Modeling anxiety and schizophrenia-related symptoms in the Roman rats: effects of neanatal stimnlation on behavioral inhibition, attentional-cognitive processes and regional brain volumes.

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Doctoral Thesis presented by

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LIST OF ABBREVIATIONS

5-CSRTT: Five choice serial reaction time task

5-HTR: 5-hydroxytryptamine receptor

ACC: Anterior cingulate cortex

ACTH: Adrenocorticotropic hormone

APO-SUS: Apomorphine susceptibility

APO-UNSUS: Apomorphine unsuceptibility

BDNF: Brain derived neurotrophic factor

BI: Behavioral inhibition

BIS: Behavioral inhibition system

CA1-3: Cornu Ammonis (hippocampal sub-field)

CSF: Cerebrospinal fluid

DHc: Dorsal hippocampus

DMTP: Delayed matching-to-place task

DRL-20: Differential reinforcement of low rates of responding

EE: Environmental enrichment

ERα: Estrogen receptor alpha

ERβ: Estrogen receptor beta

ET: Embryo transfer

Hc: Hippocampus

HPA-axis: Hypothalamic-pituitary-adrenal axis

LSD: Lysergic acid diethylamide

LV: Lateral ventricles

mGluR: Metabotropic glutamate receptor

mPFc: Medial prefrontal cortex

MRI: Magnetic Resonance Image

MWM: Morris water maze

NAc: Nucleus accumbens

NADPH: Nicotinamide adenine dinucleotide phosphate

NGF: Nerve growth factor

NH: Neonatal handling

NMDA: N-metyl-D-aspartate

NOE: Novel object exploration

NT-3: Neurotrophin-3

PCP: Phencyclidine

PnC: Pontine reticular nucleus

PND: Postnatal day

PPI: Prepulse inhibition

PT: Place task

RHA: Roman High-avoidance

RLA: Roman Low-avoidance

RT: Reversal task

SPF: Specific pathogen free

SE: Standard environment

St: Striatum

TT: Transfer test

VHc: Ventral hippocampus

VTN: Ventro tegmental nucleus

ZM: Zero-maze

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CONTENTS

Modeling anxiety and schizophrenia-related symptoms in the *Roman* rats: effects of neonatal stimulation on behavioral inhibition, attentional-cognitive processes and regional brain volumes.

ABSTRACT

The present Doctoral Thesis reports a series of studies dealing with the effects of neonatal handling (NH) treatment on anxiety/stress and attentional-cognitive processes. All the studies were performed using the inbred *Roman* rat strains, which have been bi-directionally selected by their very good (RHA) vs extremely poor (RLA) acquisition of the two-way active avoidance response in the shuttle-box task. RLA rats are more anxious/fearful than their hypoemotional RHA counterparts, which in turn display higher impulsivity and worse attentional/cognitive performance than the former. Our work was aimed to address two main objectives: First, to evaluate the potential NH-improving effects on both, the anxiety/stress and attentional/cognitive profiles of both strains of rats. Secondly, to evaluate regional volumetric differences (using structural MRI) between both rat strains in brain areas related to anxiety, stress and cognition, as well as the eventual long-lasting effects of NH on anxiety- and attention/cognition-related brain regions. To this aim, three studies were conducted: Study 1 and Study 2 were mainly focused on anxiety/stress responses. Thus, we evaluated the effects of NH on anxiety/fear phenotypes, on post-stress hormonal responses and on the volume of specific brain areas involved in anxiety/stress processes (e.g. hippocampus, amygdala). Study 3 was aimed at evaluating some attentional/cognitive processes in RHA and RLA rats, as well as the potential NH effects on sensorimotor gating (prepulse inhibition, PPI) and executive functions (spatial learning/memory and working memory) in both rat strains. The volume of relevant brain areas (medial prefrontal cortex, anterior cingulate, nucleus accumbens, lateral ventricles) was also measured through MRI. Our results showed that: i) NH reduced anxiety and stress hormone responses in both strains of rats, with some effects being more marked in RLA rats, whereas NH also improved PPI and working memory in RHA rats; (ii) compared with their RHA counterparts, RLA rats showed enlarged amygdala, hippocampus and medial prefrontal cortex, and a dramatic reduction in the volume of the lateral ventricles; iii) NH induced a global reduction of amygdala volume in both strains, and also reduced hippocampus volume in RLA rats. In conclusion, NH induced improving effects on both strains of rats (anti-anxiety/stress effect in the RLA strain, and improvement of PPI and working memory in RHA rats). On the basis of PPI, cognitive and brain volumetric measures, it is proposed that the RHA rat strain could be a useful tool for the study of, at least some, relevant symptoms or neurobiological features relevant to schizophrenia.

RESUMEN

La presente Tesis Doctoral presenta una serie de estudios centrados en los efectos de la manipulación neonatal temprana (NH, "*neonatal handling"*) sobre respuestas de ansiedad/estrés y atencionales-cognitivas. Todos los estudios se llevaron a cabo utilizando las cepas de ratas *Romanas* consanguíneas, que han sido seleccionadas bi-direccionalmente por su excelente (RHA) vs extremadamente pobre (RLA) adquisición de la respuesta de evitación activa en dos sentidos. La cepa de ratas RLA es más ansiosa/temerosa que las hypoemocionales RHA, quienes a su vez presentan mayor impulsividad y peor rendimiento atencional-cognitivo. Nuestro trabajo está dirigido a dos objetivos principales: (i) evaluar posibles efectos positivos del NH en respuestas de ansiedad/estrés y en procesos atencionales/cognitivos en ambas cepas de ratas. (ii) evaluar diferencias volumétricas (resonancia magnética estructural) entre ambas cepas de ratas en regiones cerebrales relacionadas con la ansiedad, el estrés y procesos cognitivos, así como los efectos a largo plazo del NH sobre tales procesos y áreas cerebrales. Se realizaron tres estudios: los estudios 1 y 2 evalúan los efectos del NH sobre respuestas de ansiedad y respuestas hormonales post-estrés, así como sobre los volúmenes de regiones cerebrales relacionadas con la ansiedad o el estrés (hipocampo, amígdala). En el estudio 3 se evaluaron los efectos del NH sobre el filtraje atencional (PPI) y funciones ejecutivas (memoria/aprendizaje espacial y memoria de trabajo) en ambas cepas de ratas. Se incluyeron también medidas de volúmenes de áreas cerebrales asociadas a dichas tareas atencionales/cognitivas (córtex prefrontal medial, córtex cingulado anterior, núcleo accumbens, ventrículos laterales). Los resultados indican que: i) el NH reduce la ansiedad y las respuestas hormonales al estrés en ambas cepas de ratas, especialmente en las RLAs, mientras que también mejora los niveles de PPI y la memoria de trabajo en las ratas RHA; ii) respecto a las ratas RHA, la cepa de ratas RLA presenta mayor volumen de amígdala, hipocampo y corteza prefrontal medial, y un volumen mucho menor de los ventrículos laterales; iii) el NH reduce el volumen de la amígdala en ambas cepas, así como el volumen hipocámpico en la cepa de ratas RLA. En conclusión, el NH induce perdurables efectos positivos en ambas cepas (efectos anti-ansiedad/estrés en las RLA, y mejora de la PPI y de la memoria de trabajo en las RHA). Los resultados de PPI, cognitivos y volumétricos permiten proponer que las ratas RHA podrían ser consideradas como una herramienta útil para el estudio de al menos algunos déficits cognitivos y aspectos neurobiológicos asociados a la esquizofrenia.

1. INTRODUCTION

1.1.- Some basic considerations on animal models in psychiatric diseases

The use of valid animal models constitutes a crucial tool for progress in the understanding of the neurobiological mechanisms involved in psychiatric diseases and/or symptoms (Del Rio et al., 2014; Lipska, 2004). These models could be used to test the plausibility of (psychological, neurobiological) theories about the origin of the diseases, to explore the mechanisms involved, to investigate therapeutic and adverse effects of the drugs used for the treatment and to develop new potential treatments. To these purposes, animal models need to fulfill different validity criteria, such as face (i.e. similarity of symptoms with the human condition), predictive (i.e. similarity of treatment effects between the model and the human condition, and potential for discovering novel treatments) and construct validity (i.e. coherence of neurobiological mechanisms underlying the animal model and the human disorder or some relevant symptoms) (Bakshi, 2002; Bourgin et al., 2015). Of note, the different types of validity can be independent from one another and, for instance, an animal model can possess predictive and construct validity without having face validity (Bakshi, 2002).

As psychiatry disorders are usually highly complex, involving different clusters of symptoms even within the same diagnostic, it is a very difficult task for an animal model to mimic all the main features of the disease. Thus, it is common that an animal model only parallels some aspects (or symptoms) of the disorder rather than the whole disease. The advantage of this approach is that complex diseases could be divided into pieces of information in a more manageable manner, thus making it easier to study single symptoms/processes and their mechanisms.

Several strategies have been used to develop animal models of psychiatric diseases. It is not the purpose of the present Thesis to go across all of them, as they have been the focus of many reviews (e.g. Nestler & Hyman, 2010; Tobeña & Fernández-Teruel, 2010; Van Haaren, 1993; Willner, 1991). What is relevant for the present Thesis is that one such strategy is the selection of animals by the differential expression of a given phenotype. Thus, for instance, a rodent selective breeding program for a specific phenotype, proceeds by bidirectional selection and breeding of animals presenting either very high or very low scores in the target phenotype. Carrying out such bidirectional selection for several generations usually leads to a “high” and a “low” rat (or mouse) line or strain for that particular phenotype. This selective breeding technique is used to

get genetically-based extreme phenotypical (e.g. behavioral, pharmacological, neurochemical) scores and to study the genetic basis of complex phenotypes or traits (e.g. Aguilar, 2002; Driscoll et al., 1998, 2009; Broadhurst, 1969; Fernández-Teruel et al., 2002a; Gomes et al., 2013; Grahame et al., 2000). The supposition is that after several generations of selection, two different lines/strains of subjects would represent bidirectional extremes of the phenotypic selection criteria, and these differences will be potentiated based on the effects of the genes involved either in the high or the low presence of the phenotype (Aguilar, 2002). Doing so, it is possible to stimulate, under controlled laboratory conditions and in an accelerated manner, the evolution of adaptive traits by means of natural selection (i.e. the selection imposed by a specific criterion).

Multiple research programs of selective breeding have been successfully carried out based on different selection criteria. Many behavioral and pharmacological phenotypes have been used for bidirectional breeding and selection of rodent lines/strains. Some examples are: i) emotional phenotypes, such as high vs. low defecations in the open field test (Broadhurst, 1969), high vs. low percentage of time in the open arms of the elevated plus-maze (Liebsch et al., 1998), high vs. low ambulatory activity scores (Fujita et al., 1994), or high vs. low ultrasonic vocalization (Brunelli et al., 1996); ii) cognitive phenotypes, such as high vs. low sensorimotor gating measured by prepulse inhibition of the startle response (PPI) (Schwabe et al., 2007); and iii) specific pharmacological responses, such as apomorphine susceptibility (APO-SUS) vs. apomorphine unsusceptibility (APO-UNSUS) (Ellenbroek & Cools, 2002), effects of ketamine on tail flick, PPI and novel object recognition (Petrovszki et al., 2013), among many others.

1.2.- Essentials of the Roman rat lines/strains: a genetically-based model of psychiatry-relevant traits

In line with the above mentioned strategy of genetically-based models obtained through selective breeding, the studies included within the present thesis were performed with the inbred *Roman* rat strains. The *Roman* High- (RHA) and Low-Avoidance (RLA) rats have been bidirectionally selected and bred for their very rapid (RHA) vs. extremely poor (RLA) acquisition of the two-way active avoidance response in a shuttle box (Bignami, 1965; see Figure 1). Bignami started with the psychogenetic selection of

these rat lines in Rome in the mid 60s (Bignami, 1965). At present, three colonies of *Roman* rats exist: the swiss colony, established in Geneva since 1998 (Driscoll & Bättig 1982; Steimer & Driscoll, 2003), the italian colony established at the University of Cagliari since 1998 (Driscoll, 1998; Giorgi et al., 2007) and the spanish colony established at the Autonomous University of Barcelona since 1996 (being the only inbred colony of Roman rats, named RHA-I and RLA-I strains) (Driscoll et al., 1998; Escorihuela et al., 1999).

One interesting finding observed with many psychogenetically breeding programs, is that an array of related phenotypes are usually co-selected. In that context, in a recent study in which the inbred *Roman* rat strains have been re-derived through embryo transfer (ET) to Sprague-Dawley females, to generate specific pathogen free (SPF) RHA-I/RLA-I rats, offspring were phenotyped at generations 1 (G1, born from Sprague-Dawley females), 3 and 5 (G3 and G5, born from RHA-I and RLA-I from G2 and G4, respectively), and compared with generation 60 from the parental (non-SPF) colony (Río-Alamos et al., 2017b, *in press*). The tests included in the phenotyping battery were two-way avoidance acquisition, context-conditioned fear, open-field, novelty-seeking (novel object exploration), baseline startle, pre-pulse inhibition (PPI) and stress-induced corticosterone (stress-hormone response was only measured in G3 and compared with G60).

Figure 1

A **B**

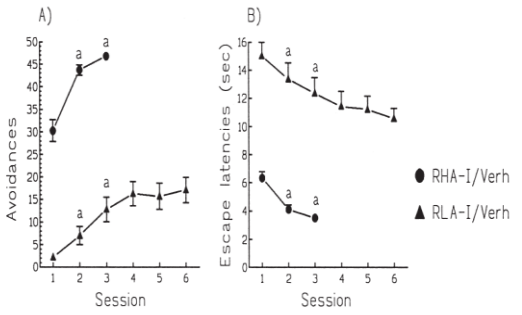

Figure 1.- (A) *Roman* rat performing the two-way active avoidance acquisition task a shuttle-box **(B)** the figure on the left shows the typical differences between RLA and RHA strains/lines, with the former performing 2-3 avoidances in the first 40-trial session compared with the 30 avoidances acquired by their RHA counterpart. On the right, the figure shows the escape latency (i.e. time elapsed to escape after the shock is delivered) by both strains/lines, with RLA rats receiving almost the 20s of the conditioned stimulus (i.e. the shock) (adapted from Escorihuela et al., 1999).

The main phenotypic characteristics of the *Roman* strains, that is those more closely linked to the selection criteria (i.e. two-way avoidance acquisition, inter-trial crossings in the shuttle box, context-conditioned freezing in the shuttle box), were conserved in G1 after embryo transfer, indicating a strong genetic basis associated with the selection trait/s. One co-selected trait, namely self-grooming (which is related to anxiety, stress and arousal) (Fernández-Teruel & Estanislau, 2016; Kalueff et al., 2016), presents also the characteristic "between-strain" differences at G1 (RLA-I > RHA-I), suggesting a possible genetic association between self-grooming and the selection traits. Conversely, typical differences between the strains in other co-selected traits (i.e. crossings in the open field test, exploration of a novel object, head-dipping in the hole-board test, baseline startle, PPI) are lost in G1 but are mostly rescued in G3 and G5, suggesting possible pre-/post-natal environmental influences on some co-selected phenotypes. Stress-induced corticosterone response, an index of the rat sensitivity to stress, is higher in RLA-I than in RHA-I rats at G3 (consistent with strain differences at G60).

In conclusion, the differences between RLA-I and RHA-I in the trait that constitutes the selection criteria (i.e. the number of avoidances in the shuttle-box) and in some closely linked phenotypes (i.e. inter-trial crossings in the shuttle box, context-conditioned freezing in the shuttle box) are present from the first generation after embryo transfer, suggesting a strong genetic basis for these traits. Moreover, the phenotypic changes observed between G1 and G3-G5 (i.e. the absence of between-strain differences in some co-selected traits at G1 but the presence of differences in these traits at G3-G5) are likely attributable to the influence of pre-/post-natal environmental factors (as a consequence of embryo transfer, at G1) (Río-Álamos et al., 2017b *in press*).

That a constellation of behavioral phenotypes tends to associate in order to shape complex traits (such as an "anxiety" trait) has also been shown in other species. For example, rhesus monkeys that have been characterized (behaviorally and physiologically) as chronically anxious showed, compared to their non-anxious counterparts, increased shyness and behavioral inhibition under novel situations, enhanced freezing responses and higher levels of corticotrophin-releasing factor and cortisol in front of humans (Kalin et al., 2001). Infant monkeys born from these fearful parents had a proneness to "inherit" the same anxious profile. Interestingly, the heritability of anxious temperament has also been documented in birds (Jones et al., 1991). These authors studied two strains of quail chicks psychogenetically selected for

showing short vs. long periods of tonic immobility (STI and LTI, respectively) when trapped by the experimenter. The selection led to stronger freezing responses, higher defecation scores and less exploratory behavior in novel situations in the LTI compared to STI. These phenotypical profiles were stable across generations of selective breeding (Jones et al., 1991).

Thus, bi-directional selection of animals for a given phenotype is possible across species, and the consistency of the selected differential (bi-directional) profiles across generations seems to be strongly influenced by genetic factors. In the case of the *Roman* rat strains/lines, the process of selective breeding for very good vs. very poor two-way avoidance acquisition has produced two differential profiles, the anxious/fearful and passive coper RLA rats and the non-anxious/impulsive and cognitively impaired RHA rats.

1.3.- The anxiety/fear-like profile of the Roman Low-avoidance (RLA) rats

As mentioned above, the rapid and very good acquisition of the two-way active avoidance response by RHA rats contrasts with the extremely poor avoidance acquisition shown by RLA animals, being this the more pronounced difference between the two strains/lines. However, the research carried out during the past four decades with the *Roman* rats has accumulated extensive evidence indicating that the differences between both strains/lines extend to many other behavioral and neurobiological phenotypes or traits. Thus, stress sensitivity, anxiety/fearfulness, coping style, impulsiveness and novelty/incentive/drug seeking are among the most prominent traits differentiating both strain/lines of rats (as we will see below) (see reviews by Driscoll et al., 1998, 2009; Giorgi et al., 2007; Steimer & Driscoll, 2003; see also Figure 2).

Figure 2

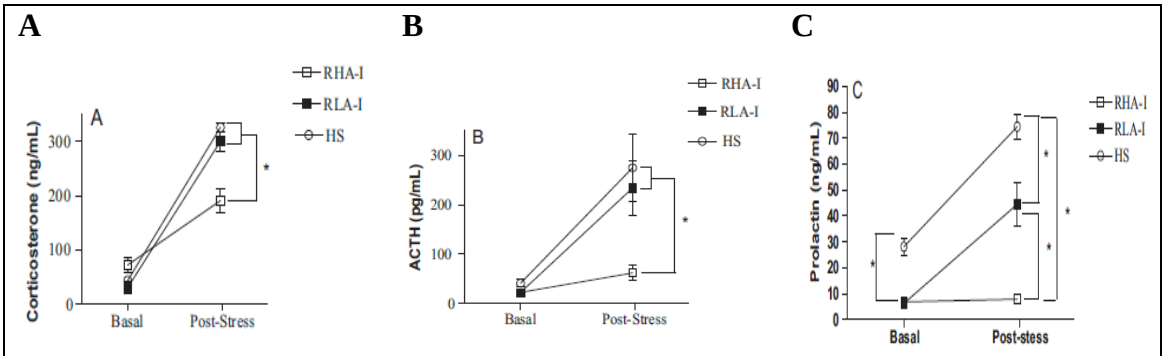

Figure 2.- Means ± SEM of basal and post-stress levels of corticosterone **(A)**, ACTH **(B)** and prolactin **(C)** between RHA-I, RLA-I and a third group of heterogeneous rats (N/Nih-Hs). RLA-I showed higher post-stress corticosterone, ACTH and prolactin concentrations in comparison to RHA-I rats, with no differences in their basal levels. (adapted from Díaz-Moran et al., 2012)

In this regard, for instance, compared to their RHA counterparts, RLA rats display elevated concentrations of stress-induced hormonal responses (ACTH, corticosterone and prolactin) (e.g. Carrasco et al., 2008; Díaz-Morán et al., 2012; Driscoll et al., 1998; Steimer & Driscoll, 2003, 2005; see Figure 2), as well as enhanced levels of anxiety/fear-related responses in several unconditioned situations, such as "novel object exploration", open field test, hole-board test, elevated plus-maze and zero-maze tests, light/dark box test and baseline acoustic startle response (e.g. Díaz-Morán et al., 2012; Driscoll et al., 1998; Fernández-Teruel et al., 2002b; Escorihuela et al., 1999; López-Aumatell et al., 2009a-b; Steimer & Driscoll 2003; see Figure 3). Moreover, RLA rats also display increased signs of fear or anxiety under conditioned procedures/tasks such as context-conditioned fear, the Vogel's punished drinking test or fear-potentiated startle (e.g. Ferré et al., 1995; López-Aumatell et al., 2009a, Vicens-Costa et al., 2011, see Figure 3). Finally, in tasks involving frustrative reward down shift, RLA rats show greater signs of frustration (and response inhibition) than RHA rats (e.g. Papini et al., 2015; Rosas et al., 2007; Torres et al., 2005).

In conclusion, the RLA rats exhibit a predominantly passive/reactive coping style (e.g. freezing behavior, self-grooming) when facing anxiogenic/stressful situations, while the hypoemotional (low anxious) RHAs display an active/proactive coping style apparently aimed at taking control over the stressor (e.g. Carrasco et al., 2008; Díaz-Morán et al., 2012; Driscoll et al., 2009; Escorihuela et al., 1995, 1999; Fernández-Teruel et al.,

2002b; Piras et al., 2010, 2014; Steimer & Driscoll, 2003; see also Table 1 for the anxiety/stress profile of RLA rats).

Initially, Bignami (1965) thought that RLA rats were "dumber" than their RHA counterparts, in part because the two-way active avoidance task was widely considered to depend on learning and memory capabilities. It is currently well known, however, that the ability to acquire/learn the two-way active avoidance response is critically dependent on anxiety levels (Fernández-Teruel et al., 1991a-c), as the task entails a "passive avoidance/active avoidance" conflict during the initial stages of acquisition (i.e. a tendency to freeze that runs against a tendency to actively cross to the opposite compartment) which is known to be mediated by anxiety (Fernández-Teruel et al., 1991a-c; Gray, 1982; Gray & McNaughton, 2000; Vicens-Costa et al., 2011). In fact, shuttle box avoidance acquisition has been proved to be inversely related to anxiety/fear, as anxiolytic drugs (and non-pharmacological treatments) accelerate, and anxiogenic drugs impair two-way avoidance acquisition (e.g. Escorihuela et al., 1994; Fernández-Teruel et al., 1991a-c; Gray, 1982; Levine, 1956; López-Aumatell et al., 2009b, Núñez et al., 1995; Vicens-Costa et al., 2011; Weiss, 1968). Moreover, some anxiolytic drug treatments have been shown to improve shuttle box acquisition/performance in RLA rats while being devoid of effects in the RHA line (Driscoll & Stübi, 1985; Escorihuela et al., 1995; Fernández-Teruel et al., 1991b).

However, although the involvement of anxiety in two-way active avoidance acquisition is clear, and the emotional differences between the *Roman* lines/strains have been firmly established, the issue on the possible differential cognitive abilities of the *Roman* lines/strains (i.e. the possible "dumbness" of the RLAs) generated an early interest which raised a series of studies. Thus, using the swiss RHA/Verh and RLA/Verh outbred lines, it was demonstrated that RLA rats displayed better spatial learning/memory, measured in the aquatic Hebb-Williams maze (Nil & Bättig, 1981), better working memory and spatial strategies in the Morris water maze (Driscoll et al., 1995; Escorihuela et al., 1995), as well as better performance in the DRL-20 bar-pressing task for food reward (Zeier et al., 1978) than their RHA counterparts. Likewise, Willig et al. (1991, 1992) also reported that RLA rats outperformed RHAs in an object recognition test and in an eight-arm radial maze. More recently, the superiority of RLA vs. RHA rats in spatial learning/memory has been replicated in our laboratory in both the outbred lines (Aguilar et al., 2002) and the inbred strains

(Martínez-Membrives et al., 2015; Oliveras et al., 2015, 2016), and it has also been reported that RLA rats perform better than RHAs in a delay-discounting instrumental task and in the five choice serial reaction time task (5-CSRTT; Klein et al., 2014; Moreno et al., 2010), thus confirming the higher impulsivity of RHA rats.

In summary, the RLA line/strain of rats has been considered as a valid animal model for the study of anxiety/fear and stress sensitivity responses, gathering some face, predictive and construct validity (e.g. Carrasco et al., 2008; Díaz-Morán et al., 2012; Driscoll & Bättig, 1982; Driscoll et al., 1998, 2009; Escorihuela et al., 1995, 1999; Fernández-Teruel et al., 1997; Ferré et al., 1995; Giorgi et al., 2003a; López-Aumatell et al., 2009a-b; Martin et al., 1982; Meyza et al., 2009; Steimer & Driscoll 2003, 2005), and thus they have been proposed as an useful tool for neurobiological and genetic studies of anxiety, fear and stress mechanisms (e.g. Carrasco et al., 2008; Driscoll et al., 2009; Fernández-Teruel et al., 1997, 2002a; Johannesson et al., 2009; Steimer & Driscoll, 2003).

Figure 3.

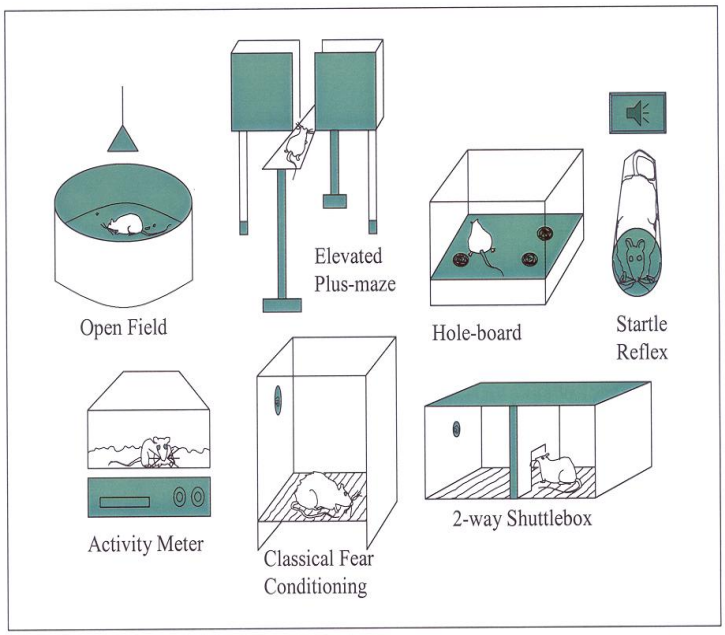

Fig. 3.- Some examples of anxiety-related tests/tasks: open field, elevated plus-maze, hole-board, startle reflex, fear conditioning, acquisition of two-way active avoidance. In all of them RLA rats display higher anxious/fearful responses/profiles than RHA rats. Conversely, in the "Activity meter", which measures baseline (non-stressed) activity, both lines/strains display similar amounts of locomotor-exploratory activity (adapted from Tobeña & Fernández-Teruel, 2010).

1.4.- The schizophrenia-like profile of the Roman High-avoidance (RHA) rats

On the other hand, different lines of research have indicated that RHA rats display several phenotypes that could be related to schizophrenia-relevant symptoms. For instance, they show impaired acquisition and retention of fear conditioning in different paradigms (Díaz-Morán et al., 2012; López-Aumatell et al., 2009a), worse performance in spatial reference and working memory tasks (Aguilar et al., 2002; Nil & Bättig, 1981) as well as in non-spatial tasks (Moreno et al., 2010; Zeier et al., 1978). They also showed higher locomotion under novelty conditions, higher preference for novelty and drugs of abuse and rewarding substances (Corda et al., 2005; Fattore et al., 2009; Fernández-Teruel et al., 2002c), impaired prepulse inhibition (Del Rio et al., 2014) and deficits in latent inhibition (Fernández-Teruel et al., 2006). In addition, they also showed augmented mesocortical dopaminergic response to stress (Giorgi et al., 2003a) and enhanced locomotor as well as mesolimbic dopaminergic sensitization to repeated dopaminergic psychostimulant administration (Corda et al., 2005; Giorgi et al., 2005, 2007; Guitart-Masip et al., 2008). From a neurochemical/neuromorphological point of view, evidence for decreased hippocampal function has also been demonstrated in RHA rats (Garcia-Falgueras et al., 2012; Meyza et al., 2009; Sallés et al., 2001).

The profile described above suggests that the RHA strain/line may have some value for modeling some of the relevant symptoms of schizophrenia (Lipska, 2004; Piontkewitz et al., 2011; Swerdlow & Light, 2015; Weiss & Feldon, 2001), as we will see below.

