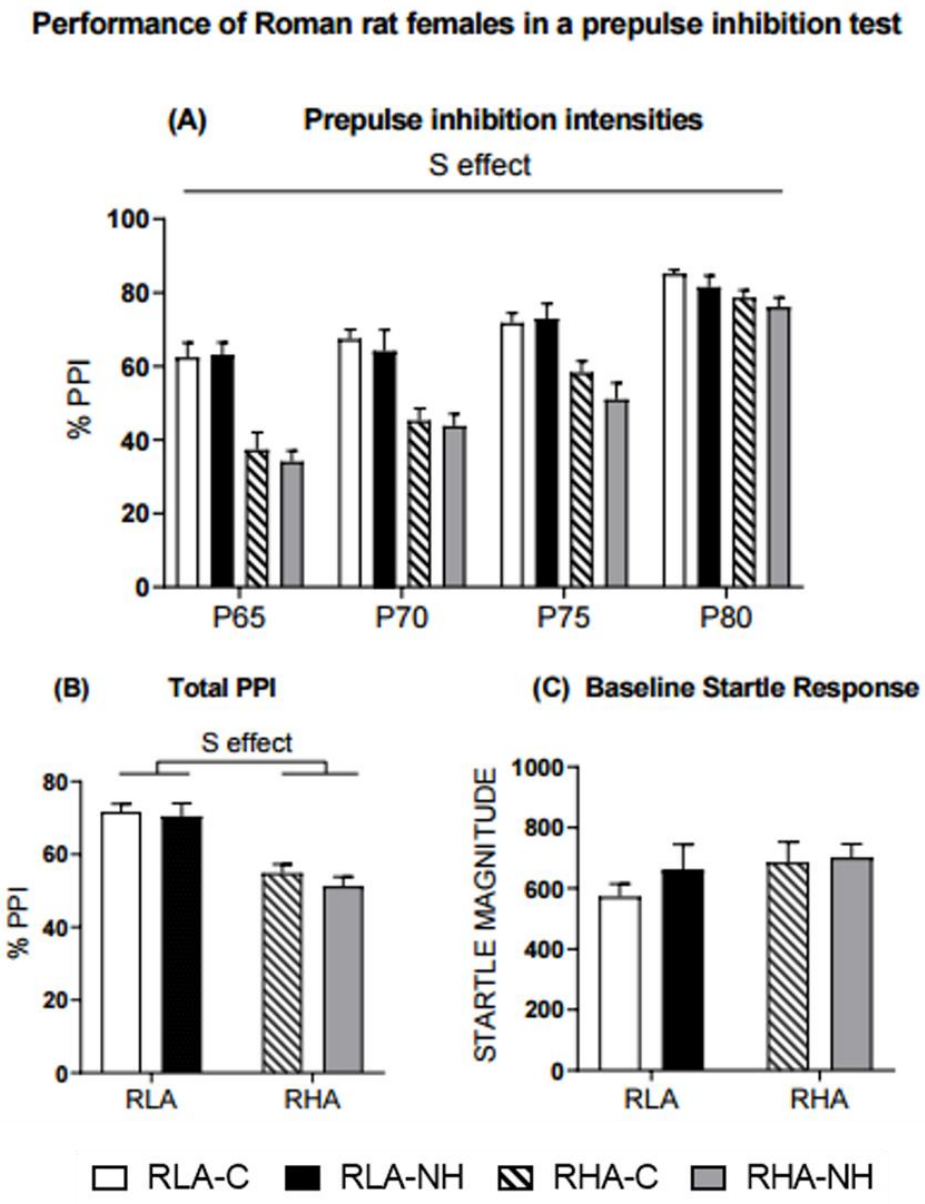


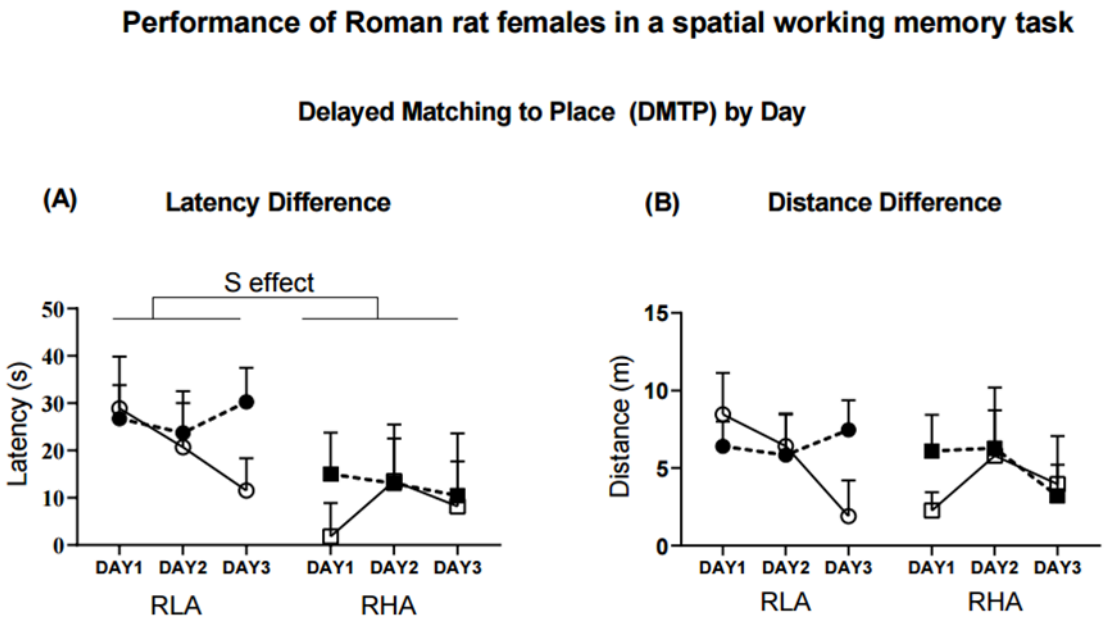
Figure 4. Means (± SEM) for the prepulse inhibition (PPI) test in female Roman rats and effects of the NH treatment on: A) %PPI for the 4 different prepulse intensities. B) The total PPI (average of the 4 prepulse intensities). C) Baseline Startle Response. Group abbreviations as in Figure 2. n = 16/group, except RLA-NH n= 14. S, strain effect (ANOVA).

3.4 Morris Water Maze

3.4.1 Delayed Matching to Place (DMTP) – Working memory

Rats showing latencies to find the platform <15 s in trial-1 of any training day were excluded from analysis. The objective was to include only rats that in each trial-1 had a significant/sufficient time for spatial acquisition. Thus, the final N=52 ("n" per group: RLA-Control=15, RLA-NH = 13, RHA-Control = 13, RHA-NH = 11).

Repeated measures factorial ANOVA was performed for the difference of the values between trials 1 and 2 across the 3 days of training (Figure 5A-B), and showed a significant "Strain" effect on "latency difference" ($F(1,48) = 6.087$; $p < 0.017$, Figure 5A), which was greater in RLA rats than in their RHA counterparts. No significant effects were found for the "distance difference" between trials 1 and 2 across the 3 days ($F(1,48) = 1.15$; $p = 0.28$; Figure 5B). In agreement with the previous analyses the repeated measures ANOVA for the mean of all the 3 first trials (Trial 1, Figure 5C-D) and the mean of the second trials (Trial 2, Figure 5C-D) revealed only "Strain" differences for latency and distance ($F(1,48) = 14.6$; $p < 0.001$, $F(1,48) = 14.5$; $p < 0.001$, respectively; Figure 5C-D). No significant effects were found for swimming speed across the 3 training days ($F(1,48) = 0.32$; $p = 0.57$).



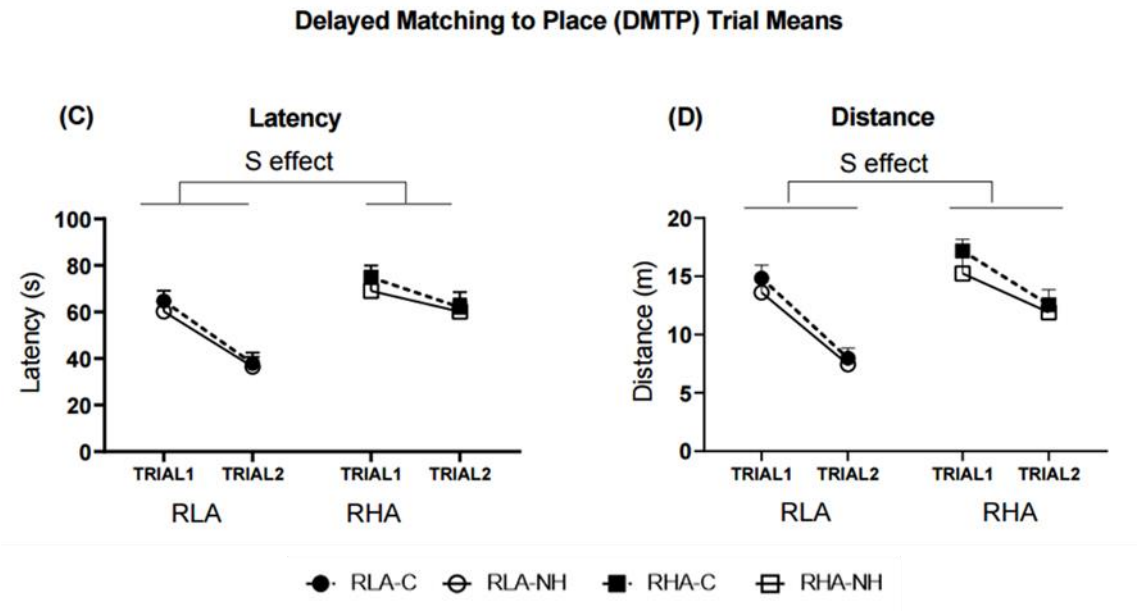


Figure 5. Means (± SEM) for delayed-matching-to-place (DMTP; working memory) in female Roman rats and effects of the NH treatment. A) Latency difference between trials 1 and 2 across the 3 days. B) Distance difference between trials 1 and 2 across days. C) Average speed for pairs of trials across the 3 days. D) Mean latency, for the 3 days, of the first (Trial 1) and second (Trial 2) trials. E) Mean distance travelled, for the 3 days, of the first (Trial 1) and second (Trial 2) trials. Group abbreviations as in Figure 2. "n" per group: RLA-Control=15, RLA-NH = 13, RHA-Control = 13, RHA-NH = 11. S, strain effect (ANOVA).

3.4.2 Place Task (PT) – Spatial Learning

There were 9 rats showing clear "passive floating" behaviour (i.e., no sign of active swimming to search the platform) that were excluded from analysis of this task ("n" per group: RLA-Control=15, RLA-NH = 13, RHA-Control = 13, RHA-NH = 12).

Repeated measures ANOVA performed on the 12 training trials (3 trials/day, 4 days) showed significant "Strain" effects for latency ($F(1,49) = 15.6$, $p<0.001$, Figure 6A) and distance ($F(1,49) = 14.4$, $p<0.001$, Figure 6B). A repeated measures ANOVA was applied to the first 9 trials, which revealed "Strain" effects on latency ($F(1,49) =17.03$, $p<0.001$) and distance ($F(1, 49) =14.9$, $p<0.001$), as well as "trial * treatment" effects on latency and distance (Huynh-Feldt $F(7.4, 362.0) = 2.2$, $p=0.03$; and $F(7.5, 352.2) =2.0$, $p=0.05$, respectively) (Figure 6A-B). Posthoc Duncan's tests showed that the second trial of the second day showed a significant difference between the RHA-Control and RHA-NH groups, both in latency and distance, meaning that the RHA-NH group performed better

in this trial. There was no significant effect of any factor or interaction effects on swimming "speed" (all F (1,49) < 1.0; p<0.75; not shown).

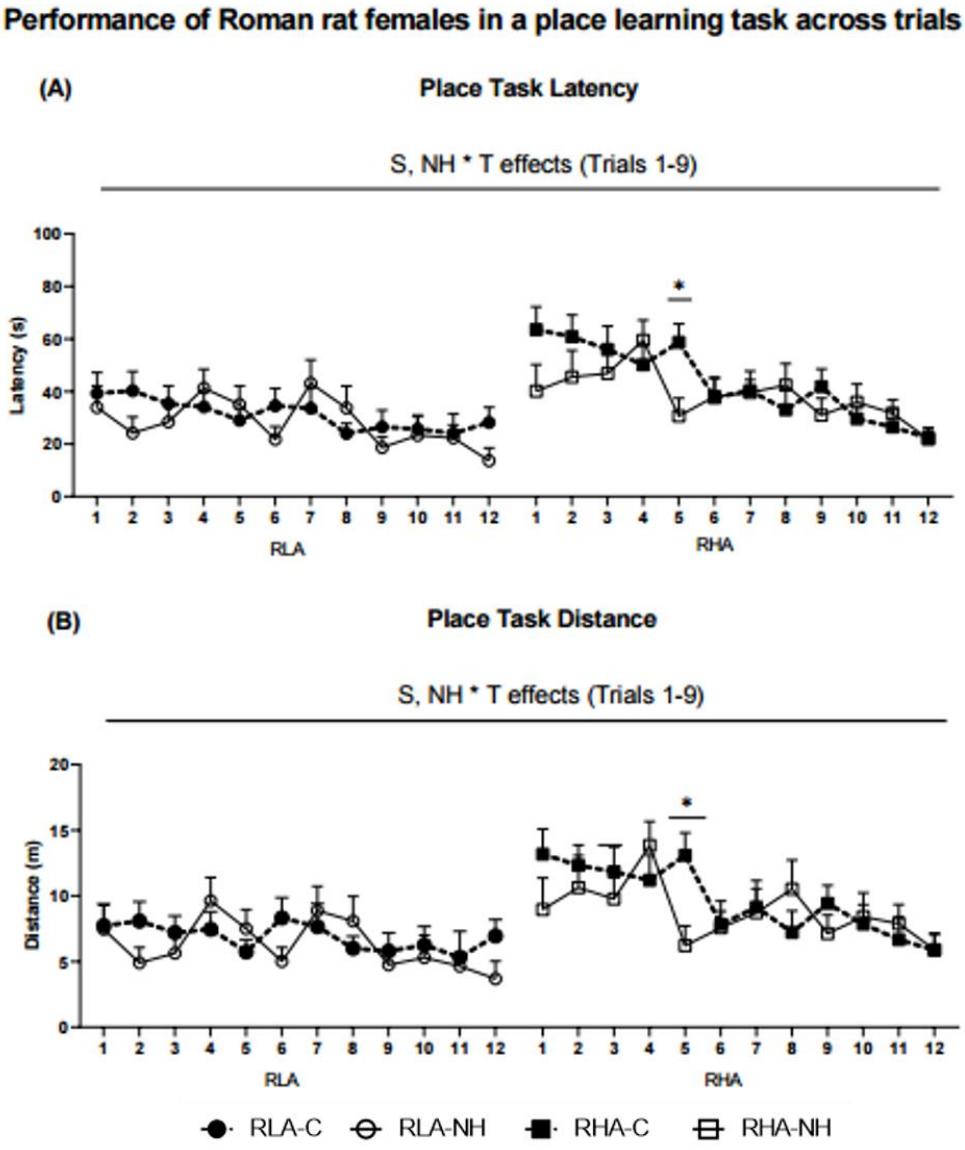


Figure 6. Means (± SEM) for each trial (1-12) of the place task (PT) in female Roman rats and effects of the NH treatment on: A) latency to find the platform for each trial. B) Distance travelled to find the platform for each trial. Group abbreviations as in Figure 2. "n" per group: RLA-Control=15, RLA-NH = 13, RHA-Control = 13, RHA-NH = 12. * p<0.05 vs. NH group from the same strain (Duncan's multiple range test). S, strain effect (ANOVA); NH; treatment effect (ANOVA); T, trial (ANOVA).

The repeated measures ANOVA on the “mean latency” and “mean distance” for each day (4 days, as within-subject factor) revealed significant “strain” effects on both measures ($F(1,49) = 9.5$; $p < 0.003$; $F(1,49) = 11.8$; $p < 0.001$, Figure 7A-B, respectively).

We calculated the differences between the last trial of each day and the first one (i.e., first trial latency or distance minus last trial latency or distance), i.e., variables “latency difference” and “distance difference” (Figure 7) to have a measure of within-session progression (i.e., short-term memory). The repeated measures ANOVA for these two variables across the 4 days approached significance for Treatment effect on” distance difference” ($F(1,49) = 3.2$; $p < 0.08$, Figure 7D). Thus, we next applied a repeated measures ANCOVA to both variables taking the first day as a covariate, since the first training day involves a certain degree of novelty and chance, and because correlations between “latency difference” and “distance difference” in the first day with the other 3 days were all non-significant (all $r < 0.17$, all $p > 0.24$). This ANCOVA showed significant “Treatment” effects on “latency difference” ($F(1,49) = 5.6$; $p = 0.022$, Figure 7C) and “distance difference” ($F(1,49) = 5.3$; $p < 0.026$, Figure 7D) in days 2-4, indicating that within-session acquisition in sessions 2 to 4 was overall significantly better in NH-treated RHA and RLA groups than in control (untreated) groups.

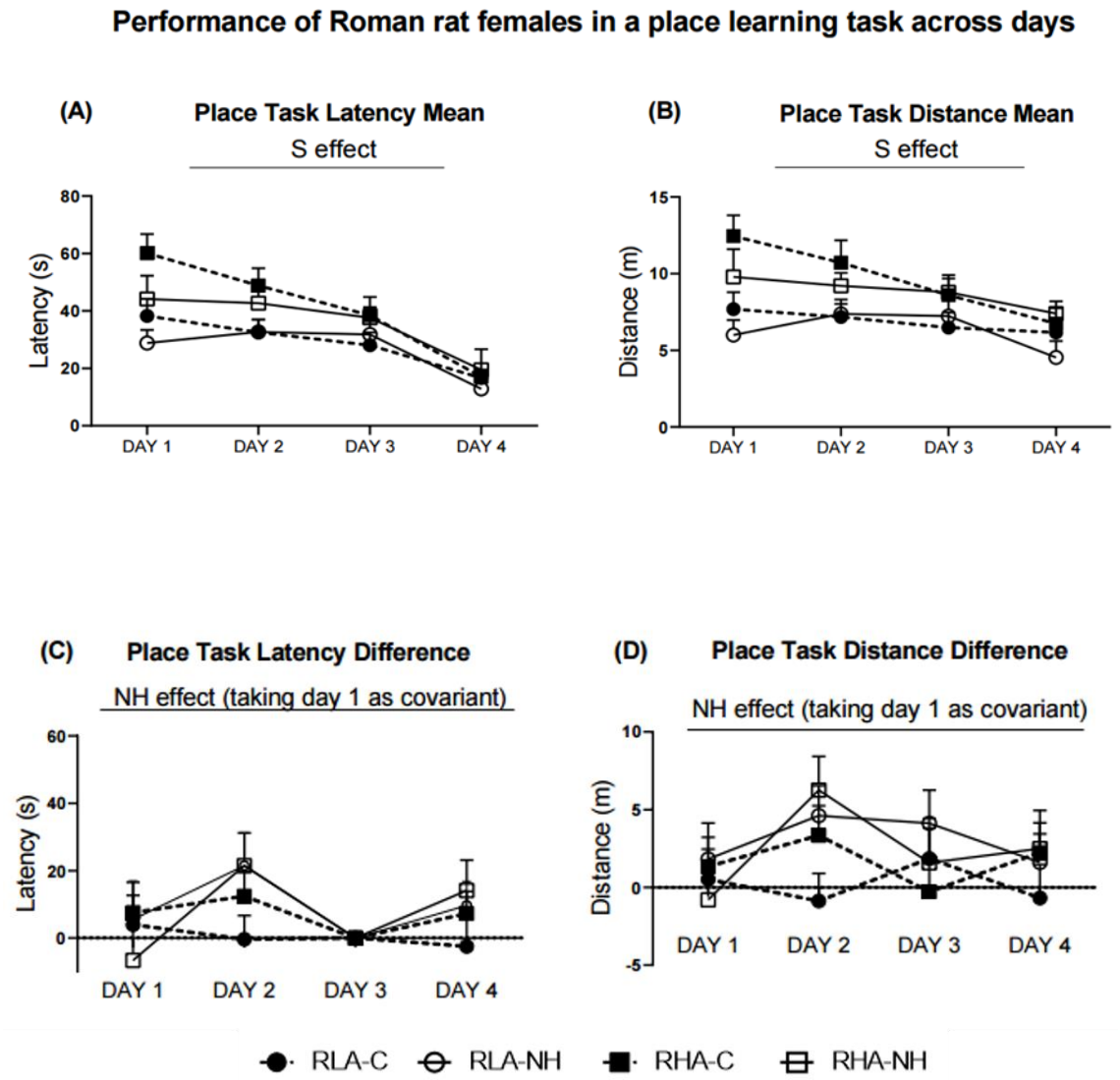


Figure 7. Means (± SEM) for the place task (PT) in female Roman rats and effects of the NH treatment on: A) The mean latency of the 3 trials/day. B) The mean distance of the 3 trials/day. C) The latency difference between the first and last trial of each day. D) The distance difference between the first and last trial of each day. Group abbreviations as in Figure 2. "n" per group: RLA-Control=15, RLA-NH = 13, RHA-Control = 13, RHA-NH = 12. NH, treatment effect (ANOVA).

3.4.3 Transfer Test (TT)

As in the previous task there were 9 rats showing clear "passive floating" behaviour (i.e., no sign of active swimming to search the platform) that were excluded from analysis of this task ("n" per group: RLA-Control=15, RLA-NH = 13, RHA-Control = 13, RHA-NH = 12).

A two-way factorial ANOVAs for the TT revealed only Strain effects on "Transfer test A_latency", "Transfer test Distance" and "Transfer test Speed" ($F(1,49) = 4.4$, $p < 0.042$;

F (1,49) = 6.9, p < 0.011; F (1,49) = 4.8, p < 0.034, respectively; Figure 8B, C, D). No significant effects were found for annulus crossings ("Transfer test A_Crossings", F (1,49) = 3.25; p < 0.77, Figure 8A).

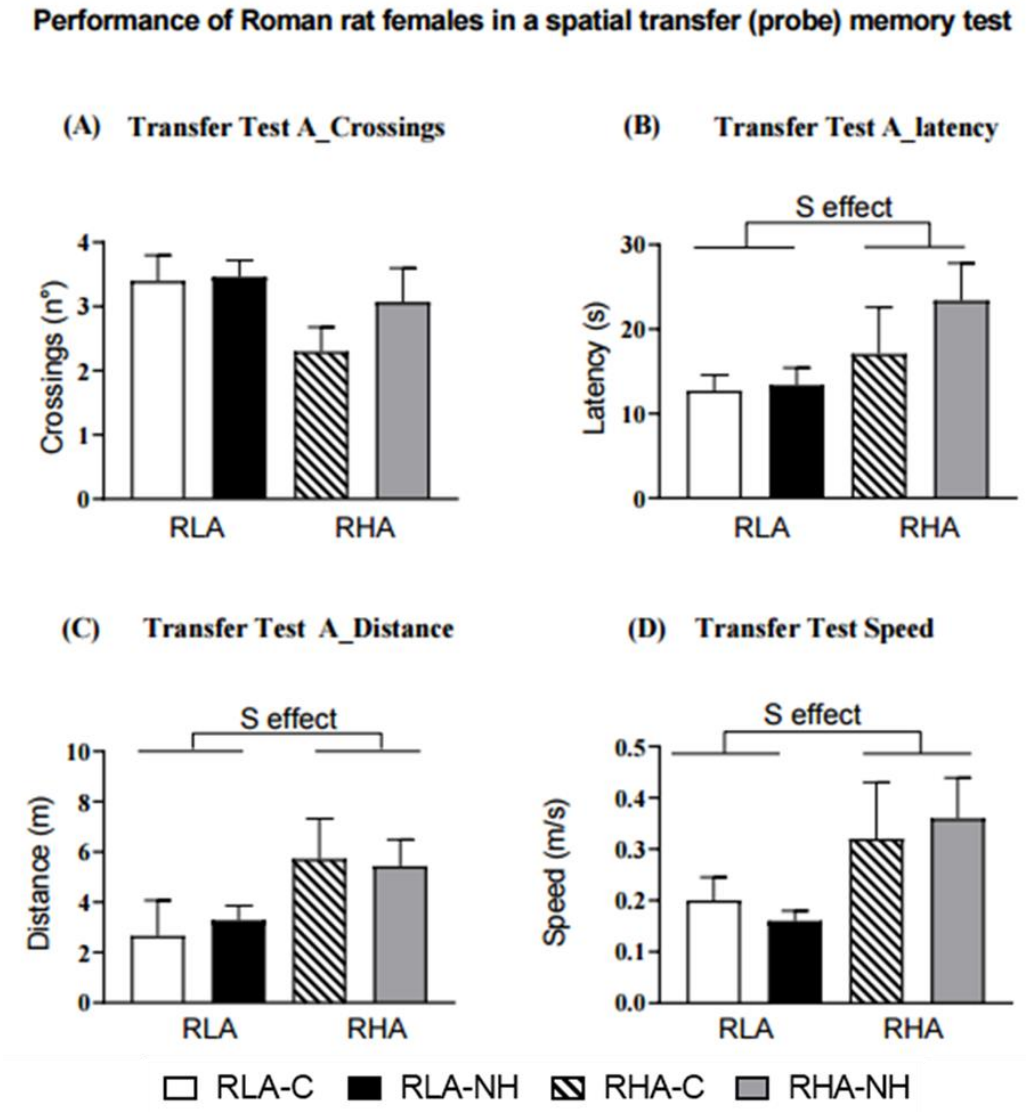


Figure 8. Means (± SEM) for transfer test (TT) in female Roman rats and effects of the NH treatment on: A) "Transfer Test A_Crossings", annulus crossings. B) "Transfer Test A_Latency", time elapsed until the first annulus crossing. C) "Transfer Test A_Distance", distance travelled until the first annulus crossing. D) "Transfer Test speed", mean swimming speed during the TT. Group abbreviations as in Figure 2. "n" per group: RLA-Control=15, RLA-NH = 13, RHA-Control = 13, RHA-NH = 12. S, strain effect (ANOVA).