Schizophrenia, affecting 1% of total population, is considered a complex multifactorial disease (involving genetic, environmental and neurodevelopmental factors) that is characterized by the presence of positive (thought disorder, hallucinations and delusions), negative (social withdrawal, anhedonia and emotionally flat) and cognitive symptoms (deficits in sensorimotor gating, latent inhibition and executive functions) (Piontkewitz et al., 2012; Weiss & Feldon, 2001).

The complexity and diversity of the schizophrenia-related symptoms make it difficult to focus on the entire constellation of symptoms. Thus an ideal animal model will not necessarily present all the schizophrenia-relevant symptoms just as schizophrenic patients do not exhibit every possible symptom. The current vision suggests that animal models should mimic several key features of the disease, and if so, it would be of special value that some of these relevant features were also associated to some

susceptibility genes or neural alterations, hypothetically relevant for the disorder (Powell & Miyakawa, 2006).

A common strategy used in schizophrenia research and modeling has relied on drugs as an indirect tool to get knowledge on possible neurochemical mechanisms involved in the disorder. Thus, if a dopaminergic psychostimulant induces locomotor sensitization or impairment in some executive function, it would suggest that dopamine might be involved in the disease. Likewise, if a serotonin agonist has psychotomimetic effects (i.e. elicits a psychotic state), this may give support to the contention that serotonin is also involved. If a drug that acts as a dopamine or serotonin antagonist alleviates schizophrenic symptoms, then this is taken as an evidence lending support to the role of these particular neurotransmitters in schizophrenia. Typical neuroleptics (chlorpromazine, haloperidol), which block dopamine D2 receptors, showed antipsychotic effects, thus leading (or contributing) to the formulation of the "dopaminergic hypothesis" (Sawa & Snyder, 2002; Snyder, 2008). The "serotonin hypothesis" comes from findings in which hallucinogens such as LSD acts as agonists at 5-HT2ARs (serotonin 2A receptors), while atypical neuroleptics (clozapine, olanzapine, risperidone) are antagonists at these receptors (González-Maeso et al., 2008; González-Maeso & Sealfon, 2009). Some antagonists of the NMDA subtype of glutamate receptor, such as phencyclidine (PCP), also mimic schizophrenic symptoms, and jointly with the anti-schizophrenic-like effects of some mGluR2 agonists (e.g. LY2140023) have led to the "glutamate hypothesis" of schizophrenia (González-Maeso et al., 2008; Patil et al., 2007).

In that context, turning back to the *Roman* rats, besides the above mentioned dopaminergic characteristics of both strains/lines (Giorgi et al., 2007; Tournier et al., 2013) it has been shown that inbred RHA-I rats exhibit increased 5-HT2A receptor binding and a dramatically reduced density of mGluR2 receptors in the prefrontal cortex and hippocampus (Klein et al., 2014). Of note, such a profile of the 5-HT2A/mGluR2 complex in RHA rats resembles that found in drug-free schizophrenic patients (e.g. Gonzalez-Maeso et al., 2008, 2009). Hence, selective breeding of RHA rats has apparently led to co-selection of some behavioral (e.g. PPI, latent inhibition, working memory deficits) and neurochemical (e.g. increased central dopaminergic tone, alterations of 5-HT2A/mGluR2 complex) traits that make this strain/line a putative model of some schizophrenia-relevant features.

Another well documented endophenotype that has been linked to some schizophrenia-related symptoms came from magnetic resonance studies. Since the first MRI study in schizophrenia (Smith et al., 1984), several studies reported robust evidence of brain abnormalities in the volume of specific brain regions that theoretically might be associated to some schizophrenia-like phenotypes (see review Shenton et al., 2001). Thus, it seem that one of the most consistent findings reported are the reductions in the amygdala, hippocampus and prefrontal cortex volume, as well as enlarged volume of the lateral ventricles (Shenton et al., 2001). In this context, rodent studies focused on the effects of excitotoxic lesions (mostly limited to hippocampus or medial prefrontal cortex), showed to decrease PPI, enhance amphetamine sensitization and locomotor activity, impair spatial learning and working memory and disrupt latent inhibition, as well as a decrease in the volume of the lesioned brain region (Bertrand et al., 2010; Black et al, 1998; Chambers et al., 1996; Déziel et al., 2015; Grecksch et al., 1999; Labbate et al., 2014; Lipska et al., 2002). In addition, excitotoxic lesions of rodent hippocampus or prefrontal cortex seems to be associated to an enlargment of the volume of the lateral ventricles (Chin et al., 2011; Schneider & Koch, 2005). On the other hand, isolation rearing studies with rodents, known to induce schizophrenia-like responses, also showed to decrease hippocampus and prefrontal cortex volume, with detrimental effects on PPI (Day-Wilson et al., 2006; Schubert et al., 2009), and neuronal activity of prefrontal cortex is reduced in rats selectively bred for deficient sensorimotor gating (Alam et al., 2015). Regarding the *Roman* rats, the RHA strain seems to cohere with the above mentioned findings as they exhibit isolation rearing-induced PPI deficits and long-term reference memory impairments (Oliveras et al., 2016) as well as decreased hippocampal and medial prefrontal cortex function (Garcia-Falgueras et al., 2012; Meyza et al., 2009; Sallés et al., 2001). In this context, we considered interesting to evaluate whether a (let us say) "positive" environment manipulation, such as neonatal handling (NH) treatment (see below), is able to induce beneficial effects on the cognitive schizophrenia-related symptoms of the RHA rats (i.e. reverse the cognitive impairments) and also evaluate the *Roman* rat strains in the volume of specific brain regions supposedly associated to these phenotypes (see Table 2 for the schizophrenia-related profile of the RHA).

TABLE 1.- Summary of the profile of RLA rats in some models, behaviors and neural aspects relevant to anxiety/fear and stress.

Test/Procedure	Type of response	Strain with higher scores
Novelty (unconditioned conflict)-based tests		
Open field	Activity	**RHA**
	Defecations	**RLA**
	Self-grooming	**RLA**
Novel cage	Defecations	**RLA**
Elevated Zero-maze and Plus-maze	Time in open sections	**RHA**
	Entries in open sections	**RHA**
	Number of head dips	**RHA**
Holeboard	Defecations	**RLA**
	Self-grooming	**RLA**
	Head-dipping duration	**RHA**
Black/White Box	Crossing latency	**RLA**
	Self-grooming latency	**RHA**
Hyponeophagia	Latency to start eating	**RLA**
	Self-groomig duration	**RLA**
	Defecations	**RLA**
Conditioned anxiety/fear, frustration and others		
Fear-potentiated startle response and baseline startle response	Acoustic startle response	**RLA**
Frustration following reward down-shift (Instrumental Successive Negative Contrast)	Decrease of instrumental responses to obtain a reward	**RLA**
Cue-conditioned and Context-conditioned Freezing (Aversive Classical Conditioning)	Defecations	**RLA**
	Freezing time	**RLA**
Hormonal and Neurochemical profile		
ACTH, Corticosterone and Prolactin response levels to stress		**RLA**
ACTH response level after CRF administration		**RLA**
Mesolimbic Dopaminergic sensitization to psychostimulants		**RHA**
Mesocortical dopaminergic response to stress		**RHA**
Neurobiology/ Neural aspects		
Hippocampal and amygdala function (*C-fos*) under different novel (anxiogenic) environments		**RLA**
Hippocampal and amygdala neuronal density		**RLA**

Table 1.- *Adapted from Fernández-Teruel et al., 1997.*

TABLE 2.- Profile of RHA rats in models, behaviors and neural aspects relevant schizophrenia.

Schizophrenia-related symptoms	Strain
Positive symptoms	
Higher locomotor activity in response to novelty	**RHA**
Higher locomotor/stereotypic responses to DA agonists (apomorphine)	**RHA**
Higher locomotor and dopaminergic sensitization to amphetamine	**RHA**
Negative symptoms	
Better nesting behavior (quality of the nests)	**RLA**
Higher aggressive behavior in resident intruder test (Social behavior)	**RLA**
Cognitive symptoms	
Worse working memory	**RHA**
Lower prepulse inhibition levels	**RHA**
Disrupted latent inhibition (LI)	**RHA**
Impaired efficiency and increase premature responses and impulsivity in the 5-choice serial reaction time test (5-CSRTT)	**RHA**
Neurochemical/neuroanatomical phenotypes	
Increased mesolimbic dopamine responses to DAergic agonists	**RHA**
Increased mesocortical dopamine responses to stress	**RHA**
Increased hippocampal function and neuronal density in CA fields/layers	**RLA**
Enhanced expression of 5-HT2A receptor in prefrontal cortex	**RHA**
Dramatic reduction of mGluR2 receptor expression in hippocampus and striatum.	**RHA**

Table 2. *Adapted from Del Rio et al., 2014*

1.5.- Neonatal handling (NH) treatment: environmentally programming behavioral and neurobiological profiles

Early-life experiences are known to potentially induce long lasting effects (positive and negative) in mammals and humans, depending on several conditions such as duration, intensity, time of appearance and level of controllability of the stressor, among other factors (Raineki et al., 2014; Sharma et al., 2016). As the interplay between environment and genes could be critical in the outcomes related to psychiatric diseases, many basic studies with laboratory animals have used environmental treatments or manipulations in order to see whether they may produce enduring beneficial or deleterious outcomes in the development of particular disorders or disease symptoms.

In our case, considering that bidirectional selection of the *Roman* rats led to co-selection of other differential phenotypes between the strains/lines (e.g. anxiety/fearfulness and stress sensitivity in RLA rats; impulsivity and attentional/cognitive impairments in RHA), we were interested to evaluate whether an environmental treatment, namely neonatal handling (NH), would be able to induce enduring beneficial effects in both strains (i.e. anxiolytic and stress-reducing effects in RLA rats and cognitive/attentional improvements in the RHA strain).

Levine´s studies were the first to demonstrate the long-term effects induced by NH treatment in rats (Levine, 1956). In these novel experiments Levine showed that brief periods (less than 15 min) of individual separation of the pups from their mother, leaving each pup individually in a novel small cage lined with paper towel, and applying such procedure daily during the first 21 days of life, were associated to permanent reductions in the HPA-axis stress response evaluated during adulthood (Denenberg et al., 1962; Fernández-Teruel et al., 2002; Levine, 1956, 1962; Levine et al., 1967). The reduction of sensitivity to stressful situations (or the increase of resistance to stressful situations), in terms of a better response and regulation of the HPA axis in rats treated with NH, are associated to a long-term reduction of fear/anxiety responses in both unconditioned or novelty situations (e.g. Bodnoff et al., 1987; Fernández-Teruel et al., 1990, 1997, 2002; Ferré et al., 1995b; Núñez et al., 1995, 1996) and aversively-conditioned or learned conflict tasks (Escorihuela et al., 1991, 1992, 1994a-b; Levine, 1956; Levine et al., 1967; Núñez et al., 1995, 1996).

Moreover, it has also been reported that NH treatment leads to better performance in cognitive tasks (working memory, spatial and reference memory) during adulthood and prevents hippocampal neurodegeneration related to aging (e.g. Aguilar et al., 2002; Cañete, 2011; Cañete et al., 2015; Escorihuela et al., 1995; Fernández-Teruel et al., 1997, 2002; Meaney et al., 1988; Raineki et al., 2014).

Thus, NH-treated rodents appear to have an improved ability to adapt, or to efficiently cope with challenging/stressful environmental conditions, and also to perform better in some cognitive tasks (such as hippocampal-dependent tasks, for example). These NH effects have been confirmed by many studies showing that the improving effects extend to a wide variety of test/tasks and to different strains/lines of rats (and mice) with remarkable long lasting effects (e.g. see Bodnoff et al., 1987; Cañete et al., 2015; Fernández-Teruel et al., 1990, 1991, 1997, 2002; Ferré et al., 1995b; McIntosh et al., 1999; Raineki et al., 2014), although absence of effects, task-specific, and sex-specific effects have also been reported (Raineki et al., 2014).

It has been proposed that three components of NH are central for the treatment to exert its positive effects over the organism's development. These three factors are: i) isolation/maternal separation, ii) exposure to a novel environment and iii) tactile stimulation. First, it seems that the mothers provide higher levels of attention to their pups (increased care behaviors such as licking and grooming, more nest building, and also more active nursing postures) once the pups are returned to the home-cage after a few minutes (usually less than 15 minutes) of separation. In addition, in second place, it has also been demonstrated that besides these changes in maternal behavior, the stimulation provided to the pups by 3-15 minutes of isolation (i.e. the decrease of body temperature, the exposure to a novel environment, etc.) plays also an important role in the long-term beneficial effects of the treatment (Macrì et al., 2008; Tang et al., 2006). Thirdly, other studies have also shown that postnatal tactile stimulations (i.e. neonatal gentle "strokes" or "massages", not including isolation nor maternal separation) are able to induce NH-like cognitive and neurobiological effects on a long-term basis in rodents (see Daskalakis et al., 2009; Denenberg, 1999; Imanaka et al., 2008; Zhang & Cai, 2008). Of note, similar "tactile/cinestesic" neonatal stimulation (also called neonatal "massage" therapy) procedures also produce long-lasting positive psychobiological/neurodevelopmental consequences in premature children (Field, 2016; Kuhn et al., 1991; Schanberg & Field, 1987).

Considering the differential genetically-based phenotypes of the *Roman* rat strain/lines, in the present Thesis we have conducted three studies aimed at evaluating whether the NH treatment would be able to induce positive long-lasting effects in the inbred *Roman* rat strains. Specifically, as RLA rats exhibit relatively high anxious and stress sensitivity traits, the aim of our studies was to evaluate whether NH treatment was able to reverse such an anxiety profile. On the other hand, we also wanted to evaluate whether NH treatment would be able to improve the cognitive impairments observed in the RHA strain. We expected either anxiolytic effects of the treatment on RHA rats, because of ground effects, nor positive cognitive outcomes of NH on the RLA strain, because of ceiling effects. To this aim, behavioral anxiety-related and schizophrenia-relevant traits, hormonal responses to stress (corticosterone and prolactin) and volumes of specific brain regions (i.e. structural MRI images) were measured in control and NH-treated *Roman* rats in the present work.

2. OBJECTIVES

2.1.- General objectives:

1. To evaluate potential effects of neonatal handling (NH) treatment on the differential anxiety/fear and stress sensitivity profiles, as well as on attentional/cognitive phenotypes/endophenotypes of the inbred *Roman* High- and Low-avoidance rat strains.

2. To characterize the inbred *Roman* rat strains with regard to the volume of brain regions relevant to anxiety and cognition, and to evaluate the effects of NH on these regions.

2.2.- Specific objectives

2.2.1.- Study 1: "Neonatal handling decreases unconditioned anxiety, conditioned fear, and improves two-way avoidance acquisition: a study with the inbred Roman high (RHA-I) and low-avoidance (RLA-I) rats of both sexes".

1. To evaluate the long-lasting effects of NH on unconditioned and conditioned anxiety/fear-related responses in the inbred *Roman* rat strains, with special emphasis on the RLA rats.
2. To evaluate, for the first time, whether NH effects on anxiety/fear in the *Roman* rat strains in both sexes, in order to study "treatment x sex" effects.

2.2.2.- Study 2: "*Neonatal handling enduringly decreases anxiety and stress responses and reduces hippocampus and amygdala volume in a genetic model of differential anxiety: Behavioral-volumetric associations in the Roman rat strains".*

1. To evaluate possible between-strain differences in the volume of specific brain regions related to anxiety/fear and stress processes in the *Roman* rat strains.
2. To evaluate whether NH treatment is able to induce changes in the volume of specific brain regions (e.g. hippocampus, amygdala) related to anxiety/fearfulness in the inbred *Roman* rat strains, with special emphasis in the RLA strain.
3. To evaluate whether NH treatment is able to enduringly reduce stress hormone responses in the *Roman* rat strains.
4. To evaluate whether there are significant associations among volumetric measures of brain regions and the different anxiety-related behavioral responses.

2.2.3.- Study 3 (Annex 1): *"Neonatal handling treatment induced differential effects on PPI and improves working memory in the Roman strain of rats"*

1. To evaluate the potential enduring positive effect of the NH treatment on attentional processes (prepulse inhibition, PPI) and cognitive performance (executive functions, spatial learning/memory) in the *Roman* rats, with special emphasis in the RHA strain.
2. To evaluate between-strain differences in the volume of brain regions related to cognition which are often affected in schizophrenia patients (e.g. medial prefrontal cortex, lateral ventricles).

3. RESULTS

3.1.- STUDY 1: "*Neonatal handling decreases unconditioned anxiety, conditioned fear, and improves two-way avoidance acquisition: a study with the inbred Roman high (RHA-I) and low-avoidance (RLA-I) rats of both sexes".*

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Neonatal handling decreases unconditioned anxiety, conditioned fear, and improves two-way avoidance acquisition: a study with the inbred Roman high (RHA-I)- and low-avoidance (RLA-I) rats of both sexes

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The present study evaluated the long-lasting effects of neonatal handling (NH; administered during the first 21 days of life) on unlearned and learned anxiety-related responses in inbred Roman High- (RHA-I) and Low-avoidance (RLA-I) rats. To this aim, untreated and neonatally-handled RHA-I and RLA-I rats of both sexes were tested in the following tests/tasks: a novel object exploration (NOE) test, the elevated zero maze (ZM) test, a "baseline acoustic startle" (BAS) test, a "context-conditioned fear" (CCF) test and the acquisition of two-way active—shuttle box—avoidance (SHAV). RLA-I rats showed higher unconditioned (novel object exploration test -"NOE"-, elevated zero maze test -"ZM"-, BAS), and conditioned (CCF, SHAV) anxiety. NH increased exploration of the novel object in the NOE test as well as exploration of the open sections of the ZM test in both rat strains and sexes, although the effects were relatively more marked in the (high anxious) RLA-I strain and in females. NH did not affect BAS, but reduced CCF in both strains and sexes, and improved shuttle box avoidance acquisition especially in RLA-I (and particularly in females) and in female RHA-I rats. These are completely novel findings, which indicate that even some genetically-based anxiety/fear-related phenotypes can be significantly modulated by previous environmental experiences such as the NH manipulation.

Keywords: neonatal handling, anxiety, inbred roman rats, two-way avoidance acquisition, coping style

Introduction

Neonatal handling (NH), typically administered to rodents during the first 3 weeks of life, is an environmental treatment that has often been used to study behavioral and neurobiological plasticity. The effects of this manipulation are well documented since the 1950s, when Seymour Levine provided the first demonstration that NH induced an enduring improvement in the ability

of rats to learn a two-way active avoidance task (Levine, 1956, 1957). These results have been confirmed by many studies showing that the improving effects of NH extend to a wide variety of tests/tasks and to different strains/lines of rats (and mice) with remarkable long lasting effects. Thus, a large amount of studies have shown that NH increases activity and specific exploratory behavior in rodents, in a variety of unconditioned anxiety/emotionality tests involving different degrees of novelty (e.g., Bodnoff et al., 1987; Escorihuela et al., 1994; Ferré et al., 1995a; Núñez et al., 1995, 1996; McIntosh et al., 1999; Fernández-Teruel et al., 2002a; Cañete et al., 2015), although absence of effects, task-specific effects, and sex-specific effects have also been reported (see review by Raineki et al., 2014). In addition, concerning its effects on conditioned fear/anxiety-related measures, studies with unselected rats have shown that NH enduringly reduces conflict-induced lick suppression and conditioned freezing (Núñez et al., 1996), accelerates two-way active avoidance acquisition (Escorihuela et al., 1992, 1994; Núñez et al., 1995), thus replicating and extending the original Levine's findings, and reduces learned helplessness (Tejedor-Real et al., 1998). NH has also been reported to decrease stress-induced corticosterone, ACTH, and prolactin secretion (e.g., Levine, 1957; Meaney et al., 1988, 1991; Núñez et al., 1996; Anisman et al., 1998; Raineki et al., 2014). Thus, from a behavioral and neuroendocrine perspective, NH-treated rodents appear to have an improved ability to adapt, or to efficiently cope with challenging/stressful environmental conditions. Finally, NH manipulation generally improves cognition in rats and mice under different spatial learning/memory paradigms, although such effects are strain- and sex-dependent (e.g., Wilson and Jamieson, 1968; Meaney et al., 1988; Zaharia et al., 1996; Fernández-Teruel et al., 2002a; Stamatakis et al., 2008; Raineki et al., 2014; Cañete et al., 2015). However, in the cognitive (learning and memory) domain there are controversial results, as with the exception of shuttle box avoidance acquisition (see refs. above), the NH procedure generally impairs aversive learning in several tasks (see review by Raineki et al., 2014).

One of the most validated genetic rat models for the study of fear/anxiety- and stress-related phenotypes is constituted by the Roman High- and Low-avoidance (RHA and RLA, respectively) rat lines/strains. They were initially selected and bred on the basis of their very good (RHA) vs. extremely poor (RLA) acquisition of the two-way active—shuttle box—avoidance response (Bignami, 1965; Driscoll and Bättig, 1982; Driscoll et al., 1998). Two inbred strains (RHA-I and RLA-I) derived from the original outbred (RHA/Verh and RLA/Verh) lines, are maintained at the Autonomous University of Barcelona since 1997 (Escorihuela et al., 1999; Driscoll et al., 2009), while colonies of the outbred RHA/RLA rat lines are maintained at Geneva (Switzerland; Dr. Steimer; e.g., Steimer and Driscoll, 2005) and Cagliari (Italy; Prof. Giorgi and Corda; e.g., Giorgi et al., 2007).

Learning a two-way avoidance task in a shuttle box involves a "passive avoidance/active avoidance" conflict during the initial stages of acquisition (i.e., a tendency to freeze–receiving the electric shock- runs against a tendency to actively cross to the opposite compartment -avoiding the insult-) which is mediated by anxiety (e.g., Wilcock and Fulker, 1973; Gray, 1982; Gray and McNaughton, 2000; Vicens-Costa et al., 2011). Accordingly, shuttle box avoidance acquisition has been shown to be inversely related to anxiety/fear (e.g., Weiss et al., 1968; Gray, 1982; Fernández-Teruel et al., 1991a,b; Escorihuela et al., 1993; Gray and McNaughton, 2000; López-Aumatell et al., 2009a,b, 2011; Vicens-Costa et al., 2011; Díaz-Morán et al., 2012). Not surprisingly, therefore, the extensive research conducted with the RLA and RHA rats over near four decades has led to the conclusion that anxiety/fearfulness and stress sensitivity are among the most prominent behavioral traits separating the two lines/strains. In fact, RLAs (both from the outbred lines and from the inbred strain) are more anxious and/or fearful than their RHA counterparts in a wide series of unconditioned and conditioned tests/tasks (e.g., Ferré et al., 1995b; Escorihuela et al., 1999; Steimer and Driscoll, 2003, 2005; Driscoll et al., 2009; López-Aumatell et al., 2009a,b; Díaz-Morán et al., 2012; Martinez-Membrives et al., 2015). Moreover, RLA rats display enhanced frustration responses following reward down-shift (e.g., Torres et al., 2005; Rosas et al., 2007; Sabariego et al., 2013) and higher stress-induced HPA-axis and prolactin responses than RHAs (e.g., Steimer and Driscoll, 2003, 2005; Carrasco et al., 2008; Díaz-Morán et al., 2012). To sum up, it is commonly accepted that, compared with RHAs, RLAs rats display increased anxiety, fearfulness, stress sensitivity, and a predominantly passive (reactive) coping style when facing situations involving conflict (e.g., Steimer and Driscoll, 2003, 2005; Díaz-Morán et al., 2012).

As mentioned earlier, NH procedure generally appears to improve the subjects' ability to adapt to, or to efficiently cope with conflicting and/or stressful conditions. However, most of the research on NH effects has been performed in one gender, usually male rats or mice. Interactions between NH and sex have been observed in some reports which evaluated NH effects in unselected rats of both sexes. To say just a few examples (see also "Discussion"): NH improved spatial learning (in the Morris Water Maze; MWM) only in males (Stamatakis et al., 2008) while, in different studies, spatial learning in the "Y" maze was improved by NH in females and impaired in males (Noschang et al., 2012), and long-term retention of inhibitory avoidance was impaired only in females (Kosten et al., 2007). The striking sex differences in the effects of NH tell us that gender must be considered as an important (or even crucial) variable in behavioral and neurobiological studies of NH induced effects and/or mechanisms.

Thus, the present study was aimed to evaluate whether the NH procedure is able to improve coping ability in both inbred Roman strains and sexes, with an especial focus on RLA-I rats. If so, we would expect that handled RLA-I rats present a more active coping style than untreated RLA-I animals, which would be reflected by unlearned and/or learned anxiety/fear measures. To this aim, non-handled (undisturbed) and NH treated inbred Roman Low- (RLA-I) and High-avoidance (RHA-I) rats of both sexes were evaluated in a test battery devoted to measure several types of unconditioned and conditioned anxiety/fear-related responses: a "novel object exploration" (NOE) test, the elevated zero-maze (ZM), a baseline acoustic startle response test (BAS), a context-conditioned fear (CCF) test and the acquisition of the two-way active avoidance (SHAV) task. This represents

the first time that the effects of NH on both unconditioned and conditioned anxiety/fear (including shuttle box avoidance acquisition) are evaluated in "inbred" Roman rats from both strains and sexes.

Materials and Methods

Animals

Pregnant inbred Roman High- (RHA-I) and Low-Avoidance (RLA-I) rats from our permanent colony at the Autonomous University of Barcelona (Medical Psychology Unit, Department Psychiatry and Forensic Medicine) were used in the present study. They were individually housed and were maintained with food and water freely available, with a 12-h light-dark cycle (light on 0800 h) and controlled temperature (22 ± 2°C). They were randomly distributed across the following experimental groups to which their offspring would be assigned: control animals, which were not disturbed until weaning (C), and animals that received neonatal handling (NH, see procedure below). All care was taken to avoid litter effects, by using a sufficiently large number of litters per group. Thus, each experimental group contained animals from at least 6 different litters. At postnatal day 1, litters were culled to a maximum of 12 pups (without any compensation for the number of males or females). After weaning (postnatal day 21st) the pups were housed in pairs of the same litter, sex and group in standard macrolon cages (50 × 25 × 14 cm) under the above conditions. Experiments were performed using 50 RLA-I and 29 RHA-I rats from the 59th generation of inbreeding. At the beginning of the experiments subjects were 2 months old (weight, 167 ± 20 g; mean ± *SD*; see **Table 1** for details of the sample). Experiments were performed during the light cycle, between 09:00 and 19:00 h in accordance with the Spanish legislation on "Protection of Animals Used for Experimental and Other Scientific Purposes" and the European Communities Council Directive (86/609/EEC) on this subject.

Procedure and Apparatus

Neonatal Handling (NH)

NH was given twice daily between postnatal days 1 and 21 (see Fernández-Teruel et al., 1992; Escorihuela et al., 1995; Steimer et al., 1998). The first daily handling session, administered in the morning (approximately between 9:30 and 10:30 h a.m.), consisted of first removing the mother from the litter and then placing the pups gently and individually in plastic cages (35 × 15 × 25 cm) lined with paper towel for a total period of 8 min. After 4 min in this situation, each pup was individually (and gently) handled and stroked for 3–4 s and returned to the same cage for the remaining 4 min. At the end of the 8-min period, each pup was gently handled for another 3–4 s and then returned to its homecage. When all the pups from one litter were back in their homecage, the mother was returned to it. The same procedure was conducted in the evening (2nd time; approximately at 5:00 h p.m.). NH was carried out in a room different from the animal room, maintaining the temperature at 24°C. NH finished at postnatal day 21. Weaning was done at postnatal day 21, after finishing the last NH session. Control (C) non-handled groups were left undisturbed, except for regular cage cleaning once a week, until weaning.

TABLE 1 | Animal samples and experimental goups.

Strain	Treatment group	Sex	Sample
RLA-I	Control (C)	♂	12 (final $n = 9$)*
	Handled (NH)	♂	17
	Control (C)	♀	12
	Handled (NH)	♀	12
RHA-I	Control (C)	♂	7 (final $n = 6$)*
	Handled (NH)	♂	8
	Control (C)	♀	7
	Handled (NH)	♀	8

**Final n = 9 and n = 6 in RLA-I and RHA-I control groups because of technical problems in several tests/tasks.*

Test 1: Novel Object Exploration Test (NOE)

In order to assess emotional reactivity (or behavioral inhibition under novelty, or "curiosity") a novel object exploration (NOE) test was conducted. The test consisted of the evaluation of the exploratory response of rats when a novel object was introduced in their home cage. Rats were 60 days-old at the beginning of the NOE test, and they were housed in pairs of the same sex, strain, and treatment condition. The test started by removing the food from the home cage (leaving only four pellets in each cage). One hour later, the novel object (graphite pencil Staedtler Noris, HB n°2) was perpendicularly introduced in their home cages through the grid cover, until it made contact with the cage bedding. To facilitate observation of the rats each individual cage was pulled from the rack about 15 cm, which allowed to score the latency to the first exploration (*LAT-NOE*; time spent until the first exploration of the novel object) and the total time (*Time-NOE*) spent exploring the pencil for each individual rat. The experimenter/observer was standing at 50 cm from the cage front. The NOE test lasted 3 min (see **Figure 1**).