3.4.4 Reversal Test (RT) – Cognitive flexibility

There were 9 rats showing clear “passive floating” behaviour (i.e., no sign of active swimming to search the platform) that were excluded from analysis of this task (“n” per group: RLA-Control=15, RLA-NH = 13, RHA-Control = 13, RHA-NH = 12).

Repeated measures factorial ANOVA for all trials (Figure 9A) revealed “Strain” effects on latency ($F(1,49) = 9.31$, $p < 0.004$, Figure 9A) and distance ($F(1,49) = 9.55$, $p < 0.003$ Figure 9B), as well as “trial * strain” effects on both measures ($F(1,49) = 2.04$, $p < 0.041$; $F(1,49) = 2.21$, $p < 0.026$, respectively; Figure 9 A-B). Posthoc Duncan’s test showed significant differences in latency between RLA-Control and RLA-NH groups in the last trial of the first day, as well as a difference in distance between RHA-Control and RHA-NH groups in the last trial of the last training day (Figure 9A-B). There were no factor or interaction effects on swimming speed (all $Fs(1, 49) < 1.32$, $p > 0.25$).

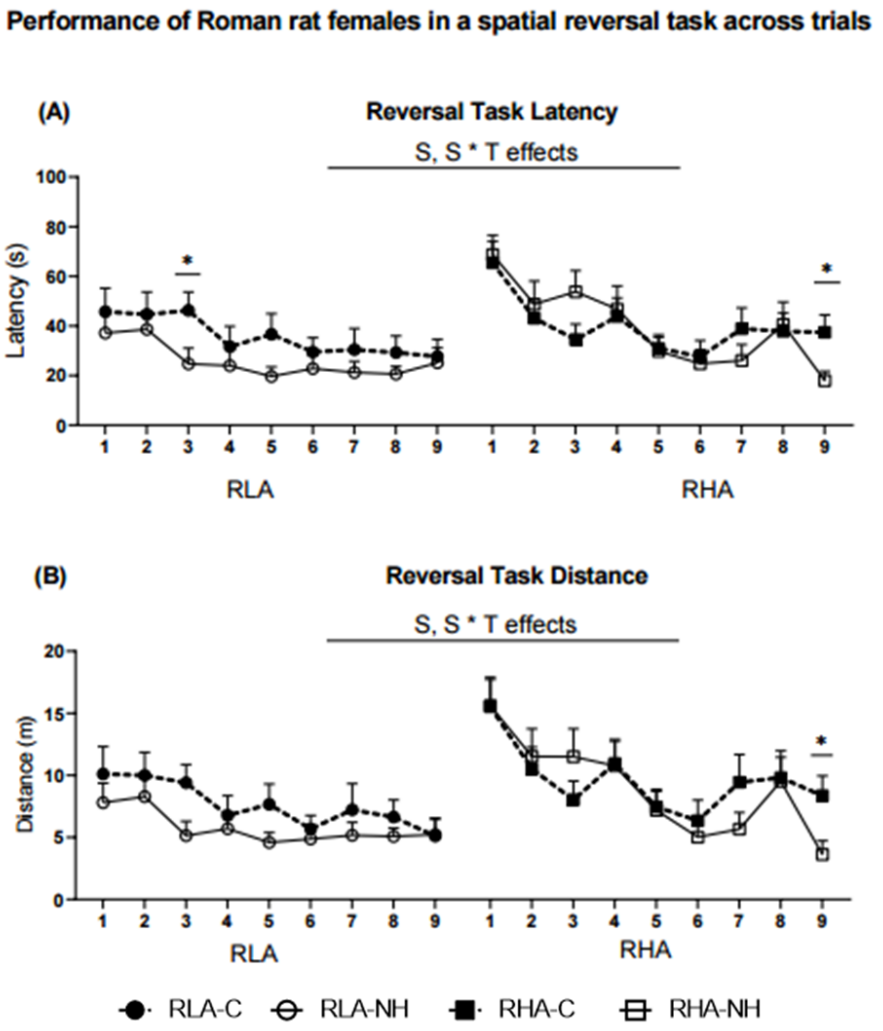


Figure 9. Means (± SEM) for each trial (1-9) of the reversal task (RT) in female Roman rats and effects of the NH treatment on: A) latency to find the platform for each trial. B) Distance travelled to find the platform in each trial. Group abbreviations as in Figure 2. "n" per group: RLA-Control=15, RLA-NH = 13, RHA-Control = 13, RHA-NH = 12. * $p<0.05$ vs. NH group of the same strain (Duncan's multiple range test). S, strain effect (ANOVA); T, trial effect (ANOVA).

ANOVA on the means of the 3 trials/day (x 3 days) revealed "Strain" effects on latencies ($F(1,49) = 4.5$, $p < 0.04$) and distances ($F(1,49) = 7.2$, $p < 0.01$), as well as a "Strain x Treatment x Day" effect on the distance travelled ($F(2, 98) = 3.2$, $p = 0.045$) (Figure 10A - B). To find the source of this interaction we performed separate ANOVA for each strain, which only revealed a significant effect of "Treatment" on distance in days 2-3 in RHA rats ($F(1, 19) = 4.8$, $p < 0.042$, taking "Day 1" distance as a covariate), since RHA-NH rats exhibited overall shorter distances in these two days than RHA-Control rats (Figure 10B).

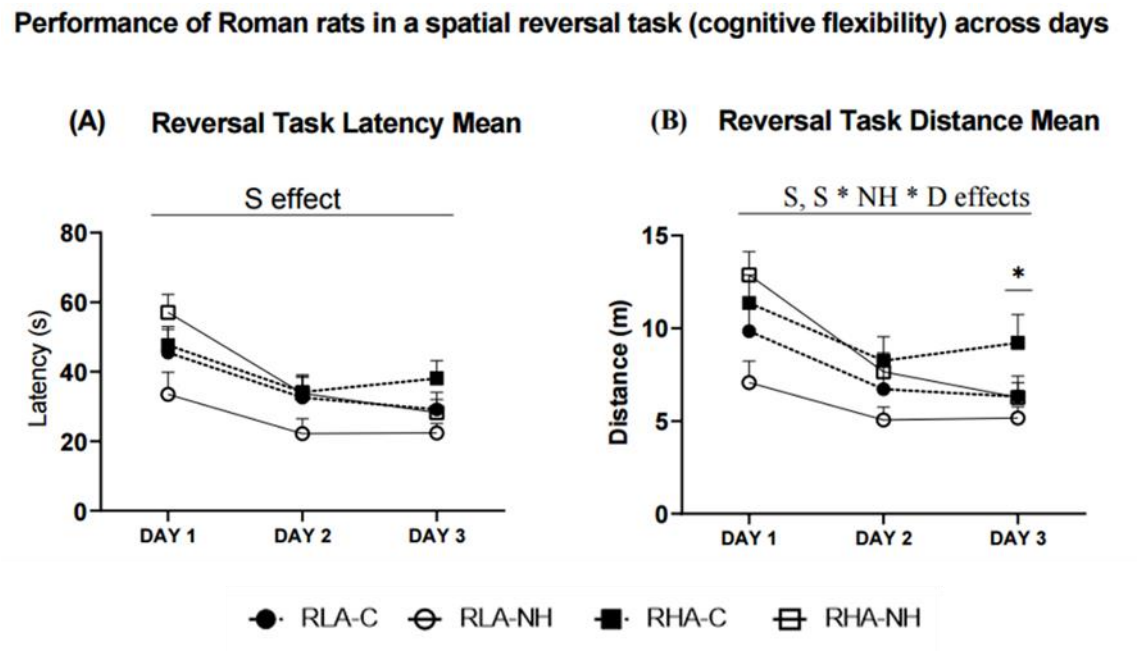


Figure 10. Means (± SEM) for the reversal task (RT) in female Roman rats and effects of the NH treatment on: A) The mean latency of the 3 trials/day. B) The mean distance of the 3 trials/day. Group abbreviations as in Figure 2. "n" per group: RLA-Control=15, RLA-NH = 13, RHA-Control = 13, RHA-NH = 12. * $p<0.05$, vs. the other 3 groups (Duncan's multiple range test). S, strain effect (ANOVA); NH, treatment effect (ANOVA); D, day effect (ANOVA).

3.4.5 Cue Task (CT)

There were 8 rats showing clear "passive floating" behaviour (i.e., no sign of active swimming to search the platform) that were excluded from analysis of this task. See the final "n" per group in Figure 11.

A repeated measures ANOVA (2 strain x 2 treatment x 8 trials) was performed for the 8 trials. No significant main effects or interactions were found on latencies (all $F(1,50) < 3.10$, $p > 0.084$, Figure 11A) or distances (all $F(1,50) < 2.17$, $p > 0.14$, Figure 11B).

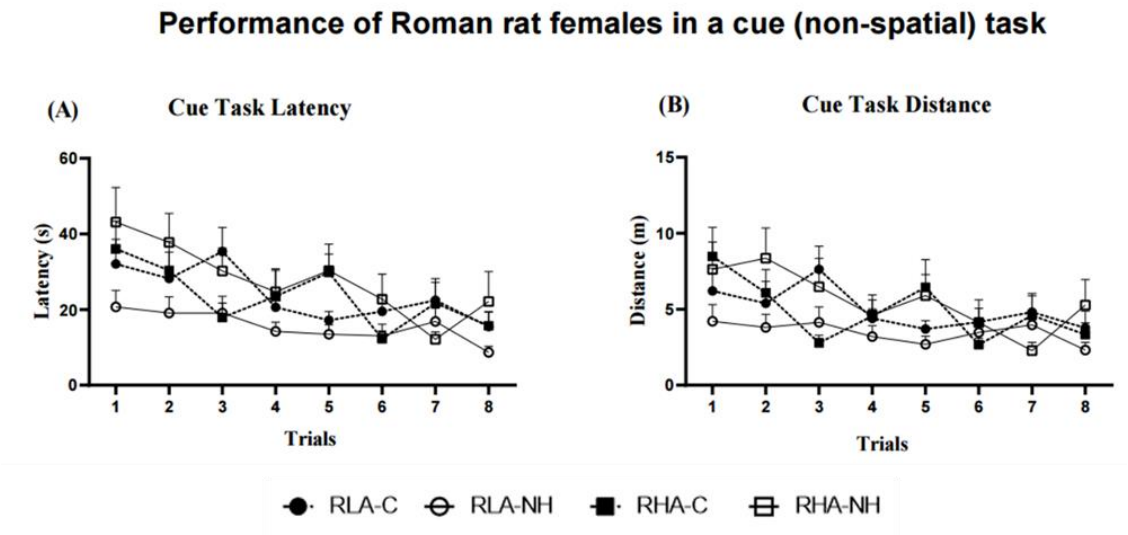


Figure 11. Means (± SEM) for each trial (1-8) of the cue task (CT) in female Roman rats and effects of the NH treatment on: A) Latencies to find the platform in each trial. B) Distance travelled to find the platform in each trial. Group abbreviations as in Figure 2. "n" per group: RLA-Control=15, RLA-NH = 13, RHA-Control = 13, RHA-NH = 12.

3.5 Correlational and factor analyses

Selection of the variables to enter in the Factor analysis was done according to the following criteria: (i) To avoid highly redundant variables, i.e., to select the best variable from two or more highly correlated variables. Thus, NOE exploration time was the variable selected from the NOE test, and "Annulus crossings" was chosen as the most representative variable (i.e., the measure that is most typically used as index of long-term reference memory) of the TT procedure (the other variables from these tests were highly correlated with the selected ones). (ii) To exclude a given variable if it is included in the calculation of another variable. Thus "total PPI" was selected and "baseline startle" was excluded because it is included in the calculation of %PPI. (iii) To choose the first part of the spatial learning tasks and the "cued task", i.e., the phase of learning prior reaching asymptotic performance in each task. That's why we selected the "mean distance" of the two first days of training in the PT and RT and the first day of the CT.

Correlational analysis with the above selected variables showed some between-task correlations (Table 1). For instance, NOE "time" moderately and negatively correlated with grooming (r=-0.43, p=0.001) and positively correlated with crossings (r=0.40, p=0.001) and non-social time (r=0.34, p=0.007) in the SI, while also showed a negative

association with PPI (r=-0.36, p=0.004). Interestingly, social time and crossings in the SI were also negatively correlated with PPI (r=-0.34, p=0.007, and r=-0.35, p=0.006, respectively), whereas grooming in the SI correlated positively with PPI (r=0.39, p=0.002). NOE and SI test variables did not correlate with learning/memory tasks, except for a low correlation of grooming with distance in the PT (r=-0.29, p=0.044). Most interestingly, the highest between-task correlation was observed between PPI and distance in the first two days of the PT (r=-0.51, p<0.001), reflecting that higher PPI levels relate to lesser distance travelled in spatial reference learning in the PT (Table 1). As for correlations among the different learning/memory tasks, distance travelled in the PT was negatively correlated with annulus crossings in the TT (r=-0.39, p=0.004) and with mean distance difference between trials-1 and trials-2 in the working memory (DMTP) task (r=-0.29, p=0.04). Finally, the distance travelled in the first day (average of the 4 trials) of the cued task (CT) was positively (r=0.43, p=0.001) and negatively (r=-0.29, p=0.039) correlated with distance in the RT and distance difference in the DMTP task, respectively (Table 1).

TABLE 1	Pearson´s correlations among the main variables										
	1	2	3	4	5	6	7	8	9	10	11
1-NOE_Time	1										
2-Non social time	**.34****	1									
3-Social time	.13	**-.31***	1								
4-Grooming	**-.43*****	**-.38****	**-.32***	1							
5-Locomotor A ctivity	**.40*****	.16	.22	**-.47*****	1						
6-Total PPI	**-.36****	-.03	**-.34****	**.39****	**-.35****	1					
7-PT (Day 1-2)	.05	.10	.20	**-.28***	.21	**-.51*****	1				
8-TT A_Crossings	.08	-.02	.02	.02	-.15	.10	**-.39****	1			
9-RT (Day 1-2)	.05	.16	.09	-.08	.12	-.02	.05	-.18	1		
10-WM (XT1-XT2)	-.12	-.16	-.18	.12	-.16	.09	**-.29***	-.00	-.08	1	
11-CT (X Day 1)	-.01	.06	.08	-.07	-.01	-.15	.19	-.03	**.43*****	**-.29***	1

Table 1. Pearson's correlation coefficients among variables of the different tests/tasks. Abbreviations: "NOE_Time", corresponds to exploration time in NOE test. "Non-social Time", "Social Time", "Grooming" and "Locomotor activity", are the corresponding variables from the SI test. "Total PPI", averaged %PPI for the four prepulse intensities. "PT (d 1-2)", corresponds to the average distance from day 1 and 2 of the place task. "TT A_Crossings", refers to number of crossings through the annulus where the platform was located during the place task. "RT (day 1-2)", refers to the average distance from day 1 and 2 of the reversal task. "WM (XT1-XT2)", corresponds to the difference between the mean distance of all trials 1 and the mean distance on all trials 2 of the DMTP (working memory) task. "CT (X day 1)", mean distance of day 1 from the cue task. Bold characters indicate significant Pearson's coefficients. *p < 0.05, **p < 0.01 *** p < 0.001 (two-tailed). N= 62 for correlations among NOE, SI and PPI variables, and N = 51 for correlations with any of the variables from the MWM tasks (after exclusion from analysis of the "passive floating" rats in the different spatial learning tasks and the CT).

To better explore associations of measures from the different tests/tasks with possible underlying factors or traits we performed obliquely-rotated factor analysis on those selected variables (Table 2). The first analysis yielded a three-fold solution as follows: (i) A first factor grouped loadings of measures from the SI test (social time, 0.56; grooming, -0.76; crossings, 0.66) and, interestingly, PPI (loading -0.73), together with distance in the PT (loading 0.64) and distance difference in the DMTP task (-0.36). (ii) A second factor involving NOE exploration time (0.80) and non-social (0.82) and social time (-0.56). (iii) A third factor with a combination of all the learning/memory tasks except the DMPT (loadings -0.57 –TT-, 0.57 –PT-, 0.76 –RT-, 0.74 –CT-). We then forced factor analysis into a two-fold solution to try to obtain a simpler structure of variable-factor associations, and a more meaningful structure of two independent factors (correlation

0.075) was obtained: (i) A first factor (tentatively named "Attention/cognition") dominated by loadings of distance in the PT (0,77) and PPI (-0.66), but with moderate contributions of distance in the RT (0.41), distance savings in the DMTP task (-0.41) and annulus crossings in the TT (0.39), while also grouping grooming (-0.64), social time (0.45) and crossings (0.56) in the SI. (ii) A second factor that mainly grouped high loadings of NOE time (0.70) with non-social (0.81) and social time (-0.61) (Table 2).

(A)	**Factor 1**	**Factor 2**	**Factor 3**	**(B)**	**Factor 1** "Attention-Cognition"	**Factor 2**
NOE_Time	-	0.80	-	NOE_Time	-	0,70
Non social Time	-	0.82	-	Non social Time	-	0,81
Social Time	0.56	-0.56	-	Social Time	**0.45**	-0,61
Grooming	-0.76	-	-	Grooming	-0.64	-
Locomotor Activity	0.66	-	-	Locomotor Activity	0.56	-
Total PPI	-0.73	-	-	Total PPI	**-0.66**	0,36
WM (XT1-XT2)	-0.36	-	-	WM (XT1-XT2)	**-0.41**	-
PT (Day 1-2)	0.64	-	0.57	PT (Day 1-2)	**0.77**	-
TT (A_Crossings)	-	-	-0.57	TT (A_Crossings)	**-0.39**	-
RT (Day 1-2)	-	-	0.76	RT (Day 1-2)	**0.41**	-
CT (X Day 1)	-	-	0.74	CT (X Day 1)	-	0,36
% of variance (cumulative)	24.1	41.6	55.7	% of variance (cumulative)	24.1	41.6
				Correlation between factors	.075	

Table 2. Obliquely-rotated factor analysis with the variables of the different tests/tasks. (A) Three-factor solution with the main behavioural variables and correlations between factors. (B) Two-factor solution and correlation between factors. Only variables with loadings >.35 are considered. Highlighted in bold are the variable loadings that are most relevant in the present study. Symbols/abbreviations as in Table 1. N = 51 (after exclusion from analysis of the passive floating" rats in the different spatial learning tasks and the CT).

4. Discussion

The present is the first study devoted to jointly investigate the differential profiles of female RHA vs. RLA rats and the effects of NH on a range of phenotypes that are thought to be relevant to schizophrenia positive, negative and cognitive symptoms. To this aim novelty exploration (NOE), social interaction and activity (SI), attentional (PPI) and spatial cognitive (WM, PT, TT, RT and CT) tests/tasks were administered to non-handled and neonatally-handled (NH) female rats of both strains.

The main results from the NOE and SI tests show that: (i) female RHA rats show much more exploration of the novel object in the NOE test than RLAs; (ii) such a between-strain difference is abolished by NH, which dramatically increases exploration of the novel object in RLA rats; (iii) female RHA rats differ from RLAs in social interaction (social time, RHA > RLA) and locomotor activity; (iv) NH treatment increases "social time" in RLAs; (v) self-grooming behaviour, an index of emotional reactivity/anxiety (Estanislau et al., 2019; Fernández-Teruel and Estanislau, 2016), is overall increased in RLA rats, and NH treatment reduces self-grooming in this strain.

Thus, relative to RHA rats, RLAs exhibit higher novelty-induced inhibition and anxiety, as suggested by the NOE test and grooming behaviour in the SI test, respectively. And, in agreement with previous findings, NH reduces such behavioural inhibition (anxiety) to RHA's levels in both measures (Río-Álamos et al., 2015, 2017; Sampedro-Viana et al., 2021). These NH effects are consistent with the well-known enduring anxiolytic-like effects of this treatment, as observed in many previous studies in RLAs and other rat strains (e.g., Fernández-Teruel et al., 1997, 2002; Núñez et al., 1996; Raineki et al., 2014; Río-Álamos et al., 2015, 2017; Steimer et al., 1998). Notably, the NH-induced reduction of self-grooming in RLA rats is paralleled by an increase of social time, while there is a significant negative correlation between both measures ($r = -0.32$; see Table 1) and there are significant loadings of both variables (with opposite sign) in the first factor of the factor analysis (see Table 2B). Collectively, these findings might be consistent with the notion that social time is negatively associated to anxiety in the present study, which is in contrast with findings obtained with RLA/RHA males in previous studies (Sampedro-Viana et al., 2021, 2023).

On the other hand, both locomotor activity and social time are increased in RHA vs. RLA rats and both variables load positively (0.56 and 0.45, respectively) in the first factor of the factorial analysis (see Table 2B). This might suggest that there is a positive association between locomotor activity and social time that might influence the global differences between the strains in both measures. However, NH did not affect activity in any rat strain, which indicates that treatment effects on social time in RLA rats does not relate to its effects on locomotion.

The overall results of social interaction (social time), showing that female RHA rats exhibit higher social time than RLAs, agree with our previous findings (Oliveras et al., 2022b). However, female results are in contrast with the finding that RHA males display lowered social preference than RLA males (Oliveras et al., 2022b; Sampedro-Viana et al., 2021). As discussed by Oliveras et al., (2022b), the above mentioned between-sex discrepancies in social behaviour of RHA vs. RLA rats seem to be in line with human studies showing that negative symptoms of schizophrenia, such as social withdrawal, are less frequent or severe in woman than in men (Oliveras et al., 2022b). Some authors have proposed that a possible intervening factor in such sexual dimorphism could be the differences in sexual hormones and their effects on brain maturation during early development (Oliveras et al., 2022b; Räsänen Sami, 2000). On the other hand, while NH significantly increases social time in male RLA and RHA rats (Sampedro-Viana et al., 2021), in the present study the treatment only had a significant effect in RLA females. In sum, these sexually dimorphic effects on social interaction deserve further studies.

We have observed that RHAs exhibit reduced PPI levels relative to RLA females, which is in agreement with previous findings (Oliveras et al., 2022a), and goes along with the differences found in RHA vs. RLA males in several studies (Oliveras et al., 2022a, 2015; Tapias-Espinosa et al., 2019, 2018). However, at variance with Río-Álamos et al., (2019) no NH-treatment effects on PPI were found in females of any strain in the present study. This discrepancy between both studies might be due to different %PPI levels shown by females in this study compared with the previous study (e.g., %PPI at prepulse intensities 65-70 dB = ~40, in the present study, vs. ~20 in RHA rats in Río-Álamos et al. study; see Figure 4 from Río-Álamos et al., 2019), and/or it might be due to the different ages of the rats in both studies (PND76 in the present study vs. PND110 in Río-Álamos et al., study).

On the other hand, this is the first time that female RHA and RLA rats have been evaluated in the above mentioned spatial cognitive tasks (WM, PT, TT, RT, CT). It is worth to highlight that, relative to RLA females, RHAs exhibit poorer spatial working memory, as well as impaired reference learning/memory (PT and TT) and cognitive flexibility (RT), and these results cohere with our previous findings with males of both

strains (Aguilar et al., 2002; Martínez-Membrives et al., 2015; Oliveras et al., 2015, 2016; Río-Álamos et al., 2019; Sánchez-González et al., 2020).

Interestingly, NH led to slight (but significant) long-lasting improvements of spatial reference learning in the PT (across sessions and within sessions) in both rat strains and, most importantly, the treatment improved cognitive flexibility in the spatial reversal task (RT), particularly in RHA rats. These findings are new in females, and seem to be relevant since they go along with previous results obtained with NH in males of both rat strains (Aguilar et al., 2002; Río-Álamos et al., 2019), thus suggesting altogether that NH treatment leads to some enduring improvements of spatial cognition and, in particular, spatial cognitive flexibility. The fact that these positive effects are more clearly observed in RHA rats, the strain presenting a relative deficit in these tasks, suggests that certain (early) environmental conditions might act as buffers against some genetically-based predispositions (or vulnerabilities) to cognitive alterations that model those that are present in schizophrenia. In this regard, it is worth to remind that the present spatial tasks are known to be related with the function of the prefrontal cortex and hippocampus, two regions that are altered in schizophrenia and in RHA rats (Elfving et al., 2019; Meyza et al., 2009; Río-Álamos et al., 2017, 2019; Tapias-Espinosa et al., 2019, 2023). Remarkably, NH treatment has consistently been reported to improve hippocampal plasticity in standard (unselected) laboratory rats (Antoniazzi et al., 2017; Boufleur et al., 2013; Fernandez-Teruel, 2022; Fernández-Teruel et al., 2002; Meaney et al., 1988; Pondiki et al., 2006; Stamatakis et al., 2008; Taschetto Vey et al., 2020). We have also observed NH effects on hippocampus volume in the Roman rat strains (Río-Álamos et al., 2017, 2019; Tapias-espinosa et al., 2019), and our recent results also show increases of c-Fos and neuroplasticity factors (e.g., brain-derived neurotrophic factor, BDNF) in the prefrontal cortex and/or hippocampus of NH-treated RHA rats (Sampedro-Viana et al., 2023; and unpublished results).

It is worth to underline that the overall pattern of results of female RHA vs. RLA rats in the present study is consistent with those observed in males (e.g., Martínez-Membrives et al., 2015; Oliveras et al., 2015; Río-Álamos et al., 2019; Tapias-Espinosa et al., 2018, 2019), as well as with the few cases in which females were used (Oliveras et al., 2022a,b; Río-Álamos et al., 2019). Such a consistency with previous studies, as well as the fact

that the period needed to complete the whole behavioural testing was around 2 months (e.g., the NOE test took 1 day, but the SI test required 2 weeks to be completed, the PPI tests required 1 week, and the MWM tasks required 4 weeks), make it most likely that the oestrus stage of the female rats had followed a randomly distributed pattern across days, tests/tasks and experimental group, rather than any systematic biased trend. Therefore, it seems unlikely that the results of the present study have been influenced by the rats' oestrus phase.