Test 2: Elevated Zero Maze (EZM)

The maze, similar to that described by Shepherd et al. (1994) (1) comprised an annular platform (i.e., a circular corridor; 105 cm diameter; 10 cm width) made of black plywood and elevated to 65 cm above the ground level. It had two open sections (quadrants) and two enclosed ones (with walls 40 cm height). The subject (80 days-old) was placed in an enclosed section facing the wall. The apparatus was situated in a black testing room, dimly illuminated with red fluorescent light, and the behavior was videotaped and measured outside the testing room. Time spent in open sections (*ZM-T*), number of entries into open sections (*ZM-E*), and number of episodes of exploratory activity at the edge of the test, namely "head dips" (*ZM-HD*), were measured for 5 min (see López-Aumatell et al., 2008, 2009a; see **Figure 1**).

Test 3: Baseline Acoustic Startle Response (BAS)

Four sound-attenuated boxes (Sr-Lab Startle Response system, San Diego Inst., San Diego, USA) diffusely illuminated (10 w)

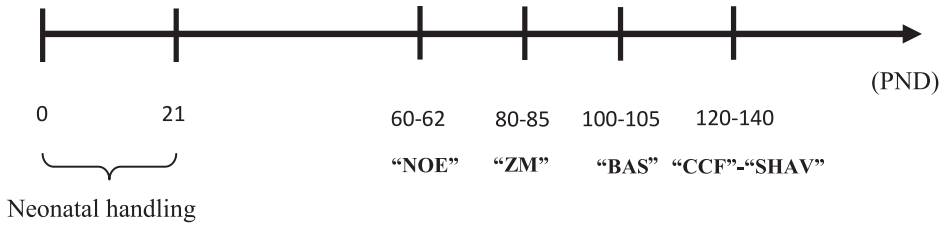

FIGURE 1 | Abbreviations: NOE, novel object exploration test; ZM, elevated zero-maze test; BAS, baseline acoustic startle; CCF, context-conditioned freezing; SHAV, two-way active –shuttle box- avoidance; PND, postnatal day.

were used (90 × 55 × 60 cm). Each box housed a Plexiglas cylinder (8.2 cm in diameter, 25 cm in length) with a grid placed in the bottom, resting on a plastic frame. For any test session each animal was placed in the cylinder, and movements of the cylinder resulting from startle responses were transduced by a piezoelectric accelerometer (Cibertec S.A. Madrid) into a voltage which was amplified, digitized and saved into a computer for analysis. The session started with 5 min of habituation. A white noise generator provided background noise of 55 dB. Then, 25 trials of acoustic startle stimuli of 105 dB and 40 ms of duration were delivered by a loudspeaker, mounted at distance of 23 cm above the plexiglas cylinder. The inter-trial interval (ITI) was 15 s in average (range 10–20 s). Startle response amplitude was defined as the maximum accelerometer voltage during the first 200 ms after the startle stimulus onset (see López-Aumatell et al., 2008; see **Figure 1**).

Tests 4 and 5: Context Conditioned Freezing (CCF) and Two-way Active—Shuttle Box—Avoidance Acquisition (SHAV)

The experiment was carried out with two identical shuttle boxes (Letica, Panlab, Barcelona, Spain) each placed within independent sound-attenuating boxes constructed of plywood. A dim and diffuse illumination was provided by a fluorescent bulb placed behind the opaque wall of the shuttle boxes. The experimental room was kept dark. The shuttle boxes consisted of two equally sized compartments (25 × 25 × 28 cm), connected by an opening (8 × 10 cm). Training consisted of a single 50-trial session for the RHA-I strain, and two 50-trial sessions, spaced 24 h apart, for RLA-I rats. RLA-I rats were trained twice as much as RHA-I rats because we did not expect any NH effect on RHA-I rats, due to roof effects (i.e., they usually attain a >60% avoidance response levels in the first 50-trial session). A 2400-Hz, 63-dB tone plus a light (from a small 7-w lamp) functioned as the CS (conditioned stimulus). The US (unconditioned stimulus) which commenced at the end of the CS, was a scrambled electric shock of 0.7 mA delivered through the grid floor. Once the rats were placed into the shuttle box, a 4-min familiarization period (without any stimulus) elapsed before training commenced. Each of the 50 (or 100 -in case of RLA-I rats-) training trials consisted of a 10-s CS, followed by a 20-s US. The CS or US was terminated when the animal crossed to the other compartment, with crossing during the CS being considered as an avoidance response and during the US as an escape response. Once a crossing had been made or the shock (US) discontinued, a 60-s inter-trial interval (ITI) was presented during which crossings (ITC) were scored within each block of trials. Freezing behavior, defined as the complete absence of movements except for breathing, was also scored (by a well-trained observer) during the 60-s inter-trial intervals of trials 2–5 as an index of context-conditioned fear (CCF; during trials 2–5 no rat made any avoidance response, i.e., all rats received electric shock in these trials). The measure of freezing during the inter-trial interval of trial 1 was excluded because it is not a proper measure of context conditioning.

The variables recorded were the number of avoidances (SHAV) and inter-trial crossings (ITCs), either grouped in blocks of 10 trials or accumulated in one (SHAV50, ITC50), or two (SHAV100, ITC100) sessions (e.g., see López-Aumatell et al., 2011; Díaz-Morán et al., 2012; see **Figure 1**).

Statistical Analysis

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

Pearson's correlation coefficients were performed among the main variables.

Factorial 2 × 2 × 2 ANOVAs ("2 strain" × "2 treatment conditions" × "2 sex") were applied to measures from NOE, ZM, and CCF tests, as well as for total measures of the shuttle box avoidance task. Appropriate repeated measures ANOVAs with "5-trial blocks" as within-subject factor were applied to BAS test ("2 strain" × "2 treatment conditions" × "2 sex" × "5 block" ANOVA), and to shuttle box avoidance acquisition with "10-trial blocks" as within-subject factor ("2 strain" × "2 treatment conditions" × "2 sex" × "10 block" ANOVAs).

Post-hoc Duncan's multiple range tests were applied to all dependent variables following significant ANOVA effects. A Student's *t*-test (independent samples) was also applied to avoidance results from male "control" and "NH" RLA-I groups, because we had the a priori hypothesis that NH treatment would improve avoidance acquisition in RLA-I rats. Significance level was set at $p \leq 0.05$.

Results

"Novel Object Exploration" Test (NOE)

The results of the NOE test (**Figures 2A,B**) showed that, compared to RHA-I rats, RLA-I animals presented higher latency

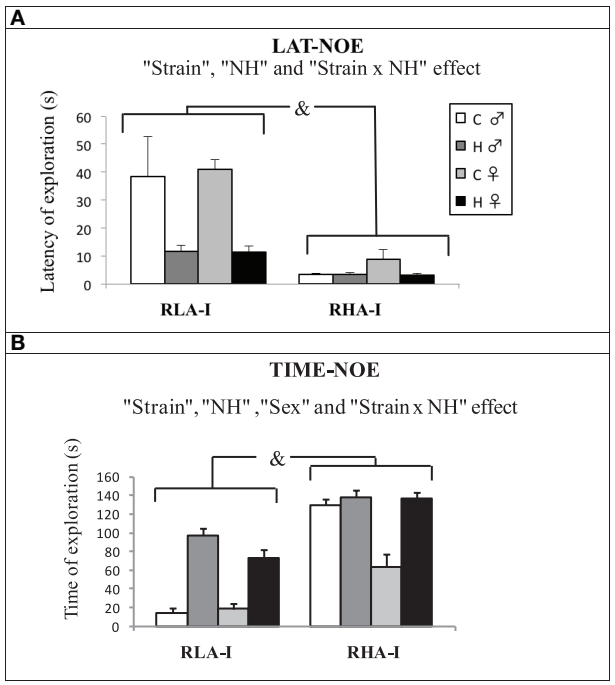

FIGURE 2 | Mean ± S.E.M of (A) "Latency –time elapsed—until the first exploration of the novel object" and (B) "Total time spent exploring the novel object" in Experiment 1 (NOE test). &, indicates the "Strain" effect, $p < 0.05$. Group symbols: C, control non-handled group; H, neonatally handled (NH) group.

(to explore for the first time the novel object; LAT-NOE) and less time spent exploring the novel object (TIME-NOE) ["Strain" effect on both parameters, $F_{(1,\ 78)} = 17.36$, $p < 0.001$, and $F_{(1,78)} = 118.30$, $P < 0.001$, respectively]. As expected, NH significantly reduced LAT-NOE and increased TIME-NOE in both rat strains ["NH" effect, $F_{(1,78)} = 9.66$, $p \leq 0.003$, and $F_{(1,78)} = 80.40$ $P < 0.001$, respectively]. A "sex" effect was found only on TIME-NOE [$F_{(1,\ 78)} = 13.08$, $p = 0.001$], indicating that females (particularly RHA-Is) spent overall less time exploring the novel object compared to males (**Figure 2B**). There were also "Strain × NH" interactions for LAT-NOE and TIME-NOE [$F_{(1,\ 78)} = 6.37$, $p \leq 0.01$, and $F_{(1,78)} = 5.32$ $P = 0.02$, respectively], as NH effects were globally stronger in RLA-I rats of both sexes.

"Elevated Zero Maze" Test (ZM)

The results of the ZM test (**Figures 3A–C**) showed "Strain" effects on ZM-E [$F_{(1,\ 78)} = 12.13$, $p \leq 0.001$], ZM-T [$F_{(1,\ 78)} = 7.29$, $p \leq 0.009$] and ZM-HD [$Fs_{(1,\ 78)} = 41.55$, $p < 0.001$], with RHA-I rats showing overall higher scores in the three parameters (**Figures 3A–C**). "NH" effects were found in ZM-T [$F_{(1,\ 78)} = 8.60$, $p \leq 0.005$] and in ZM-HD [$F_{(1,\ 78)} = 11.85$, $p \leq 0.001$], reflecting that neonatally-handled groups globally spent more time in open sections and performed more head dips than untreated animals (**Figures 3B,C**; see also Duncan's tests in **Figures 3B,C**).

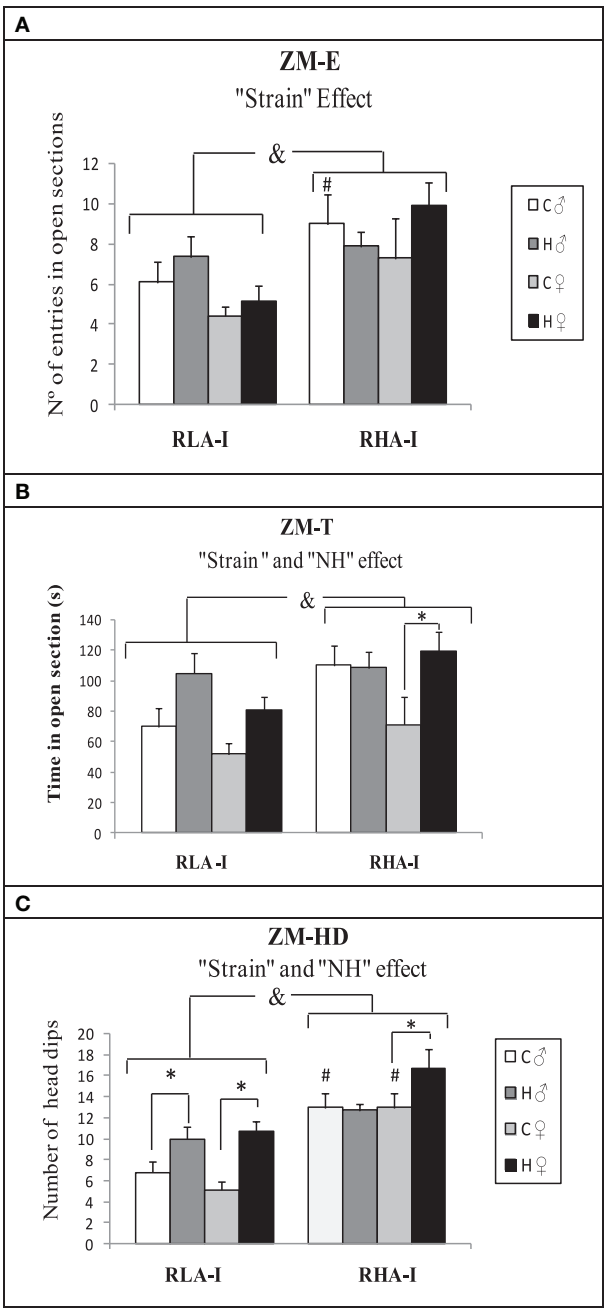

FIGURE 3 | Mean ± S.E.M. of (A) "Number of entries (ZM-E)," (B) "Time spent in open sections (ZM-T)" and (C) "Number of head dips (ZM-HD)" in Experiment 2 ("Elevated zero maze" test," ZM). &, indicates the "Strain" effect (see text for significance); *$p < 0.05$ between the groups indicated (Duncan's multiple range tests following significant ANOVA effects); #$p < 0.05$ vs. respective control (C) group of the RLA-I strain (Duncan's multiple range tests following significant ANOVA effects). Group symbols: C, control non-handled group; H, neonatally handled (NH) group.

"Baseline Acoustic Startle Response" Test (BAS)

Figure 4 shows the results of the BAS test. The repeated measures ANOVA ("2 strain" × "2 treatment conditions" × "2 sex" × "5 blocks of trials") indicated a "strain" effect, as taking the session

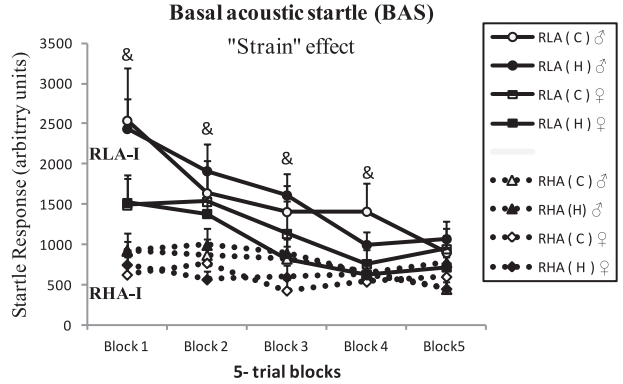

FIGURE 4 | Mean ± S.E.M of baseline acoustic startle response (BAS, in arbitrary units) across 5-trial blocks (Block1–Block5). &, significant ($p < 0.05$) "Strain" effects (factorial 2 × 2 × 2 ANOVAs) in these blocks. The overall repeated measures ANOVA also yielded a significant "Strain" effect, as indicated in the figure ($p < 0.001$, see text for more details). Group symbols: C, control non-handled group; H, neonatally handled (NH) group.

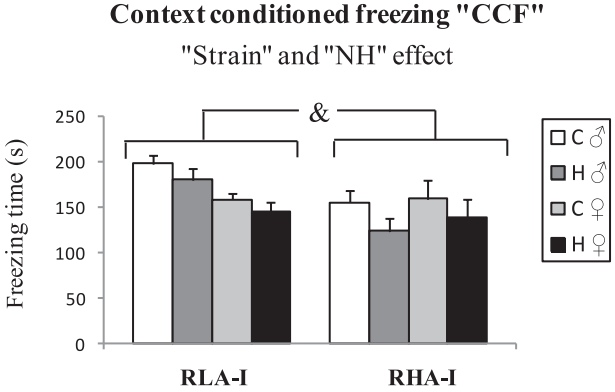

FIGURE 5 | Means ± S.E.M. of "Context conditioned freezing (CCF) in Experiment 4 ("The two-way active shuttle box avoidance acquisition"). &, indicates the "Strain" effect (see text for significance). Group symbols: C, control non-handled group; H, neonatally handled (NH) group.

as a whole, the RLA-I strain displayed higher acoustic startle response than the RHA-I strain ["Strain" effect, $F_{(1,\ 71)} = 12.26$, $p \leq 0.001$]. ANOVA also showed significant "Block" and "Block × Strain" effects [$F_{(3,\ 222)} > 22.22$, $p < 0.001$, and $F_{(3,\ 222)} = 9.11$, $p < 0.001$, respectively], indicating both an habituation effect (on both strains) as well as that such a habituation is relatively more marked in RLA-I rats (**Figure 4**). Further One-Way ANOVAs per each 5-trial block showed between-strain differences (i.e., overall higher BAS scores in RLA-I than RHA-I rats) in all blocks except in the last one [Block1–Block4, all $Fs_{(7,\ 78)} > 2.10$, all $p \leq 0.05$; see **Figure 4**]. No NH effect was observed.

"Context-Conditioned Freezing" ("CCF") and "Two-way Active—Shuttle Box—Avoidance Acquisition" Test ("SHAV")

Results of the "context conditioned freezing" (CCF) test are shown in **Figure 5**. One-Way ANOVA showed a global "Strain" effect, with the RLA-I groups performing more freezing behavior than the RHA-I strain ["Strain" effect, $F_{(1,\ 76)} = 6.79$, $p \leq 0.011$, **Figure 5**]. Interestingly, a global "NH" effect was also present, as NH decreased the time spent freezing in both strains ["NH" effect $F_{(1,\ 76)} = 4.11$, $p = 0.046$; **Figure 5**]. There was also a "Strain × Sex" effect, mainly because there was a trend for RLA-I female groups to show lesser freezing than their respective male groups, and that tendency was not present in RHA-I rats ["Strain × Sex" effect, $F_{(1,\ 76)} = 5.39$, $p = 0.023$; **Figure 5**].

Figures 6A–C shows the results of two-way avoidance (SHAV) acquisition. The repeated measures ANOVA applied to results from the first 50-trial session ("2 Strain" × "Treatment conditions" × "2 Sex" × "5 blocks of 10 trials") showed that RHA-I performed more avoidance responses than RLA-I rats ["Strain" effect, $F_{(1,\ 76)} = 462.7$, $p < 0.001$; **Figures 6A,B**] and also a global "NH" effect [$F_{(1,\ 76)} = 6.10$, $p = 0.016$; **Figures 6A,B**], with neonatally-handled animals performing overall more avoidances than untreated/control rats (**Figures 6A,B**). Duncan's test showed statistical differences between control and handled RHA-I females (in several 10-trial blocks—**Figure 6A** as well as in the whole 50-trial session—**Figure 6B**) as well as between control and handled RLA-I females (in several 10-trial blocks—**Figure 6A** and in the whole 50-trial session—**Figure 6B**), while a Student's *t*-test for independent samples showed differences between control and handled RLA-I males in the whole 50-trial session [$t_{(24)} = 2.68$, $p = 0.014$; **Figure 6B**. This *t*-test was applied because we had the—directed– a priori hypothesis that NH procedure would improve avoidance acquisition in RLA-I rats].

The repeated measures ANOVA of the first 50-trial session also showed "Block" and "Block × Strain" effects [both ANOVAs, $Fs_{(4,\ 69)} > 93.96$, $p \leq 0.001$; **Figure 6A**], thus respectively reflecting (i) the overall significant learning curves as well as (ii) that RHA-I rats learned much faster than RLA-I rats. There was also a "NH × Sex" effect [$F_{(1,\ 76)} = 5.24$, $p = 0.025$; **Figure 6B**], mainly because NH induced positive effects on avoidance acquisition of all groups except RHA-I males (**Figure 6B**).

Analysis of the whole 100 acquisition trials (i.e., the two training sessions) in RLA-I groups (repeated measures ANOVA, "2 treatment conditions" × "2 sex" × "10 blocks of 10 trials" as within-subject factor; SHAV100 trials in **Figure 6C**) showed a "NH" effect [$F_{(1,\ 46)} = 10.68$, $p = 0.002$; **Figures 6A,C**], with handled animals performing overall better than control rats (**Figure 6C**), and a "NH × Sex" effect [$F_{(1,\ 46)} = 4.24$, $p = 0.045$], as NH more markedly increased the number of avoidances in RLA-I females (see Duncan's test in **Figure 6C**) than in males. ANOVA also showed "Block," "Block × NH," and "Block × Sex" effects [all $Fs_{(6,\ 266.11)} > 2.18$, $P \leq 0.05$](**Figure 6A**), thus respectively indicating that (i) RLA-I rats show a significant acquisition curve along the 100 training trials, (ii) such an acquisition curve depends on the treatment condition (as NH-induced acquisition improvements are different depending on which 10-trial block is taken into account), and (iii) such an acquisition curve depends on the gender (particularly because of the pronounced NH effect on females, across different 10-trial blocks, which is not present in RLA-I males) (see **Figure 6A**).

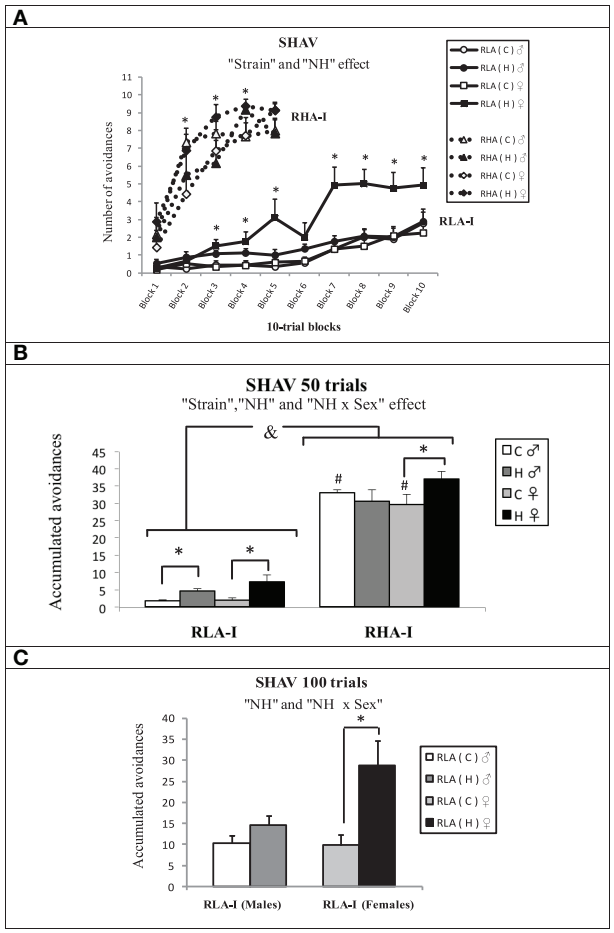

FIGURE 6 | Mean ± S.E.M. of (A) "Avoidances" in RHA-I (50 trials) and RLA-I (100 trials) grouped in blocks of 10 trials each; (B) "Accumulated avoidances" in the first 50-trial session for both rat strains; (C) "Accumulated avoidances" for the RLA-I strain in the total 100 shuttle box acquisition trials. (A) $^*p < 0.05$ vs. the respective control (C) group of the same sex (Duncan's tests after significant ANOVA effects). **(B,C)** &, indicates the "Strain" effect (see text for significance); $^*p < 0.05$ between the groups indicated (Duncan's tests following significant ANOVA effects); #$p < 0.05$ vs. respective control (C) group of the RLA-I strain (Duncan's tests following significant ANOVA effects). Group symbols: C, control non-handled group; H, neonatally handled (NH) group.

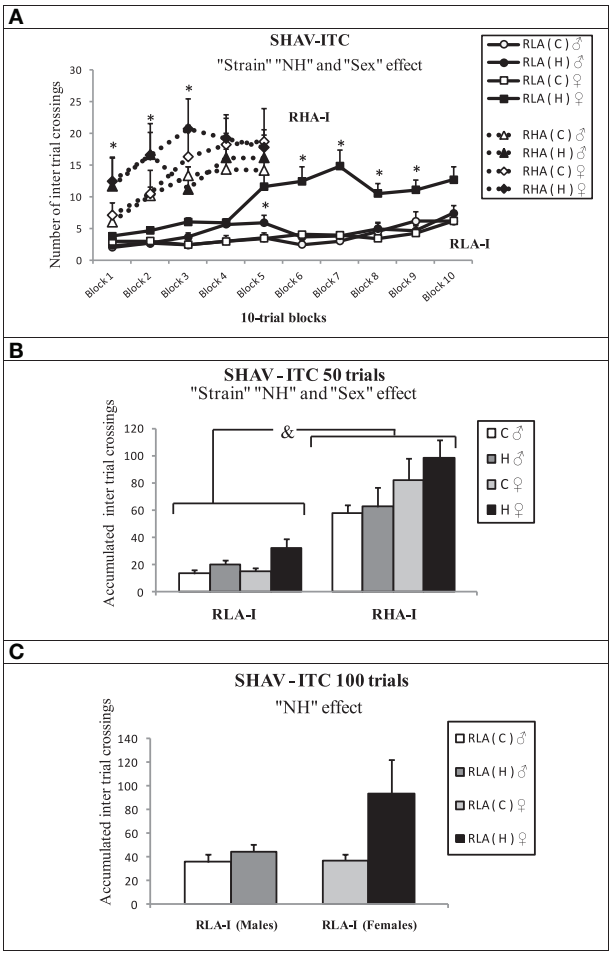

FIGURE 7 | Mean ± S.E.M. in (A) "Inter trial crossings (ITC)" in RHA-I (50 trials) and RLA-I (100 trials) grouped in blocks of 10 trials each. (B) "Accumulated Inter trial crossings" in the first 50-trial session for both strains; **(C)** "Accumulated Inter trial crossings" for the RLA-I strain in the total 100 shuttle box acquisition trials. **(A)** $^*p < 0.05$ vs. the respective control **(C)** group of the same sex (Duncan's tests after significant ANOVA effects). **(B)** &, indicates the "Strain" effect (see text for significance); $^*p < 0.05$ between the groups indicated (Duncan's tests following significant ANOVA effects). Group symbols: C, control non-handled group; H, neonatally handled (NH) group.

Figures 7A,B shows ITCs (inter-trial crossings) results during avoidance acquisition training. The repeated measures ANOVA applied to results from the first 50-trial session ("2 Strain" × "Treatment conditions" × "2 Sex" × "5 blocks of 10 trials") showed that RHA-I performed more ITCs than RLA-I rats ["Strain" effect, $F_{(1,\ 76)} = 96.4$, $p < 0.001$; **Figures 7A,B**], a global "NH" effect [$F_{(1,\ 76)} = 5.3$, $p = 0.024$; **Figures 6A,B**], with neonatally-handled animals performing overall more ITCs than untreated rats (**Figures 7A,B**), and a "Sex" effect [$F_{(1,\ 76)} = 7.5$, $p = 0.008$] indicating that females of both strains performed more ITCs than male rats (**Figures 7A,B**). Similar to SHAV50 results, there were also "Block" and "Block × Strain," as well as "Block × Sex" effects on ITCs [for all parameters, $Fs_{(4,\ 252.63)} > 2.89$, $p \leq 0.03$].

Analysis of ITCs along the whole 100 training trials (repeated measures ANOVA, "2 treatment conditions" × "2 sex" × "10 blocks of 10 trials" as within-subject factor; SHAV-ITC 100; **Figure 7C**), only in the RLA-I groups, showed a NH effect [$F_{(1,\ 46)} = 4.75$, $p = 0.035$], as neonatally-handled animals performed more ITCs than untreated ones (see **Figure 7C**). There was also a "Block" effect [$F_{(3,\ 122.06)} = 5.96$, $p = 0.001$] (**Figure 7A**), reflecting the overall ascending progression of ITCs across successive 10-trial blocks.

Correlations among Variables

Pearson correlations are shown in **Table 2**. The most relevant trends to highlight are between-test correlations. In this regard, significant correlations are observed between ZM and NOE variables (from $r = -0.26$ to $r = 0.53$), indicating that both tests

TABLE 2 | Pearson correlation coefficients are shown.