Finally, the associations among behavioural measures from the different tests/tasks, evidenced by the correlational and factor analyses, deserve some further comments. These analyses intended to explore whether NOE and/or SI measures might be related to PPI, and whether PPI levels might be associated with spatial working memory or reference learning/memory or cognitive flexibility, as suggested by previous studies with males (Oliveras et al., 2015; Río-Álamos et al., 2019). In essence, what is most informative is the result of the two-fold factor analysis (Table 2B), as it shows a first factor, tentatively named "Attention/cognition" factor, because it clusters PPI with PT (spatial reference learning), both with the highest loadings (-0.66 and 0.77), and RT distance (cognitive flexibility; 0.41), annulus crossings (TT; long-term spatial memory; -0.39) and working memory (DMTP task; -0.41). This cluster of variables suggests that PPI is positively associated with performance in the aforementioned four cognitive tasks, which is congruent with our previous studies with males (Oliveras et al., 2015; Río-Álamos et al., 2019). In addition, grooming (-0.64), crossings (0.56) and social time (0.45) also load in this first factor. It is worth noting that less grooming and more crossings (activity) relate to worse PPI, which is also in line with our previous findings with males (Tapias-Espinosa et al., 2018). Remarkably, the loadings of the first factor indicate that locomotor activity is negatively associated with PPI and with efficiency in the different cognitive tasks in the water maze. This seems in good agreement with the observed strain differences since, compared with RLAs, the more active RHA rats are impaired at PPI and in all the spatial learning/memory tasks. The second factor is defined by high loadings of NOE time (0.70), non-social (0.81) and social (-0.61) time, with marginal loadings of distance in the CT (0.36) and PPI (0.36). Thus, this is predominantly an "Exploration" factor (see Table 2B).

According to what the first factor indicates, it may be suggested that relatively high levels of activity (i.e., crossings) and lowered grooming and PPI levels strongly relate to worsened performance in the place task (spatial reference learning/memory), and are also associated (though with lower loadings) to impaired cognitive flexibility and working memory (DMTP task). This seems quite in concordance with the differences observed here in RHA vs. RLA rats, i.e., relative to RLAs, female RHA rats show higher activity, lowered grooming and impaired PPI and spatial cognition, which also coheres with previous findings with males (Oliveras et al., 2015; Río-Álamos et al., 2019; Tapias-Espinosa et al., 2018). Therefore, in accordance with these studies in males, the present multi-task approach has enabled to study relations among the different tests/tasks in female RHA/RLA rats, leading to the conclusion that relatively low grooming, and high crossings and high social interaction relate to worse PPI, and PPI is in turn positively associated with spatial cognition.

The Roman rat lines/strains are known to differ in various aspects of the function of central DA, 5-HT and glutamate systems (particularly NMDA and mGlu2 receptors), as well as neuroplasticity factors and markers of neural/synaptic activity in frontocortical, hippocampal, mesocorticolimbic and other limbic regions (e.g., Meyza et al., 2009; Sanna et al., 2019, 2022; Serra et al., 2022, 2023; Fernández-Teruel et al., 2021; Sampedro-Viana et al., 2023). Some of these neurochemical, neurotrophic and synaptic changes might be involved in (or associated to) the emotional, behavioural and cognitive differences observed between the Roman rat lines/strains (e.g., Elfving et al., 2019; Fernandez-Teruel et al., 2021; Giorgi et al., 2019; Klein et al., 2014; Río-Álamos et al., 2017, 2019; Sønderstrup et al., 2023; Tapias-Espinosa et al., 2019, 2023). Also interestingly, some of the above mentioned neurochemical and neuroplasticity factors have been reported to be sex-dependently affected by NH in standard laboratory rat strains (e.g., Katsouli et al., 2014; Panagiotaropoulos et al., 2004; Peters et al., 1991; Stamatakis et al., 2009). Therefore, future research should address NH and sex-dependent effects on those neurochemical/neuroplasticity processes in RHA vs. RLA rats.

To summarize, in the present study we have found evidence that RHA female rats may be, as already shown for males (Fernández-Teruel et al., 2021; Giorgi et al., 2019; Río-

Álamos et al., 2019; Tapias-Espinosa et al., 2019), a model with face validity for some schizophrenia-relevant phenotypes, such as locomotor activity, attentional and cognitive traits. Nevertheless, comparison with previous studies in males' points to some differences between sexes in the RHA vs. RLA profiles, particularly regarding social interaction (Oliveras et al., 2022b). Also, we show positive NH effects on novel object exploration, self-grooming and social behaviour (predominantly in RLA rats) and, for the first time we report that NH treatment has some positive effects on spatial place learning and improves cognitive flexibility (reversal spatial learning) in females of the Roman rat strains.

Author contributions

A.F-T. A.T., T.C., D.S-V and N.P-V. conceived and/or designed the experiments. N.P-V., C.R-A., P.G-F., T.C., D.S-V and A.F-T. conducted the experiments. I.O. assisted in the behavioral experiments. A.F-T., D.S-V, N.P-V., P.G-F., A-T., I.O. and T.C. analyzed the data or assisted with data analyses. A.F-T. and N.P-V. wrote the original manuscript. A.T., T.C., I.O., D.S-V., C.R-A. and P.G-F. provided critical review of the original draft. All authors read and approved the manuscript.

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Conflict of interest

The authors present no conflict of interest.

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ESTUDIO 3

Neonatal handling enhances cognitive domains related to executive function and frontocortical synaptic maturation in a rat model of schizophrenia-like behaviour

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(SOMETIDO a CONSIDERACIÓN)

Abstract

We investigated the potentially positive neurodevelopmental and enduring effects of neonatal handling (NH) on specific behavioral processes and gene expression in the prefrontal cortex of RHA versus RLA rats. Additionally, we explored associations between gene expression and behavioral responses. Male rats from both the RLA and RHA strains received NH or were left untreated (controls). Two different age groups were considered: adolescent and adults. The assessment encompassed exploratory behavior, social behavior, sensorimotor gating (PPI), and the analysis of gene expression associated with synaptic processes, cortical maturation, and neuroplasticity. In adolescent rats, NH increased novelty exploration and activity, and reduced anxiety-related self-grooming in RLAs, whereas it improved PPI in RHAs. In adult rats, NH increased novelty-induced activity in both strains, reduced self-grooming in RLA rats, and enhanced social interaction and PPI in RHAs. NH produced significant effects on gene expression in adolescent RHA rats. These effects were observed at the presynaptic level by a reduction of *Snap25* and increases of *Cables1* and *Cdk5*, and at the postsynaptic level by increases of *Grin2b, Homer1* and *Nrg1*, as well as by a NH-induced enhancement of *Bdnf*. NH also increased *Nrg1* and *Bdnf* expression in adult RLA rats. These findings show for the first time that NH treatment is able to modulate several genetically-linked behavioral and synaptic/neuroplasticity alterations in RHA/RLA rats, in line with the wide evidence on the positive long-lasting effects of NH on many emotional responses, social behavior, attention-related processes and cognition.

Key words. - Schizophrenia; Neonatal handling; Roman rats, Genetic expression, ontogeny

1. Introduction

Schizophrenia is a complex and severe mental disorder which is thought to depend on gene-environment interactions across development. Genome-wide association studies (GWAS) have shown that multiple genes may be related to psychotic and schizophrenia symptoms, while epigenetic evidence points to the importance of interactions between genes and environment across development in shaping risk for psychosis and schizophrenia later in life. Identifying the risk-associated genetic background is therefore of high relevance for implementing preventive programs. In this context, animal models exhibiting positive, negative and cognitive phenotypes related to the disorder may be of particular translational value to identify genetic, epigenetic and neurodevelopmental factors that may confer vulnerability to, or protection against schizophrenia-related traits or symptoms.

One such models is constituted by the inbred Roman high-avoidance (RHA) rats, which compared with their Roman low-avoidance (RLA) counterparts and other rat strains exhibit impaired executive/cognitive functions, attentional processes and social behavior, as well as novelty- and psychostimulant-induced hyperactivity and locomotor sensitization (Esnal et al., 2016; Martínez-Membrives et al., 2015; Moreno et al., 2010; Oliveras et al., 2015; Río-Álamos et al., 2019; reviewed by Giorgi et al., 2019; Fernández-Teruel et al., 2021, 2023; and Oliveras et al., 2023). Underlying these behavioral phenotypes, RHA rats display alterations in dopamine, serotonin, GABA and glutamate neurotransmitter systems (Fernandez-Teruel et al., 2021; Giorgi et al., 2019; Fomsgaard et al., 2018; Klein et al., 2014; Tapias-Espinosa et al., 2023), grey and white matter structures (Río-Álamos et al., 2017, 2019), prefrontal cortex (PFC) activation (Tapias-Espinosa et al., 2019, 2023), as well as increased sensitivity to neonatal handling and social isolation rearing (Oliveras et al., 2016; Sánchez-González et al., 2020; Peralta-Vallejo et al., 2023; Río Álamos et al., 2019; Sampedro-Viana et al., 2021, 2023). These neurobehavioral phenotype profiles lend face and construct validity to RHA rats as a model of schizophrenia-relevant features, while psychopharmacological findings add predictive validity to the RHA rat model (Oliveras et al., 2017; Sampedro-Viana et al., 2023).

In this regard, we have recently reported differential expression of synaptic markers during neurodevelopment and increased density of thin (immature) dendritic spines in frontal cortex (FC) of RHA vs. RLA rats, pointing to an immature FC of RHA rats (Elfving et al., 2019; Sánchez-González et al., 2021). A failure to reach a final stage of cortical maturation in at-risk individuals has been proposed to be at the core of schizophrenia (Catts et al., 2013; Gao et al., 2022). According to this neurodevelopmental hypothesis we recently mapped the FC transcriptome in adult Roman (RHA vs. RLA) rats and investigated whether there is a shift in the RHA compared to the RLA strain in functional gene networks differentially expressed during early neurodevelopment (Sønderstrup et al., 2023). We found a different maturational expression profile of synaptic components in the RHA (vs. RLA) strain, supporting a shift in FC maturation underlying schizophrenia-relevant behavioural traits and adding construct validity to this strain as a neurodevelopmental model (Sønderstrup et al., 2023). The altered expression of several synaptic markers indicated that aberrant cortical maturation during adolescence in RHA rats may lead to permanent neurodevelopmental anomalies present during adulthood in this strain, which may underlie their cognitive/attentional dysfunctions (Sønderstrup et al., 2023).

The above findings suggest that it may be room for intervention during early neurodevelopmental stages to influence synaptic and behavioral maturation in the Roman (particularly in RHA) rats. To this purpose, we intended to explore the possible positive neurodevelopmental and long-lasting effects of neonatal handling on target behavioral processes and gene expression in prefrontal cortex of both rat strains.

Neonatal handling (NH; administered to rat pups during the first 2-3 weeks of life) has been shown to long-lastingly improve emotional profiles and stress coping ability, and to enhance cognitive and attentional performance in laboratory rats and mice (e.g., Fernández-Teruel et al., 2002; Raineki et al., 2014; Río-Álamos et al., 2017, 2019). In addition, NH treatment enduringly reduces stress hormone responses and influences neuroplasticity and many neurotransmitter systems (e.g., Fernández-Teruel 2022; Fernández-Teruel et al., 2002; Fu and Depue 2019; Katsouli et al., 2014; Meaney et al., 1988, 1989; Panagiotaropoulos et al., 2004; Pondiki et al., 2006; Stamatakis et al., 2009; Taschetto-Vey et al., 2020). Many of these positive NH effects have also been

demonstrated in the Roman rat lines/strains (e.g., Fernández-Teruel et al., 1997, 2002; Río-Álamos et al., 2017, 2019; Sampedro-Viana et al., 2021, 2023; Steimer et al., 1998).

Therefore, the present study was aimed at investigating whether NH treatment may influence adolescent and adult exploratory behavior, social behavior, sensorimotor gating (prepulse inhibition of the startle response –PPI) and PFC gene expression of target genes in RHA vs. RLA rats, and whether there could be associations among gene expression and behavioral responses. The studied target genes were selected from those that were related to synaptic processes, cortical maturation and neuroplasticity in our previous studies (Elfving et al., 2019; Sønderstrup et al., 2023).

Our hypotheses were the following: 1) Compared with RLAs, RHA rats show higher exploration of a novel object and lowered social interaction, and NH treatment will increase exploration of the novel object in adolescent RLA rats (maturational effect) and social interaction in RHA rats. 2) Locomotor activity is increased in adult vs. adolescent rats and in RHA vs. RLA rats, and NH will enhance activity in adolescent rats (maturational effect), particularly in the RLA strain. 3) Self-grooming is higher in RLA rats of both ages (relative to RHAs), although more clearly in adult rats, and NH will decrease grooming in this strain at both ages (maturational effect). 4) Prepulse inhibition (PPI) levels is lower in adolescent vs. adult rats, and in RHA vs. RLAs, and NH will have a stronger effect in the RHA strain. 5) Accompanying these effects of NH interventions, we will see gene expression changes related to synaptic plasticity.

2. Materials and methods

2.1 Animals

The animals used for this study were 112 males of the inbred Roman high- (RHA, n=56) and low-avoidance (RLA, n=56) rats. All rats came from the permanent colonies maintained at the laboratory of the Medical Psychology Unit, Dept. Psychiatry and Forensic Medicine (School of Medicine, Autonomous University of Barcelona, Spain), since 1996. Each experimental group (see below) consisted of rats from 9 different litters (1-2 rats per litter). Litters were culled to 10 pups (approximately half of each sex) one day after birth (postnatal day 1, PND1), the day in which the neonatal handling (NH) treatment started (see NH procedure below). Rats were maintained under a 12:12 h

light-dark cycle (lights on at 08:00 a.m.), with controlled temperature (22 + 2º C) and humidity (50-70%). They had food and water available ad libitum.

All testing was carried out between 09:00 and 14:00h. All procedures were carried out in accordance with the Spanish Legislation (Royal Decree 53/2013, 1st February 2013) and the current regulation related to "Protection of Animals used for Scientific Purposes" established by the European Union (2010/63/UE, 22 September 2010).

2.2 Neonatal Handling (NH) Treatment

NH was administered twice daily (at 9.30 and 17.00 h) between PND1-21, in a room with a temperature of 22 ± 2 ◦C. Each handling session consisted of first removing the mother from the litter and then placing the pups gently and individually in plastic cages (35 x 15 x 25 cm) lined with paper towel for a total period of 8 min. Each pup was gently stroked for 3–4 s at 0, 4 and 8 min with bare hands, after which they were returned to their home-cage with their mother and litter. Non-handled (Control, C) rats were left undisturbed except for the weekly cleaning of their home cages. Rats were weaned at PND 21, and were housed in pairs of the same experimental group in regular (50 x 25 x 14 cm) macrolon cages.

2.3 Novel Object Exploration (NOE) test

This test was used to test anxiety-related exploratory behaviour in response to novelty. Food was removed from the home cage (except 4 food pellets left in each cage) and the cage was pulled away from the rack 20 cm to allow direct observation of the rats' behaviour. One hour later, the novel object (graphite pencil Staedtler Noris, HB nº2) was introduced vertically in the cage through the grid cover until it reached the cage bedding, and then the "Latency to the first exploration" (NOE latency time) and the "total time of exploration" (NOE exploration time) were measured for 3 minutes by a well-trained researcher standing approximately 40-50 cm away from the cage front. Exploration of the novel object was defined as approaching the nose to it at 1 cm distance (or less) or touching/manipulating the object.

2.4 Social Interaction (SI) test

For the social interaction (SI) test, two acrylic boxes (65 x 23 x 20 cm) were placed in front of each other at 12 cm, to prevent physical contact between the animals, in a red-lit room with a camera on the roof connected to a monitor outside the room to record the test. Each box had two 3 cm diameter holes at its ends. The hole facing to the room wall (distal hole) was named as “non-social hole”, while the one facing the other box was named as “social hole” (Sampedro-Viana et al., 2021).

The day before the test, animals were habituated to the room and the box in a 30-min habituation session. During this habituation period, the two holes of each box were covered with white tape and a cardboard barrier was placed between the boxes to prevent exploration of the other box. A pair of non-familiar weight-matched animals of the same strain were placed into the testing boxes (one rat in each box) for the 30-min habituation period.

In the test day, for SI assessment, the holes were opened and two weight-matched non-familiar rats (maximum weight difference 20 g) of the same strain were placed (one rat in each box) into the set-up for a 15-min test. The time spent exploring the social hole (social time) and the non-social hole (non-social time), time spent self-grooming (grooming time) and total number of crossings (crossings) were recorded (each box was divided into three equal squares by lines painted on the floor to measure horizontal activity –crossings-) by two well-trained observers (between-observer reliability r = 0.95) watching the TV monitor outside the testing room. The SI boxes were cleaned with 70% ethanol solution after each test and dried with paper towel.

2.5 Prepulse inhibition of the acoustic startle response (PPI)

The PPI test was conducted using two sound attenuated startle boxes (SR-Lab Startle Response System, San Diego Instruments, US). Animals were individually placed 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 delivered via a speaker mounted 15 cm above the cylinder. The data are transduced by an accelerometer into a voltage which is amplified, digitized, and saved into a computer for further analysis.

The session consisted of 5 minutes of habituation, after which 10 “pulse-alone” trials (105 dB(A), SPL, 40 ms) were delivered in order to obtain a stable baseline of startle responses (BL1). Next, six types of trials were randomly administered 10 times (60 trials): (i) Pulse-alone trials (105 dB(A), SPL, 40 ms; BL2; used to calculate the percentage of PPI); (ii) prepulses of 65/70/75/80 dB (A), SPL (20 ms) followed by the pulse stimulus (105 dB (A), SPL, 40 ms) with an inter-stimulus interval of 100 ms; or (iii) no-stimulus trials (background noise of 55 dB). The inter-trial interval was 15 s (range 10–20 s). The %PPI for each prepulse intensity was calculated with the following formula: %PPI = [100−(startle amplitude on prepulse trials/startle amplitude on “BL2” pulse-alone trials × 100)].

For each trial, the startle amplitude magnitude was measured by an accelerometer and calculated as an integrated response of the enclosure vibrations caused by the whole rat’s body startle reaction after startle stimulus onset (recording range: 200 ms). Startle response amplitude was defined as the maximum accelerometer voltage during the first 200 ms following the startle stimulus onset.

2.6 Experimental design

The study was carried out with male rats of both the RHA and RLA strains, receiving neonatal handling (NH) or no treatment (controls, C), and including two ages, adolescent and adult rats (see experimental and timeline design in Figure 1)..

Behavioural evaluation started at PND32-36 with the NOE test, which was administered to all rats from C (n=56, 28 per strain) and NH (n=56, 28 per strain) groups (see timeline in Figure 1).

Next, adolescent (PND36-40) and adult (PND92-96) rats were tested in parallel for social interaction (SI). In adolescent rats the n=10 in each of the 4 groups (RLA-C, RLA-NH, RHA-C, RHA-NH), and in adult rats the n=18 in each of the 4 groups (RLA-C, RLA-NH, RHA-C, RHA-NH) (see timeline in Figure 1). Two rats from the adult RHA-C group had to be excluded from the experiment because of technical problems (thus, adult RHA-C group had a definitive n=16). Body weights were 64.8 + 12.7 in adolescent rats and 298.7 + 30.2 g in adult rats (mean + standard deviation).

Twenty-four hours after the SI test, at PND38-42 and PND94-98 in adolescent and adult rats, respectively, the PPI test was carried out (Figure 1). For reasons of capacity of the laboratory the PPI test was conducted in all adolescent rats and in n=10 rats/group from each of the 4 experimental adult groups (randomly selecting these 10 rats from each of the four adult groups). Two rats from the adult RHA-C group, and one from each of the adolescent RHA-NH and RLA-NH groups, had to be excluded from the experiment due to failure of the startle apparatus.

Between 10 to 15 days after finishing the PPI test (PND47-57, adolescent; PND109-118, adults), randomly selected rats (n=8) from each of the 8 groups were anesthetized under isofluorane and brain samples were taken and deep frozen for future gene expression studies (Figure 1).

Every effort was made to counterbalance the 8 groups of rats (2 strains x 2 ages x 2 treatments) for testing day, hour of testing and experimental group in each of the above behavioural tests.

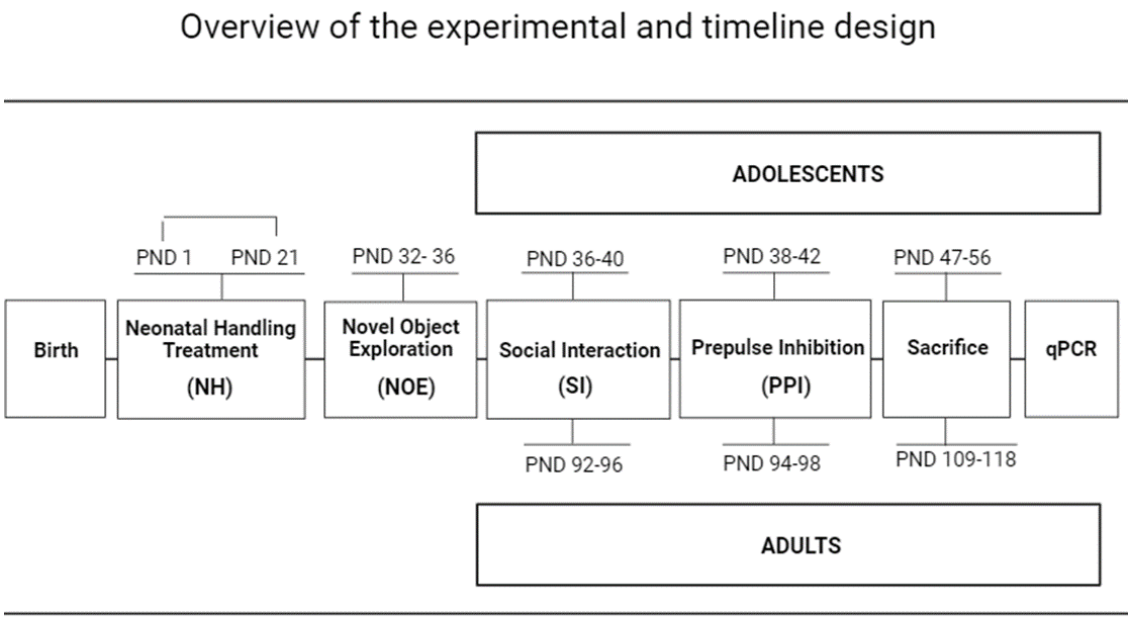


Figure 1. Overview of the experimental schedules (see "Materials and methods"). The study was carried out with male rats of both the RHA and RLA strains, receiving neonatal handling (NH) or no treatment (controls, C), and including two ages, PND40 (adolescent) and PND90 (adults). Test 1: NOE test, was carried out at PND36 in rats (n=56, 28 per group and strain). Test 2: SI was carried out in parallel in adolescent and adult rats of the corresponding C and NH treatment groups of both strains. In PND40 the n=10 in each of the 4 groups (RLA-C, RLA-NH, RHA-C, RHA-NH), and in PND90 the n=18 in each of the 4 groups (RLA-C, RLA-NH, RHA-C, RHA-NH). Test 3: Twenty-four hours after the SI test, at PND42 and PND92 in adolescent and adult rats, respectively, the PPI test was carried out n=10 rats/group. Two weeks after finishing the PPI test, at PND56 (adolescent) and PND106 (adults), randomly selected rats (n=8) from each of the 8 groups were anesthetized under isofluorane and brain samples were taken and deep frozen for future gene expression studies.

2.7 RNA extraction and sample preparation

The PFC from each selected rat was dissected out, immediately frozen in liquid nitrogen, and stored at − 80 ◦C. RNA was extracted from the PFC tissue as described earlier in (Tapias-Espinosa et al., 2021) with minor modifications. Briefly, RNA from tissue samples (around 30 µg) was extracted using the miRNeasy mini kit (Qiagen; cat. no. 217, 084) in a RNAase-free environment. RNA samples were subjected to DNAse treatment using the RNaesy-Free DNase (Qiagen; cat. No. 79254). The RNA concentration and the purity were determined by a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific). The purified RNA (200 ng) was transcribed into complementary DNA (cDNA) using qScript cDNA SuperMix kit (Quanta Biosciences, cat. no. 95,048), according to the

manufacturer instructions. The cDNA products were diluted 1:5 with RNase/DNase-free water, and stored at 80°C.

2.8 Real-time PCR

Real-time qPCR was performed on individual samples in duplicate in 96-well PCR-plates using the Stratagene Mx3005p qPCR System (Agilent Technologies) and Fast SYBR Green Master Mix (Applied Biosystems, cat. no. 438,512). 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. The expression of 2 reference genes (Gapdh, Rpl13A) and 7 target genes (Grind2b, Snap25, Nrg1, Homer1, Bdnf, Cables1, Cdk5, Table 1) were investigated. Briefly, each SYBR Green reaction (10 µl total volume) contained 1 × 5 µl Fast SYBR Green Master Mix (Applied Biosystems, cat. no. 438,512), 0.3 µM primer pairs, and 2 µl of diluted cDNA. The thermal conditions for the PCR were 20 s at 95 °C, followed by 40 cycles of 5 s denaturation at 95 °C, 30 s annealing at 60 °C, and 1 cycle of 60s at 95 °C, 30s at 55 °C and 60 s at 95 °C. Each run was completed by dissociation curve analysis to confirm the amplification specificity and absence of primer dimers. We generated a standard curve, performed in duplicate, on each plate. 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 (Pfaffl, 2001).

3. Results

3.1 NOE test

The factorial ANOVA (2 strain x 2 NH) showed strain ($F(1,108)= 170.7$ $p<0.001$), NH ($F(1,108)= 24.5$ $p<0.001$) and “strain x NH” ($F(1,108)= 22.2$ $p<0.001$) effects on the latency to the first exploration of the novel object. Posthoc Duncan’s test showed that RLA-Control group was different from both RLA-NH and RHA-Control (Figure 2A). Regarding the exploration time (time spent exploring the novel object) the ANOVA revealed strain ($F(1,108)= 21.6$ $p<0.001$) and NH ($F(1,108)= 73.5$ $p<0.001$) effects.

Posthoc Duncan's test revealed that RHA rats explored more the object and NH increased exploration time in both rat strains (see Duncan's test; see Fig. 2B).