	LAT-NOE	TIME-NOE	ZM-E	ZM-T	ZM-HD	BAS1_5	BAS21_25	BAS	CCF	SHAV1	ITC1	SHAV2 (*)	ITC2 (*)	TOTAL-SHAV (*)
LAT-NOE	1													
TIME-NOE	**−, 60*** **	1												
ZM-E	**−, 26*** *	**,32****	1											
ZM-T	**−, 30****	**,41*****	**,82*****	1										
ZM-HD	**−, 33****	**,53*****	**,51*****	**,59*****	1									
BAS1_5	**,22*** *	−,19	−,06	−,03	**−,33****	1								
BAS21_25	**,26*** *	−,11	**−,25*** *	−,20	−,19	**,67*****	1							
BAS	**,28*** *	−,17	−,15	−,10	**−,29****	**,92*****	**,87*****	1						
CCF	,14	−,18	−,11	−,06	−,20	,17	,13	,17	1					
SHAV1	**−,39*****	**,58*****	**,34****	,25*	**,56*****	**−,39*****	**−,25*** *	**−,36****	**−,40*****	1				
ITC1	**−,30****	**,45*****	**,29****	,21	**,50*****	**−,30****	−,17	**−,27*** *	**−,40*****	**,88*****	1			
SHAV2 (*)	−,11	,17	−,04	,03	,20	−,13	−,13	−,16	**−,36*** *	**,75*****	**,66*****	1		
ITC2 (*)	−,11	,15	−,02	,05	**,29*** *	−,11	−,07	−,12	**−,37****	**,72*****	**,78*****	**,74*****	1	
TOTAL-SHAV (*)	−,17	,23	−,05	,02	,20	−,10	−,13	−,14	**−,40****	**,88*****	**,74*****	**,98*****	**,77*****	1

*Significant values are in bold. *p < 0.05; **p < 0.01; ***p < 0.001 (two-tailed). (*) Refers to the RLA-I groups only (thus n = 50), which were the only groups performing 100 trials in the shuttle box avoidance task.*

might be partly measuring similar anxiety-related traits. There are also low but significant correlations among both NOE and ZM variables with BAS parameters (NOE with BAS variables, from $r = 0.22$ to $r = 0.28$. ZM with BAS variables: $r = -0.25$ between ZM-E and BAS21-25; $r = -0.29$ between ZM-HD and BAS; see **Table 2**). Most importantly, there were very relevant correlations among ZM variables and SHAV and ITC (ranging from r = 0.29 to r = 0.56; **Table 2**), as well as between NOE variables and SHAV and ITCs (ranging from $r = -0.30$ to $r = 0.58$; **Table 2**) and between BAS (acoustic startle) responses and SHAV and ITCs (ranging from $r = -0.25$ to $r = -0.39$; **Table 2**), thus suggesting that unconditioned anxiety-related trait is negatively associated with two-way avoidance acquisition, i.e., the higher the unconditioned anxiety levels in those three tests the poorer the acquisition levels in the avoidance task.

Discussion

In the present study we have investigated, for the first time: (1) NH effects in inbred RHA-I/RLA-I rats of both sexes, (2) by using a test battery which included both unconditioned (NOE, ZM, and BAS) anxiety/fear tests and -most importantly- a context-conditioned fear test and shuttle box avoidance acquisition (i.e., the trait which constitutes the basis of genetic selection of RLA-I and RHA-I rats). We have found that, compared with their RHA-I counterparts, RLA-I rats show higher unconditioned anxiety/fear-related responses in the novel object exploration (NOE) and elevated zero-maze (ZM) tests, as well as in the baseline acoustic startle (BAS) test. These results agree with previous reports showing similar differences between the RLA-I and RHA-I strains in a variety of novelty/conflict tests (e.g., Driscoll et al., 2009; López-Aumatell et al., 2009a,b; Díaz-Morán et al., 2012; Martinez-Membrives et al., 2015; see "Introduction" for further references). As expected, and also in agreement with previous reports, RLA-I rats also displayed an overall increase of context-conditioned freezing and markedly impaired acquisition of the two-way active avoidance response compared with RHA-I rats (e.g., López-Aumatell et al., 2009a,b; Díaz-Morán et al., 2012; Martinez-Membrives et al., 2015; see "Introduction").

The main novel findings of the present study concern the effects of neonatal handling. Thus, regarding the unconditioned tests, i.e., NOE and ZM, we have found that NH increases exploration of both the novel object (NOE) and the open sections of the ZM test in both rat strains, although in the NOE test such effects are apparently more marked in RLA-I rats, which are more behaviorally inhibited (i.e., more anxious) than RHA-I rats in both tests (compare untreated rats of both strains in **Figures 2**, **3**). Actually, the levels attained by NH-treated RLA-I rats in NOE measures tend to approach the response levels of untreated RHA-I rats. These NH effects are overall in agreement with those previously reported on several (unconditioned) novelty/anxiety-related traits in unselected rats (e.g., Escorihuela et al., 1994; Ferré et al., 1995a; Núñez et al., 1995, 1996; Fernández-Teruel et al., 2002a; Raineki et al., 2014; see further references in the "Introduction") as well as in the Roman rats from the "outbred" lines (e.g., Fernández-Teruel et al., 1992; Steimer et al., 1998).

Importantly, the present study is the first demonstration that NH enduringly improves two-way avoidance acquisition in "inbred" RLA-I rats of both sexes and in female RHA-I rats (see "NH × sex" interactions in "Results"). The positive effect of NH manipulation on avoidances in RLA-I rats is also more pronounced in females, as reflected by significant "NH × sex" effects on SHAV100 (see and **Figures 6A–C**). NH also induced a significant increase of ITCs, both considering all groups (see **Figures 7A,B**) or only RLA-I groups (see **Figure 7C**). We have to remind here that the relevant literature shows that ITCs are positively related with (and are a positive predictor of) two-way avoidance acquisition, i.e., ITCs are "pseudoavoidance" responses indicating that animals are developing active coping strategies to

solve the "passive avoidance/active avoidance" conflict involved in the task (for review see Castanon et al., 1995; Aguilar et al., 2004), as it is also suggested by the positive SHAV-ITC correlations observed here (see **Table 2**). In parallel to these results, NH overall decreased context-conditioned freezing (i.e., classically conditioned fear) in both rat strains. Fear to the context during the initial stages of shuttle box avoidance training is known to be inversely related to effective avoidance acquisition (e.g., López-Aumatell et al., 2011; Vicens-Costa et al., 2011; Díaz-Morán et al., 2012; Martinez-Membrives et al., 2015). The negative correlations between context-conditioned freezing and number of avoidances and ITCs (see **Table 2**) give further support to that contention.

In the only previous study with Roman rats in which NH effects were evaluated on shuttle box avoidance, only "outbred" RLA males (from the swiss RLA/Verh outbred line) were used (Escorihuela et al., 1995). This study indicated a slight trend toward a positive treatment effect on avoidance responses, which failed to be significant according to overall ANOVA (Escorihuela et al., 1995). Therefore, the present study in inbred Roman rats of both sexes is the first demonstration of a significant NH-induced modulation of the trait which is the criterion for selection of the RLA-I and RHA-I strains (i.e., shuttle box avoidance acquisition).

Conditioned freezing and two-way avoidance acquisition (as well as ITCs) are apparently less affected by NH than the unconditioned anxiety measures (NOE, ZM). This would be congruent with the view that, in the Roman rat strains, two-way avoidance acquisition and conditioned freezing are more strongly linked to their genetic constitution than unconditioned anxiety/fearfulness traits (e.g., Castanon et al., 1995; Fernández-Teruel et al., 2002b; Steimer and Driscoll, 2003, 2005; Driscoll et al., 2009). Related to that, it was reported already in early behavioral genetic studies in rats that two-way active avoidance acquisition is probably among the types of behavioral traits having the highest heritability coefficients (e.g., Wahlsten, 1972; Wilcock and Fulker, 1973; Wilcock et al., 1981; see also Castanon et al., 1995; Fernández-Teruel et al., 2002b; Johannesson et al., 2009; Baud et al., 2013, 2014). With regard to the Roman rats, it has been suggested that the "warm up" phase, i.e., the performance during initial 10–20 trials of each shuttle box training session, is the aspect that most markedly differentiates both lines/strains (e.g., Driscoll and Bättig, 1982; Fernández-Teruel et al., 1991b; Escorihuela et al., 1995, 1999; Ferré et al., 1995b; Driscoll et al., 2009). In particular, the extremely slow "warm up" effect typically shown by RLA rats seems to stem from their proneness for fear conditioning (e.g., Escorihuela et al., 1995; López-Aumatell et al., 2009a,b; Estanislau et al., 2013), thus to freeze when facing an aversively-conditioned context (as it is the case during the initial trials in the shuttle box task), which is known to run against actively searching for a more adaptive (active) response like escape or avoidance (e.g., Weiss et al., 1968; Wilcock and Fulker, 1973; Fernández-Teruel et al., 1991a,b; Gray and McNaughton, 2000; López-Aumatell et al., 2009a,b; Vicens-Costa et al., 2011; Díaz-Morán et al., 2012). Hence, it seems possible that a more proactive (or less reactive) coping style of NH-treated RLA-I rats (as suggested by NH effects on conditioned freezing and ZM and NOE tests) might be partly responsible for their improved ability to acquire the two-way avoidance task.

As said in the Introduction, some studies on NH that have used rats of both sexes have shown that "treatment × gender" interactions are common, and either NH effects are often observed in just one gender or handling effects show divergent patterns in both sexes. As a few examples of this: (1) Stamatakis et al. (2008) reported that in acutely-stressed (Wistar) males rats NH manipulated showed better place learning performance than females, while no sex differences were observed in a spatial memory trial. (2) Likewise, handling-induced changes in hippocampal mineralocorticoid receptors were found in males only (Stamatakis et al., 2008). (3) Learning of a spatial "Y" maze task was impaired by NH in males and improved in female Wistar rats (Noschang et al., 2012) while, in the same study, (4) only NH-treated females (but not males) showed a decreased SOD/CAT (superoxide dismutase/catalase) ratio in prefrontal cortex. (5) Impairing NH effects on long-term retention of inhibitory avoidance were observed in female, but not male Sprague-Dawley rats (Kosten et al., 2007). (6) In another study, NH produced sex-dependent effects on stress-induced corticosterone and brain *c-fos* expression in adolescent Sprague–Dawley rats (Park et al., 2003). (7) Furthermore, Papaioanou et al. (2002) reported that NH treatment interacts with stress type (i.e., short-term or long-term) and with sex to induce changes in the concentration and turnover of brain serotonin and dopamine in Wistar rats. In this context, it is remarkable that also in the present study the positive effects of NH on avoidance acquisition have been shown to be divergent depending on gender. Thus, there are significant "NH × sex" effects on SHAV50 and SHAV100 (avoidances after 50 or 100 trials, respectively), which reflect the fact that NH improved avoidance acquisition more markedly in female rats of both strains during the first 50 trials (SHAV50; see **Figure 6B**) or in RLA-I females (compared with RLA-I males) after completing the 100 trials (SHAV100; see **Figure 6C**).

There is evidence, from factor-analytical studies using very large samples of F2 rats (derived from the "outbred" Roman lines, $n = 800$; Aguilar et al., 2003) or heterogeneous NIH-HS rats ($n = 1600$; López-Aumatell et al., 2011) that females' responses when facing conflicting situations might be more driven by activity-related responses (i.e., more "proactive" responses) than males' responses, which would be more driven by anxiety/freezing (i.e., "reactive" coping strategies; e.g., Fernandes et al., 1999; Aguilar et al., 2003; López-Aumatell et al., 2011). In this connection, it is tempting to suggest that the more marked NH effects observed in females, particularly in the two-way avoidance task, might be partly due to the fact that NH is able to disinhibit conflict-induced behavior (i.e., so changing a "reactive" to a more "proactive" coping strategy) more easily in females than in males.

The present positive results of NH on two-way avoidance acquisition are in contrast with several lines of research carried out by using psychogenetically-selected strains/lines of rats possessing divergent abilities to acquire shuttle box avoidance (i.e., Mausdley reactive vs. non-reactive rats, Levine and Broadhurst, 1963; RLA/Lu v.s RHA/Lu rats, Satinder and Hill, 1974), which failed to show acquisition improvements

following neonatal handling. Possible reasons to explain the different results of these and the present study could be the more intensive neonatal handling procedure used here (i.e., two handling sessions/day in the present study v/s one session/day in those studies), or the fact that the present shuttle box training parameters (i.e., composite "light + tone" CS; CS, US and inter-trial interval of longer durations than in those studies; no overlapping between CS and US) were specifically selected to facilitate the emergence of escape (or avoidance) responses and to minimize the presence of "response failures" (see details in Escorihuela et al., 1995).

The observed between-strain differences in baseline acoustic startle (BAS) are in agreement with previous reports (e.g., López-Aumatell et al., 2009a,b). Notably, however, neonatal handling did not affect BAS responses in any rat strain. The baseline acoustic startle is a reflex response that is mediated by a fast "cochlear root nucleus—caudal pontine reticular nucleus" pathway (e.g., see review by Koch and Schnitzler, 1997). To the best of our knowledge the effects of NH treatment on BAS have been evaluated for the first time in the present study, and the absence of changes in NH-treated rats, which contrasts with the positive effects observed in the other tests/tasks, suggests that brainstem-mediated reflex responses (i.e., BAS) are less sensitive to (NH) manipulation influences than more cognitively elaborated conflict-based responses (like NOE, ZM, CCF, or SHAV), which are thought to be under hippocampal control (e.g., Gray and McNaughton, 2000; López-Aumatell et al., 2008, 2009a, and references therein). Possibly in line with that contention, in a study in which rats were treated with environmental enrichment (EE) for several months, the treatment produced the expected long-lasting positive effects on several stress/anxiety-related and cognitive responses, but EE did not affect baseline acoustic startle (Peña et al., 2009).

A more active/functional hippocampus has been related to increased anxiety when facing "approach-avoidance" or "passive avoidance/active avoidance" conflict situations (such as the cases of NOE-ZM and CCF tests and the SHAV task, respectively) (Gray and McNaughton, 2000). In line with that, it is remarkable that the high anxious (and passive/reactive coper) RLA-I rat strain has a more functional hippocampus than the (low anxious) RHA-I strain (Meyza et al., 2009; Garcia-Falgueras et al., 2012). It would be interesting to investigate how hippocampal function during (unconditioned or conditioned) conflict could be affected by neonatal handling and how such an effect on hippocampus would be relevant for the H-induced changes in RLA-I rats. Would NH manipulation influence septo-hippocampal function in a manner similar to anxiolytic drugs—i.e., benzodiazepine agonists, which reduce conflict and improve shuttle box avoidance acquisition? (e.g., Fernández-Teruel et al., 1991a; Gray and McNaughton, 2000). A number of effects of neonatal handling on different neurobiogical aspects within the hippocampal formation have been reported, for example: (i) increased hippocampal long-term potentiation (e.g., Wilson et al., 1986) and decreased hippocampal neuronal loss with age in H-treated rats (e.g., Meaney et al., 1988; see reviews by Fernández-Teruel et al., 1997, 2002a); (ii) enhanced hippocampal type II glucocorticoid receptors, linked to decreased HPA-axis responses to stress (e.g., Meaney et al., 1988); (iii) increased GAP-43 (growth associated protein 43) expression in rat pups (Zhang et al., 2012); (iv) increases in hippocampal but not cortical 5-HT and 5-HIAA in rats (e.g., reviewed by Anisman et al., 1998; Fernández-Teruel et al., 2002a), as well as in hippocampal nerve growth factor mRNA (Mohammed et al., 1993; Pham et al., 1997); (v) enhancement of NADPHdiaphorase-positive neurons (a potential marker of nitric oxide-producing neurons) (Vaid et al., 1997); (vi) increases of central benzodiazepine and GABA-A receptors (Bodnoff et al., 1987; Bolden et al., 1990; see review by Raineki et al., 2014). Preliminary results from our laboratory suggest that RLA-I rats have reduced content of hippocampal PSA (polysialic acid, related to neural cell adhesion molecules -NCAM-), which is raised to RHA-I levels by NH. Thus, provided that all these forms (and others not listed here) of hippocampal plasticity have been shown to be sensitive to NH effects, it does not seem unreasonable to expect that hippocampal function during conflict (i.e., under anxiety-inducing, conditioned or unconditioned) situations could also be enduringly modulated by neonatal handling, thus inducing changes on coping strategies/responses. Testing such a hypothesis should be matter of further research.

In summary, in the present study, several long-lasting effects of NH are reported for the first time: (i) NH manipulation is able to partially counteract the genetically-based two-way avoidance acquisition deficit of (inbred) RLA-I rats, being the effect more evident in females. (ii) NH manipulation improves acquisition in females (but not males) of the RHA-I strain. (iii) NH effects on shuttle box avoidance acquisition are paralleled by a treatment-induced reduction of context-conditioned freezing (during inter-trial intervals 2–5 of the training session) also in both rat strains, which may suggest that the treatment has produced some change toward more adaptive (i.e., proactive) coping strategies, and that such an effect may underlie (at least partly) the avoidance acquisition improvement, particularly in RLA-I rats. (iv) The positive effects of NH on SHAV, ITCs, CCF, NOE, and ZM test measures, also agree with the contention that the treatment induces changes toward more proactive coping strategies. (v) Baseline acoustic startle is not influenced by NH, in line with findings obtained with other anxiety-reducing environmental treatments (Peña et al., 2009), thus suggesting that brainstem-mediated responses like BAS could be less sensitive to chronic treatment influences than conflict-based hippocampus-mediated responses.

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References

Aguilar, R., Gil, L., Fernández-Teruel, A., and Tobeña, A. (2004). Genetically-based behavioral traits influence the effects of Shuttle Box avoidance overtraining and extinction upon intertrial responding: a study with the Roman rat strains. *Behav. Process.* 30, 63–72. doi: 10.1016/j.beproc.2004.01.002

Aguilar, R., Gil, L., Gray, J. A., Driscoll, P., Flint, J., Dawson, G. R., et al. (2003). Fearfulness and sex in F2 Roman rats: males display more fear though both sexes share the same fearfulness traits. *Physiol. Behav.* 78, 723–732. doi: 10.1016/S0031-9384(03)00043-X

Anisman, H., Zaharia, M. D., Meaney, M. J., and Merali, Z. (1998). Do early-life events permanently alter behavioral and hormonal responses to stressors? *Int. J. Dev. Neurosci.* 16, 149–164. doi: 10.1016/S0736-5748(98)00025-2

Baud, A., Flint, J., and Fernandez-Teruel, A., and The Rat Genome Sequencing Mapping Consortium. (2014). Identification of genetic variants underlying anxiety and multiple sclerosis in heterogeneous stock rats. *World J. Neurosci.* 4, 216–224. doi: 10.4236/wjns.2014.43025

Baud, A., Hermsen, R., Guryev, V., Stridh, P., Graham, D., McBride, M. W., et al. (2013). Combined sequence-based and genetic mapping analysis of complex traits in outbred rats. *Nat. Genet.* 45, 767–775. doi: 10.1038/ng.2644

Bignami, G. (1965). Selection for high rates and low rates of avoidance conditioning in the rat. *Anim. Behav.* 13, 221–227. doi: 10.1016/0003-3472(65)90038-2

Bodnoff, S. R., Suranyi-Cadotte, B., Quirion, R., and Meany, M. J. (1987). Postnatal handling reduces novelty-induced fear and increases ^{3}H-flunitrazepam binding in rat brain. *Eur. J. Pharmacol.* 144, 105–107 doi: 10.1016/0014-2999(87)90016-1

Bolden, S. W., Hambley, J. W., Johnston, G. A., and Rogers, L. J. (1990). Neonatal stress and long-term modulation of GABA receptors in rat brain. *Neurosci. Lett.* 111, 258–262. doi: 10.1016/0304-3940(90)90271-A

Cañete, T., Blázquez, G., Tobeña, A., Giménez-Llort, L., and Fernández-Teruel, A. (2015). Cognitive and emotional alterations in young Alzheimer's disease (3xTgAD) mice: effects of neonatal handling stimulation and sexual dimorphism. *Behav. Brain Res.* 281, 156–171. doi: 10.1016/j.bbr.2014.11.004

Carrasco, J., Márquez, C., Nadal, R., Tobeña, A., Fernández-Teruel, A., and Armario, A. (2008). Characterization of central and peripheral components of the hypothalamus-pituitary-adrenal axis in the inbred Roman rat strains. *Psychoneuroendocrionology* 33, 433–437. doi: 10.1016/j.psyneuen.2008.01.001

Castanon, N., Pérez-Díaz, F., and Mormede, P. (1995). Genetic analysis of the relationship between behavioral and neuroendocrine traits in Roman high and low avoidance rat lines. *Behav. Genet.* 25, 371–383. doi: 10.1007/BF02197288

Díaz-Morán, S., Palència, M., Mont-Cardona, C., Cañete, T., Blázquez, G., Martínez-Membrives, E., et al. (2012). Coping style and stress hormone responses in genetically heterogeneous rats: comparison with the Roman rat strains. *Behav. Brain Res.* 228, 203–210. doi: 10.1016/j.bbr.2011.12.002

Driscoll, P., and Bättig, K. (1982). "Behavioral, emotional and neurochemical profiles of rats selected for extreme differences in active, two-way avoidance performance," in *Genetics of the Brain,* ed I. Lieblich (Amsterdam: Elsevier), 95–123.

Driscoll, P., Escorihuela, R. M., Fernández-Teruel, A., Giorgi, O., Schwegler, H., Steimer, T., et al. (1998). Genetic selection and differential stress responses. The Roman lines/strains of rats. *Ann. N.Y. Acad. Sci.* 851, 501–510.

Driscoll, P., Fernández-Teruel, A., Corda, M. G., Giorgi, O., and Steimer, T. (2009). "Some guidelines for defining personality differences in rats," in *Handbook of Behavior Genetics,* ed K. Yong-Kyu (New York, NY; Springer), 281–300.

Escorihuela, R. M., Fernández-Teruel, A., Gil, L., Aguilar, R., Tobeña, A., and Driscoll, P. (1999). Inbred Roman high- and low-avoidance rats: differences in anxiety, novelty-seeking, and shuttlebox behaviors. *Physiol. Behav.* 67, 19–26.

Escorihuela, R. M., Fernández-Teruel, A., Núñez, F. J., Zapata, A., and Tobeña, A. (1992). Infantile stimulation and the role of the benzodiazepine receptor system in adult acquisition of two-way avoidance behavior. *Psychopharmacology (Berl.)* 6, 282–284.

Escorihuela, R. M., Fernández-Teruel, A., Zapata, A., Núñez, J. F., and Tobeña, A. (1993). Flumazenil prevents the anxiolytic effects of diazepam, alprazolam and adinazolam on the early acquisition of two-way active avoidance *Pharmacol. Res.* 28, 53–58.

Escorihuela, R. M., Tobeña, A., Driscoll, P., and Fernández-Teruel, A. (1995). Effects of training, early handling, and perinatal flumazenil on shuttle box acquisition in Roman Low-Avoidance Rats: toward overcoming a genetic deficit. *Neurosci. Biobehav. Rev.* 19, 353–367.

Escorihuela, R. M., Tobeña, A., and Fernández-Teruel, A. (1994). Environmental enrichment reverses detrimental action of early inconsistent stimulation and increases the beneficial effects of postnatal handling on shuttlebox learning in adult rats. *Behav. Brain Res.* 61, 169–173.

Estanislau, C., Díaz-Morán, S., Cañete, T., Blázquez, G., Tobeña, A., and Fernández-Teruel, A. (2013). Context-dependient differences in grooming behavior among the NIH heterogeneous stock and the Roman high- and low-avoidance rats. *Neurosci. Res.* 77, 187–201. doi: 10.1016/j.neures.2013.09.012

Fernandes, C., González, M. I., Wilson, C. A., and File, S. E. (1999). Factor analysis shows that female rat behavior is characterised primarily by activity, male rats are driven by sex and anxiety. *Pharmacol. Biochem. Behav.* 64, 731–738. doi: 10.1016/S0091-3057(99)00139-2

Fernández-Teruel, A., Escorihuela, R. M., Castellano, B., González, B., and Tobeña, A. (1997). Neonatal handling and environmental enrichment on emotionality, novelty/reward seeking, and age-related cognitive and hippocampal impairments: focus on the Roman rat lines. *Behav. Genet.* 27, 513–526.

Fernández-Teruel, A., Escorihuela, R. M., Gray, J. A., Aguilar, R., Gil, L., Giménez-Llort, L., et al. (2002b). A quantitative trait locus influencing anxiety in the laboratory rat. *Genome Res.* 12, 618–626. doi: 10.1101/gr.203402

Fernández-Teruel, A., Escorihuela, R. M., Núñez, J. F., Gomà, M., Driscoll, P., and Tobeña, A. (1992). Early stimulation effects on novelty-induced behavior in two psychogenetically-selected rat lines with divergent emotionality profiles. *Neurosci. Lett.* 137, 185–188.

Fernández-Teruel, A., Escorihuela, R. M., Núñez, J. F., Zapata, A., Boix, F., Salazar, W., et al. (1991a). The early acquisition of two-way (shuttle-box) avoidance as an anxiety-mediated behavior: psychopharmacological validation. *Brain Res. Bull* 6, 173–176.

Fernández-Teruel, A., Escorihuela, R. M., Tobeña, A., and Driscoll, P. (1991b). Stress and putative endogenous ligands for benzodiazepine receptors: the importance of characteristics of the aversive situation and of differential emotionality in experimental animals. *Experientia* 47, 1051–1056.

Fernández-Teruel, A., Giménez-Llort, L., Escorihuela, R. M., Gil, L., Aguilar, R., Steimer, T., et al. (2002a). Early-life handling stimulation and environmental enrichment: Are some of their effects mediated by similar neural mechanisms? *Pharmacol. Biochem. Behav.* 73, 233–245. doi: 10.1016/S0091-3057(02)00787-6

Ferré, P., Nuñez, J. F., García, E., Tobeña, A., Escorihuela, R. M., and Fernández-Teruel, A. (1995a). Postnatal handling reduces anxiety as measured by emotionality rating and hyponeophagia test in female rats. *Pharmacol. Biochem. Behav.* 51, 199–203.

Ferré, P., Fernández-Teruel, A., Escorihuela, R. M., Driscoll, P., Corda, M. G., Giorgi, O., et al. (1995b). Behavior of the Roman/Verh high- and Low-Avoidance rat lines in anxiety tests: relationship with defecation and self grooming. *Physiol. Behav.* 58, 1209–1213.

Garcia-Falgueras, A., Castillo-Ruiz, M. M., Put, T., Tobeña, A., and Fernández-Teruel, A. (2012). Differential hippocampal neuron density between inbred Roman high- (low anxious) and low-avoidance (high anxious) rats. *Neurosci. Lett.* 522, 41–46. doi: 10.1016/j.neulet.2012.06.011

Giorgi, O., Piras, G., and Corda, M. G. (2007). The psychogenetically selected Roman high- and low-avoidance rat lines: a model to study the individual vulnerability to drug addiction. *Neurosci. Biobehav. Rev.* 31, 148–163. doi: 10.1016/j.neubiorev.2006.07.008

Gray, J. A. (1982). *The Neuropsychology of Anxiety: An Inquiry into the Functions into the Septo-hipoccampal System*. New York, NY: Oxford University Press.

Gray, J. A., and McNaughton, N. (2000). *The Neuropsychology Of anxiety: An Enquiry into the Functions of the Septo-hippocampal System, 2nd edn.* Oxford: Oxford University Press.

Johannesson, M., López-Aumatell, R., Stridh, P., Diez, M., Tuncel, J., Blázquez, G., et al. (2009). A resource for the simultaneous high-resolution mapping of multiple quantitative trait loci in rats: the NIH heterogeneous stock. *Genome Res.* 19, 150–158. doi: 10.1101/gr.081497.108

Koch, M., and Schnitzler, H. U. (1997). The acoustic startle response in rats-circuits mediating evocation, inhibition and potentiation. *Behav. Brain Res.* 89, 35–49. doi: 10.1016/S0166-4328(97)02296-1

Kosten, T., Lee, H., and Kim, J. (2007). Neonatal handling alters learning in adult male and female rats in a task-specific manner. *Brain Res.* 1154, 144–153. doi: 10.1016/j.brainres.2007.03.081

Levine, S. (1956). A further study on infantile handling and adult avoidance learning. *J. Pers.* 25, 70–80 doi: 10.1111/j.1467-6494.1956.tb01289.x

Levine, S. (1957). Infantile experience and resistance to physiological stress. *Science* 126, 405–406. doi: 10.1126/science.126.3270.405

Levine, S., and Broadhurst, P. L. (1963). Genetic and ontogenetic determinants of adult behavior in the rat. *J. Comp. Physiol. Psychol.* 56, 243–248 doi: 10.1037/h0040285

López-Aumatell, R., Blázquez, G., Gil, L., Aguilar, R., Cañete, T., Giménez-Llort, L., et al. (2009b). The Roman High- and Low-Avoidance rat strains differ in fear-potentiated startle and classical aversive conditioning. *Psicothema* 21, 27–32.

López-Aumatell, R., Guitart-Masip, M., Vicens-Costa, E., Giménez-Llort, L., Valdar, W., Johannesson, M., et al. (2008). Fearfulness in a large N/Nih genetically heterogeneous rat stock: differential profiles of timidity and defensive flight in males and females. *Behav. Brain. Res.* 188, 41–55. doi: 10.1016/j.bbr.2007.10.015

López-Aumatell, R., Martínez-Membrives, E., Vicens-Costa, E., Cañete, T., Blázquez, G., Mont-Cardona, C., et al. (2011). Effects of environmental and physiological covariates on sex differences in unconditioned and conditioned anxiety and fear in a large sample of genetically heterogeneous (N/Nih-HS) rats. *Behav. Brain Funct.* 7:48. doi: 10.1186/1744-9081-7-48

López-Aumatell, R., Vicens-Costa, E., Guitart-Masip, M., Martínez-Membrives, E., Valdar, W., Johannesson, M., et al. (2009a). Unlearned anxiety predicts learned fear: a comparision among heterogeneous rats and the Roman rat strains. *Behav. Brain Res.* 202, 92–101. doi: 10.1016/j.bbr.2009.03.024

Martinez-Membrives, E., Lopez-Aumatell, R., Blázquez, G., Cañete, T., Tobeña, A., and Fernández-Teruel, A. (2015). Spatial learning in the genetically heterogeneous NIH-HS rat stock and RLA-I/RHA-I rats: revisiting the relationship with unconditioned and conditioned anxiety. *Physiol. Behav.* 144, 15–25. doi: 10.1016/j.physbeh.2015.03.003

McIntosh, J., Anisman, H., and Merali, Z. (1999). Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adults rats: gender-dependent effects. *Dev. Brain Res.* 113, 97–106. doi: 10.1016/S0165-3806(99)00005-X

Meaney, M. J., Aitken, D. H., van Berkel, C., Bhatnagar, S., and Sapolsky, R. M. (1988). Effect of neonatal handling on age-related impairments associated with the hippocampus. *Science* 12, 766–768. doi: 10.1126/science.3340858

Meaney, M. J., Mitchell, J. B., Aitken, D. H., Bhatnagar, S., Bodnoff, S. R., Iny, L. J., et al. (1991). The effects of neonatal handling on development of the adrenocortical response to stress: implications for neuropathology and cognitive deficits in later life. *Psychoneuroendocrinology* 16, 85–103. doi: 10.1016/0306-4530(91)90072-2

Meyza, K. Z., Boguszewski, P. M., Nikolaev, E., and Zagrodzka, J. (2009). Diverse sensitivity of RHA/Verh and RLA/Verh rats to emotional and spatial aspects of a novel environment as a result of a distinct pattern of neuronal activation in the fear/anxiety circuit. *Behav. Genet.* 39, 48–61. doi: 10.1007/s10519-008-9234-z

Mohammed, A. H., Henricksson, B. G., Söderström, S., Ebendal, T., Olsson, T., and Seckl, J. R. (1993). Environmental influences on the central nervous system and their implications for the aging rat. *Behav. Brain Res.* 57, 183–191 doi: 10.1016/0166-4328(93)90134-C

Noschang, C., Krolow, R., Arcego, D. M., Toniazzo, A. P., Huffell, A. P., and Dalmaz, C. (2012). Neonatal handling affects learning, reverseal learning and antioxidant enzymes activities in a sex-specific manner in rats. *Int. J. Dev. Neurosci.* 30, 285–291. doi: 10.1016/j.ijdvneu.2012.01.010

Núñez, J. F., Ferré, P., Escorihuela, R. M., Tobeña, A., and Fernández-Teruel, A. (1996). Effects of postnatal handling of rats on emotional, HPA-axis, and prolactin reactivity to novelty and conflict. *Physiol. Behav.* 60, 1355–1359.

Núñez, J. F., Ferré, P., García, E., Escorihuela, R. M., Fernández-Teruel, A., and Tobeña, A. (1995). Postnatal handling reduces emotionality ratings and accelerates two-way active avoidance in female rats. *Physiol. Behav.* 57, 831–835.

Papaioanou, A., Dafni, U., Alikaridis, F., Bolaris, S., and Stylianopoulou, F. (2002). Effects of neonatal handling on basal and stress-induced monoamine levels in the male and female rat brain. *Neuroscience* 114, 195–206. doi: 10.1016/S0306-4522(02)00129-X

Park, M., Hoang, T., Belluzzi, J. D., and Leslie, F. M. (2003). Gender specific effect of neonatal handling on stress reactivity of adolescent rats. *J. Neuroendocrinol.* 15, 289–295. doi: 10.1046/j.1365-2826.2003.01010.x

Peña, Y., Prunell, M., Rotllant, D., Armario, A., and Escorihuela, R. M. (2009). Enduring effects of environmental enrichment from weaning to adulthood on pituitary-adrenal function, pre-pulse inhibition and learning in male and female rats. *Psychoneuroendocrinology* 34, 1390–1404. doi: 10.1016/j.psyneuen.2009.04.019

Pham, T. M., Söderström, S., Henrisckson, B. G., and Mohammed, A. H. (1997). Effects of neonatal stimulation on later cognitive function and hippocampal nerve growth factor. *Behav. Brain Res.* 86, 113–120 doi: 10.1016/S0166-4328(96)02252-8

Raineki, C., Lucion, A. B., and Weinberg, J. (2014). Neonatal handling: an overview of the positive and negative effects. *Dev. Psychobiol.* 56, 1613–1625. doi: 10.1002/dev.21241

Rosas, J. M., Callejas-Aguilera, J. E., Escarabajal, M., Gómez, M. J., de la Torre, L., Agüero, A., et al. (2007). Succsessive negative effect in instrumental runway behavior: a study with Roman high-(RHA) and low-(RLA) avoidance rats. *Behav. Brain Res.* 185, 1–8. doi: 10.1016/j.bbr.2007.07.027

Sabariego, M., Morón, I., Gómez, M. J., Donaire, R., Tobeña, A., Fernández-Teruel, A., et al. (2013). Incentive loss and hippocampal gene expression in inbred Roman high- (RHA-I) and Roman low-(RLA-I) avoidance rats. *Behav. Brain Res.* 257, 62–70. doi: 10.1016/j.bbr.2013.09.025

Satinder, K. P., and Hill, K. D. (1974). Effects of genotype and postnatal experience on activity, avoidance, shock threshold, and open-field behavior of rats. *J. Comp. Physiol. Psychol.* 86, 363–374. doi: 10.1037/h0035975

Shepherd, J. K., Grewal, S. S., Fletcher, A., Bill, D. J., and Dourish, C. T. (1994). Behavioural and pharmacological characterisation of the elevated zero-maze as an animal model of anxiety. *Psychopharmacology (Berl.)* 116, 56–64. doi: 10.1007/BF02244871

Stamatakis, A., Pondiki, S., Kitraki, E., Diamantopoulou, A., Panagiotaropoulos, T., Raftogianni, A., et al. (2008). Effect of neonatal handling on adult rat spatial learning and memory following acute stress. *Stress* 11, 148–159. doi: 10.1080/10253890701653039

Steimer, T., and Driscoll, P. (2003). Divergent stress responses and coping styles in psychogenetically selected Roman high-(RHA) and low-(RLA) avoidance rats: behavioural, neuroendocrine and developmental aspects. *Stress* 6, 87–100. doi: 10.1080/1025389031000111320

Steimer, T., and Driscoll, P. (2005). Inter-individual vs. line/strain differences in psychogenetically selected Roman High-(RHA) and Low-(RLA) Avoidance rats: neuroendocrine and behavioural aspects. *Neurosci. Biobehav. Rev.* 29, 99–112. doi: 10.1016/j.neubiorev.2004.07.002

Steimer, T., Escorihuela, R. M., Fernández-Teruel, A., and Driscoll, P. (1998). Long-term behavioral and neuroendocrine changes in Roman high-(RHA/Verh) and Low-(RLA/Verh) avoidance rats following neonatal handling. *Int. J. Dev. Neurosci.* 16, 165–174. doi: 10.1016/S0736-5748(98)00032-X

Tejedor-Real, P., Costela, C., and Gibert-Rahola, J. (1998). Neonatal handling reduces emotional reactivity and suceptibility to learned helplessness. Involvement of catecholaminergic systems. *Life Sci.* 62, 37–50. doi: 10.1016/S0024-3205(97)01036-9

Torres, C., Cándido, A., Escarabajal, M. D., de la Torre, L., Maldonado, A., Tobeña, A., et al. (2005). Successive negative contrast in one-way avoidance learning in female Roman rats. *Physiol. Behav.* 85, 377–382. doi: 10.1016/j.physbeh.2005.02.023

Vaid, R. R., Yee, B. K., Shalev, U., Rawlins, J. N. P., Weiner, I., Feldon, J., et al. (1997). Neonatal nonhandling and *in utero* prenatal stress reduce the density of NADPH-diaphorase-reactive neurons in the fascia dentata and Amon's horn of rats. *J. Neurosci.* 17, 5599–5609.

Vicens-Costa, E., Martínez-Membrives, E., López-Aumatell, R., Guitart-Masip, M., Cañete, T., Blázquez, G., et al. (2011). Two-way avoidance acquisition is negatively related to conditioned freezing and positively associated with startle reactions: a dissection of anxiety and fear in genetically heterogeneous rats. *Physiol. Behav.* 103, 148–156. doi: 10.1016/j.physbeh.2010.12.009

Wahlsten, D. (1972). Genetic experiments with animal learning: a critical review. *Behav. Biol* 7, 143–182. doi: 10.1016/S0091-6773(72)80197-4

Weiss, J. M., Krieckhaus, E. E., and Conte, R. (1968). Effects of fear conditioning on subsequent avoidance behavior and movement. *J. Comp. Physiol. Psychol.* 65, 413–421. doi: 10.1037/h0025832

Wilcock, J., Fulker, D., and Broadhurst, P. L. (1981). Analysis of two two-way escape-avoidance conditioning measures from a diallel cross of eight strains of rats. *Behav. Genet.* 11, 339–358. doi: 10.1007/BF01070816

Wilcock, J., and Fulker, D. W. (1973). Avoidance learning in rats: genetic evidence for two distinct behavioral processes in the shuttle box. *J. Comp. Physiol. Psychol.* 82, 247–253. doi: 10.1037/h0033914

Wilson, D. A., and Jamieson, J. L. (1968). Infantile handling and the facilitation of discrimination and reversal learning. *Q. J. Exp. Psychol.* 20, 197–199 doi: 10.1080/14640746808400149

Wilson, D. A., Willner, J., Kurz, E. M., and Nadel, L. (1986). Early handling increases hippocampal long-term potentiation in young rats. *Behav. Brain Res.* 21, 223–227. doi: 10.1016/0166-4328(86)90240-8

Zaharia, M. D., Kulczycki, J., Shanks, N., Meaney, M. J., and Anisman, H. (1996). The effectsof early postnatal stimulation on Morris water-maze acquisition in adult mice: genetic and maternal factors. *Psychopharmacology* 128, 227–239. doi: 10.1007/s002130050130

Zhang, Z., Zhang, H., Du, B., and Chen, Z. (2012). Neonatal handling and environmental enrichment increase the expression of GAP-43 in the hippocampus and promote cognitive abilities in prenatally stressed rat offspring. *Neurosci. Lett.* 522, 1–5. doi: 10.1016/j.neulet.2012.05.039

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3.2.- **STUDY 2:** ***"****Neonatal handling enduringly decreases anxiety and stress responses and reduces hippocampus and amygdala volume in a genetic model of differential anxiety: Behavioral-volumetric associations in the Roman rat strains".*

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Neonatal handling enduringly decreases anxiety and stress responses and reduces hippocampus and amygdala volume in a genetic model of differential anxiety: Behavioral-volumetric associations in the Roman rat strains

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Abstract
The hippocampus and amygdala have been proposed as key neural structures related to anxiety. A more active hippocampus/amygdala system has been related to greater anxious responses in situations involving conflict/novelty. The Roman Low- (RLA) and High-avoidance (RHA) rat lines/strains constitute a genetic model of differential anxiety. Relative to RHA rats, RLA rats exhibit enhanced anxiety/fearfulness, augmented hippocampal/amygdala c-Fos expression following exposure to novelty/conflict, increased hippocampal neuronal density and higher endocrine responses to stress. Neonatal handling (NH) is an environmental treatment with long-lasting anxiety/stress-reducing effects in rodents. Since hippocampus and amygdala volume are supposed to be related to anxiety/fear, we hypothesized a greater volume of both areas in RLA than in RHA rats, as well as that NH treatment would reduce anxiety and the volume of both

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structures, in particular in the RLA strain. Adult untreated and NH-treated RHA and RLA rats were tested for anxiety, sensorimotor gating (PPI), stress-induced corticosterone and prolactin responses, two-way active avoidance acquisition and *in vivo* 7 T 1H-Magnetic resonance image. As expected, untreated RLA rats showed higher anxiety and post-stress hormone responses, as well as greater hippocampus and amygdala volumes than untreated RHA rats. NH decreased anxiety/stress responses, especially in RLA rats, and significantly reduced hippocampus and amygdala volumes in this strain. Dorsal striatum volume was not different between the strains nor it was affected by NH. Finally, there were positive associations (as shown by correlations, factor analysis and multiple regression) between anxiety and PPI and hippocampus/amygdala volumes.

1. Introduction

Previous studies have shown positive associations between anxiety-related temperament/responses and hippocampus and amygdala activity/connectivity and volume in healthy humans (Barros-Loscertales et al., 2006; Cherbuin et al., 2008; Hahn et al., 2010; Levita et al., 2014), as well as between anxious temperament and activation of both structures in rhesus monkeys (Oler et al., 2010). Remarkably, a causal role for the hippocampus in solving (anxiety-mediated) approach-avoidance conflicts has been shown by both positive associations between hippocampal activity and behavioural inhibition in healthy volunteers, and by passive avoidance deficits in hippocampus-lesioned patients (Bach et al., 2014).

The septo-hippocampal system, in close interplay with the amygdala, are key neural mechanisms in the regulation of anxiety as a crucial part of the behavioral inhibition system (Davis and Whalen, 2001; Gray and McNaughton, 2000; LeDoux 2000). Gray's theory has recruited wide support from animal lesion and pharmacological studies, confirming that the more active the hippocampus and amygdala the greater the anxious/fearful responses (Gray and McNaughton, 2000; Mc Naughton, Corr, 2004), but results on associations between anxiety-hippocampus volume in untreated rats and in rat lines selected for divergent anxiety have been inconclusive (Kalisch et al., 2006).

A well-characterized model of anxiety are the Roman High- (RHA) and Low-avoidance (RLA) rat lines/strains, psychogenetically selected for their good (RHA) vs poor (RLA) acquisition of the two-way active avoidance response, a "passive avoidance/active avoidance" conflict that involves anxiety (e.g. Driscoll et al., 1998, 2009; Escorihuela et al. 1999; Fernández-Teruel et al., 1991; Gray and McNaughton, 2000). Compared with RHAs, RLA rats display increased anxiety/stress-related responses (e.g., Carrasco. et al., 2008; Díaz-Morán et al., 2012; Estanislau et al., 2013; López-Aumatell et al., (2009a, 2009b), decreased GABA-A receptor function (Bentareha et al., 1998; Giorgi et al., 1994), enhanced anti-anxiety effects of anxiolytic substances/treatments (Martin et al., 1982; Steimer and Driscoll 2003; Torres et al., 2007) and a more functional/active hippocampus (Garcia-Falgueras et al., 2012; Meyza et al., 2009). Hippocampal volume is supposed to reflect its overall activity (see discussion in e.g. Barros-Loscertales et al., 2006; Kalisch et al., 2006), thus we would expect increased hippocampal volume in RLAs as compared with RHA rats.

An environmental treatment with well-known long-lasting anxiolytic effects is neonatal handling (NH), which enduringly reduces anxiety/stress responses in a variety of tests/tasks in rodents, including RLA/RHA rats (e.g. Anisman et al., 1998; Fernández-Teruel et al., 1997, 2002; Meaney et al., 1988; Steimer et al., 1998; Raineki et al., 2014). As the hippocampus and amygdala are crucial in the regulation of anxiety, we expected that an enduring reduction of anxiety in NH-treated RLA rats would be paralleled by a decrease in volume of these structures. We also expected no changes in the dorsal striatum, which we have used as a "control" brain region not related with anxiety. In separate batches of control and NH-treated animals we have also evaluated stress hormone responses (corticosterone, prolactin) and two-way avoidance acquisition (as an index of anxiety; see Fernández-Teruel et al., 1991; Gray and McNaughton, 2000). Post-stress levels of corticosterone and prolactin were assessed as independent markers of stress sensitivity and of NH effects, because both hormonal responses are known to reflect the levels of perceived stress and, prolactin, in particular, has even been related to the levels of anxiety in rats (for review see Díaz-Morán et al., 2012, and references therein).

2. Experimental procedures

2.1. Animals

Pregnant inbred Roman High- (RHA, $n=16$) and Low-Avoidance (RLA, $n=17$) female rats (Autonomous University of Barcelona), from the 60th inbreeding generation were used. They were individually housed and were maintained with food and water freely available, 12-h light-dark cycle (light on at 0800 h) and controlled temperature (22 ± 2 °C). They were randomly distributed across the following four experimental groups to which their offspring would be assigned: RHA and RLA control (C), nonhandled rats, and RHA and RLA animals that received neonatal handling (NH) treatment. After weaning (postnatal day 21st) the pups were housed in pairs of the same experimental group in standard macrolon cages ($50\times25\times14$ cm^3).

For the first experiment (Batch 1) 32 RHA and 32 RLA male rats (16 in each of the 4 experimental groups) were used for behavioral evaluation between 2 and 4 months of age (see below), and for structural MRI assessment at 6 months of age. Each of the 4 experimental groups was composed of 16 male rats representing

5-6 different litters, with 3-4 pups coming from each litter. For MRI studies (see below), each of the 4 experimental groups was formed by 8 male rats that were pseudorandomly selected from the corresponding experimental group of 16 animals, in a manner that the original 5-6 different litters (1-2 rats per litter) were still represented.

A second, independent batch (Batch 2) of naive control and NH-treated male animals, were used only to evaluate pre- and post-stress corticosterone (Cort) and prolactin (PRL) responses at 6 months of age (total n=37-30). Each of the 4 experimental groups was composed of 8-10 male rats representing 5 different litters, with 1-2 pups coming from each litter.

A third, independent batch (Batch 3) of naive control and NH-treated male rats (total n=50) were tested for two-way avoidance acquisition (see below) at 6 months of age. Control and NH-treated RLA rats (n=15 and n=23, respectively) came from 5 to 6 different litters/group, with 3-4 rats coming from each litter. Control and NH-treated RHA rats (n=5 and n=7, respectively) came from 5 litters/group, with 1-2 pups coming from each litter.

Batch 1 of animals was evaluated at 60-120 days of age for behavior, and at 6 months of age for MRI volumetry. In order to evaluate the long- lasting effects induced by the treatment (NH) and to avoid confounding effects of training, the other two batches (Batch 2 and Batch 3) of experimentally "naive" animals were evaluated at the same age as animals from Batch 1 were submitted to MRI assessment. Thus, rats from batches 2 and 3 were used as an independent test of the long-lasting anti-stress and anti-anxiety effects of NH.

Experiments were performed between 09:00 and 19:00 h in accordance with the Spanish legislation on "Protection of Animals Used for Experimental and Other Scientific Purposes" and the European Communities Council Directive (86/609/EEC) on this subject.

2.2. Procedure and apparatus

2.2.1. Neonatal handling (NH)

NH was given twice daily (at 9:30 and 17:00 h) between postnatal days 1-21. Each handling session consisted of first removing the mother from the litter and then placing the pups gently and individually in plastic cages ($35 \times 15 \times 25$ cm^3) lined with paper towel for a total period of 8 min. At minutes 0, 4 and 8 of this 8-min period, each pup was gently handled by the experimenter's hands (without wearing gloves) while being softly stroked from the head to the tail for 3-4 s. At the end of the 8-min period, each pup was returned to its homecage. When all the pups from one litter were back in their homecage, the mother was also returned to it. Control (C) non-handled groups were left undisturbed, except for regular cage cleaning once a week, until weaning. This neonatal handling procedure has been used because it has been shown to induce long lasting anxiety/stress-reducing effects in previous works (Escorihuela et al., 1995; Fernández-Teruel et al., 1992a, 1992b; Río-Alamos et al. 2015).

2.2.2. Batch 1: Novel object exploration test (NOE)

To assess novelty-induced behavioral inhibition, a novel object exploration (NOE) test was conducted, consisting in the evaluation of the exploratory response of rats when a novel object was introduced in their home-cage. Rats were 60 days old at the beginning of the test. The test started by removing the food from the home-cage (leaving only four pellets in each cage). One hour later, the novel object (graphite pencil Staedtler Noris, HB no.2) was perpendicularly introduced in their home cage through the grid cover, until it made contact with the cage bedding. To facilitate observation, each cage was pulled from the rack about 20 cm. Latency to the first exploration (NOE-L; time elapsed until the first exploration of the novel object) and the total time (NOE-T) spent exploring the pencil for each individual rat were scored in a 3-min test. The experimenter/observer was standing at 50 cm from the cage front (see Río-Alamos et al., 2015).

2.2.3. Batch 1: Elevated zero maze (ZM)

The maze comprised a circular corridor (105 cm diameter; 10 cm width) made of black plywood, elevated to 65 cm above the ground, having two open sections and two enclosed ones (walls 40 cm height), and was situated in a black-painted testing room, dimly illuminated with red fluorescent light. Rats were 90 days old at the beginning of ZM testing. Each rat was placed in an enclosed section of the ZM facing the wall and behavior was videotaped and measured outside the testing room for 5 min. Measures were the time spent into open sections (ZM-T), number of head-dips through the border/edge of the maze (ZM-HD) and number of line crossings (ZM-LC) (Río-Alamos et al., 2015).

2.2.4. Batch 1: Prepulse inhibition of the acoustic startle response (PPI)

Four sound attenuated boxes (SR-Lab Startle Response System, San Diego Instruments, USA) were used. Each box consists of a Plexiglas cylinder situated on the top of a platform with a sensor that detects the strength made by the rat in each trial. Two speakers situated 15 cm from each side of the cylinder deliver the acoustic stimuli and a white noise generator provides the background noise. Each box was constantly lit by a 10 W lamp. The data were transduced by an accelerometer into a voltage which is amplified, digitized and saved into a computer for analysis.

Subjects were 110-120 days old at the beginning of testing. The session started with a 5 min habituation period in the startle chambers. Then, 10 "pulse-alone" trials (105 dB, 40 ms) were delivered in order to obtain a stable baseline of startle. After this, each one of the six different types of trials are randomly administered 10 times (60 trials in total):

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

At the end, in order to measure the habituation to the startle stimulus, five "pulse-alone" trials were delivered (105 dB, 40 ms).

The interval between trials was 10-20 s with a mean of 15 s. The startle magnitude was recorded during 200 ms after the onset of the pulse.

The %PPI for each prepulse intensity was calculated by applying the following formula: %PPI=100-(startle amplitude on prepulse trial/startle amplitude on pulse trial) $\times$ 100 (Oliveras et al., 2015).

2.2.5. Batch 1: Magnetic resonance image (MRI) volumetry

In vivo 1H-Magnetic resonance studies were performed, when rats (n=32, randomly selected from the original sample of n=64; Batch 1) were 6 months old, at the joint NMR facility of the Autonomous University of Barcelona and CIBER-BBN (Cerdanyola del Vallès, Spain), using a 7-Tesla horizontal magnet (*BioSpec 70/30*, Bruker BioSpin, Ettlingen, Germany) equipped with actively shielded gradients (B-GA20S) using a dedicated rat brain quadrature receive surface coil, actively decoupled from a transmit volume coil with 72 mm inner. Rats were positioned in the scanner bed, which allowed localized delivery of anesthesia (isoflurane, 1.5-2.5% in O2 at 1 L/min; respiratory frequency monitored with a pressure probe and kept between 60 and 80 breaths/min). A recirculation

water system, integrated in the animal bed, was used to control the body temperature as measured with a rectal probe (37 ± 1 °C).

T2-weighted fast spin-echo were initially obtained in axial, sagittal and coronal planes to be used as reference scout images for accurate slice selection of the axial planes through the measured areas of the brain. Imaging parameters for these images were: effective echo time (TEeff) = 36 ms, repetition time (TR) = 2 s, echo train length (ETL) = 8, field of view (FOV) = 3.5 × 3.5 cm^2, matrix size (MTX) = 256 × 256, slice thickness (ST) = 0.5 mm. Using these scout images high resolution T2-weighted images were acquired in the axial plane with the following parameter: TEeff = 39 ms, TR = 4.5 s, ETL = 8, FOV = 3.2 × 3.2 cm^2, MTX = 320 × 320 and ST = 0.5 mm.

Using ImageJ software, brain volume (BV); left, right and total hippocampus (THc); dorsal hippocampus (DHc); ventral hippocampus (VHc), dorsal striatum (St) and amygdala (Am) were manually outlined by two raters blinded to group status (between-rater reliability $r \geq 0.89$). Briefly, this software allows the user to outline the boundaries of the region of interest (ROI) on a MRI and afterward to calculate the corresponding area using the formula that is shown below. All ROI's were delimitated from rostral to caudal (Figure S1 A-F).

In the present study, the hippocampus and its sub-divisions, amygdala and dorsal striatum (St) borders were defined according to the rat brain atlas (Paxinos and Watson, 1998; Wolf et al., 2002). For total hippocampus, the starting rostral slice was defined by the cornu ammonis (CA) and dentate gyrus (DG) and coincided with the dorsal hippocampal commissure to a level of approximately -1.92 mm from Bregma. The caudal boundary was defined by the absent of DG and the clear separation of the two cerebral hemispheres. Moreover, the aqueduct opened up and became a clearly visible, large, round circle. The last hippocampal slice included corresponded to a level of approximately -6.72 mm from Bregma. 10 consecutive slices from each animal were scored. Dorsal hippocampus was defined from -1.92 until -3.72 from bregma. Ventral hippocampus was defined from -4.80 until -6.60. One or two slices of total hippocampus, falling between -3.72 and -4.80 from bregma, were not included into DHc/VHc due to unclarity of the boundaries.

The rostral amygdala slice included corresponds to a level of approximately -1.20 mm from bregma, were the postero-anterior commissure linked the striatum with the amygdala, which at this point presents a kind of triangular shape. The most caudal slice included corresponds to a level of approximately -5.04 mm from Bregma where the ventral subiculum can still be differentiated from the Amygdala nuclei. 9 consecutive slices from each animal were scored.

The first dorsal striatum slice included corresponds to a level of approximately 2.28 from bregma, where lateral ventricles begin to appear. In this slice, dorsal striatum is laterally surrounded by the external capsule and in the rostral region is surrounded by the genu of the corpus callosum. The ventral part of the dorsal striatum is traced by a diagonal imaginary line that connects the bottom of the lateral ventricle with the final point of the external capsule. The most caudal slice included corresponds to a level of approximately -0.96 from bregma, in which dorsal striatum can be clearly separated from the globus pallidus and the internal capsule. 6 consecutive slices from each animal were scored.

For the delineation of the brain volume (BV) the most anterior brain slice included was the first slice in which prefrontal cortex appears (approximately 5.16 from Bregma). The most posterior brain slice included corresponded to a level of approximately -9.60 from Bregma. The entire included brain tissue was distributed over 35 consecutive slices in each individual animal (for delimitations of ROI´s see Figure S1 A-F).

Volumes of delimitated area on each slice were calculated using the following formula:

[(Field of view (FOV)/Matrix size (MTX)) × Slice thickness] × number of pixels included in delimitated area.