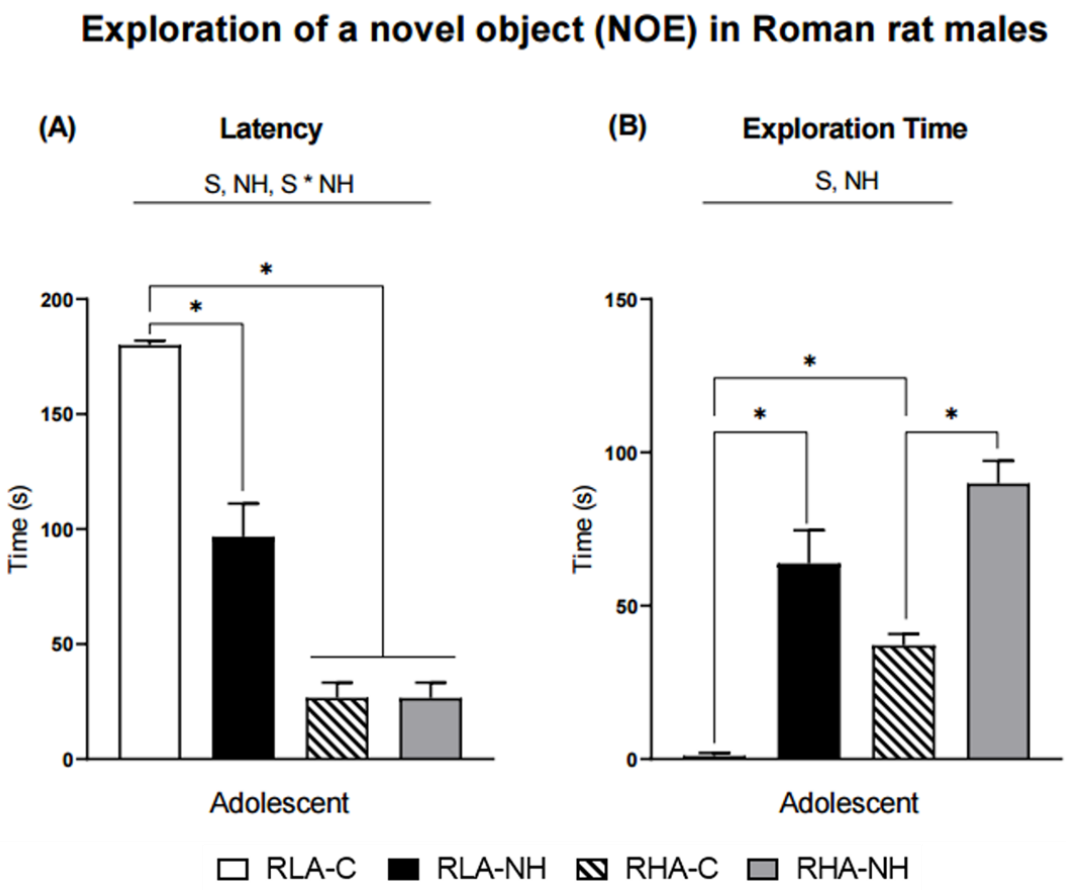
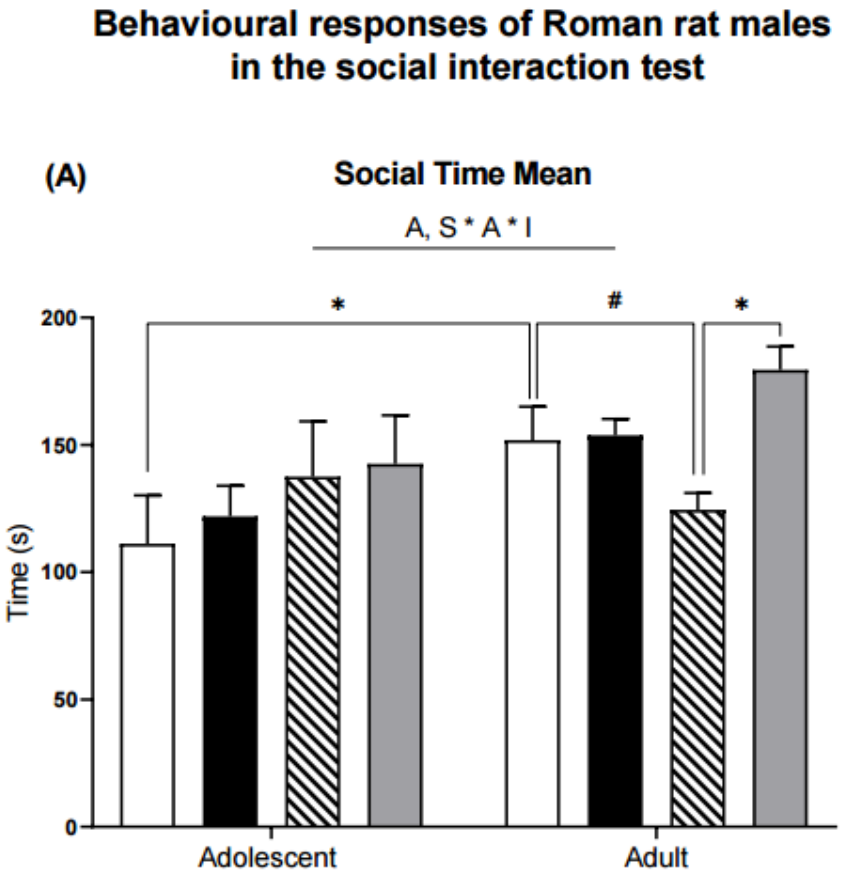


Figure 2. Means (± SEM) for the novel object exploration (NOE) test in male RLA and RHA rats and effects of the NH treatment on: A) "Latency", as time elapsed until the first exploration of the novel object. B) "Exploration Time", spent exploring the novel object. n = 28/group. * p<0.05, Duncan's multiple range test. S, strain effect (ANOVA); NH, treatment effect (ANOVA).

3.2 Social Interaction (SI) test

The overall ANOVA, including strain, NH and age as between-subject factors, and interval (0-5, 5-10 and 10-15 min intervals) as within-subject factor, revealed "strain x age x interval" and age effects on social time ($F(2,204)= 3.43$ $p=0.034$, and $F(1,102)= 6.81$ $p=0.01$, respectively) (Fig. 3A), as well as an age effect on non-social time ($F(1,102)= 27.07$ $p<0.001$) (Fig. 4A). Thus, further analyses were carried out for each age separately.

In adolescent rats the ANOVAs (2 strain x 2 NH x 3 interval) did not reveal significant effect of any factor or interaction for social time (all Fs < 1.64, all p > 0.21; Fig. 3B) or non-social time (all Fs < 2.98, all p > .09; Fig. 3B). In adults, ANOVA showed significant NH (F (1,66) = 5.49 p=0.022), "Strain x NH" (F (1,66) = 5.77 p=0.019) and "strain x interval" (Huynh-Feldt (df 2,134) = 3.15 p=0.047) effects on social time (Fig. 2B), but no significant effects of any factor or interaction were found for non-social time (all Fs < 1.95, all p > .16; Fig. 4B). Posthoc Duncan's tests for social time showed that adult RLA rats exhibited more social time than RHAs, whereas NH treatment significantly increased this measure in the RHA strain (Fig. 3A-B).



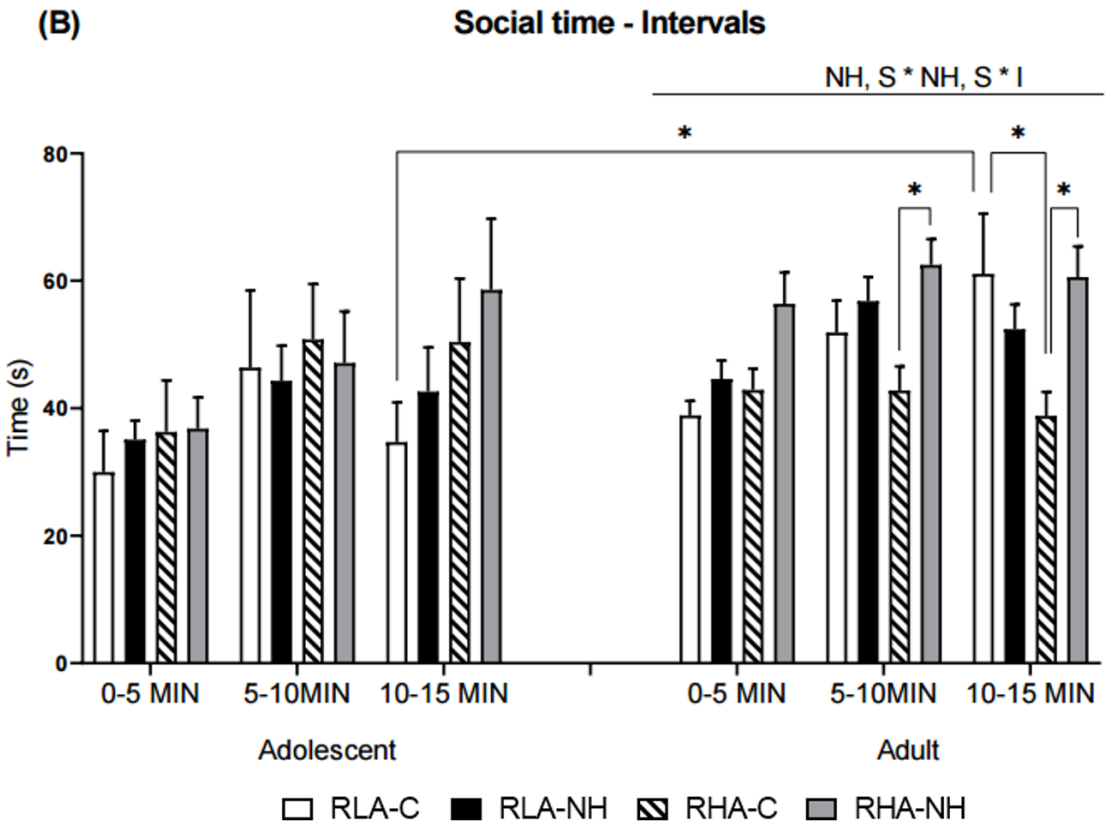


Figure 3. Means (± SEM) for the social interaction (SI) test in male Roman rats and effects of the NH treatment on A) Social Time Mean showed a significant difference between ages and strain*age*interval effect. B) Social Interaction intervals showed, treatment effect, strain*treatment effect, and strain*interval effect in PND 90 group. n = 10/ PND 40 group, n = 18/ PND 90 group. * $p<0.05$, Duncan's multiple range test. # $p<0.037$ (Duncan's multiple range test; one-tailed, as there was a directed hypothesis on that difference based on previous studies). S, strain effect (ANOVA); NH, treatment effect (ANOVA); D, day effect (ANOVA); I, interval effect (ANOVA).

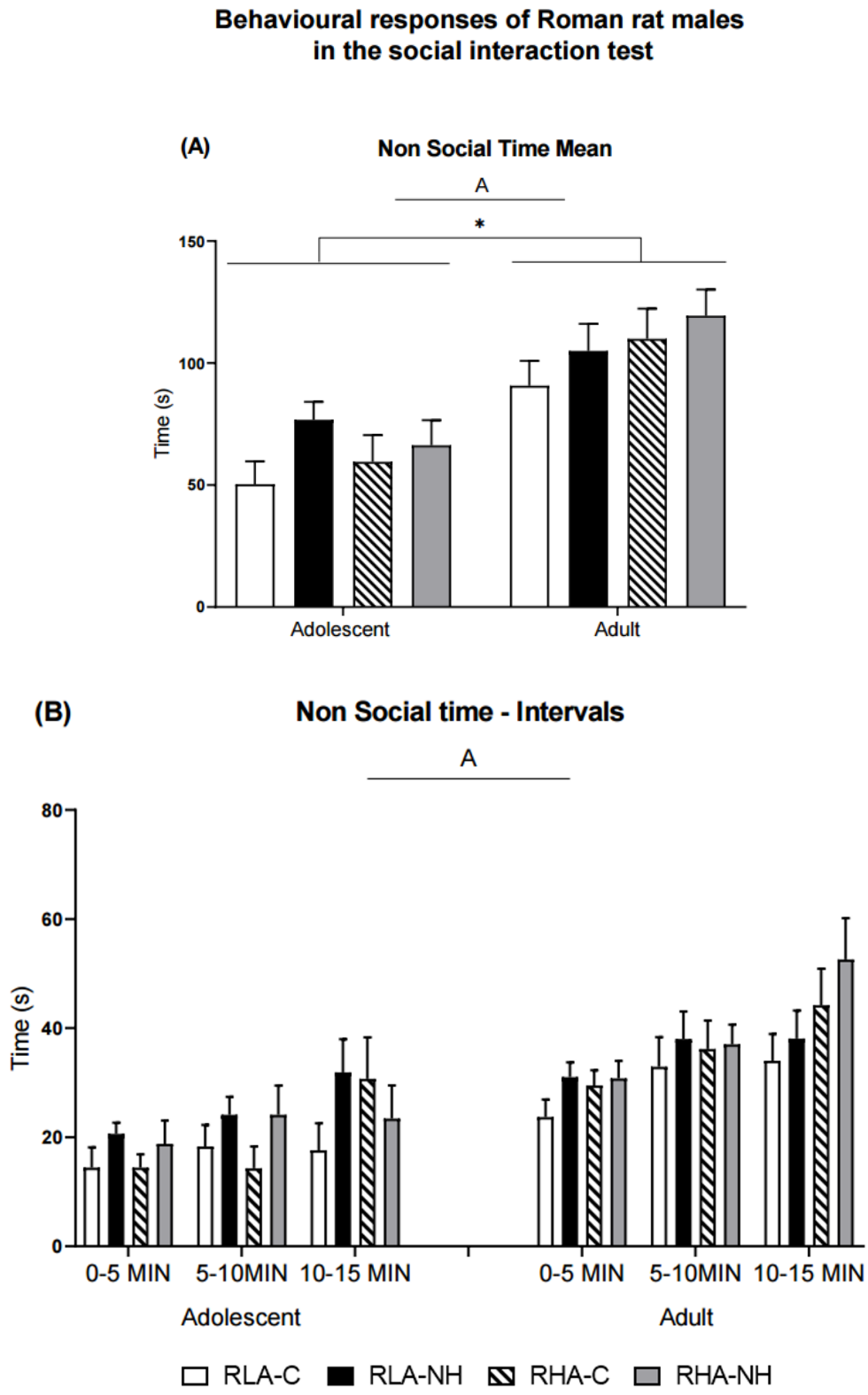


Figure 4. Means (± SEM) for the social interaction (SI) test in male Roman rats and effects of the NH treatment on A) Total Non-Social Time showed a significant ages effect. B) Social Interaction intervals showed, age effect. n = 10/ PND 40 group, n = 18/ PND 90 group. A, age effect (ANOVA).

Regarding self-grooming behaviour in the SI test, the factorial ANOVA (2 strain x 2 NH x 2 age) revealed strain ($F(1,102)= 17.95$ $p<0.001$) and NH ($F(1,102)= 7,0$ $p=0.01$) main effects, since RLA rats showed overall enhanced time spent grooming and NH markedly reduced it (Fig. 5A). There also appeared "strain x NH" ($F(1,102)= 14.31$ $p<0.001$), "strain x age" ($F(1,102)= 4.52$ $p=0.036$) and "NH x age" ($F(1,102)= 4.51$ $p=0.036$) effects (Fig. 5A), indicating, as confirmed by posthoc Duncan's tests, that NH-reducing effects on grooming were particularly marked in RLA rats and especially in adults of this strain (Fig. 5A). When analysing the data with separate ANOVA for each age, it was found that in adolescent rats there was a "strain x NH" effect ($F(1,36)= 5.40$ $p=0.026$), since the NH-reducing effect is observed only in RLA rats (see Duncan's test in Fig. 5A left). In adults, the ANOVA revealed strain ($F(1,66)= 26.66$ $p<0.001$), NH ($F(1,66)= 14.94$ $p<0.001$) and "strain x NH" ($F(1,66)= 10.45$ $p=0.002$), indicating a particularly marked reduction of grooming by NH in RLA rats (see Duncan's test in Fig. 5A right).

As for locomotor activity (crossings) in the SI test, ANOVA revealed strain ($F(1,102)= 65.1$ $p<0.001$), NH ($F(1,102)= 29.99$ $p<0.001$), age ($F(1,102)= 16.72$ $p<0.001$) and "strain x age" ($F(1,102)= 6.89$ $p=0.01$) effects (Fig.5B). Overall, these effects indicate that RHAs are more active than RLA rats, NH increases activity, adults are more active than adolescent rats, but such an effect is particularly evident in RHA rats (Fig. 5B).

We run ANOVA analyses for each age, and strain and NH effects were found in adolescents rats ($F (1,36) = 11.9$ $p<0.001$, and $F (1,36) = 11.6$ $p=0.002$, respectively) as well as in adult rats ($F (1,66) = 77.9$ $p<0.001$, and $F (1,66) = 10.6$ $p<0.002$, respectively) (Fig. 5B).

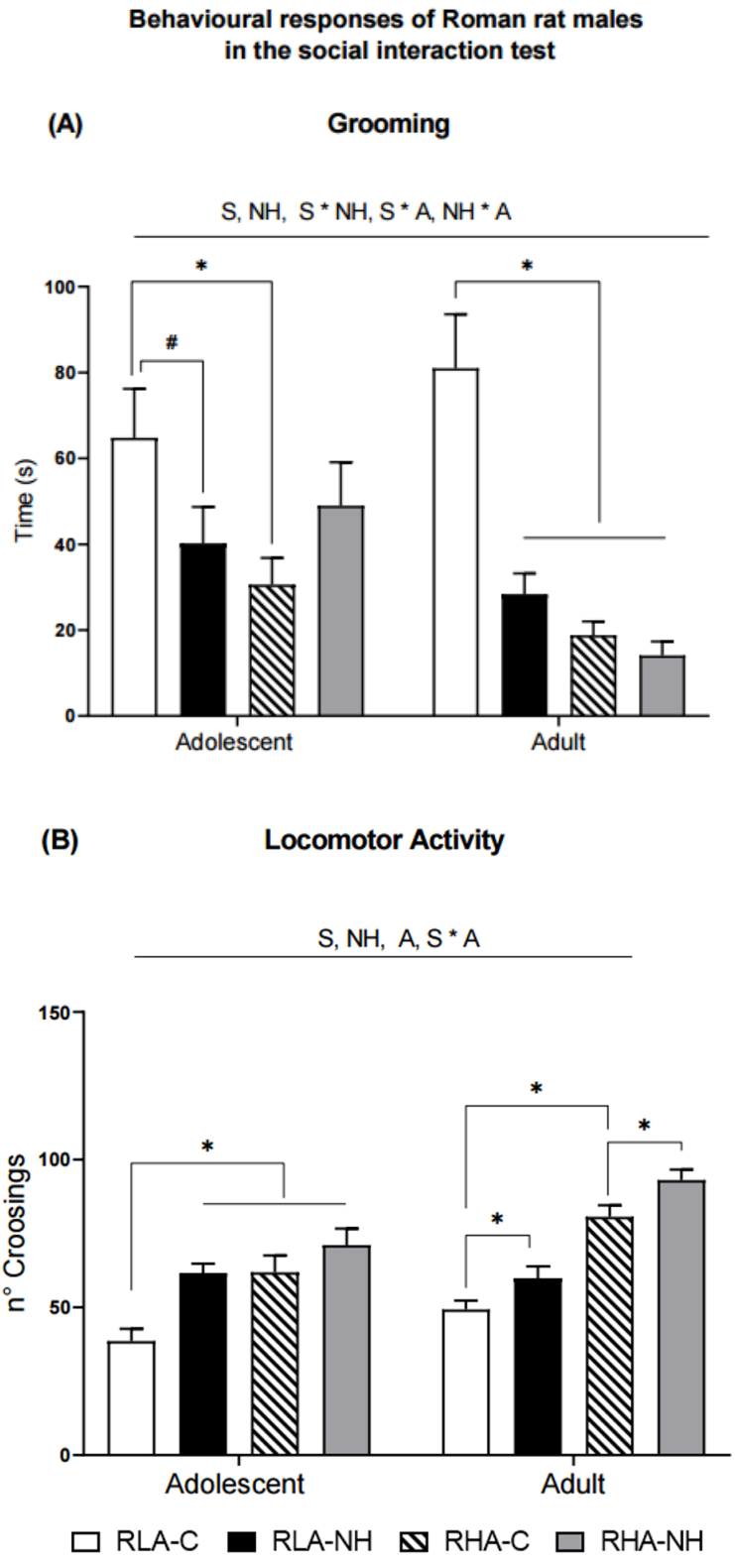


Figure 5. Means (± SEM) for the social interaction (SI) test in male Roman rats and effects of the NH treatment on A) Grooming and B) Locomotor Activity. n = 10/ PND 40 group, n = 18/ PND 90 group. * $p<0.05$, Duncan's multiple range test. # $p<0.035$ (Duncan's multiple range test; one-tailed, as there was a directed hypothesis on that difference based on previous studies) S, strain effect (ANOVA); NH, treatment effect (ANOVA); D, day effect (ANOVA).

3.3 Prepulse inhibition

A factorial repeated measures ANOVA (2 strain x 2 NH x 2 age x 4 prepulse intensities, the latter as within-subject factor) revealed strain ($F(1,70)= 28.57$ $p<0.001$) and age ($F(1,70)= 7.03$, $p=0.01$) effects, and an almost significant "strain x NH" ($F(1,7)= 3.95$ $p=0.051$) effect (Fig. 6A-B). The analysis also yielded a significant "strain x NH x age x intensity" interaction effect (Huynh-Feldt $(2.6,184.14)= 3.98$ $p=0.012$), indicating that strain and NH effects were dependent upon the other factors (Fig. 6A). In order to dissect better the source of that interaction effect, separate ANOVA for each age were carried out.

For adolescent rats, the ANOVA (2 strain x 2 NH x 4 prepulse intensities) revealed strain ($F(1,36)=12.04$ $p<0.001$) and "strain x NH x intensity" ($F(3,108)= 3.10$ $p=0.03$) effects. According to the posthoc Duncan's comparisons this interaction reflects the fact that RHA-C rats exhibited significantly lower PPI than RLA-C rats at several prepulse intensities and in Total-PPI, whereas RHA-NH rats showed a trend to an increase of PPI that was significant at the 70 dB prepulse intensity and was also reflected by the absence of difference between RHA-NH and RLA-C groups in Total-PPI (see Duncan's tests in Fig. 6A-B).

For adult rats, the ANOVA (2 strain x 2 NH x 4 intensities) yielded strain ($F(1,34)= 16.42$ $p<0.001$), "strain x NH" ($F(1,34)= 4.32$ $p=0.045$) and "strain x intensity" (Huynh-Feldt$(1.85,63.01)= 7.34$ $p=0.002$) effects on PPI levels. Based on the posthoc Duncan's comparisons, these interaction effects indicate that NH significantly increased PPI at 65-70 dB prepulse intensities, as well as Total-PPI, in RHA rats, while not affecting these measures in RLA rats (see comparison of RHA-C vs. RHA-NH and RLA-C groups in Fig. 6A-B).

Regarding the baseline startle measure, ANOVA (2 strain x 2 NH x 2 age) only showed a global age effect ($F(1,102)= 86.3$ $p<0.001$), reflecting that baseline startle levels in adult rats were much higher than the same measure in adolescent rats (Fig. 6C).

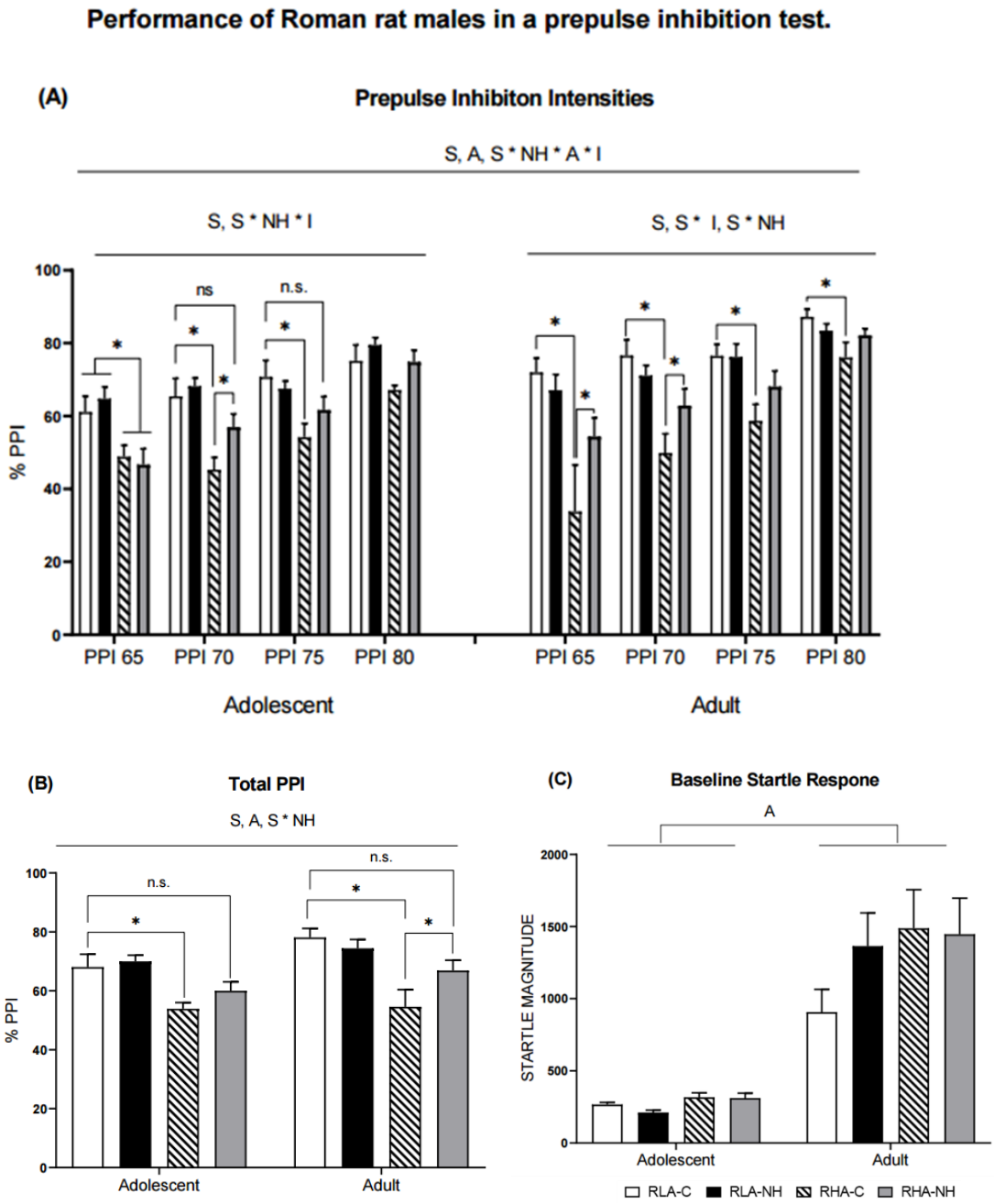


Figure 6. Means (± SEM) for the Prepulse inhibition (PPI) test in male Roman rats and effects of the NH treatment on: A) The PPI intensities. B) The total PPI. C) Baseline Startle Response. n = 10/ group. * $p<0.05$, Duncan's multiple range test. A, age effect (ANOVA).