2.2.6. Batch 2: Hormone measurements

Control and NH-treated rats from both strains were evaluated for their pre- and post-stress hormone responses at 6 months of age. Blood samples were taken (between 9:30 and 12:30 h) by tail-nick procedure in resting conditions, in order to evaluate basal and post-stress hormone levels. One week after basal blood sampling (for baseline hormonal measurements), rats were individually exposed to a novel environment (plexiglas cage, 40 cm × 40 cm × 40 cm) for 20 min in a novel room (white-painted walls, fluorescent illumination), and post-stress blood samples (for post-stress hormonal measurements) were taken by tail-nick immediately following the 20 min exposure to the novel cage. All care was taken to get each animal's blood sample in less than 2 min after removal from its home cage (baseline sampling) or from the novel cage (post-stress sampling). The animals were returned to their home cages in the animal room after each blood sampling. The tail-nick consisted of gently wrapping the animals with a cloth, making a 2 mm incision at the end of one of the tail arteries and then massaging the tail while collecting approximately 200 μL of blood into ice-cold Safe-lock 2.0 ml tubes (Eppendorf, Hamburg, Germany). Plasma obtained after centrifugation was stored at −80 °C until processing. Enzyme-linked immuno sorbent assay (competitive ELISA), determined plasma corticosterone and prolactin levels. EMS Reader MF V.2.9-0 was the reader make used. Corticosterone EIA (Inmunodiagnostic System Ltd., IDS Ltd.; Boldon, United Kingdom) was the corticosterone reagent used, ProlactinRat ELISA (Demeditec Diagnostics, GmbH; Germany) was the prolactin reagent used. Finally,the Immulite® 1000 (Siemens) was the apparatus used to obtain the results.

2.2.7. Batch 3: Two-way active, shuttle box avoidance (SHAV) acquisition

Control and NH-treated rats from both strains were evaluated for two-way active -shuttle box- avoidance acquisition at 6 months of age.The experiment was carried out with 2 identical shuttle boxes (Letica, Panlab, Barcelona, Spain), each placed within independent, sound-attenuating boxes. A dim and diffuse illumination (50 lx inside the shuttle boxes) was provided by a fluorescent bulb placed behind the opaque wall of the shuttle boxes. The experimental room was kept dark. The shuttle boxes consisted of two equally sized compartments (25 cm × 25 cm × 28 cm), connected by an opening (8 cm × 10 cm). A 2400-Hz, 63-dB tone plus a light (from a small, 7-W lamp) functioned as the conditioned stimulus (CS). The unconditioned stimulus (US) which commenced at the end of the CS, was a scrambled electric shock of 0.7 mA delivered throu-
gh the grid floor. Once the rats were placed into the shuttle box, a 4-min familiarization period elapsed before training commenced. Each training trial consisted of a 10-s CS, followed by a 20-s US (no CS-US overlapping). The CS or US was terminated when the animal crossed to the other compartment, with crossing during the CS being considered as an avoidance response (SHAV) and during the US as an escape response. A 60-s inter-trial interval (ITI) was used. Training consisted of a single 50-trial session.

2.3. Data analysis

Statistical analyses were performed using the "Statistical package for social science" (SPSS, version 17). All variables were normally distributed (Kolmogorov-Smirnov test) except NOE-L, which was Ln transformed to achieve normality ($p > 0.05$). As ANOVA analysis revealed that total brain volume is greater in RLA than in RHA rats, the volume of every structure was corrected and expressed as a percentage (%) of brain volume, which was used for analysis.

Factorial 2 × 2 ANOVAs ("2 Strain" × "2 treatment conditions") were applied to measures from the "novel object exploration", "elevated zero-maze", "prepulse inhibition" and two-way active

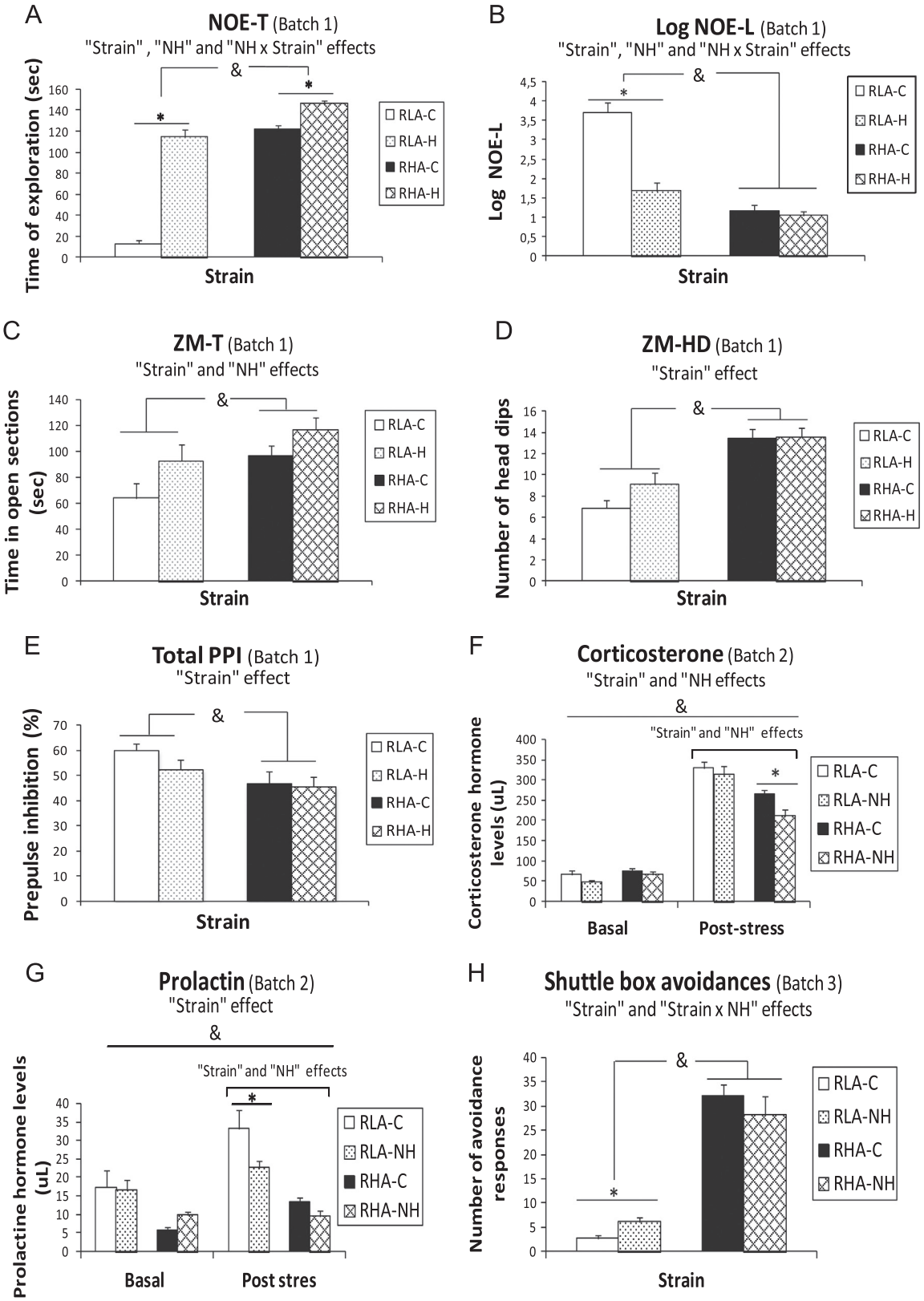

Figure 1 Mean ± S.E.M of: (A) total time spent exploring the novel object (NOE-T) and (B) latency to explore (time elapsed to begin exploration of) the novel object (transformed into their logarithm values; "Log NOE-L") in the NOE test; (C) time spent in open sections (ZM-T), (D) number of head dips (ZM-HD) and (E) total % prepulse inhibition (% PPI) averaged for the four prepulse intensities (65, 70, 75, 80 dB). (F) Corticosterone hormone levels; (G) Prolactine hormone levels; (H) number of avoidance responses in the two-way avoidance acquisition, the shuttle box test. &, indicates the "Strain" effect; *$p<0.05$ between groups indicated (Duncan's multiple range tests following significant ANOVA effects). Group symbols: C, control non-handled group; H, neonatally handled (NH) group. A-E (Batch 1) $n=64$; 16/group. F, G (Batch 2) $n=37$ for Corticosterone ($n=8$-10/group) and $n=30$ for Prolactin ($n=6$-8/group). H (Batch 3) $n=50$; RLA, Control $n=15$, NH $n=23$; RHA, Control $n=5$, NH $n=7$ (the "n" difference between the strains being due to the fact that no effect of NH treatment was expected in male RHA rats according to previous results; see Rio-Álamos et al., 2015).

avoidance tests, as well as to MRI measures. Repeated measures factorial ANOVAs, with "basal and post stress hormone levels" as within-subject factor, were applied to corticosterone and prolactin levels. As between strain and "pre-/post-stress" factors were found, factorial 2 × 2 ANOVAs were applied independently for each, basal and post stress hormone levels. Post-hoc Duncan's multiple range tests were applied to all dependent variables following significant ANOVA effects. Significance level was set at $p \leq 0.05$.

In Batch 1, Pearson's correlation coefficients and forward stepwise multiple regressions were performed with behavioral measures and MRI volumes as dependent/independent variables. Factor analysis (direct oblimin rotation method) was also performed.

3. Results

In the "novel object recognition" test (Batch 1), RLA rats showed higher latency to explore the novel object and less time exploring it than RHAs, ["Strain" effects, both $Fs(1, 60) \geq 69.74$ and $p<0.001$]. NH significantly reduced latency and increased time of exploration ["NH" effects, both $Fs(1, 60) \geq 32.13$ and $p<0.001$]. There were also "Strain × NH" interactions, as NH effects were globally stronger in RLA rats ["Strain × NH" effects, both $Fs(1, 60) \geq 71.48$ and $p<0.001$] (Figure 1A, B; including Duncan's tests).

In the "elevated zero maze" test, RLA rats showed decreased time spent in open sections, less number of entries in open sections and also less number of head dips and line crossings compared to RHA rats ["Strain" effects, all $Fs(1, 60) \geq 5.00$ and $p \leq 0.03$]. NH-treated animals spent more time in open sections than control rats ["NH" effects, $F(1, 69)=9604.0$ and $p=0.027$] (Figure 1C,D, Table S1).

No significant effects were observed in "baseline startle" (Table S1). Compared to RHAs, RLA rats showed higher levels of prepulse inhibition [$F(1, 60) =6.48$, $p=0.013$] (see Figure 1E, and Table S1).

Regarding MRI measures, RLA rats showed larger brain volume than the RHAs ["Strain" effect, $F(1, 28)=26.08$, $p<0.001$] (see Figure 2A). RLA rats also showed greater total hippocampus volume (THc: +11,7%), as well as larger ventral hippocampus (VHc: +18.2%) and amygdala (Am: +14%) than RHA rats ["Strain" effects in all parameters, $Fs(1, 28) \geq 57.53$ and $p<0.001$] (Figure 2B,D,E including Duncan´s test). NH-treated animals also showed a global decrease of amygdala volume ["NH" effect, $F(1, 28)=8.43$, $p=0.007$], that was especially pronounced in RLA rats (see Figure 2E and Duncan's test). Finally, "NH × Strain" effects were observed on total hippocampus and dorsal hippocampus volume ["Strain x NH" effects, both $Fs(1, 28) \geq 5.86$ and $p \leq 0.02$], as NH significantly reduced the volume of both regions in RLA but not in RHA rats (see Figure 2B,C). No effects were observed on striatum volume (see Figure 2F).

Summarizing the most important associations between volumetric measures and behavior, significant (Pearson's) correlations (Table S2) were observed between THc and VHc volume and "latency to explore the novel object" ($r=0.57$ and $r=0.43$ respectively), "time exploring the novel object" ($r=-0.61$ and $r=-0.44$ respectively) and head-dipping in the elevated zero-maze ($r=-0.51$ and -0.49 respectively). THc volume also correlates with %PPI ($r=0.39$). Total amygdala (Am) volume showed correlations with head-dipping in the elevated zero-maze ($r=-0.43$), "latency to explore the novel object" ($r=0.69$), "time exploring the novel object" ($r=-0.68$). Striatum volume did not show correlations with any behavioral measure (see further correlations in Table S2).

High positive correlations between THc and Am volume were observed ($r=0.726$) and also between VHc and Am ($r=0.76$) (Table S2), whereas striatum volume was not correlated with hippocampus nor amygdala volume but showed correlation with dorsal hippocampus ($r=0.40$) (see further correlations in Table S2).

Consistent with these correlational patterns multiple regression analyses showed that "latency to explore the novel object" and "time exploring the novel object" are predicted by amygdala volume, while head-dipping in the elevated zero-maze and PPI are predicted by THc volume. When region volumes were taken as dependent variables and behavioral measures as predictors, significant regression models were observed for THc volume, which is jointly predicted by NOE-T and ZM-HD (Table 1A), and also for VHc volume, which is predicted by ZM-HD (Table 1A; Figure 3A-F). Further supporting these analyses, factor analysis showed: 1) a first factor mainly grouping THc, VHc and amygdala volumes (loadings >0.80 in all cases) with three anxiety measures (loadings of -0.57, -.0.83, 0.81); 2) a second factor grouping zero-maze and PPI variables, and 3) a third factor with loadings of St and DHc volume (Table 1B).

In Batch 2, the repeated measures factorial ANOVA indicated that RLA rats showed overall higher corticosterone and prolactin levels than RHAs ["Strain effect" in both parameters, $Fs(1, 31) \geq 14.62$ and $p \leq 0.001$]. NH-treated animals showed decreased levels of corticosterone compared to control animals ["treatment" effect, $F(1, 31)=7.55$, $p=0.01$]. "Strain × pre-/post-stress" effects were observed in both parameters [both $Fs(1, 31) \geq 15.4$ $p \leq 0.001$]. Thus, separate 2 × 2 ANOVAs for "pre-stress" and "post-stress" measures indicated no significant effects for pre-stress levels but revealed "strain" [$Fs(1,31) \geq 27.49$ and $p<0.001$] and "NH" effects on post-stress corticosterone and prolactin [both $Fs(1, 31) \geq 5.14$ and $p \leq 0.03$], as RLA rats showed increased post-stress hormone responses and NH led to overall reductions of both hormones (see Duncan's tests in Figure 1 F,G).

In Batch 3, the 2 × 2 ANOVA on avoidances showed "strain" [$F(1, 46)=207.48$, $p<0.001$] and "strain × NH" effects [$F(1, 46)=4.24$, $p<0.05$], as RHA rats made many more avoidances than RLAs, while NH increased two-way avoidance responses in RLA rats without significantly affecting avoidance acquisition in RHA rats (see Duncan's tests in Figure 1H].

4. Discussion

The present study shows that, compared with RHAs, RLA rats are more anxious, present increased PPI levels and enhanced endocrine responses to stress, in agreement with the notion that RLA rats are a genetic model of increased anxiety and stress responses (e.g. Carrasco. et al., 2008; Díaz-Morán et al., 2012; Driscoll et al., 2009; Steimer and Driscoll 2003). NH treatment markedly decreased unconditioned anxiety (without affecting overall activity, as indicated by line crossings in the zero-maze) in RLA rats, reduced conditioned anxiety (as shown by the increase of two-way avoidance responses in RLAs), and decreased post-stress hormone responses, which is in line with our previous reports of NH anxiolytic and anti-stress effects in different tests (e.g. Fernández-Teruel et al., 2002; Rio-Alamos et al., 2015; Steimer et al., 1998). Of note, NH anti-stress effects on hormone responses and anxiolytic-like effects on two-way avoidance acquisition were observed in separate batches (batches 2 and 3) of naive rats at 6 months of

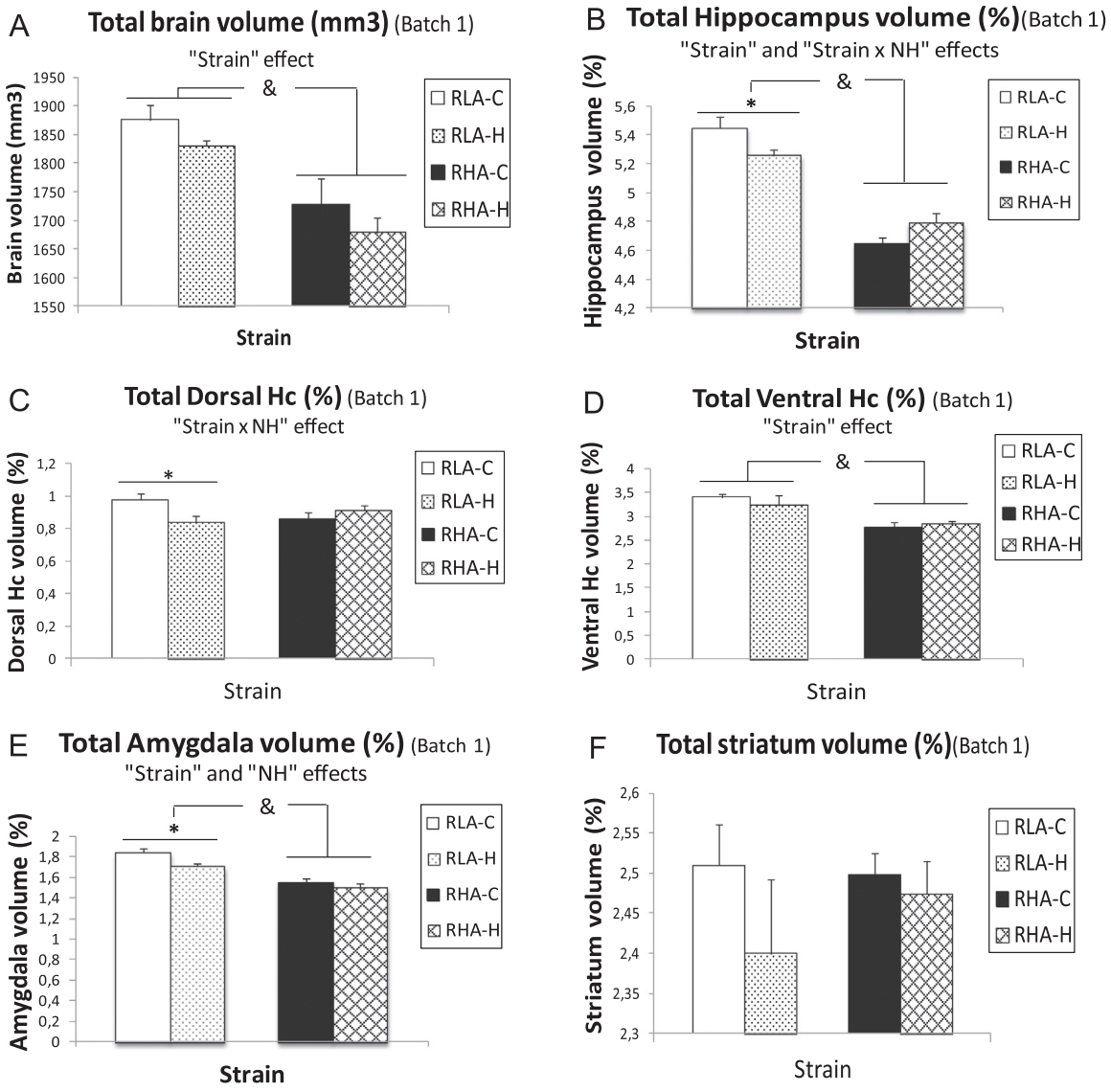

Figure 2 Mean ± S.E.M of (A) total brain volume (BV) in mm^3(B) total hippocampus volume (THc %); (C) dorsal hippocampus volume (DHc %); (D) ventral hippocampus volume (VHc %); (E) amygdala volume (Am %) and (F) striatum volume (St %). & indicates the "Strain" effect (see text for significance); *$p<0.05$ between groups indicated (Duncan´s multiple range tests following significant ANOVA effects). Group symbols: C, control non-handled group; H, neonatally handled (NH) group. ($n=32$; 8/group, randomly taken from the total sample of Batch 1, $n=64$).

age, thus providing independent evidence that NH anti-anxiety and anti-stress effects are present at 6 months of age, the time in which MRI measurements were taken in Batch 1.

Previous studies addressing the effects of environmental treatments/manipulations on hippocampus/amygdala volume and anxiety/stress have essentially focused on the consequences of chronic stressors (Bourgin et al., 2015; Delgado y Palacios et al., 2011; Lee et al., 2009; Schubert et al., 2009), which lead to hippocampal atrophy (Bourgin et al., 2015; Lee et al., 2009) and amygdala hypertrophy (Bourgin et al., 2015; Salm et al., 2004), supposedly as a consequence of the stress-induced chronic hypercortisolemia and overstimulation of these limbic areas (e.g. Bourgin et al., 2015; Mc Ewen et al., 2015). However, the present is the first study evaluating changes in hippocampus and amygdala volume after an infantile anxiety/stress-reducing treatment in a genetically-based rat model of anxiety (Río-Alamos et al., 2015). According to Gray's theory on the neuropsychology of anxiety (Gray and McNaughton 2000) and the reviewed studies we predicted that: 1) RLA rats would present greater hippocampal/amygdala volumes (in parallel to higher anxiety and PPI levels) than RHA rats; 2) NH should decrease anxiety and hippocampus/amygdala volume in RLA rats; 3) there would be positive associations (as assessed through correlations, regression and factor analyses) between hippocampus/amygdala volume and anxiety and PPI.

The main novel findings reported in the present study are: i) RLA rats have larger brain, hippocampus and amygdala volumes than RHA rats; ii) NH reduces anxiety and hippocampal and amygdala volume in RLA rats in parallel to a reduction of anxiety; iii) hippocampal volume

Table 1 (A) Linear regressions (forward stepwise method) between amygdala volume (Am %, as independent variable, predictor) and (1) latency to explore the novel object (Log NOE-L, as dependent variable) and (2) time spent exploring the novel object in the NOE test (NOE-T, as dependent variable); and between T-Hc volume (T-Hc %, as independent variable, predictor), and (3) number of head dips in the ZM test (ZM-HD, as dependent variable) and (4) % total prepulse inhibition (% PPI) in the PPI test (as dependent variable). (5) Multiple linear regression taking all behavioral variables as potential predictors (or independent variables; forward stepwise method) and total hippocampus volume (THc %) as dependent variable. Log NOE-L and NOE-T are predicted by T-Am volume (%); ZM-HD and total %PPI are predicted by THc volume (%). THc volume is jointly predicted by NOE-T and ZM-HD, and (6) VHc is predicted by ZM-HD. When using dorsal striatum as independent variable no correlations were present. (B) Two-factor solution and correlation between factors. Loadings ≥ 0.30 are shown. Symbols: Log NOE-L and NOE-T, Ln transformed latency and time of exploration in novel object exploration test (respectively); ZM-T, ZM-HD and ZM-LC, time in open section; numbers of head dips and numbers of line crossings in elevated zero maze test (respectively); %PPI, percentage of prepulse inhibition; THc, DHc and VHc, Total, dorsal and ventral hippocampus volume; Am, amygdala volume; St, striatum volume ($n=32$).

(A) Forward stepwise multiple regressions

Dependent variable	Method	Step	Predictor variables	r	r^2	$p \leq$
(1)-Log NOE-L	Forward Stepwise	1	T-Am volume (%)	0.69	0.48	0.001
(2)-NOE-T	Forward Stepwise	1	T-Am volume (%)	-0.68	0.46	0.001
(3)-ZM-HD	Forward Stepwise	1	T-Hc volume (%)	-0.51	0.26	0.003
(4)-% PPI	Forward Stepwise	1	T-Hc volume (%)	0.39	0.15	0.027
(5)-THc volume (%)	Forward Stepwise	2	NOE-T; ZM-HD	-0.67	0.45	0.001
(6)-VHc volume (%)	Forward Stepwise	1	ZM-HD	0.49	0.24	0.005

(B) Factorial analysis (oblimin direct rotation) with behavioral and volumetric variables. Loadings ≥ 0.30 are shown

Dependent variable	Factor 1	Factor 2	Factor 3
Log NOE_L	0.81	-	-
NOE-T	-0.83	-	-
ZM-T	-	0.88	-
ZM-HD	-0.57	0.80	-
ZM-LC	-	0.80	-
Baseline startle	-	-0.55	-
% PPI	0.39	-0.66	-
THc volume (%)	0.89	-	-
DHc volume (%)	0.34	-	0.68
VHc volume (%)	0.81	-	-
Am volume (%)	0.88	-	-
St volume (%)	-	-	0.88
% of variance (cumulative)	39.78	56.47	67.99
Correlations between factors			
1	1		
2	-0.30	1	
3	0.07	0.05	1
$N=$	32		

is positively associated with anxiety measures as well as with PPI levels; and, iv) amygdala volume is also associated with anxiety responses but not with PPI. Conversely, dorsal striatum volume, which was taken as a control area and was expected not to be associated to anxiety, does not differ between both rat strains, is not affected by NH and does not show associations with anxiety measures. Thus, it does not seems that in RLA rats "everything within the brain is bigger", but some specific anxiety-related areas are proportionally bigger than in RHA rats. The present anxiety-volumetric associations (factor analysis, regression), which are specifically observed with hippocampus and amygdala volumes but not with striatal volume, are consistent with the contention that the two former structures are related to the anxiety profiles and the NH effects observed.

Concerning hippocampus volume and anxiety in rats, the study by Kalisch et al. (2006) is, to our knowledge, the only previous related work. The HAB ("high anxiety behaviour") rat line had larger hippocampal volumes than their LAB ("low anxiety behaviour") counterparts. There was also a positive correlation between anxiety behaviour and hippocampus volume which, as the authors themselves recognize, was likely a biased and inconclusive result, due to the dichotomous distribution of the behavioural and volumetric

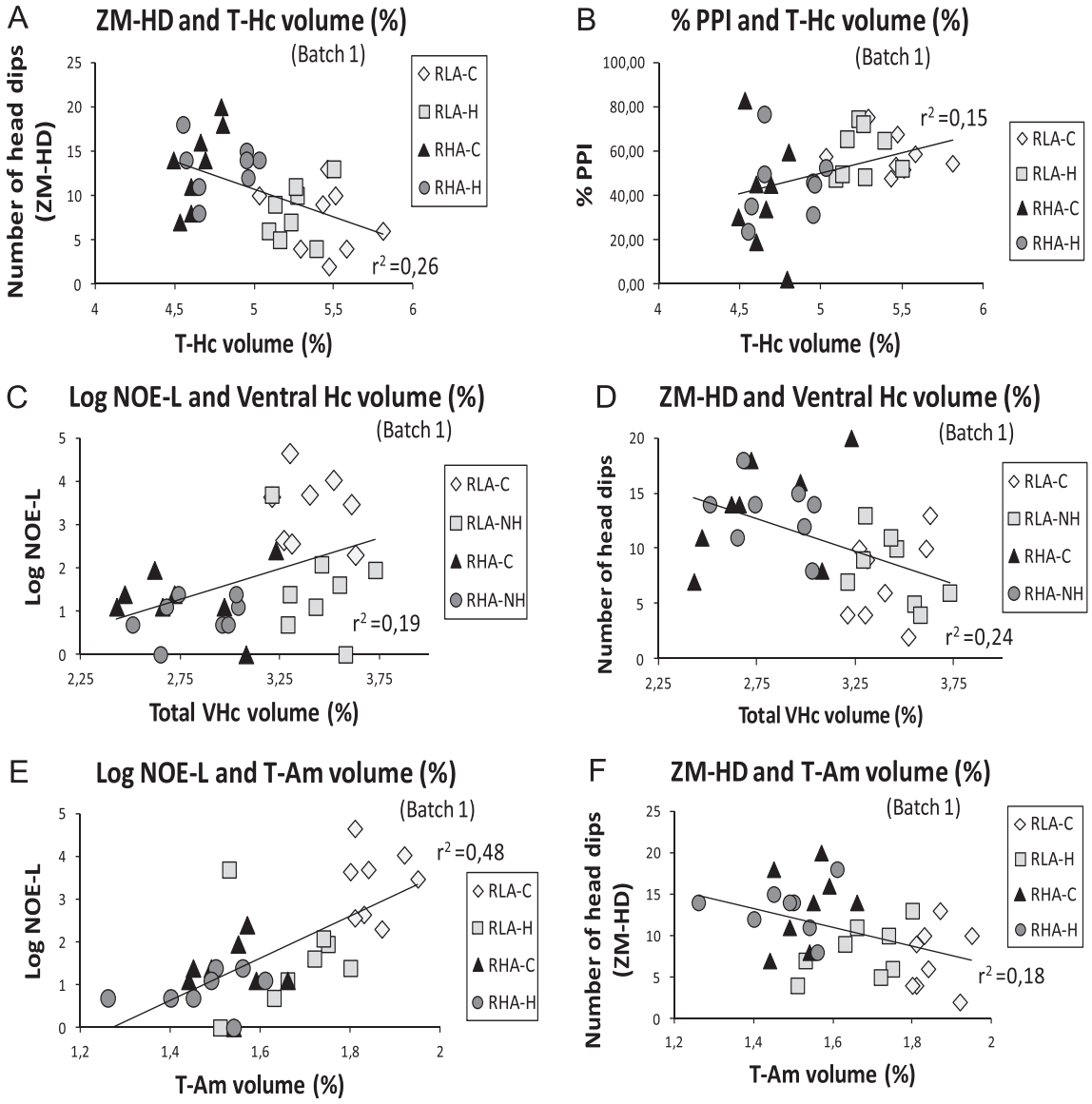

Figure 3 Linear regressions between (A) number of head dips in the "elevated zero maze" test (ZM-HD) and (B) percentage of total prepulse inhibition in the "prepulse inhibition" test (PPI) as independent variables (predictors) and total hippocampus volume (THc %) as a dependent variable, (C) latency of time elapsed in begin the exploration of a novel object in NOE test and (D) number of head dips in the "elevated zero maze" test (ZM-HD) as independent variables (predictors) and ventral hippocampus volume (VHc %) as a dependent variable, and (E) latency of time elapsed in begin the exploration of a novel object in NOE test and (F) number of head dips in the "elevated zero maze" test (ZM-HD) and amygdala volume (AM %) as a dependent variable. Group symbols: C, control non-handled group; H, neonatally handled (NH) group ($n=32$; 8/group).

measures. Our results, instead, show significant correlations between hippocampal (and amygdala) volume and anxiety, with the data in all cases fitting normal (rather than dichotomous) distributions.