3.4 Differentially expressed genes in the PFC

A three-way factorial ANOVAs (2 strain x 2 NH x 2 Age) for the Cables1 expression levels showed, strain ($F(1,58)= 21,41$ $p<0.001$), NH ($F(1,58)= 6,94$ $p<0.011$) effects, and a nearly significant "NH x Age" ($F(1,58)= 3,70$ $p=0.060$) effect. Posthoc Duncan's tests showed that RHA-NH rats exhibited more expression levels than RHA-C, in adolescent group, and expression levels in RHA-C are higher compared to RLA-C in adult group (Figure 7A).

Three-way factorial ANOVA (2 strains x 2 NH x 2 Age) on the expression levels of CDK5 revealed NH ($F(1,54)= 8,23$ $p= 0.006$) and "strain x NH" ($F(1,54)= 6,10$ $p=0.017$) effects (Figure 6B). Posthoc Duncan's tests showed that adolescent RHA-C rats exhibited lower expression levels than RLA-C and RHA-NH (Figure 7B).

The three-way factorial ANOVA for Snap25 expression, showed strain ($F(1,55)= 13,22$ $p<0.001$), NH ($F(1,55)= 8,53$ $p=0.005$), and "strain x NH" ($F(1,55)= 13,87$ $p<0.001$) effects (Figure 7C). Posthoc Duncan's tests showed that, at both ages the RHA- C group exhibited much higher expression levels than the respective RLA-C and RHA-NH groups (Figure 7C).

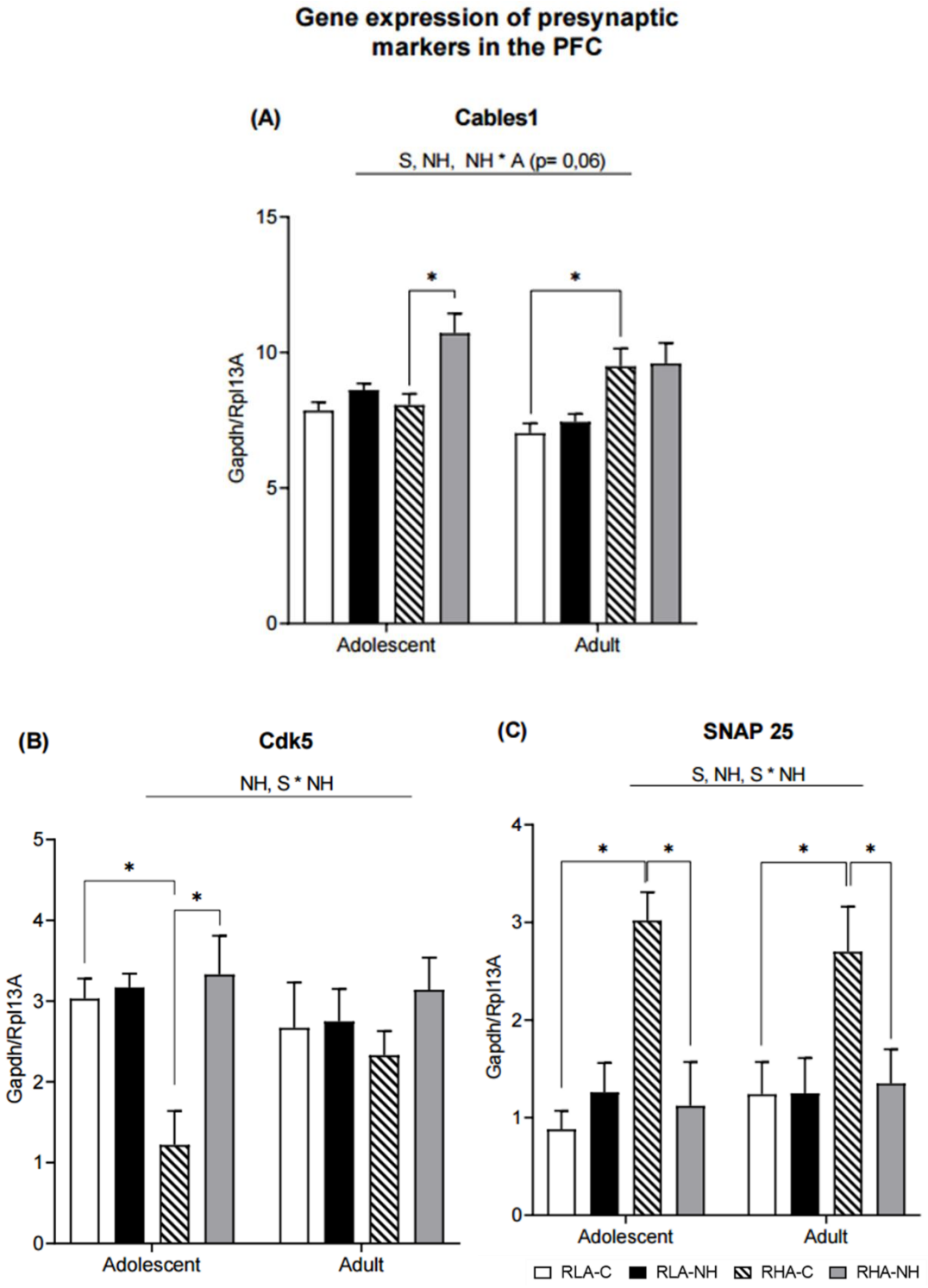


Figure 7. Means (± SEM) for gene expression levels of presynaptic market in the PFC of male Roman rats. (A) Cables1 gene. (B) Cdk5 gene. (C) Snap25 gene. n = 5-8/ group, *p<0.05 Duncan's multiple range test. S, strain effect (ANOVA); NH, treatment effect (ANOVA); A, Age effect (ANOVA).

Expression levels of genes related to postsynaptic activity (Nrg1, Homer1) were measured in PFC between adolescent and adult RHA and RLA rats. ANOVA (2 strain x 2 NH x 2 Age) results for Nrg1, showed NH ($F(1,59)= 14,69$ $p<0.001$) and "strain x NH x

age" (F(1,59)= 14,55 p<0.001) effects (Figure 8A). Posthoc Duncan's tests revealed a decreased expression of Nrg1 in adolescent RHA-C vs. RLA-C and RHA-NH rats, as well as decreased expression in adult RLA-C relative to RLA-NH and RHA-C groups (Figure 8A).

ANOVA (2 strain x 2 NH x 2 Age) on the expression levels of Homer1 revealed strain (F(1,55)= 12,69 p=0.001), age (F(1,55)= 59,67 p<0.001) and "strain x NH x age" (F(1,55)= 7,00 p=0.011) effects (Figure 8B). Duncan's tests were performed, showing that adult RHA-C rats show an overexpression compared to RLA-C rats. In addition, the effect of NH is observed in adolescent rats, where RHA-NH rats increase the level of expression compared to their control RHA-C. Finally, adult RHA-C rats show an overexpression compared to adolescent RHA-C rats (Figure 8B).

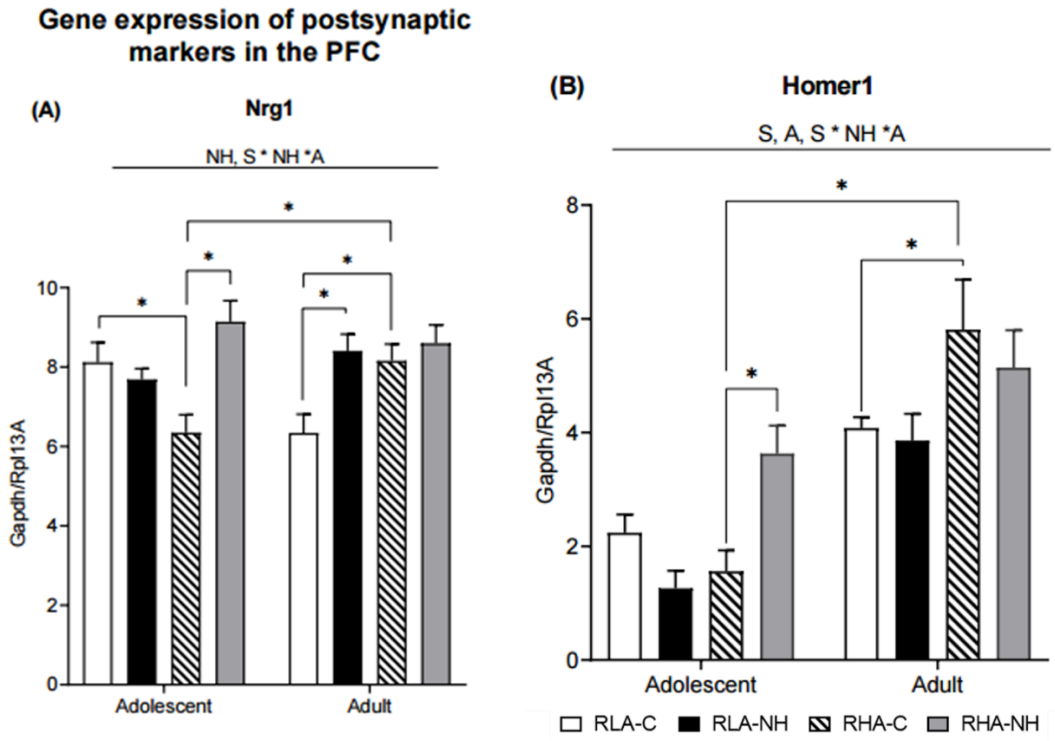


Figure 8. Means (± SEM) for gene expression levels of presynaptic market in the PFC of male Roman rats. (A) NRG1 gene. (B) Homer1 gene. n = 5-8/ group, *p<0.05 Duncan's multiple range test. S, strain effect (ANOVA); NH, treatment effect (ANOVA); A, Age effect (ANOVA).

ANOVA (2 strain x 2 NH x 2 Age) on the expression levels of Bdnf revealed strain (F(1,52)= 4,96 p=0,031), NH, (F(1,52)= 7,09 p=0.011), and "strain x NH x Age" (F(1,52)= 4,92 p=0.032) effects (Figure 9). Posthoc Duncan's tests showed that, in adults, RHA-C rats exhibit a higher level of expression than RLA-C rats, the treatment led to increased expression in RLA-NH vs. RLA-C rats and, adolescent RHA-NH rats show higher

expression than their control RHA-C. Furthermore, there is overexpression in adult RHA-C rats compared to adolescent RHA-C rats (Figure 9).

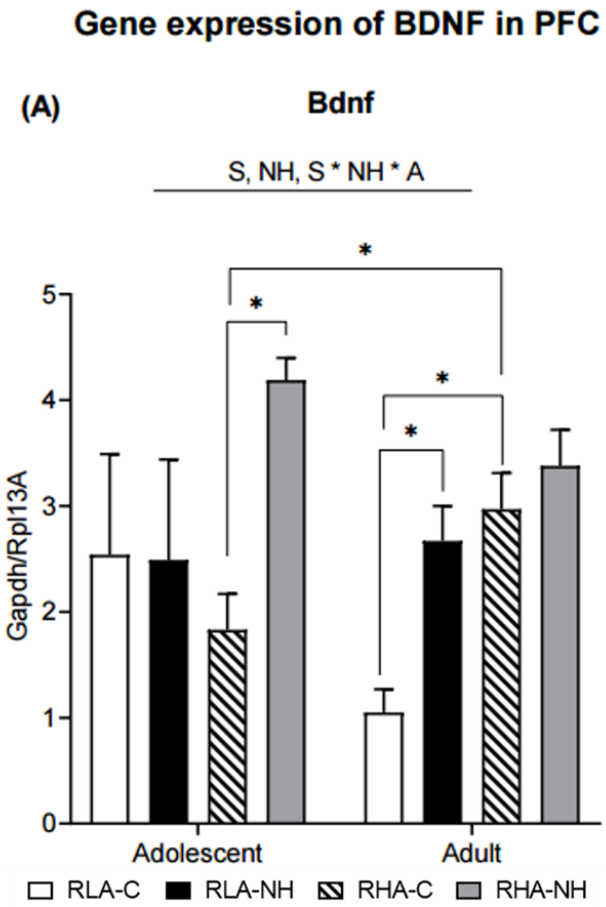


Figure 9. Means (± SEM) for BDNF gene expression levels of presynaptic market in the PFC of male Roman rats. n = 6-8/ group, *p<0.05 Duncan's multiple range test. S, strain effect (ANOVA); NH, treatment effect (ANOVA); A, Age effect (ANOVA).

The mRNA expression levels of selected receptor genes, Grin2b and Drd1, are given in Fig. 10A-B. ANOVA for Grin2b data (2 strain x 2 NH x 2 Age) showed strain ($F(1,58)= 7.83$ $p=0.007$) and age ($F(1,58)= 39,69$ $p<0.001$) effects. Posthoc Duncan's tests showed that adolescent RHA-NH rats have more expression levels than RHA-C (Fig. 10A). Regarding Drd1 expression, ANOVA (2 strain x 2 NH x 2 Age) showed age ($F(1,63)= 21,41$ $p<0.001$) and "strain x NH x age" ($F(1,63)= 5,56$ $p=0.022$) effects. Posthoc Duncan's tests revealed that adolescent RHA-NH exhibited higher expression levels than RHA-C rats, whereas adult RHA-C showed increased expression relative to adolescent RHA-C rats (Fig. 10B).

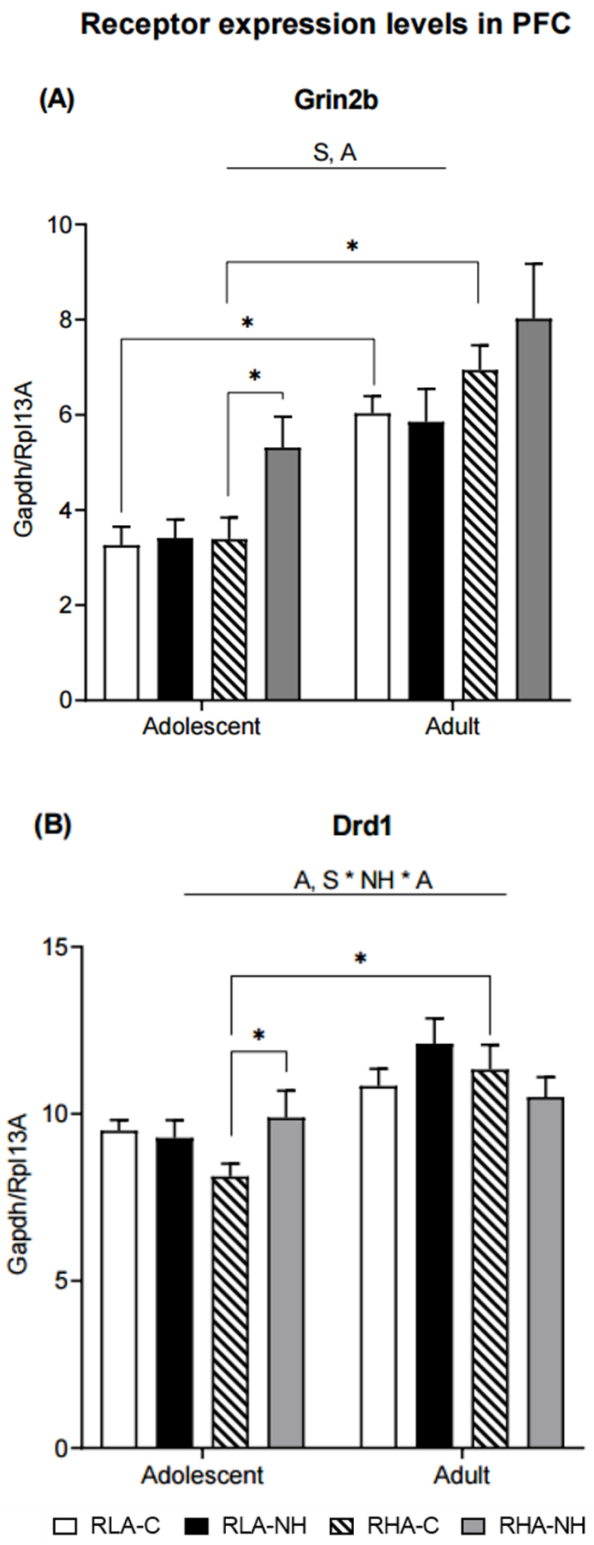


Figure 10. Means (± SEM) for gene expression levels of receptors market in the PFC of male Roman rats. (A) Grin2b gene. (B) Drd1 gene. n = 6-8/ group, *p<0.05 Duncan's multiple range test. S, strain effect (ANOVA); NH, treatment effect (ANOVA); A, Age effect (ANOVA).

4. DISCUSSION

The present study addressed, for the first time in RHA and RLA rats, both the possible maturational effects (in adolescent rats) and long-term effects (in adult rats) of NH treatment on behaviors related to novelty-induced inhibition/exploration (NOE), anxiety (grooming), locomotor activity (SI test), social interaction (SI test) and attention/sensorimotor gating (PPI test). In addition, also for the first time, we analyzed differential gene expression of targets involved in neurodevelopment, neuroplasticity and synaptic processes as a function of strain, neonatal treatment and age.

Between-strain behavioral differences across development, and developmental effects of neonatal handling as a function of the rat strain and age

The results show that novelty exploration (NOE) is different between the strains (RHA>RLA) during early adolescence (PND32-36), in line with previous findings with older rats (PND52-60) from both strains (PND52-60; Cuenya et al., 2016; Río-Álamos et al., 2015, 2017a,b; Sampedro-Viana et al., 2021). Horizontal exploratory behavior (locomotion) and grooming are different between the strains already from adolescence, suggesting that between-strain differences in both behaviours mature very early during development. Strain differences in both grooming (RHA<RLA) and locomotion (RHA>RLA) become larger in adulthood (see Figure 4A-B).

Between-strain differences in social interaction (social time) only appeared in adult rats. Conversely, RLA rats showed superior PPI performance than RHAs already during adolescence. This strain difference is still more marked in adulthood (see Fig. 5A). This is in general agreement with previous studies from our group (Oliveras et al., 2015, 2022a; Río-Álamos et al., 2017b; Tapias-Espinosa et al., 2018, 2019).

As for neonatal handling effects, the treatment increases novelty exploration (NOE test) and locomotor activity (in the SI test), particularly in adolescent RLA rats, while also reducing anxiety-related grooming. In the PPI test, NH treatment also shows a trend to increase %PPI levels at various prepulse intensities, in adolescent RHA rats (but not in RLAs), that becomes significant at the 70 dB prepulse intensity (see Fig. 6A).

NH effects on NOE time, locomotor activity, grooming and PPI in adolescent Roman rats, are novel findings. It is outstanding that NH-treated RLA rats exhibit NOE, activity and

grooming levels that approach –or even match- the scores of RHA rats (see Fig. 2A, 5A-B). This suggests that NH treatment has some developmental effects on coping-linked and anxiety-related responses (i.e., grooming; Fernández-Teruel and Estanislau 2016) that begin to operate very early during development. As suggested by many reports, these NH effects likely involve early neurodevelopmental changes in neural circuits, neurotransmitters and neuroendocrine factors that play relevant roles in shaping those behavioural responses and coping profiles (e.g., Antoniazzi et al., 2014; Meaney et al., 1988, 1989; Monroy et al., 2010; Papaioannou et al., 2002; Park et al., 2003).

On the other hand, in adult rats NH increases novelty-induced locomotor activity in both strains, reduces grooming in RLA rats, and enhances social interaction and PPI in RHAs. Such NH effects are in line with studies on adult rats from other strains, and with our previous findings suggesting that NH might enduringly improve cognition/attention-related processes particularly in RHA rats (e.g., Aguilar et al., 2002; Fernández-Teruel et al., 1997, 2002; Fu and Depue, 2019; Raineki et al., 2014; Río-Álamos et al., 2015, 2017b, 2019; Sampedro-Viana et al., 2021, 2023; Steimer et al., 1998).

Modulation of gene expression as a function of strain, age and neonatal handling: associations between gene expression and behavior

We observed an upregulation of expression of Cables 1 by NH treatment in adolescent RHA rats, and increased expression of the gene in control adult RHA rats vs. RLAs. The result in adolescent RHA rats is at variance with our previous findings (Sønderstrup et al., 2023), which could be due to the different age of the rats in both studies (PND44-46 in Sønderstrup et al. study vs. PND47-56 in the present study) and/or to the fact that here the rats underwent behavioural testing (between PND36-42 in adolescent rats, and PND92-98 in adult rats) that ended 10-15 days before sacrifice, whereas in Sønderstrup et al. study the rats were experimentally naive. Another important environmental difference between both studies was that the control (untreated) rats from the present study, although they were not neonatally handled (except for cage cleaning once a week) they were actually much more disturbed than control rats from Sønderstrup et al. report. Thus, since in the present study all rats from control and NH groups were in the same animal room, control rats were also exposed to the fact that between PND1 and PND21 two experimenters entered the animal room several times every day (ten

times in the morning and ten times in the afternoon) to take or leave the cages of the rats that were receiving neonatal handling.

Cables 1 is an activator of Cdk5 (cyclin-dependent kinase 5), and expression of the latter gene was reduced in adolescent control RHA rats but, as observed for Cables 1, it was increased by NH. Actually, the correlation between Cables 1 and Cdk5 in adolescent rats was positive (r=0.52, p=0.005), thus indirectly supporting their related brain function. Cdk5 is an important modulator of synaptic plasticity through regulation of NRB2 (NMDA2B) degradation (Hawasli et al., 2007) as well as a stabilizer of dendritic spines into maturational states (Huang et al., 2017). Interestingly, we report that expression level of Grin2b gene (coding for NMDA2B receptor subunit) is increased by NH treatment in adolescent RHA rats. Lowered expression of Cdk5 may therefore indicate impaired synaptic maturation in the adolescent RHA strain. Cdk5 is also involved in recruitment and assembly of presynaptic proteins during synaptogenesis (Easley-Neal et al., 2013), so that the increased expression of the SNARE component Snap25 in adolescent control RHA rats might be related to a compensatory mechanism to the reduced expression of Cdk5. Remarkably, however, NH treatment normalized to RLAs' levels the altered expression of both Snap25 and Cdk5 in adolescent RHA rats, suggesting a maturational effect of neonatal handling on these two genes. Likewise, in adult RHA rats Snap25 was overexpressed, and was also normalized to RLAs' levels by NH treatment.

As said earlier, Cdk5 and Snap25 have important roles in synaptic plasticity and synaptogenesis, and alterations of Cdk5 and upregulation of presynaptic SNARE components, such as Snap25, have been related to schizophrenia and schizophrenia-linked phenotypes such as impaired prepulse inhibition and memory deficits (Jeans et al., 2007; Engmann et al., 2011; McKee et al., 2010; Oliver and Davies 2009; Ramos-Miguel et al., 2015). In addition, RHA rats are considered a model of schizophrenia-relevant traits (Fernández-Teruel et al., 2021; Oliveras et al., 2023), exhibiting deficits of working and reference memory, attention and sensorimotor gating (as seen in the present study), and also showing impaired frontocortical synaptic maturation (Elfving et al., 2019; Sánchez-González et al., 2021; Sønderstrup et al., 2023; Oliveras et al., 2015; Río-Álamos et al., 2019; Tapias-Espinosa et al., 2019, 2023). On the basis of the above findings, it seems outstanding that Cdk5 and Snap25 alterations in adolescent and adult

RHA rats are “normalized” (to RLAs’ levels) by neonatal handling in the present study, and Snap25 expression levels correlate negatively with PPI in adolescent rats (r= -0.37, p=0.044). It is worth noting that these findings are paralleled by the NH-induced improvement of PPI in both adolescent and adult RHA rats from this strain as well as an increase of social behavior in adult rats from this strain. It is also interesting that age affected Snap25 expression, in interaction with NH, and this was reflected by a lower gene expression in adult vs. adolescent RHA rats, also supporting the findings that social interaction and PPI were globally higher in adults than adolescent rats.

Dysregulation of neurotrophic factors such as BDNF has long been associated with the cellular, cytoarchitectural and volumetric alterations seen in schizophrenia (Angelucci et al., 2005). Human postmortem studies have reported significant alterations in BDNF mRNA and protein levels in brains from patients with schizophrenia in both cortical and hippocampal regions (Durany et al., 2001; Reinhart et al., 2015; Takahashi et al., 2000). In humans, the Val (66) Met single-nucleotide polymorphism in the BDNF gene is associated with schizophrenia-risk (Lencz et al., 2009; Ursini et al., 2016), and when introduced in an animal model, this genotype leads to deficits in sensorimotor gating processes (Notaras et al., 2017). The RHA strain presents both frontocortical and hippocampal volumetric alterations (Rio-Alamos et al., 2017, 2019; Tapias-Espinosa et al., 2019) and sensorimotor gating deficits (Oliveras et al., 2015; Rio-Alamos et al., 2018; Esnal et al., 2016), which may be related to the Bdnf gene and protein alterations in adult RHA rats observed here and in previous studies (Elfving et al., 2019; Sanna et al., 2022; Serra et al., 2022, 2023; Sønderstrup et al., 2023).

BDNF is important for synaptic plasticity and synaptic communication during neurodevelopment (Favalli et al., 2012; Kowianski et al., 2018; Nieto et al., 2013), and synaptic alterations have been reported in schizophrenia brains (Focking et al., 2015). We report here altered expression levels of synaptic components in the RHA rats, including Nrg1 and the postsynaptic density (PSD) protein Homer1. Expression of both genes tends to be reduced in adolescent RHA (vs. RLA) rats and, in agreement with our previous findings, both are increased in adult RHA rats relative to RLAs (Elfving et al., 2019; Sonderstrup et al., 2023). It is remarkable that (similar to Snap25) Homer1, Nrg1 and Bdnf show an influence of age in interaction with strain and NH (see the “strain x

NH x age" interactions), i.e., while expression levels of the three genes are equal in adolescent vs. adult RLA rats, they are clearly increased in adult vs. adolescent RHA rats, and NH treatment increases expression levels of the three genes in adolescent RHAs. This seems consistent with the notion that aberrant (or delayed) synaptic maturation of the PFC is a characteristic of RHA rats (Elfving at al., 2019; Sánchez-González et al., 2021; Sønderstrup et al., 2023), and that such neurodevelopmental alteration/s might be to some extent "normalized" by neonatal handling.

Abnormalities in the PSD are linked to neurodevelopmental disorders such as autism spectrum and schizophrenia (Kaizuka and Takumi, 2018). Homer1 protein mediates the glutamatergic, serotonergic and dopaminergic receptor signaling at the postsynaptic site (de Bartolomeis et al., 2013a; de Bartolomeis et al., 2013b; Iasevoli et al., 2011), and Homer1 polymorphisms are associated with differential response to treatment in schizophrenia (Spellmann et al., 2011). Nrg1 has a role in regulating the excitatory/inhibitory neurotransmission at the PSD and is linked to schizophrenia risk (Mostaid et al., 2016). Nrg1 modulates sensorimotor gating, as seen in animal (Rhein et al., 2013; Karl et al., 2011) and human studies (Roussos et al., 2011; Hong et al., 2008). Disruption of Nrg1 signaling impairs hippocampal-prefrontal synchrony and top-down attention (Tan et al., 2018), and as already reported previously (as well as here), impairment of hippocampal function, sensorimotor gating and attention are important traits in the RHA rats (Meyza et al., 2009; Moreno et al.,2010; Klein et al., 2014; Oliveras et al., 2015, 2022; Tapias-Espinosa et al., 2019). It is therefore outstanding that expression of these tree genes, i.e, Bdnf, Homer 1 and Nrg1, is increased by NH treatment in adolescent RHA rats, while adult control RHA rats show enhanced expression relative to RLAs. These results lend support to the notion that NH produces some positive maturational neuroplasticity and synaptic effects in adolescent RHA rats that might be related to the behavioral changes in NOE and PPI observed in NH-treated juvenile RHA rats. In RLAs, in turn, these genes were not affected by NH in adolescence, but the treatment led to increased Nrg1 and Bdnf levels in adulthood. Interestingly, Nrg1 and Bdnf expression levels in adult rats correlated negatively ($r=-0.44$, $p=0.016$; $r=-0.58$, $p=0.002$) with NOE latency, and Bdnf expression correlated positively with NOE time ($r=0.40$, $p=0.04$). Moreover, Bdnf and Nrg1 showed positive correlations with crossings

in the SI test (r=0.66, p<0.001; and r=0.45, p=0.012, respectively) and correlated negatively with grooming (r=-0.39, p=0.05; and r=-0.64, p<0.001, respectively). These correlational patterns cohere with the findings that NH increased NOE time and crossings, and reduced grooming in adult RLA rats, whereas both groups of adult RHA rats showed enhanced NOE time and locomotion, and lowered grooming relative to adult RLAs. This consistent pattern of associations in adult rats is further reinforced by the negative correlation of Homer1 expression with NOE latency (r=-0.49, p=0.009) and the positive correlation with crossings (r=0.38, p=0.049), as well as by the positive correlations of Nrg1 with Bdnf (r=0.46, p=0.019) and Homer1 (r=0.65, p<0.001). Altogether, it would seem that the NH and strain effects on expression of these three genes in adult RHA and RLA rats are part of some common neural mechanism/s (or pathway), that in turn might be related to the more emotional aspects of the behaviors measured here, i.e., novelty-induced responses such as novel object exploration, locomotion and grooming under novelty conditions (in the SI test). Neonatal handling treatment has well-known and enduring anti-anxiety (and anti-stress) consequences, represented here by the increase of both novel object exploration (NOE test) and locomotion under novelty conditions (in the SI test), and by the reduction of novelty-induced self-grooming (Fernández-Teruel and Estanislau 2016; Fernández-Teruel et al., 2002; Fu and Depue 2019; Raineki et al., 2014). Therefore, the present NH and strain-related findings, together with the aforementioned behavior-gene expression correlations, suggest that expression of these three genes (in particular, Bdnf and Nrg1) is negatively related to anxiety (i.e., to the behavioral inhibition produced by novelty).

On the other hand, at variance with our previous findings (Elfving et al., 2019; Sønderstrup et al., 2023), we did not observe differences between the strains at any age regarding Drd1 expression, although there was a global increase of expression with age which is roughly in line with Sønderstrup et al., (2023) findings. At present, the only explanation we have for the discrepancy between both studies regarding the between-strain comparison of control (untreated) groups relates to the above-mentioned important differences in environmental conditions, behavioral testing and age between the rats used in the present study and the rats used in Elfving et al. (2019) and Sønderstrup et al. (2023) studies.

In summary, we report the first time that NH produces several effects on gene expression during adolescence only in RHA rats, which constitute the strain exhibiting abnormal schizophrenia-linked behavioral phenotypes. These effects are observed at the presynaptic level by a reduction of Snap25 and increases of Cdk5 expression levels, and at the postsynaptic level by increases of Grin2b, Homer1 and Nrg1 expression, as well as by a NH-induced enhancement of Bdnf expression in adolescent RHA rats. Of these effects, those observed on Snap25 are the ones that seem to better parallel the NH-induced effects on PPI in adolescent and adult RHA rats, as also supported by the negative correlation between Snap25 expression and PPI in the whole sample of adolescent rats. The strain and NH effects on Snap25 are in line with findings that upregulation of presynaptic SNARE components (such as Snap25) seem to be related to schizophrenia and schizophrenia-linked phenotypes such as impaired prepulse inhibition and memory deficits (Jeans et al., 2007; Engmann et al., 2011; McKee et al., 2010; Oliver and Davies 2009; Ramos-Miguel et al., 2015). The increases of Cdk5, Bdnf, Homer1, Nrg1 and Grin2b induced by NH treatment in PFC of adolescent RHA rats may also be related to treatment-induced changes in maturational synaptic/neuroplastic processes that in turn might underlie the observed changes in several behaviors and improvement in sensorimotor gating observed in this strain. Conversely, there are no NH-induced changes on gene expression in adult RHAs, whereas the treatment increases Nrg1 and Bdnf expression in adult RLA rats, in parallel to the decrease of anxiety-related behavioral inhibition in this strain during adulthood.

To conclude, the findings of the present study add support to the notion of an aberrant neurodevelopment and immature frontal cortex in RHA rats, consistent with their profile of schizophrenia-relevant behavioral phenotypes. Most importantly, we report for the first time that a “positive” early environmental treatment, such as NH, may have the capacity to modulate such a genetically-linked synaptic/neuroplasticity alterations in RHA rats, in line with the large amount of evidence on the positive long-lasting effects of NH on many emotional responses, social behavior, attention-related processes and cognition (e.g., Aguilar et al., 2002; Fernández-Teruel 2022; Fernández-Teruel et al., 2002; Peralta-Vallejo et al., 2023; Raineki et al., 2014; Río-Álamos et al., 2017, 2019; Sampedro-Viana et al., 2021, 2023; and present results).

Author contributions

A.F-T. A.T., T.C., D.S-V and N.P-V. conceived and/or designed the experiments. N.P-V., C.R-A., P.G-F., T.C., D.S-V and A.F-T. conducted the experiments. I.O. assisted in the behavioral experiments. A.F-T., D.S-V, N.P-V., P.G-F., A-T., I.O. and T.C. analyzed the data or assisted with data analyses. A.F-T. and N.P-V. wrote the original manuscript. A.T., T.C., I.O., D.S-V., C.R-A. and P.G-F. provided critical review of the original draft. All authors read and approved the manuscript.

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Conflict of interest

The authors present no conflict of interest

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4. DISCUSIÓN

Los objetivos de esta Disertación Doctoral se centran principalmente en dos áreas. Por un lado, se investigó la asociación entre pruebas cognitivas, atencionales y conductuales que reflejan fenotipos relevantes para la esquizofrenia, en ratas HS y *Roman*. Por otro lado, se evaluaron los efectos a largo plazo del tratamiento de estimulación neonatal (NH) en procesos cognitivos y conductuales, así como en la expresión de genes relacionados con la función sináptica, la neuroplasticidad y el neurodesarrollo en ratas *Roman*.

A modo de sumario, los principales resultados observados en la presente Tesis podrían resumirse como sigue a continuación.

ESTUDIO 1

Partiendo de la hipótesis de que el deterioro de los procesos relacionados con la atención podría compartir mecanismos neurobiológicos subyacentes con la conducta social, así como con los procesos de aprendizaje-memoria espacial, y con la flexibilidad cognitiva, este estudio presenta la caracterización combinada de la prueba de PPI, interacción social, memoria espacial de trabajo, memoria de referencia, y flexibilidad cognitiva en ratas genéticamente heterogéneas, HS. En este estudio se encontró que el subgrupo de ratas HS que exhiben niveles más bajos de PPI también muestra ciertos déficits en memoria de trabajo espacial, flexibilidad cognitiva y memoria de referencia, así como menor interacción social. Estos resultados sugieren una asociación positiva entre las diferencias individuales en PPI y algunos rasgos relevantes para la esquizofrenia (tanto negativos como cognitivos) en ratas no seleccionadas, genéticamente heterogéneas.

ESTUDIO 2

Se realizó un estudio conductual, atencional y de memoria en ratas hembra de las cepas *Roman*. Los resultados muestran que las ratas RHA presentan mayor conducta exploratoria, actividad locomotora y tiempo de interacción social, así como deficiente respuesta sensoriomotora (PPI) y menor acicalamiento (*grooming*). Además, se muestran efectos positivos del tratamiento de NH sobre el comportamiento exploratorio, el acicalamiento y la interacción social y, por primera vez, se informa que el tratamiento con NH tiene algunos efectos positivos en el aprendizaje espacial y

mejora la flexibilidad cognitiva en las hembras de la cepa de rata RHA. Estos resultados aportan evidencia de que las ratas hembra RHA pueden constituir un modelo con validez aparente para algunos fenotipos relevantes para la esquizofrenia, aunque también muestran diferencias respecto a los resultados de estudios previos realizados en machos de ambas cepas (p. ej., el patrón de diferencias en interacción social entre las cepas en las hembras de aquí respecto los machos de estudios previos).

ESTUDIO 3.

Se muestra por primera vez que los rasgos conductuales de exploración de la novedad (NOE; RHA > RLA), la locomoción (test IS; RHA > RLA), la ansiedad (acicalamiento; RHA < RLA) y el filtraje sensoriomotor (PPI; RHA < RLA) son diferentes entre las cepas incluso desde la adolescencia, y la diferencia es aún más marcada en la adultez. Por otro lado, las diferencias en interacción social entre cepas (RHA < RLA) solo se observan en la etapa adulta.

Se observaron, en ratas RHA y RLA, efectos del tratamiento de NH sobre los comportamientos asociados con la exploración inducida por la novedad (NOE), la ansiedad (acicalamiento), la actividad locomotora (prueba IS), la interacción social (prueba IS) y la PPI. Así, el NH incrementó la exploración de la novedad y redujo la conducta de acicalamiento (*grooming*) en ratas RLA. Por otra parte, el NH incrementó el tiempo de interacción social en ratas RHA, y mejoró los niveles de PPI en esta cepa. Además, por primera vez, se observó un efecto del NH sobre la expresión de genes relacionados con el neurodesarrollo y plasticidad sináptica mayoritariamente en ratas adolescentes RHA.

Estudio 1.- Estratificación de la PPI y asociación con la conducta social y función cognitiva en ratas HS

Un paradigma frecuentemente utilizado para valorar el filtraje sensoriomotor es la inhibición de la respuesta de sobresalto por un prepulso, o PPI. De manera prácticamente idéntica a como se hace con animales, esta prueba se emplea en personas con esquizofrenia (y otros síntomas o trastornos), debido a que los pacientes con este trastorno son incapaces de filtrar de manera óptima los estímulos sensoriales

irrelevantes o intrusivos**.** Braff y colaboradores (Braff et al., 1999; Swerdlow et al., 2008) usaron escalas de calificación para medir la severidad de los síntomas en pacientes con esquizofrenia, y fueron los primeros en identificar una correlación entre la PPI y los síntomas positivos y negativos de la enfermedad.

La investigación básica se ha valido de modelos animales, y pruebas que reflejan procesos relevantes, para el estudio de relaciones entre fenotipos asociados a la esquizofrenia, tales como el filtraje sensoriomotor y otros rasgos que se observan en los pacientes con este trastorno (Goktalay et al., 2014; Shoemaker et al., 2003; Swerdlow et al., 2008, 2006). La comprensión de los procesos subyacentes a (o implicados en) las variaciones naturales y espontáneas en la PPI, y su posible asociación con la variación en otros rasgos (o fenotipos) relacionados con la esquizofrenia, puede facilitar la investigación y comprensión de los posibles mecanismos neurobiológicos compartidos (o no) entre aquel endofenotipo (PPI, filtraje sensoriomotor) y el resto de síntomas-fenotipos típicos del trastorno.

En este contexto, el objetivo del estudio 1 fue simplemente el de explorar la posible relación entre la PPI y la conducta social (IS), así como también con diferentes procesos cognitivos como la memoria de trabajo espacial (*spatial working memory*, SWM), aprendizaje/memoria de referencia (*Place task*, PT, y *Transfer test*, TT) y flexibilidad cognitiva (*Reversal task*, RT) en ratas genéticamente heterogéneas, HS.

Los resultados muestran que las ratas estratificadas como "baja PPI" exhiben una menor IS en comparación con su contraparte, el subgrupo de "alta PPI". Esto es consistente con los resultados de un estudio previo en ratas Wistar, en el que las ratas estratificadas por "baja PPI" tenían un peor desempeño en IS (Goktalay et al., 2014), y resultados congruentes con esos se observaron en otro estudio con ratas de la cepa Sprague-Dawley (Futamura et al., 2003) que, sin embargo, no es comparable al presente estudio porque Futamura et al. manipularon los niveles de neurotrofinas de sus ratas durante el periodo neonatal. Así mismo, nuestro estudio se diferencia del de Goktalay et al., (2014) en que estos autores usaron una "n" por grupo muy reducida (n = 5/grupo), comparada con la del presente estudio, y utilizaron un procedimiento de IS en el que podían tener cierta contaminación de los niveles de actividad locomotora.

Esta posible relación entre la PPI y la IS podría deberse a que los mecanismos neurobiológicos subyacentes a ambos procesos estarían directa o indirectamente relacionados. Es conocido que parte del circuito de la PPI está modulado por varias áreas corticales y subcorticales, incluyendo la corteza orbitofrontal, la corteza cingulada anterior, la corteza prefrontal medial, el núcleo accumbens, la amígdala basolateral y el HPC (Kohl et al., 2013). Estas son regiones que se asume que intervienen en la conducta social y en algunos de los síntomas positivos y cognitivos de la esquizofrenia. Se ha observado que ratas con lesiones a nivel cortical y subcortical (límbico) presentaban déficit en la PPI (Shoemaker et al., 2003; Swerdlow et al., 2006). En segundo lugar, en roedores, el déficit en la PPI es provocado por diferentes mecanismos, que incluyen la estimulación de los receptores de dopamina D2 mediante sustancias como la anfetamina o la apomorfina, la activación de sistemas serotoninérgicos a través de liberadores de serotonina (5-HT) o agonistas directos en múltiples receptores de serotonina, y el bloqueo de los receptores de N-metil-D-aspartato (NMDA) mediante fármacos como la fenciclidina (PCP) o el MK801 (Geyer et al., 2001; Oliveras et al., 2017; Oral and Göktalay, 2021). Por otra parte, Østerbøg et al. (2020) hallaron que la expresión en CPF de genes de los receptores metabotrópicos del glutamato (mGlu2), de la dopamina (D2) y, en menor medida, de la serotonina, se relacionaba con el desempeño en la PPI. Estos receptores intervienen directamente en la neurotransmisión de la dopamina y el glutamato en la corteza prefrontal, por lo que una alteración de estos mecanismos podría estar implicada en la etiología y/o sintomatología de la esquizofrenia (Coyle, 2006; Lerner et al., 2021; Stahl, 2018; Tsai and Coyle, 2002).

Está por explorar más exhaustivamente en qué medida dichos mecanismos neuroquímicos o circuitos neurales, o algunos de ellos, podrían estar implicados tanto en la PPI como en la conducta social y en otros fenotipos (o síntomas) como los aspectos cognitivos investigados aquí, aunque algunos efectos farmacológicos sugieren que ciertos mecanismos neurales pueden estar involucrados en esos tres tipos de síntomas o procesos. En este sentido, cabe decir que (i) la administración de antagonistas de los receptores glutamatérgicos NMDA (como la ketamina, fenciclidina, o el MK801) en ratas produce síntomas-fenotipos relevantes para la esquizofrenia, tales como alteraciones de la conducta motora, déficits en la PPI, empeoramiento de la conducta social y

deterioro en procesos de aprendizaje y memoria de trabajo y de referencia espacial. Esto sugiere que los mecanismos glutamatérgicos centrales estarían involucrados en fenotipos relacionados con la sintomatología positiva, negativa y cognitiva de la esquizofrenia. Por otra parte, (ii) esas alteraciones inducidas por antagonistas de los receptores NMDA son revertidas o atenuadas por al menos algunos antipsicóticos atípicos (de segunda y tercera generación), cuyos efectos terapéuticos se atribuyen principalmente a sus acciones sobre receptores D2, 5-HT2A y 5-HT1A (Adell et al., 2012; Oliveras et al., 2017; Sampedro-Viana et al., 2023b). Esto sugeriría que los sistemas glutamatérgicos, dopaminérgicos (mesolímbico y mesocortical, probablemente) y serotoninérgicos podrían estar implicados, en alguna medida, en los tres tipos de fenotipos o síntomas mencionados.

Con respecto a la asociación de la PPI con las funciones cognitivas, nuestros resultados muestran una relación positiva de los niveles de PPI con el desempeño cognitivo, lo que concuerda con los resultados de Oliveras et al., (2015), donde las ratas HS estratificadas por su baja PPI exhibieron peor ejecución en la tarea de memoria espacial de trabajo (tarea SWM) que las ratas con alta PPI. Además, los presentes resultados amplían estos hallazgos, pues las ratas con baja PPI muestran un deterioro de la flexibilidad cognitiva y empeoramiento de la memoria espacial a largo plazo.

Globalmente, lo dicho arriba es consistente también con lo mostrado por otros autores, en el sentido de que ratas genéticamente seleccionadas por su baja PPI, o por otros criterios, pero que conllevan un empeoramiento de la PPI (por ejemplo, las ratas RHA), muestran a su vez déficits de flexibilidad cognitiva en diferentes tareas (Freudenberg et al., 2007; Oliveras et al., 2015; Río-Álamos et al., 2019).

Esta perturbación a nivel cognitivo podría deberse en parte a la actividad GABérgica deteriorada en la ruta cortico-hipocampal (McGarrity et al., 2016). Existe evidencia que vincula la desinhibición neural hipocampal (por ejemplo, tras inyectar picrotoxina, un antagonista GABA-A, intra-HPC) con el deterioro del rendimiento de la memoria dependientes del HPC (Andrews-Zwilling et al., 2012; Koh et al., 2010; McGarrity et al., 2016). Además, considerando la conectividad del HPC-CPF, está desinhibición también afectaría la función cognitiva dependiente del CPF, como lo es la atención (McGarrity et al., 2016). Esta hipótesis podría explicar los hallazgos recientes que muestran que la

hiperactividad intrínseca del HPC se correlaciona con los déficits de memoria y atención en la esquizofrenia (Tregellas et al., 2014). Y podría suponer una hipótesis a verificar a fin de comprobar hasta qué punto similares alteraciones en la función HPC-CPF se dan en ratas que espontáneamente muestran déficits atencionales -de PPI- asociados a disminuciones de la conducta social y empeoramiento de la memoria (y/o flexibilidad cognitiva) espacial.

Estudio 2.- ***Efecto de la NH sobre la sintomatología negativa y cognitiva en ratas hembras adultas RHA vs RLA***

Los resultados de la prueba de NOE muestran que las ratas RHA realizan más exploración del objeto novedoso que las RLAs, pero esta diferencia entre cepas se elimina con el NH, puesto que el tratamiento aumenta drásticamente la exploración del objeto novedoso en las ratas RLA. Por otro lado, las ratas RHA hacen mayor "tiempo social" y actividad locomotora en la prueba de IS, y se observa que el tratamiento de NH aumenta el "tiempo social" en las RLAs. Finalmente, el comportamiento de *grooming* (acicalamiento) es mayor en las ratas RLA, y el tratamiento de NH lo reduce, sugiriendo una reducción de la ansiedad (Fernández-Teruel and Estanislau, 2016).

Estos resultados indican que las RLA presentan una mayor inhibición conductual inducida por la novedad (NOE) y ansiedad (acicalamiento), mientras que el tratamiento de NH tiende a reducir esta inhibición conductual (ansiedad) a niveles similares a los de su contraparte, las ratas RHA, para ambas medidas. Estos hallazgos coinciden con los reportados en estudios previos con machos de ambas cepas (Río-Álamos et al., 2015; Sampedro-Viana et al., 2021)**.** En cuanto al test IS, se observa que las ratas hembra RHA exhiben un tiempo social más alto que las RLAs, lo cual coincide con investigaciones anteriores (Oliveras et al., 2022b). El tratamiento de NH no tiene un efecto significativo sobre las RHA, pero sí se observa un aumento de la conducta social en las ratas RLA. Estos resultados de IS contrastan con los observados en machos RHA, donde la preferencia social es menor que en las ratas RLA (Oliveras et al., 2022b; Sampedro-Viana et al., 2021). Esta diferencia en el comportamiento social entre las ratas RHA y RLA según el sexo tiene una cierta consistencia con hallazgos en humanos que indican una menor

frecuencia o severidad de los síntomas negativos de la esquizofrenia, como el retraimiento social, en mujeres en comparación con hombres (revisado por Oliveras et al., 2022b). Algunos investigadores sugieren que las diferencias en las hormonas sexuales y su influencia en el desarrollo cerebral durante la infancia-adolescencia podrían ser un factor subyacente en este dimorfismo sexual (Oliveras et al., 2022b).

Con respecto a los resultados de las pruebas cognitivas, las hembras RHA presentan niveles más bajos de PPI que las hembras RLA, lo cual concuerda con investigaciones anteriores (Oliveras et al., 2022a) y se alinea con los hallazgos encontrados en estudios con machos (Oliveras et al., 2015, 2022a; Río-Álamos et al., 2019; Tapias-Espinosa et al., 2018, 2019). Sin embargo, a diferencia de lo encontrado en el estudio de Río-Álamos et al., (2019), nuestros resultados no muestran efectos del tratamiento de la NH en la PPI de hembras de ninguna de las cepas. Esta diferencia podría deberse a los diferentes niveles de respuesta en el %PPI observadas en las hembras de este estudio en comparación con el estudio mencionado (por ejemplo, %PPI a intensidades de prepulso de 65-70 dB = aproximadamente a 40 en nuestro estudio, respecto a aproximadamente 20 en ratas RHA en el estudio de Río-Álamos et al., 2019). Además, las ratas en nuestro estudio son sustancialmente más jóvenes (DPN 76) que las utilizadas por Río-Álamos et al. (2019) (DPN 110), lo que podría generar la discrepancia en los resultados.

En lo referente a las tareas cognitivas se encontró que, en comparación con las hembras RLA, las ratas RHA muestran una memoria de trabajo espacial deficiente, así como memoria de referencia deteriorada y una flexibilidad cognitiva disminuida. Estos resultados concuerdan con hallazgos previos en machos de ambas cepas (Aguilar et al., 2002; Escorihuela et al., 1995; Fernández-Teruel et al., 1997; Martínez-Membrives et al., 2015; Oliveras et al., 2015; Río-Álamos et al., 2019; Sánchez-González et al., 2020). El tratamiento de la NH demostró mejorar ligeramente el aprendizaje de referencia espacial en ambas cepas de ratas, así como –más claramente- la flexibilidad cognitiva en la tarea de reversión espacial (*reversal task*), especialmente en las ratas RHA. Estos resultados son novedosos en hembras y sugieren que el tratamiento de la NH podría ofrecer mejoras duraderas en la cognición espacial y/o flexibilidad cognitiva, especialmente en aquellas cepas (o individuos) con relativos déficits cognitivos.

Diversos estudios convergen en mostrar profundos y perdurables efectos del NH sobre la función neural y la neuroplasticidad. Así, el NH, entre otros muchos efectos en sistemas de neurotransmisión, **(i)** reduce perdurablemente la ansiedad y las respuestas de estrés mediadas por el eje hipotálamo-pituitario-adrenal (entre otras), HPC y amígdala, **(ii)** mejora procesos atencionales y cognitivos (p. ej., memoria de trabajo, memoria espacial) dependientes del HPC y del CPF, **(iii)** mejora la potenciación a largo plazo *("long term potentiation"*, o LTP) en el HPC, **(iv)** previene el estrés oxidativo neuronal e incrementa la neurogénesis en el HPC, **(v)** disminuye la función y respuestas dopaminérgicas dependientes del núcleo accumbens (respuestas a psicoestimulantes u otras drogas de abuso) y reduce la densidad dendrítica en este último, **(vi)** altera el volumen del HPC y la amígdala, y **(vii)** genera incrementos de densidad de espinas dendríticas piramidales (esto es, sinapsis) en el HPC y CPF (revisado por Fernández-Teruel, 2021). Todos estos efectos, y otros sobre diversos sistemas de neurotransmisión y factores neurotróficos (Fernández-Teruel, 2022; Fu and Depue, 2019), se han observado en ratas o ratones de laboratorio, y muchos de ellos aparecen ya durante la adolescencia (ver revisión en Fernández-Teruel 2021).