Our present findings, observed for the first time in rats, are therefore consistent with Gray's theory of septo-hippocampal system and anxiety and with studies in healthy humans and nonhuman primates indicating positive associations between hippocampal/amygdala volume and anxiety (Barros-Loscertales et al., 2006; Cherbuin et al., 2008; Levita et al., 2014; Oler et al., 2010). However, it may seem at first sight surprising that an anxiety-reducing treatment such as NH produces hippocampal volume reduction, because hippocampal atrophy has also been observed following chronic stress (e.g. Bourgin et al., 2015; Lee et al., 2009; Mc Ewen et al., 2015), which produces behavioural (e.g. increased signs of anxiety, depression-like features) and endocrine (enhanced hormone responses to acute stressors) effects opposite to those observed after NH. Nevertheless, it has to be kept in mind that chronic stress and NH would reduce hippocampal volume through essentially different mechanisms. First, the deleterious effects of chronic stressful events on the hippocampus are thought to critically depend on a chronic and "abnormal" state of neuronal overstimulation by glucocorticoids (Mc Ewen et al., 2015), which leads to

neurotoxicity (i.e. neuronal functional and/or structural damage in the hippocampus) and atrophy. Second, trait anxiety, which is reduced by NH (present behavioural results), refers to a psychological/cognitive dimension which is qualitatively different from "chronic stress", and relates to "normal" stable emotional/cognitive processes involved in monitoring threat and conflict, a "normal" function in which the hippocampus is known to be crucial (e.g. Bach et al. 2014; Gray and McNaughton, 2000). Thus, as far as volume is thought to be related with function (e.g. Taubert et al., 2012; Woollett and Maguire, 2011), the decreased hippocampal activity in relatively less anxious subjects (Bach et al., 2014; Gray and McNaughton 2000) would be associated with decreased volume (Barros-Loscertales et al., 2006; Kalisch et al., 2006) and, accordingly, enduringly reducing trait anxiety (e.g. through NH) might be expected to reduce hippocampal volume. This would actually cohere with the anxiolytic effects of hippocampal lesions or administration of anxiolytic drugs, which reduce hippocampal activity (Gray and McNaughton, 2000). Hypothetically, NH would enduringly reduce hippocampal volume through an enduring decrease of some aspects of its threat monitoring function (i.e. anxiety-related activity) in RLA rats (which have a "hyperfunctional" hippocampus, compared with RHA rats; see "Introduction"), and this would lead to an enduring reduction of anxiety.

Studies on the functional specialization of hippocampal subregions have led to the proposal that the dorsal part of the hippocampus would preferentially be involved in mnemic functions and spatial information processing while the ventral hippocampus is supposed to be mostly related to emotional processing (i.e. anxiety, acquisition of fear conditioning; Bannerman et al., 2004, 2014). Notably, in this regard, our present findings show that ventral hippocampus volume is different between the strains (RLA > RHA), while NH reduces dorsal (but not ventral) hippocampus volume in RLA rats. Since NH induces a long-lasting reduction of unconditioned anxiety in RLAs we would have expected a reduction of ventral hippocampus by NH, but this was not the case. That result may seem paradoxical compared with the above mentioned dominant notion on dorsal and ventral hippocampus functional specialization (e.g. Bannerman et al. 2004, 2014), although the following findings concerning dorsal hippocampus manipulations and anxiety should also be taken into account: 1) When administered into the dorsal hippocampus, the GABA-A agonist muscimol, the CCK2 receptor antagonist LY225910 and the Avpr1b receptor antagonist SSR 149415 (all of them showing anxiolytic-like effects in different tests) produced anxiolytic effects in the elevated plus maze test (Engin and Treit 2008; Rezayat et al. 2005), whereas administration (into the dorsal hippocampus) of the CCK2 receptor agonist CCK8s induced anxiogenic effects in the same test (Rezayat et al. 2005). Moreover dorsal and ventral hipppocampal muscimol similarly disrupted contextual fear conditioning (Zhang et al. 2014). 2) Temporary inactivation of the dorsal hippocampus by lidocaine induced a decrease of fear behaviour during a retention session (24 h following the initial "training" session) in the shock-probe burying test, while lidocaine infused in the ventral hippocampus impaired fear acquisition in the first session without affecting retention (McEown and Treit 2009). On the other hand, inactivation of the dorsal hippocampus by tetrodotoxin induced anxiolytic-like effects in the shock-probe burying test (Degroot and Treit 2004). 3) Injection of the acetylcholinesterase inhibitor physostigmine (which increases acetylcholine availability and has anxiolytic-like effects) in either the dorsal or ventral hippocampus had anxiolytic effects in the elevated plus maze (Degroot and Treit 2002). 4) The interaction of dorsal hippocampus with basolateral amygdala has been shown to be involved in processing some of the consequences of controllability vs. uncontrollability of stress (Hadad-Ophir et al., in press).

Hence, although anxiety has preferentially been related to ventral hippocampal function (e.g. Bannerman et al. 2004, 2014), the above reviewed findings suggest that (at least in some instances) the dorsal hippocampus has also a role on anxiety/fear processing. Our finding that NH reduces dorsal hippocampus volume in RLA rats would be in keeping with that evidence. Nevertheless, the present correlational, factor and regression analyses reveal that anxiety-related behaviors are more strongly associated with ventral than dorsal hippocampus volume (see Figure 3C,D, Table 1A,B; Table S2), consistent with the proposed involvement of ventral hippocampus in anxiety (e.g. Bannerman 2004, 2014).

Dorsal and ventral hippocampus present different connectivity with subcortical structures. Thus, for instance, the ventral hippocampus is known to have more dense connections with the amygdala (and hypothalamus) than the dorsal hippocampus, although the latter has also connections with amygdaloid lateral regions (Grigoryan and Segal 2016). Given the interplay between both hippocampal subregions and amygdala, and the recognized role of the latter on emotion/stress regulation, we expected between-strain differences and NH effects on amygdala volume that would parallel the observed effects on anxiety. Remarkably, the high-anxious RLA rats showed increased amygdala volume, which is consistent with findings of increased neuronal density and novelty-induced c-Fos in this strain (Gómez et al., 2008; Meyza et al., 2009). Amygdala-anxiety associations were also observed (Figure 3E,F; Table 1A,B; Table S2), this being in line with two studies in humans that have reported similar positive correlations (Barros-Loscertales et al., 2006; Rahman et al., 2014). Interestingly, the associations between amygdala and hippocampus volume were positive and stronger with the ventral than dorsal hippocampus (Table 1B; Table S2), which is consistent with the known stronger connectivity of amygdala with ventral hippocampus than with the dorsal part. On the other hand, it is known that prolonged stress leads to enhanced dendritic branching, increased spine density and hypertrophy in the amygdala (Bourgin et al., 2015; Mc Ewen et al., 2015). Remarkably, however, the present is the first report of a long-lasting reduction of amygdala volume as a consequence of an environmental infantile anxiety/stress-reducing treatment (NH). Thus, the genetically-based differential amygdala and hippocampus volume in RLA vs RHA rats may be enduringly modulated by environment. Moreover, the present results represent a proof of concept of amygdala-anxiety association, by showing that such a relationship holds also true in the other direction, i.e. reduction of anxiety and stress sensitivity paralleled by reduction of amygdala volume.

Although not affected by NH, PPI shows positive associations with hippocampal volume (see Table 1A,B; Table S1). This appears to be in line with the proposal that hippocampus modulates PPI (e.g. Koch and Schnitzler 1997; Swerdlow et al., 2001). In fact, there are studies showing that PPI is

positively correlated with hippocampal volume in untreated rats and in humans (Kumari et al., 2005; Schubert et al., 2009). It seems then paradoxical that NH affects hippocampal volume without influencing PPI, so further investigation is warranted on this issue.

In summary, the present study shows for the first time that RLA rats have greater hippocampal and amygdala volumes than RHA rats, which coheres with the higher trait anxiety levels of the former (e.g. Rio-Alamos et al., 2015) and with Gray's model on the role of the septo-hippocampal system in anxiety (e.g. Barros-Loscertales et al., 2006; Gray and McNaughton, 2000). Most importantly, we have shown that NH, a treatment that induces permanent reductions of anxiety (see also Rio-Alamos et al. 2015, and references therein), leads to long-lasting decreases of hippocampus and amygdala volume in RLA rats, which constitutes a sort of proof of concept that both structures are related to anxiety. These are particularly outstanding findings, as previous works in rats have mostly focused on the effects of different forms of chronic stress treatments on hippocampal and/or amygdala volumes on a short term basis, but no study had thus far evaluated the long-lasting consequences of an anxiety/stress-reducing treatment – such as NH – on the volume of these structures. While the marked strain-related divergences in hippocampus and amygdala volume point to the possible genetic basis of these structural differences (Oler et al., 2010), the observed NH treatment effects indicate that significant modulation/plasticity induced by environmental conditions is possible even on a long-term basis. The positive relationships among anxiety measures and hippocampus/amygdala volumes, as shown by factor and regression analysis, further support the observed treatment effects and are consistent with the hypothesized role of the septo-hippocampal system, including the amygdala, in anxiety temperament and responses (e.g. Bach et al., 2014; Barros-Loscertales et al., 2006; Gray and McNaughton, 2000).

5. Conclusions

In conclusion, we present the first evidence that, 1) genetically-based differential anxiety (i.e. RLA > RHA) is paralleled by (and associated with) specific differences in relative hippocampus and amygdala volume (RLA > RHA), but not in relative dorsal striatum volume, and 2), a neonatal anxiety/stress-reducing treatment (NH) leads to long-lasting dorsal hippocampus and amygdala volume reductions (in the high-anxious RLA rats) which may be involved in the observed NH behavioral effects. This paradigm may be useful to study the morphological/plasticity and molecular/functional changes produced by NH within these brain structures, and whether such changes underlie long-lasting behavioral effects (e.g. anxiety/stress-related, and possibly other, responses or traits).

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The funding agencies had no role in the study design, collection, analysis and interpretation of data, writing of the report, or the decision to submit the paper for publication.

Contributors

C.R-A, A.F-T and A.T. conceived and designed the experiments. C.R-A, I.O., C.G., M.A.P., E.M-M., S.L-P., A.F-T, T.C. and G.B. performed the experiments. C.R-A, R.T., A.F-T analyzed and interpreted the data. C.R-A and A.F-T wrote the paper. All authors revised the manuscript, made contributions and approved the final manuscript.

Conflict of interest

The authors have no potential conflicts of interest to declare in relation to the work described.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.euroneuro.2016.12.003.

References

Anisman, H., Zaharia, M.D., Meaney, M.J., Merali, Z., 1998. Do early-life events permanently alter behavioral and hormonal responses to stressors? Int. J. Dev. Neurosci. 16, 149–164.

Bach, D.R., Guitart-Masip, M., Packard, P.A., Miró, J., Falip, M., Fuentemilla, L., Dolan, R.J., 2014. Human hippocampus arbitrates approach-avoidance conflict. Curr. Biol. 24 (5), 541–547.

Bannerman, D.M., Rawlins, J.N., McHugh, S.B., Deacon, R.M., Yee, B.K., Bast, T., 2004. Regional dissociations within the hippocampus-memory and anxiety. Neurosci. Biobehav Rev. 28 (3), 273–283.

Bannerman, D.M., Sprengel, R., Sanderson, D.J., McGHugh, B., Rawlins, J.N., Monyer, H., Seeburg, H., 2014. Hippocampal synaptic plasticity, spatial memory and anxiety. Nat. Rev. 15, 181–192.

Barros-Loscertales, A., Meseguer, V., Sanjuan, A., Belloch, V., Parcet, M.A., Torrubia, R., 2006. Behavioral Inhibition System activity is associated with increased amygdala and hippocampal gray matter volume: a voxel-based morphometry study. Neuroimage 33 (3), 1011–1015.

Bentareha, R., Araujo, F., Ruano, D., Driscoll, P., Escorihuela, R.M., Tobeña, A., 1998. Pharmacological properties of the GABA (A) receptor complex from brain regions of (hypoemotional) Roman high- and (hyperemotional) low-avoidance rats. Eur. J. Pharmacol. 354 (1), 91–97.

Bourgin, J., Cachia, A., Boumezbeur, F., Diemaï, B., Bottlaender, M., Duchesnav, E., Mériaux, S., 2015. Hyper-responsivity to stress in rats is associated with a large increase in amygdala volume. A 7T MRI study. Eur. Neuropsychoapharmacol. 25 (6), 828–835.

Carrasco, J., Márquez, C., Nadal, R., Tobeña, A., Fernández-Teruel, A., Armario, A., 2008. Characterization of central and

peripheral components of the hypothalamus-pituitary-adrenal axis in the inbred Roman rat strains. Psychoneuroendocrionology 33, 433–437.
Cherbuin, N., Windsor, Td, Anstey, K.J., Maller, J.J., Meslin, C., Sachdev, P.S., 2008. Hippocampal volume is positively associated with behavioural inhibition (BIS) in a large community-based sample of mid-life adults: the PATH through life study. Soc. Cogn. Affect. Neurosci. 3 (3), 262–269.
Davis, M., Whalen, P.J., 2001. The amygdala: vigilance and emotion. Mol. Psychiatry 6, 13–34.
Degroot, A., Treit, D., 2004. Anxiety is functionally segregated within the septo-hippocampal system. Brain Res. 1001, 60–71.
Degroot, A., Treit, D., 2002. Dorsal and ventral hippocampal cholinergic systems modulate anxiety in the plus-maze and shock probe tests. Brain Res. 949, 60–70.
Delgado y Palacios, R., Campo, A., Henningsen, K., Verhoye, M., Dijkstra, J., Van, Audekerke, 2011. Magnetic resonance imaging and spectroscopy reveal differential hippocampal changes in anhedonic and resilient subtypes of the chronic mild stress rat model. Biol. Psychiatry 70 (5), 449–457.
Díaz-Morán, S., Palència, M., Mont-Cardona, C., Cañete, T., Blázquez, G., Martínez-Membrives, E., 2012. Coping style and stress hormone responses in genetically heterogeneous rats: comparison with the Roman rat strains. Behav. Brain Res. 228, 203–210.
Driscoll, P., Escorihuela, R.M., Fernández-Teruel, A., Giorgi, O., Schwegler, H., Steimer, T., 1998. Genetic selection and differential stress responses. The Roman lines/strains of rats. Ann. N. Y. Acad. Sci. 851, 501–510.
Driscoll, P., Fernández-Teruel, A., Corda, M.G., Giorgi, O., Steimer, T., 2009. In: Yong-Kyu, K. (Ed.), Some guidelines for defining personality differences in rats. In Handbook of Behavior Genetics. Springer, New York, NY, pp. 281–300.
Engin, E., Treit, D., 2008. Dissociation of the anxiolytic-like effects of Avpr1a and Avpr1b receptor antagonists in the dorsal and ventral hippocampus. Neuropeptides. 42, 411–412.
Escorihuela, R.M., Tobeña, A., Driscoll, P., Fernández-Teruel, A., 1995. Effects of training, early handling, and perinatal flumazenil on shuttle box acquisition in Roman Low-avoidance Rats: toward overcoming a genetic deficit. Neurosci. Biobehav. Res. 19, 353–367.
Escorihuela, R.M., Fernández-Teruel, A., Gil, L., Aguilar, R., Tobeña, A., Driscoll, P., 1999. Inbred Roman high- and low-avoidance rats: differences in anxiety, novelty-seeking, and shuttlebox behaviors. Physiol. Behav. 67, 19–26.
Estanislau, C., Díaz-Morán, S., Cañete, T., Blázquez, G., Tobeña, A., Fernández-Teruel, A., 2013. Context-dependent differences in grooming behavior among the NIH heterogeneous stock and the Roman high- and low-avoidance rats. Neurosci. Res. 77, 187–201.
Fernández-Teruel, A., Escorihuela, R.M., Núñez, J.F., Zapata, A., Boix, F., Salazar, W., 1991. The early acquisition of two-way (shuttle-box) avoidance as an anxiety-mediated behavior: psychopharmacological validation. Brain Res. Bull. 6, 173–176.
Fernández-Teruel, A., Escorihuela, R.M., Núñez, J.F., Gomà, M., Driscoll, P., Tobeña, A., 1992a. Early stimulation effects on novelty-induced behavior in two psychogenetically-selected rat lines with divergent emotionality profiles. Neurosci. Lett. 137, 185–188.
Fernández-Teruel, A., Escorihuela, R.M., Driscoll, P., Tobeña, A., Bättig, K., 1992b. Differential effects of early stimulation and/or perinatal flumazenil treatment in young Roman low- and high-avoidance rats. Psychopharmacology (Berl.) 108 (1–2), 170–176.
Fernández-Teruel, A., Escorihuela, R.M., Castellano, B., González, B., Tobeña, A., 1997. Neonatal handling and environmental enrichment on emotionality, novelty/reward seeking, and age-related cognitive and hippocampal impairments: focus on the Roman rat lines. Behav. Genet 27 (6), 513–526.
Fernández-Teruel, A., Giménez-Llort, L., Escorihuela, R.M., Gil, L., Aguilar, R., Steimer, T., 2002. Early-life handling stimulation and environmental enrichment: are some of their effects mediated by similar neural mechanisms? Pharmacol. Biochem. Behav. 73, 233–245.
Garcia-Falgueras, A., Castillo-Ruiz, M.M., Put, T., Tobeña, A., Fernández-Teruel, A., 2012. Differential hippocampal neuron density between inbred Roman high- (low anxious) and low-avoidance (high anxious) rats. Neurosci. Lett.. 522, 41–46.
Giorgi, O., Orlandi, M., Escorihuela, R.M., Driscoll, P., Lecca, D., Corda, M.G., 1994. GABAergic and dopaminergic transmission in the brain of Roman high-avoidance and Roman low-avoidance rats. Brain Res. 638 (1–2), 133–138.
Gómez, M.J., Morón, I., Torres, C., Esteban, F.J., de la Torre, L., Cándido, A., 2008. One-way avoidance acquisition and cellular density in the basolateral amygdala: strain differences in Roman high- and low-avoidance rats. Neurosci. Lett. 450 (3), 317–320.
Gray, J.A., McNaughton, N., 2000. The Neuropsychology of Anxiety: An Enquiry into the Functions of the Septo-hippocampal System 2nd edn. Oxford University Press, Oxford.
Grigoryan, S., Segal, M., 2016. Lasting differential effects on plasticity induced by prenatal stress in dorsal and ventral hippocampus. Neural Plast. http://dx.doi.org/10.1155/2016/2540462.
Hadad-Ophir, O., Ardi, Z., Brande-Eliat, N., Kehat, O., Anunu, R., Ritcher-Levin, G., 2016. Exposure to prolonged controllable or uncontrollable stress affects GABAergic function in sub-regions of the hippocampus and the amygdala. Neurobiol. Learn. Mem. (in press), http://dx.doi.org/10.1016/j.nlm.2016.06.009.
Hahn, T., Dresler, T., Plichta, M.M., Ehlis, A.C., Ernst, L.H., Markulin, F., Polak, T., 2010. Functional amygdale-hippocampus connectivity during anticipation of aversive events is associated with Gray's trait sensitivity to punishment. Biol. Psychiatry 68 (5), 459–464.
Kalisch, R., Schubert, M., Jacob, W., Kessler, M.S., Hemauer, R., Wigger, A., 2006. Anxiety and hippocampus volume in the rat. Neuropsychopharmacology 5, 925–932.
Kumari, V., Zachariah, E., Ettinger, U., Sharma, T., Reduced prepulse inhibition in unaffected siblings of schizophrenia patients, *Psychophysiology* 42, 2005, 588-94.
Koch, M., Schnitzler, H.U., 1997. The acoustic startle response in rats-circuits mediating evocation, inhibition and potentiation. Behav. Brain Res. 89, 35–49.
LeDoux, J.E., 2000. Emotion circuits in the brain. Ann. Rev. Neurosci. 23, 155–184.
Lee, T., Jarome, T., Li, S.J., Kim, J.J., Helmstetter, F.J., 2009. Chronic stress selectively reduces hippocampal volume in rats: a longitudinal magnetic resonance imaging study. Neuroreport 20 (17), 1554–1558.
Levita, L., Bois, C., Healey, A., Smyllie, E., Papakonstantinou, E., Hartley, T., Lever, C., 2014. The behavioural inhibition system, anxiety and hippocampal volume in a non-clinical population. Biol. Mood Anxiety Disord. 4 (1), 4.
López-Aumatell, R., Vicens-Costa, E., Guitart-Masip, M., Martínez-Membrives, E., Valdar, W., Johannesson, M., 2009a. Unlearned anxiety predicts learned fear: a comparison among heterogeneous rats and the Roman rat strains. Behav. Brain Res. 202, 92–101.
López-Aumatell, R., Blázquez, G., Gil, L., Aguilar, R., Cañete, T., Giménez-Llort, L., 2009b. The Roman High- and low-avoidance rat strains differ in fear-potentiated startle and classical aversive conditioning. Psicothema 21, 27–32.
Martin, J.R., Oettinger, R., Driscoll, P., Buzzi, R., Bättig, K., 1982. Effects of chlordiazepoxide and imipramine on maze patrolling within two different maze configurations by psychogenetically selected lines of rats. Psychopharmacology (Berl.) 78 (1), 58–62.
McEown, K., Treit, D., 2009. The role of the dorsal and ventral hippocampus in fear and memory of a shock-probe experience. Brain Res. 1251, 185–194.

Mc Ewen, B.S., Bowles, N.P., Gray, J.D., Hill, M.N., Hunter, R.G., Karatsoreos, I.N., 2015. Mechanisms of stress in the brain. Nat. Neurosci. 18 (10), 1353–1363.

Mc Naughton, N., Corr, P.J., 2004. A two-dimensional neuropsychology of defense: fear/anxiety and defensive distance. Neurosci. Biobehav. Rev. 28 (3), 285–305.

Meaney, M.J., Aitken, D.H., van Berkel, C., Bhatnagar, S., Sapolsky, R.M., 1988. Effect of neonatal handling on age-related impairments associated with the hippocampus. Science 12, 766–768.

Meyza, K.Z., Boguszewski, P.M., Nikolaev, E., Zagrodzka, J., 2009. Diverse sensitivity of RHA/Verh and RLA/Verh rats to emotional and spatial aspects of a novel environment as a result of a distinct pattern of neuronal activation in the fear/anxiety circuit. Behav. Genet. 39 (1), 48–61.

Oler, J.A., Fox, A.S., Shelton, S.E., Rogers, J., Dyer, T.D., Davidson, R.J., 2010. Amygdalar and hippocampal substrates of anxious temperament differ in their heritability. Nature 466 (7308), 864–868.

Oliveras, I., Rio-Alamos, C., Cañete, T., Blázquez, G., Martinez-Membrives, E., Giorgi, O., 2015. Prepulse inhibition predicts spatial working memory performance in the inbred Roman high- and low-avoidance rats and in genetically heterogeneous NIH-HS rats: relevance for studying pre-attentive and cognitive anomalies in schizophrenia. Front Behav. Neurosci. 18 (9), 213.

Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates, Compact 6th ed. CA. Academic Press, San Diego.

Rahman, A.S., Xu, J., Potenza, M.N., 2014. Hippocampal and amygdalar volumetric differences in pathological gambling: a preliminary study of the associations with the behavioral inhibition system. Neuropsychopharmacology 39 (3), 738–745.

Raineki, C., Lucion, A.B., Weinberg, 2014. Neonatal handling: an overview of the positive and negative effects. Dev. Psychobiol.. 56, 1613–1625.

Rezayat, M., Roohbakhsh, A., Zarrindast, M.-R., Massoudi, R., Djahanguiri, B., 2005. Cholecystokinin and GABA interaction in the dorsal hippocampus of rats in the elevated plus-maze test of anxiety. Physiol. Behav. 84, 775–782.

Río-Alamos, C., Oliveras, I., Cañete, T., Blázquez, G., Matínez-Membrives, E., Tobeña, A., Fernández-Teruel, A., 2015. Neonatal handling decreases unconditioned anxiety, conditioned fear, and improves two-way avoidance acquisition: a study with the inbred Roman high (RHA-I)- and low-avoidance (RLA-I) rats of both sexes. Front. Behav. Neurosci. 9, 174.

Salm, A.K., Pavelko, M., Krouse, E.M., Webster, W., Kraszpulski, M., Birkle, D.L., 2004. Lateral amygdaloid nucleus expansion in adult rats is associated with exposure to prenatal stress. Brain Res. Dev. Brain Res. 148 (2), 159–167.

Schubert, M.I., Porkess, M.V., Dashdori, N., Fone, K.C., Auer, D.P., 2009. Effects of social isolation rearing on the limbic brain: a combined behavioral and magnetic resonance imaging volumetry study in rats. Neuroscience 159 (1), 21–30.

Steimer, T., Escorihuela, R.M., Fernández-Teruel, A., Driscoll, P., 1998. Long-term behavioral and neuroendocrine changes in Roman high- (RHA/Verh) and Low-(RLA/Verh) avoidance rats following neonatal handling. Int. J. Dev. Neurosci.. 16, 165–174.

Steimer, T., Driscoll, P., 2003. Divergent stress responses and coping styles in psychogenetically selected Roman high-(RHA) and low-(RLA) avoidance rats: behavioural, neuroendocrine and developmental aspects. Stress 6, 87–100.

Swerdlow, N., Geyer, M., Braff, D., 2001. Neural circuit regulation of prepulse inhibition of startle in the rat: current knowledge and future challenges. Psychopharmacology 156, 194–215.

Taubert, M., Villringer, A., Ragert, P., 2012. Learning-related gray and white matter changes in humans: an update. Neuroscientist 18, 320–325.

Torres, C., Escarabajal, M.D., Cándido, A., de la Torre, L., Gómez, M.J., Maldonado, A., 2007. One-way avoidance learning and diazepam in female roman high-avoidance and low-avoidance rats. Behav. Pharmacol. 18 (3), 251–253.

Wolf, O.T., Dyakin, V., Vadasz, C., de Leon, M.J., McEwen, B.S., Bulloch, K., 2002. Volumetric measurement of the hippocampus, the anterior cingulate cortex, and the retrosplenial granular cortex of the rat using structural MRI. Brain Res. Brain Res. Protoc. 10 (1) (411)(:6).

Woollett, K., Maguire, E.A., 2011. Acquiring “the Knowledge” of London's layout drives structural brain changes. Curr. Biol. 21, 2109–2114.

Zhang, W.-N., Bast, T., Xu, Y., Feldon, J., 2014. Temporary inhibition of dorsal or ventral hippocampus by muscimol: distinct effects on measures of innate anxiety on the elevated plus maze, but similar disruption of contextual fear conditioning. Behav. Brain Res. 262, 47–56.

3.3.- STUDY 3: ***"****Neonatal handling induces strain-dependent effects on limbic brain areas and prepulse inhibition, and improves working memory in the roman rat strains".*

(see Annex 1)

EXPERIMENT 1

1.3.1. Introduction

As said in the "Introduction" of the present Thesis (see also references in Study 1 and Study 2) NH has shown to long-lastingly improve cognitive processes in rodents, particularly in hippocampal-dependent tasks (for reviews see, for example, Fernández-Teruel et al., 2002b; Raineki et al., 2014).

In order to evaluate the potential enduring NH effects on attentional and cognitive abilities, and related brain regions (other than those used in Study 2) in the inbred *Roman* rats, we conducted the present Study 3.