Los hallazgos presentados sugieren que ciertas condiciones ambientales tempranas "positivas" (como el NH o similares) podrían mitigar las vulnerabilidades genéticas a alteraciones cognitivas relacionadas con la esquizofrenia. Los efectos positivos del NH parecen estar relacionados con la mejora de la neuroplasticidad del HPC y la corteza prefrontal, regiones cerebrales implicadas, entre otros procesos, en la cognición espacial, y que aparecen alteradas en la esquizofrenia y en las ratas RHA (Elfving et al., 2019; Klein et al., 2014; Río-Álamos et al., 2019, 2017; Sánchez-González et al., 2021; Sønderstrup et al., 2023; Tapias-Espinosa et al., 2023, 2019).

Asociaciones entre PPI, IS y funciones cognitivas: comparación entre los estudios 1, 2 y trabajos previos.

El análisis de componentes principales en ratas HS (Tabla 2) muestra un primer factor con asociación entre la PPI (-0,58) con el tiempo social (-0,55) y la memoria de referencia durante la prueba de TT (-0,34), indicando una asociación de mayor PPI con mayor IS y

mejor memoria de referencia. Por otro lado, el factor 1 también muestra que la distancia en el ensayo 2 de la tarea WM en el día 1 (0,71) y la distancia recorrida durante los ensayos 1-4 de la tarea de RT (0,63) están negativamente asociadas con la PPI, indicando que una mayor PPI está asociada con mejor memoria de trabajo espacial y mejor flexibilidad cognitiva en el primer día de la tarea de aprendizaje.

El análisis factorial para ratas *Roman* muestra que la PPI se agrupa con la distancia en la PT, ambos con las cargas más altas (-0.66 y 0.77), la distancia en RT (0.41), TT (-0.39) y con WM (-0.41). Este grupo de variables sugiere que la PPI está positivamente asociado con el rendimiento en las cuatro tareas cognitivas mencionadas. Además, la PPI se relaciona con variables de la IS, pues el acicalamiento (-0.64), actividad locomotora (0.56) y el tiempo social (0.45) también cargan en este primer factor. Esto indica que menor acicalamiento, mayor actividad locomotora y mayor tiempo social se relacionan con peor desempeño en la PPI (Tabla 2).

Para facilitar su lectura e interpretación, en la Tabla 2 se han destacado en negrita las variables que representan procesos que coinciden en el sentido de sus cargas (o "saturaciones", indicadas por los números) dentro del Factor 1 de ambos estudios (PPI, en color rojo), mientras que la variable que discrepa entre ambos estudios, el "Tiempo social", se ha marcado en cursiva y subrayada. En esencia, el primer factor de ambos estudios indica que a peor "%PPI total" peor es el rendimiento en la tarea de "memoria espacial de trabajo", en "memoria espacial a largo plazo" (TT) y en "flexibilidad cognitiva" (la tarea RT), mientras que el "Tiempo social" no se comporta igual, sino de forma opuesta, entre ambos estudios.

Tabla 2

ESTUDIO 1			ESTUDIO 2		
Análisis factorial de ratas macho de la cepa HS			Análisis factorial de hembras de las cepas *Romans*		
	Factor 1	**Factor 2**		**Factor 1**	**Factor 2**
%PPI Total	**-0,58**	0,69	Tiempo NOE	-	0,70
			Tiempo no Social	-	0,81
Tiempo Social	*-0,55*	-0,64	Tiempo Social	*0,45*	-0,61
Dist WM-Trial 1	0,79	0,31	Acicalamiento	-0,64	-
Dist WM-Trial 2	**0,71**	-	Actividad Locomotora	0,56	-
Dist RT (1-4)	**0,63**	-	%PPI Total	**-0,66**	0,36
TT- Time in - Quadrant	**-0,34**	0,64	Dist WM (XT1-XT2)	**-0,41**	-
			Dist PT (Day 1-2)	**0,77**	-
			TT (Annulus crossings)	**-0,39**	-
			Dist RT (Day 1-2)	**0,41**	-
			CT (X Day 1)	-	0,36
% de varianza (acumulado)	38,0	61,4	% de varianza (acumulado)	24,1	41,6
Correlación entre factores	0,09		Correlación entre factores	0,075	

Tabla 2.- Análisis de factores con las variables de los diferentes tests/tareas del estudio 1 y estudio 2. Resaltadas en negrita están las cargas de variables que son más relevantes en el presente estudio. Se incluyen solo variables con saturaciones (cargas) > 0,30. Abreviaturas estudio 1: "%PPI Total", porcentaje total de inhibición de la respuesta de sobresalto (PPI) en ensayos de "pre-pulso + pulso". "Tiempo Social"; tiempo dedicado a explorar el agujero social en el test de interacción social (IS). "Dist WM-Trial 1", "Dist WM-Trial 2"; distancia recorrida durante el ensayo 1 y el ensayo 2, respectivamente, en el primer día de la tarea de memoria de trabajo espacial (WM). "Dist RT (1-4)"; distancia promedio recorrida en los ensayos 1-4 de la tarea de RT. "TT- Time in Quadrant"; tiempo pasado en el cuadrante objetivo (es decir, el cuadrante donde se colocó la plataforma durante la tarea de aprendizaje de reversión) en el test de (TT). Abreviaciones estudio 2: "Tiempo NOE", corresponde al tiempo de exploración en el test NOE. "Tiempo No Social", "Tiempo Social", "Acicalamiento" y "Actividad Locomotora", son las variables correspondientes del test IS. "% PPI Total", promedio de %PPI para las cuatro intensidades del prepulso. " Dist WM (XT1- XT2)", corresponde a la diferencia entre la distancia media de todos los ensayos 1 y la distancia media en todos los ensayos 2 de la tarea de memoria de trabajo. "Dist PT (Day 1-2)", corresponde a la distancia promedio de los días 1 y 2 de la tarea de aprendizaje/memoria de referencia (PT). "TT (Annulus crossings)", se refiere al número de cruces a través del anillo donde estaba ubicada la plataforma durante la tarea PT. "Dist RT (Day 1-2)", se refiere a la distancia promedio de los días 1 y 2 de la tarea de reversión espacial. "CT (X Day 1)", distancia media del día 1 de la tarea guiada.

En una muestra de ratas macho de las cepas HS y *Roman*, Oliveras et al. reportaron una asociación positiva entre el %PPI y la WM (Oliveras et al., 2015). Nuestros resultados aportan un número mayor de tareas (funciones) cognitivas, y por tanto de asociaciones con la PPI en ratas HS, lo cual apoya los resultados de Oliveras et al. respecto a la WM y los extiende a otras funciones o procesos, tales como la memoria de referencia a largo plazo y la flexibilidad cognitiva, además de la IS.

Río-Álamos et al. (2019) muestran también, en machos RHA/RLA, que "alta PPI" (ratas RLA) se relaciona con mejor ejecución en las tareas espaciales PT, TT y RT, aunque estos autores no realizaron un análisis correlacional ni factorial entre las diferentes tareas. Nuestros resultados extienden a ratas hembra RHA/RLA las diferencias (RHA vs RLA) en tareas cognitivas espaciales, los efectos de la estimulación neonatal sobre procesos cognitivos, y las asociaciones entre dichos procesos y la PPI sugeridas en aquel trabajo. Dichas asociaciones son, además, bastante convergentes (con excepción de la IS) con las observadas en ratas macho HS en el Estudio 1, y en ratas macho HS y RHA/RLA del trabajo de Oliveras et al. (2015).

No deja de ser destacable que, a pesar de que los 4 trabajos citados (es decir, los estudios 1-2 presentes, y los de Oliveras et al. y Río-Álamos et al.) utilizan diferentes

tipos/cepas de ratas y sexos, e incluso parcialmente diferentes tareas espaciales, los resultados de los estudios correlacionales y factoriales indican con una cierta consistencia asociaciones positivas entre niveles de PPI, memoria de trabajo espacial, memoria de referencia espacial y flexibilidad cognitiva espacial. Como se ha comentado, las asociaciones entre la PPI y esos procesos cognitivos, en términos de correlaciones y análisis factoriales, solo han sido exploradas y mostradas en ratas en los presentes estudios 1-2 y en el de Oliveras et al. (2015), y ofrecen coherencia en los resultados. El hecho de que, además, dichos resultados se han observado utilizando tres cepas diferentes de ratas, una de ellas genéticamente heterogénea (HS), da más fuerza a los hallazgos, y sugiere que quizá ciertos mecanismos neurobiológicos comunes pueden estar jugando un papel relevante en esas diferentes tareas y funciones. Ciertamente, el hipocampo, cuya función es sabido que se halla alterada en la esquizofrenia, y que (modula o) interviene tanto en la PPI como en la memoria de trabajo y memoria espacial (y otros procesos cognitivos), es una región claramente candidata para la investigación de mecanismos y procesos neurobiológicos que puedan ser comunes a determinados clusters (o agrupaciones) de síntomas o fenotipos relacionados con este trastorno.

Estudio 3.- Conducta inducida por la novedad, IS y PPI: efectos de la cepa (RHA vs. RLA) y de la edad (adolescentes vs. adultos) en ratas macho

En el estudio 3, se investigaron por primera vez en ratas RHA y RLA tanto los posibles efectos madurativos (en ratas adolescentes) como los efectos a largo plazo (en ratas adultas) del tratamiento con la NH en comportamientos relacionados con la inhibición/exploración inducida por novedad (NOE), ansiedad (medida mediante el acicalamiento durante la prueba IS), actividad locomotora (evaluada a través de la prueba IS), conducta social (evaluada mediante la prueba IS) y atención/filtraje sensoriomotor (evaluada mediante la prueba PPI). Además, también se analizó por primera vez la expresión diferencial de genes “diana” implicados en el neurodesarrollo, la neuroplasticidad y los procesos sinápticos, teniendo en cuenta la cepa de las ratas, el tratamiento neonatal y la edad.

Los resultados muestran que la exploración de un objeto novedoso (NOE) es diferente entre las cepas (RHA>RLA) durante la adolescencia temprana (DPN32-36). Este resultado es consistente con hallazgos previos obtenidos con ratas mayores de ambas cepas, en la adolescencia tardía (DPN52-58) (Cuenya et al., 2016) o en la adultez temprana (DPN60) (Río-Álamos et al., 2017, 2015; Sampedro-Viana et al., 2021). Además, se muestra que la actividad locomotora y el acicalamiento son diferentes entre las cepas desde la adolescencia. Estos hallazgos novedosos en las ratas *Roman* sugieren que las diferencias entre las cepas en ambos comportamientos, que son típicas en las ratas *Roman* adultas, maduran muy temprano durante el desarrollo. Sin embargo, parece claro, de acuerdo con los resultados, que las diferencias entre cepas en acicalamiento (RHA<RLA) y actividad locomotora (RHA>RLA) se acentúan en la adultez. Además, algunas diferencias entre las cepas en la IS (tiempo social) solo aparecieron en ratas adultas. Por otra parte, las ratas RLA mostraron un rendimiento superior en PPI que las ratas RHA durante la adolescencia, lo que se ha observado aquí por primera vez. Esta diferencia entre cepas es aún más marcada en la adultez. Esto está en general en línea con estudios previos (Oliveras et al., 2015, 2022a; Río-Álamos et al., 2017; Tapias-Espinosa et al., 2018, 2019). Es destacable también el hecho de que se observan efectos consistentes de la edad sobre las diferentes variables conductuales. En concreto, a excepción de la conducta de acicalamiento, todas las demás conductas o variables, tanto del test de IS como del test de PPI (%PPI y Startle basal) se incrementan con la edad.

Estudio 3.- Conducta inducida por la novedad, IS y PPI: efectos del NH en función de la cepa (RHA vs. RLA) y de la edad (adolescentes vs. adultos) en ratas macho

En cuanto a los efectos del NH, los resultados obtenidos en ratas adolescentes indican que el NH incrementa la exploración de novedad (prueba NOE) y la actividad locomotora (en la prueba IS), particularmente en ratas RLA, y reduce el tiempo de acicalamiento, lo cual sugiere claramente una reducción de la ansiedad (Fernández-Teruel and Estanislau, 2016). En la prueba de PPI, el NH también muestra efectos de interacción con la cepa y la intensidad del prepulso en ratas adolescentes RLA y RHA, debido a la tendencia del NH a aumentar los niveles de %PPI en varias intensidades de prepulso en ratas RHA

adolescentes (pero no en las RLA), siendo significativo a la intensidad de prepulso de 70 dB.

Los resultados de los efectos del NH sobre el tiempo de NOE, la actividad locomotora, el acicalamiento y la PPI en ratas R*oman* adolescentes, constituyen hallazgos novedosos. Es particularmente destacable que las ratas RLA tratadas con NH exhiban niveles de NOE, actividad y acicalamiento que se acercan o incluso igualan los puntajes de las ratas RHA. Esto sugiere que el tratamiento de NH tiene efectos durante el neurodesarrollo (maduración) sobre respuestas vinculadas al afrontamiento y relacionadas con la ansiedad, que son rasgos estrechamente vinculados a la genética de las ratas RLA, y que comienzan a operar muy temprano durante el desarrollo (ver Fernández-Teruel et al., 2021; Giorgi et al., 2019, y referencias en dichos trabajos). Estos efectos del NH probablemente involucren cambios a nivel de los circuitos neuronales, neurotransmisores y factores neuroendocrinos durante el neurodesarrollo temprano, los cuales desempeñan funciones relevantes en la configuración de las respuestas comportamentales y de los perfiles de afrontamiento.

Consistente con lo anterior, en ratas macho de las cepas Wistar y Sprague Dawley, adolescentes (PND40) y pre-adolescentes (PND30), respectivamente, se ha informado que la estimulación neonatal (NH) reduce las respuestas del eje HPA al estrés y aumenta los niveles de 5-HT en varias regiones del cerebro, incluido el HPC, que es una región conocida por su papel en la regulación de la ansiedad y las respuestas neuroendocrinas al estrés (Papaioannou et al., 2002; Park et al., 2003). Consistentemente, el tratamiento de NH tiene efectos ansiolíticos en la prueba del laberinto en cruz elevado y un efecto protector contra el daño oxidativo en la corteza, el HPC y el estriado en ratas adolescentes. Relacionado con lo anterior, el NH administrado durante los primeros 12 días de vida también ha mostrado aumentar la densidad de espinas dendríticas piramidales en el HPC ventral y la corteza prefrontal, así como la longitud dendrítica en la corteza prefrontal en ratas Sprague Dawley adolescentes (Monroy et al., 2010). Por lo tanto, dado que el HPC y la corteza prefrontal están relacionados con la ansiedad, la inhibición conductual inducida por novedad y la (modulación de la) PPI, y que estas regiones se ven afectadas por el NH en ratas de laboratorio (adolescentes, o incluso

prepuberales), los efectos presentes del NH en ratas *Roman* adolescentes parecen estar en línea con esos hallazgos.

Por otro lado, en ratas adultas hemos encontrado que el NH aumenta la actividad locomotora inducida por novedad (test IS) en ambas cepas, reduce el acicalamiento en ratas RLA y mejora la IS y la PPI en las RHA. Tales efectos del NH en ratas *Roman* adultas se alinean globalmente con estudios en ratas adultas de otras cepas (revisiones de Fernández-Teruel et al., 2002; Raineki et al., 2014; Río-Álamos et al., 2019; Sampedro-Viana et al., 2021, y referencias en dichos artículos), y también están en línea con nuestros hallazgos previos que sugieren que el NH podría mejorar de manera duradera los procesos relacionados con la cognición/atención, particularmente en ratas RHA (Aguilar et al., 2002; Fernández-Teruel et al., 1997, 2002; Río-Álamos et al., 2015, 2017,2019).

Estudio 3.- Expresión de genes presinápticos (Cdk5, Cables1, Snap25): efectos de cepa, edad y NH

Los resultados muestran que existe un aumento de la expresión de Cables1 en ratas RHA adolescentes tratadas con NH, y un aumento de la expresión del gen en las RHA controles adultas frente a las ratas RLA. El resultado en ratas RHA adolescentes difiere de los resultados previos de Sønderstrup et al., (2023), posiblemente debido a la diferencia de edad de las ratas en ambos estudios (DPN44-46 en Sønderstrup et al. vs. DPN47-56 en el presente estudio) y/o al hecho de que aquí se realizaron pruebas de comportamiento (entre DPN36-42 en ratas adolescentes y DPN92-98 en ratas adultas) que finalizaron 10-15 días antes del sacrificio, mientras que en el estudio de Sønderstrup et al., las ratas no habían pasado por experimentación alguna ni pruebas comportamentales. Otra diferencia ambiental importante es que nuestras ratas controles, aunque no fueron manipuladas neonatalmente (excepto por la limpieza de las jaulas una vez a la semana), fueron mucho más “perturbadas” que las ratas de control del estudio de Sønderstrup et al. (2023). Esto fue debido a que todas las ratas de los grupos de control y NH en nuestro estudio estaban en la misma sala de estabulación, lo que implicaba que todas ellas estuvieron sometidas a unas 20 entradas (diariamente,

por la mañana y por la tarde) de al menos una persona en la sala de estabulación, para coger de los racks las cajas con las ratas que iban a recibir el NH y unos 10 minutos después para devolverlas a su rack. Por tanto, las ratas control de este estudio estuvieron sometidas a esa perturbación ambiental durante los primeros 21 días de vida, cosa que no ocurrió en el estudio de Sønderstrup et al. (2023).

CABLES1 es un activador de CDK5, el cual está relacionado principalmente con la modulación de la citoestructura y migración neuronal del SNC (Dhavan and Tsai, 2001; Shah and Rossie, 2018). La expresión de *Cdk5* se redujo en ratas adolescentes RHA de control, pero, como se observó para *Cables1*, aumentó en las que habían recibido NH. La reducción de la expresión de *Cdk5* puede indicar una maduración sináptica alterada en la cepa RHA adolescente. CDK5 también está involucrado en el reclutamiento y ensamblaje de proteínas presinápticas durante la sinaptogénesis (Easley-Neal et al., 2013), por lo que el aumento de la expresión del componente SNARE, *Snap25*, en ratas adolescentes RHA control podría estar relacionado con un mecanismo compensatorio para la reducida expresión de *Cdk5*. Sin embargo, es especialmente destacable que el tratamiento con NH normalizó (hasta niveles del grupo RLA) la expresión alterada tanto de *Snap25* como de *Cdk5* en ratas adolescentes RHA, lo que sugiere un efecto madurativo de la estimulación neonatal sobre estos dos genes. Así mismo, en ratas adultas RHA, la sobre-expresión de *Snap25* se normalizó a los niveles de las RLAs tras el tratamiento con NH.

Estudio 3.- Expresión de genes postsinápticos (Nrg1, Homer1, Bdnf): efectos de cepa, edad y NH

Los resultados muestran que existe un aumento de la expresión de *Bdnf* como consecuencia del tratamiento con NH en ratas RHA adolescentes, un aumento de la expresión del gen en ratas RHA adultas de control frente a las ratas RLA, y un incremento de expresión en estas últimas tras administrar NH. BDNF es importante para la plasticidad sináptica y la comunicación sináptica durante el neurodesarrollo (Favalli et al., 2012; Kowiański et al., 2018; Nieto et al., 2013), y alteraciones de esta neurotrofina se han relacionado con la esquizofrenia (Angelucci et al., 2005; Favalli et al., 2012; Nieto

et al., 2013; Ursini et al., 2016). De hecho, en coherencia con ello, se ha informado de alteraciones sinápticas en cerebros de pacientes esquizofrénicos (Föcking et al., 2015) y en modelos animales de fenotipos relevantes para la esquizofrenia (Angelucci et al., 2005; Sanna et al., 2019; Taschetto Vey et al., 2020). En línea con todo ello, en el presente estudio se muestran niveles de expresión alterados de componentes sinápticos en las ratas RHA, incluyendo *Nrg1* y la proteína de densidad postsináptica (PSD) *Homer*1. La expresión de ambos genes tiende a reducirse en ratas RHA adolescentes (vs. RLA) y, de acuerdo con nuestros hallazgos previos, ambos están aumentados en ratas RHA adultas en comparación con las RLAs (Elfving et al., 2019; Sønderstrup et al., 2023). Es notable que (similar a Snap25) *Homer1, Nrg1 y Bdnf* muestran una influencia de la edad en interacción con la cepa y el NH, debido a que la expresión de los tres genes tiende a estar aumentada en ratas adultas vs. adolescentes RHA, y el tratamiento con NH aumenta los niveles de expresión de los tres genes en las RHAs adolescentes, mientras que no se observa un efecto claro de la edad (teniendo en cuenta los tres genes) en el caso de las RLA y el efecto positivo del NH aparece sobre *Nrg1 y Bdnf* en RLA adultas (pero no en adolescentes). Esto parece consistente con la idea de que la maduración sináptica aberrante (o alterada) de la corteza prefrontal es una característica de las ratas RHA (Elfving et al., 2019; Fernández-Teruel et al., 2021; Sánchez-González et al., 2021; Sønderstrup et al., 2023), y que tales alteraciones de maduración cortical podrían ser en alguna medida "normalizadas" por la estimulación neonatal ya desde la adolescencia.

Las anomalías en la PSD han sido vinculadas a desórdenes del neurodesarrollo como los trastornos del espectro autista y la esquizofrenia (Kaizuka and Takumi, 2018). La proteína HOMER1 media la señalización de los receptores glutamatérgicos, serotoninérgicos y dopaminérgicos postsinápticos (de Bartolomeis et al., 2013a, 2013b). NRG1 tiene un papel en la regulación de la neurotransmisión excitatoria/inhibitoria en la PSD y parece estar vinculada al riesgo de esquizofrenia (Mostaid et al., 2016). NRG1 modula el filtrado sensoriomotor (la PPI), como se ha observado en estudios en animales (Olaya et al., 2018) y humanos (Hong et al., 2008; Roussos et al., 2011). La interrupción de la señalización de NRG1 afecta la sincronización hipocampo-prefrontal y procesos atencionales (Tan et al., 2018) y, como ya se mencionó previamente, el deterioro en el filtrado sensoriomotor y las alteraciones atencionales son rasgos importantes en las

ratas RHA, probablemente junto a una disminución de la función hipocámpica en esta cepa (Fernández-Teruel et al., 2021, 2023; Giorgi et al., 2019; Klein et al., 2014; Meyza et al., 2009; Moreno et al., 2010; Oliveras et al., 2015; Sallés et al., 2001; Tapias-Espinosa et al., 2019). Por lo tanto, es destacable que la expresión de estos tres genes, *Bdnf, Homer1 y Nrg1*, aumenta tras el tratamiento con NH en ratas RHA adolescentes, mientras que las ratas RHA adultas de control muestran una expresión aumentada en relación con las RLAs. Estos resultados respaldan la idea de que el NH puede producir efectos positivos sobre la maduración cortical y la plasticidad sináptica en las ratas RHA adolescentes, que podrían estar relacionados con los cambios de comportamiento en NOE y PPI observados en ratas RHA juveniles y adultas tratadas con NH. En las RLAs, a su vez, estos genes no se vieron afectados por el NH en la adolescencia, pero el tratamiento condujo a niveles aumentados de *Nrg1 y Bdnf* en la edad adulta.

Estudio 3.- Expresión de genes de receptores de membrana (Grin2b, Drd1): efectos de cepa, edad y NH

El gen *Grin2b* codifica una de las subunidades del receptor NMDA, el cual es responsable del control de la migración y conexión neuronal durante la formación del SNC y, posteriormente, su función es la mediación de la neurotransmisión excitatoria del glutamato (Myers et al., 2019; Sabo et al., 2023). En los resultados presentes encontramos que el nivel de expresión del gen *Grin2b* se incrementa con el tratamiento de estimulación neonatal en ratas RHA adolescentes, lo que concuerda con lo discutido anteriormente sobre el potencial efecto modulador de la maduración cortical que tendría el tratamiento de NH. Por otro lado, en contraste con nuestros hallazgos previos (Elfving et al., 2019; Sønderstrup et al., 2023), no observamos diferencias en expresión de *Drd1* entre los controles de ambas cepas de ratas (en ninguna edad), aunque hubo un aumento global de la expresión con la edad, lo que en líneas generales concuerda con los hallazgos de Sønderstrup et al. (2023). Actualmente, la única explicación que tenemos, para la discrepancia entre ambos estudios respecto a la comparación entre cepas (sin tratamiento), se relaciona con las diferencias importantes (mencionadas anteriormente) en condiciones ambientales, pruebas de comportamiento y edad entre

las ratas utilizadas en el presente estudio y las ratas utilizadas en los estudios de (Elfving et al., 2019; Sønderstrup et al., 2023).

Estudio 3.- Procesos moleculares sinápticos y de neuroplasticidad influidos por la estimulación infantil: relación entre la expresión génica y los resultados conductuales-atencionales

En la Figura 4 (y su leyenda) intentamos resumir en qué procesos sinápticos y de neuroplasticidad están involucrados los genes cuya expresión se ha medido en el presente Estudio 3, a fin de tener una imagen algo más integrada de los procesos que las variaciones en dichos genes pueden estar afectando en la sinapsis.

La actividad de CDK5 en la célula está estrictamente regulada en los niveles de expresión proteica, modificación post-traduccional y por sus activadores. La CDK5 monomérica no muestra actividad enzimática y requiere asociarse con un sustrato regulatorio p35 para su activación (Dhavan and Tsai, 2001; Huang et al., 2017). La activación ocurre cuando la proteína c-Abelson (C-ABL) conduce a la fosforilación de tirosina de CDK5, y esta fosforilación es potenciada por CABLES (Zukerberg et al., 2000). La fosforilación de CDK5 por C-ABL estimula la actividad de la kinasa p35/Cdk5 (Zukerberg et al., 2000). La relación fisiológica entre CABLES y CDK5 es coherente con la correlación positiva de sus genes tanto en adolescentes como en adultos ($r=0,520$, $p < 0.01$ y $r=0,456$, $p< 0.05$; $n= 27$), respectivamente (ver Figura 4).

Otra de las funciones presinápticas de CDK5 es la regulación de la neurotransmisión (Dhavan and Tsai, 2001). Dentro de este proceso existen tres proteínas sustrato para CDK5, Synapsin 1, MUNC18 y Anfifisina. La proteína MUNC18 (mammalian uncoordinated-18) y el complejo de proteínas SNARE (Sinaptobrevina, Sintaxina, SNAP25) participan en la fusión de la vesícula sináptica y son cruciales para la exocitosis de neurotransmisores y hormonas. MUNC18 interacciona con sintaxina; esta última tiene dos conformaciones, una auto-inhibida (cerrada) y otra abierta. La conformación cerrada impide su interacción con los dominios SNARE de sinaptobrevina y SNAP25, y se mantiene cerrada gracias a MUNC18, que actúa a modo de capuchón y evitando el paso de la sintaxina al estado abierto. La fosforilación de Munc18 por CDK5 resulta en la

disociación del complejo MUNC18-sintaxina 1, permitiendo la formación del complejo SNARE y subsecuente mecanismo de neurosecreción (ver Figura 4). Nuestros resultados muestran una correlación negativa entre CDK5 y SNAP25 (r=-0,810, p < 0.01, n=27), y entre Cables1 y SNAP25 (r=-0,408 p< 0.05, n= 27) en adolescentes. Esta relación respalda la idea de un mecanismo compensatorio entre la menor actividad regulatoria de CDK5 y la mayor actividad de SNAP25 para la formación del complejo SNARE. Además, hemos observado una correlación negativa entre SNAP25 y la PPI (r = -0,371, p< 0.05; n= 27) en adolescentes. La hiperactividad de SNAP25 con las proteínas del complejo SNARE impide su disociación, y por tanto el reciclaje de vesículas y la reducción en la amplitud de los potenciales postsinápticos excitatorios corticales. Lo anterior se ha observado en modelos de ratones con hiperactividad de SNAP25, que muestran ataxia y empeoramiento del filtrado sensoriomotor, o PPI (Jeans et al., 2007).

Con respecto a los genes postsinápticos los niveles de expresión de *Nrg1 y Bdnf* en ratas adultas correlacionaron negativamente (r=-0.44, p=0.016; r=-0.58, p=0.002) con la latencia en la prueba de exploración de objetos nuevos (NOE), y, en coherencia con ello, la expresión de *Bdnf* se correlacionó positivamente con el tiempo de NOE (r=0.40, p=0.04). Además, *Bdnf y Nrg1* mostraron correlaciones positivas con la actividad horizontal en la prueba de interacción social (IS) (r=0,66, p<0.001; y r=0,45, p=0.012, respectivamente), y correlacionaron negativamente con el acicalamiento (r=-0,39, p=0.05; y r=-0,64, p<0.001, respectivamente). Estos patrones de correlación concuerdan con los hallazgos de que el NH aumentó el tiempo de NOE y la actividad horizontal, y redujo el acicalamiento en ratas adultas RLA, mientras que ambos grupos de ratas adultas RHA mostraron un aumento en el tiempo de NOE y la locomoción, y una reducción en el acicalamiento en comparación con las RLA adultas. Este patrón consistente de asociaciones en ratas adultas se refuerza aún más por la correlación negativa de la expresión de *Homer1* con la latencia en el NOE (r=-0,49, p=0.009) y la correlación positiva con la actividad (r=0,38, p=0.049), así como por las correlaciones positivas de *Nrg1* con *Bdnf* (r=0,46, p=0.019) y *Homer1* (r=0,65, p<0.001). En conjunto, parecería que los efectos de la estimulación neonatal y de la cepa, sobre la expresión de estos tres genes en ratas adultas (RHA y RLA), forman parte de algún mecanismo/patrón neural común, que a su vez podría estar relacionado con los aspectos más emocionales

de los comportamientos medidos aquí (respuestas inducidas por la novedad como la exploración de objetos nuevos –NOE-, y la locomoción y el acicalamiento en la prueba de IS).

El tratamiento de estimulación neonatal tiene efectos anti-ansiedad (y anti-estrés) bien conocidos y duraderos, representados aquí por el aumento tanto de la exploración de objetos nuevos (prueba de NOE) como de la locomoción bajo condiciones de novedad (en la prueba de IS), y por la reducción del acicalamiento inducido por la novedad. Por lo tanto, los hallazgos actuales relacionados con la estimulación neonatal y la cepa, junto con las correlaciones mencionadas entre comportamiento y expresión génica, sugieren que la expresión de estos tres genes (en particular, *Bdnf y Nrg1*) está relacionada negativamente con la ansiedad (es decir, con la inhibición del comportamiento producida por la novedad).

Finalmente, nuestros resultados muestran una correlación entre los genes *Drd1 y Grin2b* que codifican para los receptores de D1 y NMDA, respectivamente, en adolescentes. La correlación positiva ($r= 0,609$, $p<0.001$; $n=27$) indica que a mayor expresión de *Drd1* hay mayor expresión de *Grin2b*. Esto es compatible con la estrecha relación fisiológica entre estos dos receptores, pues en estudios previos se ha demostrado que la estimulación del receptor D1 potencia la actividad del receptor NMDA en la corteza prefrontal, a través de un mecanismo postsináptico dependiente del calcio. Además, el receptor D1 activo induce el aumento del receptor NMDA en la membrana postsináptica, aumentando la expresión superficial de este último (Dunah and Standaert, 2001; Tseng et al., 2009). En contraste, se ha observado que, en el HPC, el receptor D1 inhibe la actividad del receptor NMDA (Tong and Gibb, 2008) y la potenciación a largo plazo dependiente del mismo (Cepeda and Levine, 2006). Sin embargo, lo que demuestran estos estudios es que la activación del receptor D1 modula las corrientes postsinápticas de NMDA y también que D1 afecta la localización subcelular del receptor NMDA (plasticidad sináptica) (Cepeda and Levine, 2006; Dunah and Standaert, 2001; Lee and Liu, 2004; Tseng et al., 2009) (Ver Figura 4).

Estudios previos, en ratas Wistar, han mostrado que el NH produjo un incremento de expresión de *Grin2b* y de densidad de receptores NMDAB (NR2B) en corteza prefrontal (prelímbica + cingulado anterior) e hipocampo dorsal de ratas adultas Wistar

(Stamatakis et al., 2009), sugiriendo que el NH incrementa la neuroplasticidad en esas regiones. Nuestros resultados se asemejan en parte, en cuanto que en ratas RHA adolescentes se observa un aumento de expresión de *Grin2b* en RHAs (y una ligera tendencia –no significativa- en el mismo sentido en las RHA adultas). El NR2B se ha relacionado consistentemente con la plasticidad cerebral (en particular en el CPF y el HPC). Dado el conocido papel que estas regiones tienen en el control de las respuestas de estrés, el afrontamiento del mismo y de situaciones de conflicto, y en procesos cognitivos espaciales y no espaciales (revisado por Stamatakis et al., 2009), nuestros datos pueden ser en parte coherentes con el hecho de que las ratas RHA que recibieron NH presenten mejoras en PPI e interacción social, al igual que en memoria de trabajo y flexibilidad cognitiva en trabajos previos (Aguilar et al., 2002; Río-Álamos et al., 2019).

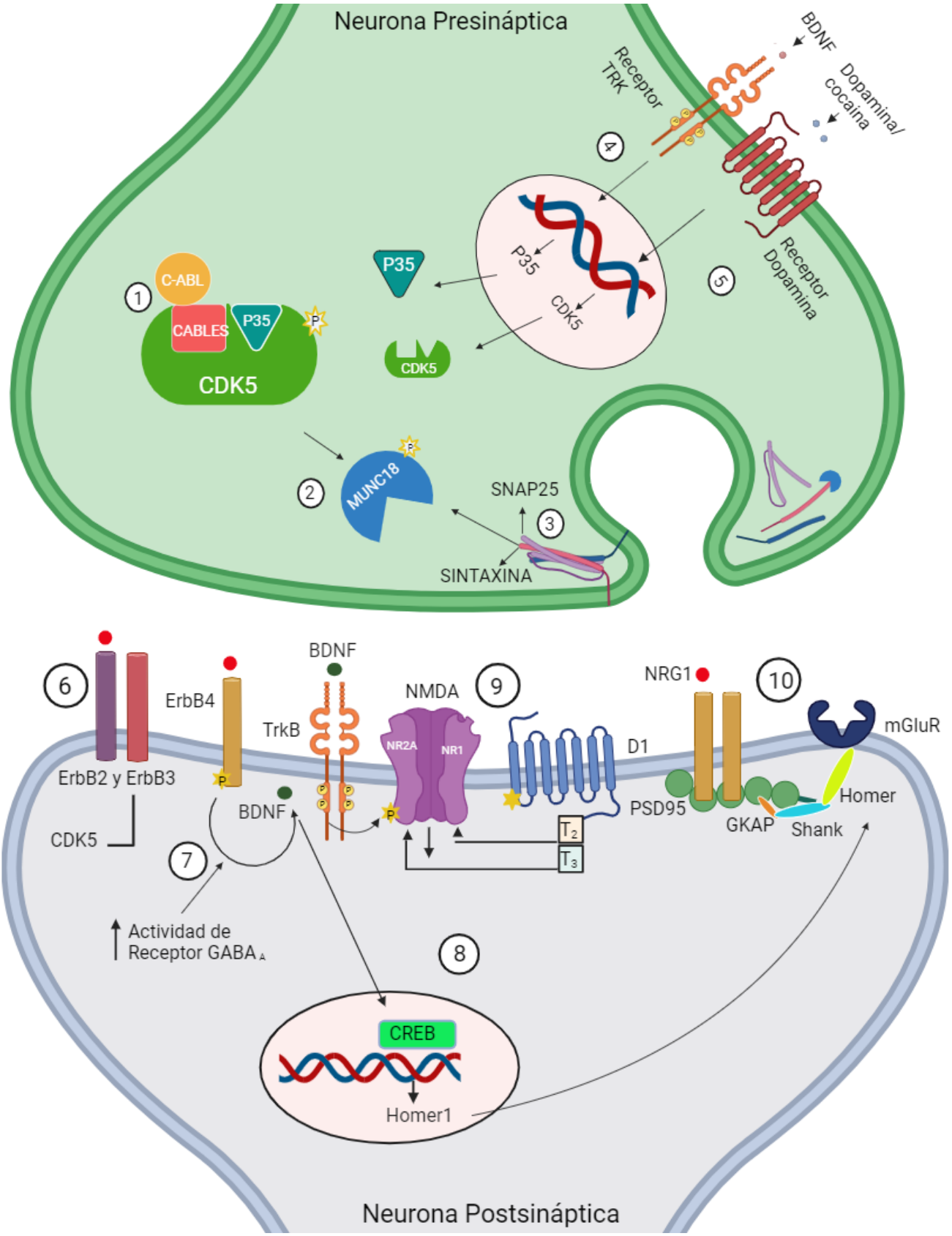


Figura 4 (ver leyenda en la página siguiente)

Figura 4. **(1)** C-ABL a través de la proteína adaptadora **CABLES** fosforila (activa) la enzima **CDK5** y a su sustrato P35 (Zukerberg et al., 2000). **(2)** La proteína MUNC18 interacciona con sintaxina; la fosforilación de Munc18 por CDK5 disocia el complejo Munc18-sintaxina, permitiendo la formación del complejo SNARE. **(3)** Formación del complejo SNARE (SINAPTOBREVINA, SINTAXINA, **SNAP-25**) para facilitar la fusión de las vesículas (representadas por la "muesca" o inflexión semicircular en la membrana presináptica), con la membrana de la neurona presináptica. **(4) BDNF** es capaz de inducir la expresión de p35 y **(5)** la estimulación de los receptores de dopamina promueve la expresión de CDK5 (Dhavan and Tsai, 2001). **(6)** La unión de receptores ErbB2 y ErbB3 permite que **NRG1** se una a estos y promueva la expresión de CDK5 postsináptico, el cual participa principalmente en la liberación de acetilcolina. (Dhavan and Tsai, 2001). **(7)** La activación de ErbB4 inducida por NRG1 en interneuronas GABAérgicas conduce a la expresión de BDNF y a la estimulación de cascadas de señalización, incluyendo TrkB. Esto lleva a la activación de receptor **NMDA** (Pandya and Pillai, 2014). **(8)** BDNF induce la expresión de **HOMER1A** a través de la cascada de señalización TrkB receptor-Ras-ERK-CREB (Serchov et al., 2016). **(9)** la estimulación del receptor **D1**, potencia la actividad del receptor NMDA en la corteza prefrontal a través de un mecanismo postsináptico dependiente del calcio (Tseng et al., 2009). **(10)** Aunque los mecanismos que subyacen a este efecto aún no están claros, a través del consorcio de las proteínas PSD95, y las del complejo GKAP-Shank-Homer, generan la activación de los receptores de ErbB4 los cuales estan involucrados en regular la función de los receptores metabotrópicos de glutamato (mGluRs) (Serchov et al., 2016).

Discusión y conclusión general, limitaciones y perspectivas

Comparación de los hallazgos en hembras y en machos RHA vs. RLA

Las pruebas comportamentales realizadas en ratas *Roman* hembras (estudio 2) y machos (estudio 3) nos permiten hacer comparaciones cualitativas de las diferencias y semejanzas entre animales de ambas cepas en ambos sexos, así como comparaciones respecto a estudios previos y sobre los efectos del tratamiento de NH (Tabla 3). Comparando con estudios en los que machos RHA y RLA se evaluaron en pruebas comportamentales y cognitivas similares a las del estudio 2 (p. ej., Aguilar et al., 2002; Martínez-Membrives et al., 2015; Oliveras et al., 2015, 2022a,b; Río-Álamos et al., 2015, 2019), podemos concluir que, a excepción de la conducta social, el perfil conductual de las RHA vs. RLA es consistente entre machos y hembras (Tabla 3), lo que indica que ambos podrían ser considerados como un modelo con validez aparente para algunos rasgos relevantes (cognitivos, atencionales) de la esquizofrenia. Además, se observa un efecto positivo del NH sobre la conducta exploratoria de la novedad (test NOE) y el aprendizaje/memoria espacial y flexibilidad cognitiva en las hembras (estudio 2), que es

coherente con resultados de estudios previos en machos de las cepas *Roman* (Aguilar et al., 2002; Río-Álamos et al., 2015, 2019) (ver Tabla 3).

	Hembras (Estudio 2)	**Efecto del NH**	**Machos (Estudio 3)**	**Efecto del NH**
Exploración de novedad (test NOE)				
Exploración objeto nuevo	RHA>RLA	**+** RHA, **++**RLA[1]	RHA>RLA[1,2,3]	**+**RHA, **++**RLA[1,2,3]
Interacción social y respuestas a la novedad (test IS)				
Exploración o preferencia por el agujero social	RHA>RLA	**+** RLA	RHA<RLA [3]	**+**RHA [3]
Acicalamiento (*grooming*)	RHA<RLA	**--** RLA	RHA<RLA [3]	**--** RLA [3]
Actividad Locomotora	RHA>RLA	**=**	RHA>RLA	**+**RLA **+**RHA
Filtraje sensoriomotor (atención –PPI-)				
Inhibición de respuesta de sobresalto por prepulso	RHA<RLA	**=** **+**RHA [2]	RHA<RLA [4]	**+**RHA [2]
Aprendizaje/memoria espacial (funciones cognitivas)				
Memoria (espacial) de trabajo	RHA<RLA	**=**	RHA<RLA [4]	**++**RHA [2,4,5]
Aprendizaje espacial de referencia (*Place tasks*)	RHA<RLA	**+**RHA	RHA<RLA[2,5,6]	**=** [2,5,6]
Flexibilidad cognitiva (*Reversal place tasks*)	RHA<RLA	**++**RHA, +RLA	RHA<RLA [2]	**+**RHA, **+**RLA [2,5]
Memoria de referencia a largo plazo (*One-trial Transfer/Probe tests*)	RHA<RLA	**=**	RHA<RLA [2]	**=** [2]

Tabla 3. Cuadro de comparación de los rasgos de conducta exploratoria (NOE), social (IS), atencional (PPI) y de memoria espacial en tareas del "laberinto acuático de Morris", entre machos y hembras RHA y RLA de los estudios de esta Tesis y otros estudios previos. "**=**", sin efecto significativo. "**--**"; disminución significativa. "**+**"; aumento significativo. "**++**"; aumento significativo de mayor magnitud. Los números junto a los acrónimos RHA/RLA se refieren a las siguientes referencias bibliográficas relacionadas: [1] Río-Álamos et al., 2015; [2] Río-Álamos et al., 2019, [3]Sampedro-Viana et al., 2021; [4] Oliveras et al., 2015, [5] Aguilar et al., 2002, 6 Martínez-Membrives et al., 2015.

Las limitaciones de espacio y materiales (equipos, salas de experimentación), temporales (debido a las edades específicas de los animales en el estudio, adolescentes y adultos) y humanas (personal de laboratorio), impidieron que el estudio con hembras (estudio 2) tuviese el mismo diseño experimental que el de machos (estudio 3), y que se pudiese llevar a cabo un estudio de comparación de ambos sexos y edades completamente en paralelo. Por ello, sería sin duda recomendable que en futuros estudios se realice la exploración de los rasgos comportamentales (y de expresión génica) en hembras de las dos edades (adolescentes y adultas), y que en el mismo diseño experimental se pudiesen incluir machos en las mismas condiciones.

Por otra parte, en el estudio 3 con machos, no fue posible incluir pruebas cognitivas (aprendizaje/memoria espacial, memoria de trabajo, flexibilidad cognitiva) como las incluidas en el estudio 2 con hembras, debido a que la ventana temporal para pruebas de conducta y toma de muestras cerebrales en las ratas adolescentes (que fue tan solo de unos 14 días) no lo permitía. Sería deseable, por supuesto, tener medidas cognitivas como aquellas también en machos adolescentes (y adultos), para poder relacionarlas con expresión génica en futuros estudios.

Expresión génica diferencial en CPF, y efectos del NH sobre la neuroplasticidad

En las ratas RHA, que parecen presentar un CPF inmaduro en la edad adulta (Elfving et al., 2019; Río-Álamos et al., 2019; Sønderstrup et al., 2023; Tapias-Espinosa et al., 2019, 2023), resulta interesante que el tratamiento con NH incrementa, durante la adolescencia, la expresión génica en la fase postsináptica -*Ngr1, Homer1, Bdnf, Drd1, Grin2b*-, así como, a nivel presináptico, la expresión de *Cables1 y Cdk5,* mientras que disminuye drásticamente (hasta el nivel de las ratas RLA control) la expresión de *Snap25*. Nuestros resultados con el *Snap25*, sin embargo, van en la línea de otros previos en los que un incremento de expresión de este gen se ha asociado a empeoramiento de la PPI (Jeans et al., 2007). Es notable el hecho de que esto coincide con el hallazgo de que las ratas RHA control (tanto adolescentes como adultas), que presentan consistentes déficits de PPI, son las que muestran expresión elevada de *Snap25*, y que las RHA que

recibieron NH, que mejoran su ejecución en la PPI, también presentan niveles reducidos (normalizados) de expresión del gen.

Globalmente, estos resultados podrían sugerir que tanto a nivel pre- como post-sináptico el NH induce cambios en función sináptica y neuroplasticidad durante la adolescencia, que tienen que ver con la maduración cortical de las ratas RHA. Ello redundaría en los efectos conductuales y atencionales positivos que se observan tanto en las RHA adolescentes como en las adultas, esto es, una moderada mejoría en la PPI e incremento de la actividad exploratoria (de la novedad) en la adolescencia, y un más marcado incremento de la PPI, la interacción social y la actividad en respuesta a la novedad (durante el test de IS) en la edad adulta, así como efectos positivos sobre la cognición espacial y otros observados en estudios previos (Aguilar et al., 2002; Fernández-Teruel et al., 1997, 2002; Río-Álamos et al., 2015, 2017, 2019).

En qué medida las consecuencias moleculares, observadas tras el NH, son cruciales para que el tratamiento produzca dichos efectos comportamentales y atencionales/cognitivos es una cuestión que solo podrá resolverse con estudios en los que se manipule experimentalmente la infra- o sobre-expresión de los genes estudiados, o/y la cantidad proteica de los genes en el CPF, y se exploren los efectos conductuales-cognitivos de tales manipulaciones. Estudios no publicados de nuestro grupo muestran, por ejemplo, que la expresión proteica de BDNF está aumentada en el CPF en ratas RHA y RLA adultas que recibieron NH (Río-Álamos et al., datos no publicados).

En conclusión, los hallazgos presentes respaldan la idea de que las ratas macho RHA presentan un neurodesarrollo alterado e inmadurez cortical, de forma consistente con lo propuesto en trabajos previos (Elfving et al., 2019; Sønderstrup et al., 2023; Tapias-Espinosa et al., 2019, 2023) y con su perfil de fenotipos conductuales relacionados con la esquizofrenia (p. ej., hiperactividad, hipersensibilidad a la sensibilización locomotora y dopaminérgica tras tratamiento crónico con psicoestimulantes, y déficits en interacción social, en PPI, y en varias tareas cognitivas). Los resultados obtenidos en el estudio 2, sugerirían que las hembras RHA podrían presentar también dicha inmadurez corticofrontal (y/o hipocámpica), dados los déficits que muestran en PPI y funciones cognitivas, aparte de otras alteraciones (p. ej., hiperactividad, empeoramiento de la conducta maternal, etc.).

Más importante aún, los resultados moleculares muestran, por vez primera, que en el global de los genes valorados el efecto del tratamiento NH es potente y significativo, particularmente en ratas RHA adolescentes. Tales efectos pueden ser interpretables como positivos, puesto que el NH mejora la PPI e incrementa la exploración en el test NOE, en coherencia con otros efectos emocionales y cognitivos hallados en trabajos previos (p. ej., Aguilar et al., 2002; Río-Álamos et al., 2015, 2017, 2019), y, a su vez, incrementa la interacción social en ratas RHA adultas (ver también Sampedro-Viana et al., 2021, 2023a).

En la medida en que está bien establecido que el NH produce positivos y perdurables efectos neuro-madurativos y de neuroplasticidad (p. ej., Antoniazzi et al., 2014; Fernández-Teruel, 2021, 2022; Meaney et al., 1988; Stamatakis et al., 2009), nuestros hallazgos sugieren que la estimulación neonatal estaría favoreciendo la maduración cortical en las ratas RHA. Además, esto está en línea con la gran cantidad de evidencia sobre los efectos positivos duraderos del NH en las respuestas emocionales, de estrés y de afrontamiento, comportamiento social, y procesos relacionados con la atención y cognición, observados en ratas y ratones de gran variedad de cepas, incluidas las RLA y RHA adultas (p. ej., Escorihuela et al., 1994, 1995; Fernández-Teruel, 2021, 2022; Fernández-Teruel et al., 1997, 2002; Fu and Depue, 2019; Meaney et al., 1988; Raineki et al., 2014; Río-Álamos et al., 2019; y referencias en dichos artículos).

5. CONCLUSIONES

1. En ratas macho HS, un déficit relativo de PPI está asociado a una menor interacción social, peor memoria espacial de trabajo, menor flexibilidad cognitiva y empeoramiento de la memoria espacial a largo plazo.

2. Es factible identificar un fenotipo de riesgo para estudiar rasgos/síntomas cognitivos y negativos relacionados con la esquizofrenia en ratas de acuerdo a la estratificación según sus puntajes de PPI.

3. Las ratas hembra RHA muestran mayor interacción social que las ratas RLA, y el tratamiento de NH incrementa la conducta social y reduce las respuestas relacionadas con la ansiedad o inhibición conductual en las RLA.

4. Las ratas hembra RHA presentan déficit de PPI, así como peor memoria espacial de trabajo y de referencia, y flexibilidad cognitiva disminuida, comparadas con las ratas hembra RLA.

5. El tratamiento de NH produce cierta mejoría en la memoria de referencia espacial y una notable mejoría en la flexibilidad cognitiva en ratas hembra RHA.

6. Las ratas hembra RHA pueden ser un modelo con validez aparente para algunos fenotipos cognitivos-atencionales y conductuales relevantes para la esquizofrenia.

7. Las diferencias típicas entre cepas, como mayor actividad locomotora y conducta exploratoria, menor ansiedad, menor interacción social y PPI deficitaria, de los machos RHA frente a los RLA, se manifiestan en general desde la adolescencia y se acentúan en la adultez.

8. La edad tiene un efecto consistente sobre las diferentes conductas o variables tanto del test de IS (excepto acicalamiento) como del test de PPI (%PPI y Startle basal), las cuales se incrementan con la edad.

9. El tratamiento de NH aumenta la actividad locomotora inducida por novedad en machos de ambas cepas, reduce el acicalamiento en ratas RLA y mejora la interacción social y la PPI en los machos RHA.

10. En ratas control adolescentes, la expresión en CPF de los genes *Cdk5* y *Nrg1* está disminuida en las RHA, mientras que la expresión de *Snap25* está marcadamente aumentada.

11. En ratas control adultas, la expresión en CPF de los genes *Cables 1, Snap25, Nrg1, Homer 1 y Bdnf* está aumentada en las RHA.

12. En los machos RHA el tratamiento de NH incrementa, durante la adolescencia, la expresión génica en la fase postsináptica -*Ngr1, Homer1, Bdnf, Drd1, Grin2b*-, así como, a nivel presináptico, la expresión de *Cables1 y Cdk5*, mientras que disminuye drásticamente (hasta el nivel de las ratas RLA control) la expresión de *Snap25* tanto en las RHA adolescentes como en las adultas.

En conclusión, los resultados son compatibles con la idea de que las ratas RHA presentan una maduración deficitaria de la corteza prefrontal, que puede tener relación con las alteraciones en rasgos fenotípicos relacionados con la esquizofrenia observadas en esta cepa. Por ello es particularmente interesante el hallazgo de que el tratamiento de estimulación neonatal (NH) induce cambios en expresión génica sobre todo en las RHA adolescentes (en todos los genes) y adultas (en *Snap25*), lo que sugiere que el NH está influyendo a largo plazo sobre la función sináptica y procesos de neuroplasticidad que, a su vez, podrían estar en la base de los cambios fenotípicos observados en las ratas RHA que recibieron el tratamiento.

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