In the first part of this study (Experiment 1) we present new additional data regarding the volumes of brain regions that have been related with attentional and cognitive/executive functions (i.e. medial prefrontal cortex, mPFc; anterior cingulated cortex, ACC; nucleus accumbens, NAc; lateral ventricles, LV). These volumetric measures were obtained from the same MRI study from which hippocampus, amygdala and striatum volumes were previously reported in Study 2.

1.3.2. Materials and methods

Animals and neonatal handling (NH) treatment

Pregnant inbred *Roman* High- (RHA) and Low-Avoidance (RLA) female rats (Autonomous University of Barcelona), from the 60^{th} inbreeding generation were randomly distributed across the following four experimental groups to which their offspring would be assigned: RHA and RLA control (C), non-handled rats, and RHA and RLA animals that received neonatal handling (NH) treatment. Each experimental group was composed of 8 male rats representing 5-6 different litters (1-2 pups per litter).

NH was administered twice daily (at 9:30 and 17:00 h) between postnatal days 1-21 following the same method as in previous works (Aguilar et al., 2002; Escorihuela et al., 1995; Fernández-Teruel et al., 1992a-b, 2002b). 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. At minutes

0, 4 and 8 of this 8-min period, each pup was gently handled/stroked for 3-4 s. Control (C) non-handled groups were left undisturbed, except for regular cage cleaning once a week, until weaning.

Magnetic Resonance Image (MRI) volumetry

In vivo 1H-Magnetic resonance studies were performed, when rats (n=32, randomly selected from the original sample of experiment 1) were 6 months old, at the joint NMR facility of the Autonomous University of Barcelona and CIBER-BBN (Cerdanyola del Vallès, Spain), using a 7-Tesla horizontal magnet (*BioSpec 70/30*, Bruker BioSpin, Ettlingen, Germany) equipped with actively shielded gradients (B-GA20S) using a dedicated rat brain quadrature receive surface coil, actively decoupled from a transmit volume coil with 72mm inner. Rats were positioned in the scanner bed, which allowed localized delivery of anesthesia (isoflurane, 1.5-2.5% in O2 at 1 L/min; respiratory frequency monitored with a pressure probe and kept between 60–80 breaths/min). A recirculation water system, integrated in the animal bed, was used to control the body temperature as measured with a rectal probe (37ºC±1ºC).

T2-weighted fast spin-echo were initially obtained in axial, sagittal and coronal planes to be used as reference scout images for accurate slice selection of the axial planes through the measured areas of the brain. Imaging parameters for these images were: effective echo time (TEeff)=36 ms, repetition time (TR)=2 s, echo train length (ETL)=8, field of view (FOV)=3,5×3,5cm^2, matrix size (MTX)=256×256, slice thickness (ST)=0.5 mm. Using these scout images high resolution T2-weigthed images were acquired in the axial plane with the following parameter: TEeff=39 ms, TR=4.5 s, ETL=8, FOV=3.2×3.2cm^2, MTX=320×320 and ST=0.5 mm.

Using ImageJ software, brain volume (BV), medial prefrontal cortex (mPFc), anterior cingulate cortex (ACC), lateral ventricles (LV) and nucleus accumbens (NAc) were manually outlined by two raters blinded to group status (between-rater reliability $r \geq 0.89$). Briefly, this software allows the user to outline the boundaries of the region of interest (ROI) on a MRI and afterward to calculate the corresponding area using the formula that is shown below. All ROI´s were delimitated from rostral to caudal.

All brain region borders were defined according to the rat brain atlas (Paxinos and Watson, 1998; Wolf et al., 2002). For the delineation of the brain volume (BV) the most anterior brain

slice included was the first slice in which prefrontal cortex appears (approximately 5.16 from Bregma). The most posterior brain slice included corresponded to a level of approximately -9.60 from Bregma. The entire included brain tissue was distributed over 35 consecutive slices in each individual animal.

The medial prefrontal (mPFc) included the prelimbic and infralimcic cortex and 4 consecutive slices were scored for each animal. The starting rostral slice was at the level of 4.20 from Bregma, at the first appearance of the forceps minor of the corpus callosum, and the caudal slice was at 2.52 from Bregma just before the decusation of the corpus callosum. The anterior cingulated cortex (ACC) included the anterior cingulate cortex area 1 and 2 and was scored on 7 consecutive slices for each animal. The starting rostral slice was at the level of 2.28 from Brgema, when corpus callosum already decussate and the caudal slice is at -1.56 from Bregma just before the appearance of the retrospenial granular cortex. Lateral ventricles (LV) were scored on 11 consecutive slices for each animal, being the most rostral at the level of 0.12 and the caudal slice at -4.80 from Bregma. Lateral ventricles can be easily recognized in T2-weighted images by the presence of a hypersignal. Nucleus accumbens (NAc) was socred on just 3 slices for each animal, with the rostral slice at 2.52 from bregma and the most caudal slice at 1.56 from Bregma.

Volumes of delimitated area on each slice were calculated using the following formula: [(Field of view (FOV) / Matrix size (MTX)) x Slice thickness] x number of pixels included in delimitated area.

Statistical analyses

Statistical analyses were performed using the "Statistical package for social science" (SPSS, version 17). As ANOVA analysis revealed that total brain volume was greater in RLA than in RHA rats, the volume of every structure was corrected and expressed as a percentage (%) of brain volume, which was used for analysis. Factorial 2 x 2 ANOVAs ("2 Strain" x "2 treatment conditions") were applied to MRI data. Post-hoc Duncan´s multiple range tests were applied to all dependent variables following significant ANOVA effects. Significance level was set at $p \leq 0.05$.

3.3.3. Results

For reasons of coherence and for a more integrated discussion of the present Experiment 1-Study 3, we present here again the most relevant volumetric results from Study 2 (hippocampus, amygdala, striatum; see Table 1) along with the new MRI results (Study 3: prefrontal cortex, anterior cingulate cortex, nucleus accumbens and lateral ventricles) from the same animals (Figure 2A-D). In summary, as shown in Table 1 (see also Study 2), RLA rats present greater hippocampus and amygdala volume than their RHA counterparts. NH treatment reduced amygdala volume in both rat strains, although more markedly in RLA rats. Likewise, NH was able to significantly reduce hippocampus volume in RLA rats. No "strain" or NH effects were found on striatum volume (see Table 1; see detailed explanation of these results in Study 2).

Table 1.- Mean, S.E.M. and F (ANOVA), in brain volumetric measures of the *Roman* high - and low-avoidance rats obtained from Study 2.

	Means (± SEM)				F p-value		
	RLA-C	RLA-NH	RHA-C	RHA-NH	"Strain"	"NH"	"Strain x NH"
BV (mm3)	1875,5 (25,1)	1830,2 (9,3)	1728,8 (45,2)[a]	1678,9 (25,2)	**26.08*** **	2.66	.006
Hc (%)	5,43 (0,080)	5,26 (0,053)[a]	4,64 (0,039)[a]	4,79 (0,068)	**104.9*** **	0.1	**7.4***
Am (%)	1,85 (0,033)	1,71 (0,033)[a]	1,56 (0,029)[a]	1,50 (0,033)	**57.5*** **	**8.4***	2.0
St (%)	2,51(0,051)	2,40 (0,091)	2,50 (0,027)	2,50 (0,040)	0.30	1.32	0.57

Table 1.- C and NH, control (non-handled) and neonatally-handled groups, respectively. **"BV"**, total brain volume. **"Hc"**, hippocampus volume. **"Am"**, amygdala volume. **"St"**, striatum volume. Bold numbers mean significant ANOVA effects for "Strain", "Treatment" or "Strain x Treatment". *p < 0.05 **p < 0.01 and *** p < 0.001. "**a",** differences vs. RLA-C (Duncan's multiple range tests). n=32 for MRI volumetry (n=8/group). See Study 2 for details.

Regarding the new MRI measures from Experiment 1 (in the present Study 3), RLA rats showed greater volume of the medial prefrontal cortex than RHA rats [*F*s(3, 28) = 5.36, p = 0,028], while RHA rats showed a marked and significant enlargement of the lateral ventricles compared to their RLA counterparts [*F*s(3, 28) = 10.21, p = 0,003] (see Fig. 1 and Fig. 2A-D).

No “strain” differences were observed on the volume of anterior cingulated cortex or nucleus accumbens. NH treatment did not affect the volume of these four brain regions.

Figure 1

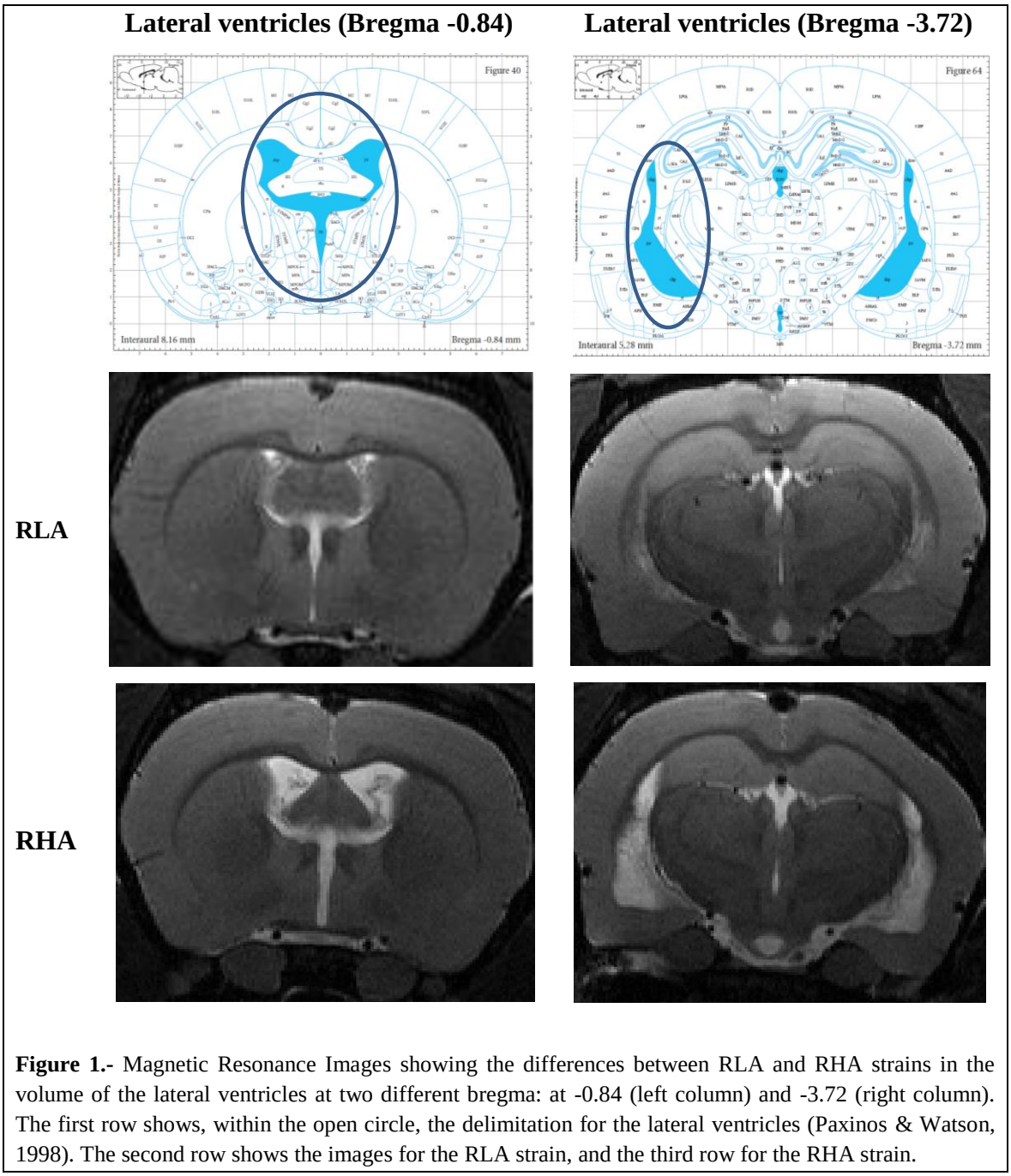

Figure 1.- Magnetic Resonance Images showing the differences between RLA and RHA strains in the volume of the lateral ventricles at two different bregma: at -0.84 (left column) and -3.72 (right column). The first row shows, within the open circle, the delimitation for the lateral ventricles (Paxinos & Watson, 1998). The second row shows the images for the RLA strain, and the third row for the RHA strain.

Figure 2

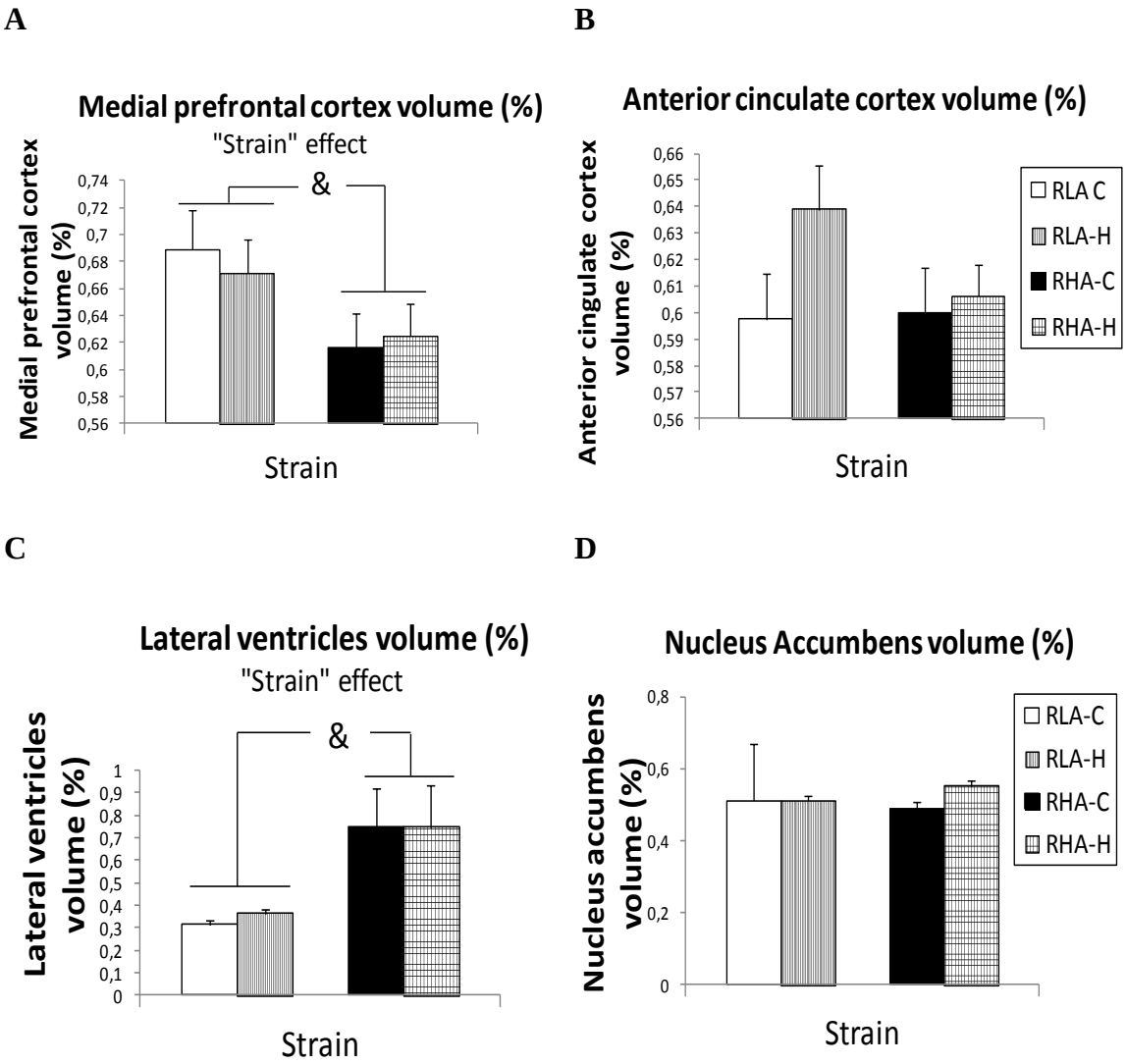

Figure 2A-D.- Mean ±S.E.M of **(A)** medial prefrontal cortex volume (%); **(B)** anterior cingulated cortex volume (%); **(C)** lateral ventricles volume (%); **(D)** nucleus accumbens volume (%). "&" indicates "Strain" effect. Group symbols: C, control non-handled group; H, neonatally handled (NH) group. (n=32; 8/group, randomly taken from total sample of animals from Experiment 1)

EXPERIMENT 2

1.3.4 Introduction

The findings that NH reduced hippocampal volume in RLA rats and the suggestive (but not significant) trend for a reduction of PPI levels by NH in this strain (in Study 2), prompted us to carry out a study (Experiment 2 of Study 3) aimed at clarifying the potential effects of NH on both PPI and executive functions (i.e. spatial learning/memory, cognitive flexibility and working memory) which are typically impaired in schizophrenia.

A large sample of male and female rats from both *Roman* strains was used, in order to increase the potency of analyses and to maximize the likelihood of observing even subtle NH effects.

1.3.5 Material and methods

Animals and neonatal handling (NH) treatment

Pregnant RLA (n=20) and RHA (n=20) rats were randomly distributed across the experimental groups (RLA; control and NH / RHA, control and NH). After weaning, all pups were housed in pairs of the same experimental group and sex in standard macrolon cages (50 x 25 x 14 cm) and were maintained with food and water freely available, 12-h light-dark cycle (light on at 0800 h) and controlled temperature (22 ± 2ºC). Eight experimental groups (Control and NH of both sexes of RHA and RLA rats) ranging from 26-38 animals per group (total sample Batch 1, n=258) were used, with each of them composed of rats representing at least 13-14 different litters (pregnant dams) with 2-4 pups coming from each litter.

Neonatal handling treatment was performed as in Experiment 1 (see section 3.3.2 above), and testing started when they were 60 days old.

Animals were submitted to a behavioral battery including NOE, PPI and several tasks performed with the Morris water maze test. NOE and PPI tests were evaluated at the same age (i.e. at PND 60 and PND110, respectively) of the animals used in Study 2. After PPI testing (Batch 1), male rats were randomly selected and divided in different batches to perform different spatial tasks in the Morris water maze test (called Batch 2 and Batch 3). In Batch 2 (n= 12 per/group) rats were consecutively submitted to a place task (place learning),

a transfer test (reference memory) and reversal place task (cognitive flexibility). In Batch 3 (n= 14-18 per/group), animals performed a delayed matching-to-place task (working memory). Finally, in Batch 4, male rats from batches 2 and 3, were pseudo randomly pooled (n=12 per/group, with half animals taken from each batch) in order to perform a non-spatial cued task.

Table 2.- Experimental sample of Experiment 2

Experimental group	NOE-PPI test (Batch 1) (PND 60-110)		PT, TT, RT (Batch 2) (PND 120)		DMTP (Batch 3) (PND 140)		Cued task (Batch 4) (PND 150)	
	♂	♀	♂	♀	♂	♀	♂	♀
RLA-C	35	32	12	-	16	-	12	
RLA-NH	38	35	12	-	18	-	12	
RHA-C	28	27	12	-	14	-	12	
RHA-NH	37	26	12	-	16	-	12	

Table 3.- C and NH, control (non-handled) and neonatally-handled groups, respectively, for RLA and RHA rat strains. **Batch 1**: "NOE", novel object exploration test. "PPI", prepulse inhibition test. **Batch 2**: "PT", place task in the Morris water maze test (MWM); (TT) transfer test in the MWM; (RT) reversal task in the MWM. **Batch 3**: "DMTP", delayed matching-to- place task in the MWM. **Batch 4**: Cued task in the MWM. "PND", post-natal day in which animals were evaluated. "♂", male rats. "♀", female rats.

Novel object exploration test (NOE): Batch 1

To assess novelty-induced behavioral inhibition/disinhibition a novel object exploration (NOE) test was conducted, consisting in the evaluation of the rats' exploratory response when a novel object was introduced in their home-cage. Rats were 60 days old at the beginning of the test. The test started by removing the food from the home-cage (leaving only four pellets in each cage). One hour later, the novel object (graphite pencil Staedtler Noris,

HB nº2) was perpendicularly introduced in their home cage through the grid cover, until it made contact with the cage bedding. To facilitate observation, each cage was pulled from the rack about 20 cm. Latency to the first exploration (NOE-L; time elapsed until the first exploration of the novel object) and the total time (NOE-T) spent exploring the pencil for each individual rat were scored in a 3-min test. The experimenter/observer was standing at 50 cm from the cage front (see Study 1 and 2).

Prepulse Inhibition of the Acoustic Startle Response (PPI): Batch 1

Four sound attenuated boxes (SR-Lab Startle Response System, San Diego Instruments, USA) were used. Each box consists of a Plexiglas cylinder situated on the top of a platform with a sensor that detects the strength made by the rat in each trial. Two speakers situated 15 cm from each side of the cylinder deliver the acoustic stimuli and a white noise generator provides the background noise. Each box was constantly lit by a 10 W lamp. The data were transduced by an accelerometer into a voltage which is amplified, digitized and saved into a computer for analysis.

Subjects were 110 days old at the beginning of testing. The session started with a 5 min habituation period in the startle chambers. Then, 10 "pulse-alone" trials (105 dB, 40ms) were delivered in order to obtain a stable baseline of startle. After this, each one of the six different types of trials are randomly administered 10 times (60 trials in total):

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

At the end, in order to measure the habituation to the startle stimulus, five "pulse-alone" trials were delivered (105 dB, 40 ms).

The interval between trials was 10-20 s with a mean of 15s. The startle magnitude was recorded during 200 ms after the onset of the pulse.

The %PPI for each prepulse intensity was calculated by applying the following formula: %PPI = 100 - (startle amplitude on prepulse trial / startle amplitude on pulse trial) x 100 (Oliveras et al., 2015; see also Study 2).

Morris water maze test (MWM)

The testing apparatus consisted of a circular pool (diameter: 150 cm, height: 60 cm), filled to a depth of 30 cm with 24 °C water. There were no local signals available within the swimming pool. Four points equally spaced around the perimeter of the tank were arbitrarily designed to serve as starting locations (N, S, E, and W). On this basis, the tank was divided into four equal quadrants. Located somewhere within one of these quadrants was a circular platform (diameter: 15 cm, height: 27 cm) whose upper surface was 2 cm below the water level. The parameters measured in all tasks were total distance travelled; latency elapsed in reached the platform and speed. Moreover, distance travelled in both, center and periphery was also scored. Animal behavior was monitored by a video camera mounted on the ceiling above the center of the pool and using a computerized tracking system (Smart v.2.5.14; PANLAB, Barcelona, Spain). Four different starting positions were equally spaced around the perimeter of the pool (Martínez-Membrives et al., 2015).

Place task (PT): Batch 2

The training session consisted of 3 consecutive trials each day, during five days, starting from one of the four starting positions. The order of starting points (N, S, E or W) was randomly determined and the platform stayed always in the same place. A trial began by placing the rat into the water facing the wall of the pool at one of the starting points and if the rat failed to escape within 90 s (i.e. reach the platform), it was gently guided to the platform by the experimenter. Once the rat reached the platform, it was allowed to stay there for 15 s. Approximately 20 min elapsed between consecutive trials (Oliveras et al., 2016) (see Figure 3A).

Transfer test (TT): Batch 2

Recall of the platform location of the place task was tested 24 h later (just one trial) over 60 sec in a test in which the platform had been removed. The starting position for every animal was the “south”, as in the last day of place task it was not used. Since the platform in this task had been removed, the parameters measured were (a) latency to reach the platform position,

(b) distance travelled until reaching the platform position, (c) number of entries into the platform position (namely, annulus crossings), (d) time spent in the platform quadrant and (e) distance travelled in platform quadrant (Oliveras et al., 2016) (see Figure 3B).

Reversal task (RT): Batch 2

After a day of rest, animals underwent a reversal task. The platform was located (and stayed fixed during the whole reversal phase) in the quadrant opposite to that used in the place task. Training consisted in 3 consecutive trials/day, during four consecutive days. Each of the 3 daily trials started from one of the four starting positions. A trial began by placing the rat into the water facing the wall of the pool at one of the starting points and if the rat failed to escape (i.e. to reach the platform) within 90 s, it was gently guided to the platform by the experimenter. Once the rat reached the platform, it was allowed to stay there for 15 s. Approximately 20 min elapsed between consecutive trials (see Figure 3C).

Figure 3

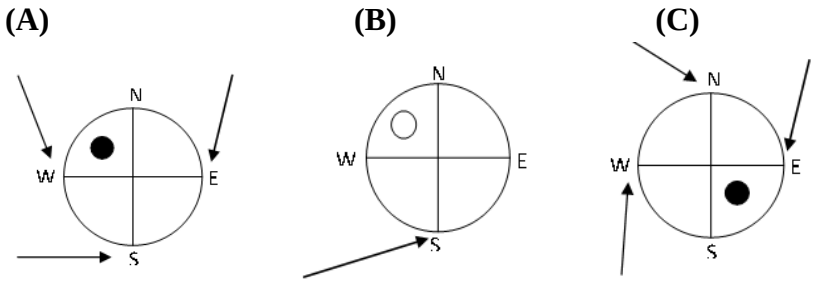

Figure 3.- The arrows indicate the starting positions for **(A)** place task, **(B)** transfer test and **(C)** reversal task. Black circles indicate the platform location; open circle indicates that the platform had been removed in the transfer test. **(A)**, **(B)** and **(C)** were performed by rats from Batch 2 (n=12 per group).

Delayed matching-to-place (DMTP): Batch 3

Animals were allowed to swim for 90 s or until they located the hidden platform. Each rat went through 2 trials per day: a sample/acquisition trial (T1) and a retention trial (T2). The two trials were separated by 30 s because the rat was allowed to stay on the platform for 15 s and then spent another 15 s in an individual cage before the second trial started. The starting position and the location of the platform were pseudorandomly varied every day, but remained constant during the two trials of each day (see Fig. 4). Several room cues were

constantly visible from the pool. The parameters measured were, total distance travelled, time elapsed (latency) until the rat reached the platform and swimming speed. Moreover, the distance travelled in both, center and periphery was also scored. The index of spatial working memory was "Mean T1-T2," i.e. distance savings in T2 vs. T1 (computed by the subtraction of T1- T2) averaged for the 3 training days (Oliveras et al., 2015).

Figure 4

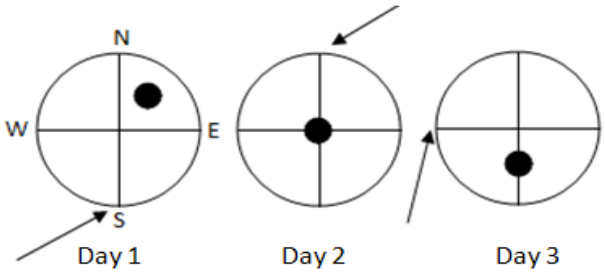

Figure 4.- The arrows indicate the starting position for the 2 trials of each training-day on the delayed matching-to-place task. Black circles indicate the platform location for each training-day. This task was performed by rats from Batch 3 (n= 14-18 animals per group)

Cued task: Batch 4

After four days of rest, animals were tested on a cued task. It consisted in 4 trials performed in a one-day test. The order of the starting positions was pseudo-randomly determined. For this test, the platform was about 1 cm above the water surface and it was cued with a small striped (black and white) flag. The platform stayed fixed for the whole training day. Approximately 15 min elapsed between consecutive trials. The parameter used in this task was the distance travelled to reach the platform in each trial (Oliveras et al., 2015) (see Figure 5).

Figure 5

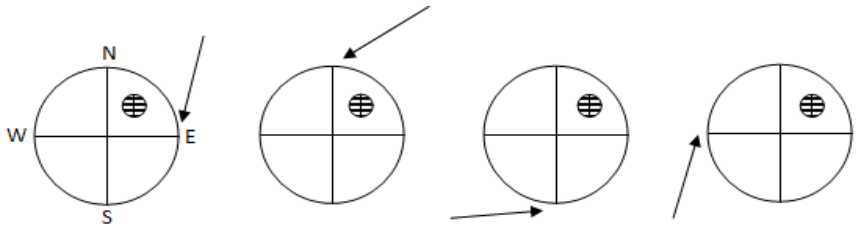

Figure 5.- The arrows indicate the four starting positions, one for each trial, in the cued task. Semi-open circles indicate that the platform was above the surface of the water and marked by a black and white flag. A pseudorandom sample of animals taken from both batches 2 and 3 were pooled to perform this task (n=12 animals per group).

Statistical analyses

Statistical analyses were performed using the "Statistical package for social science" (SPSS, version 17). Factorial 2 x 2 x 2 ANOVAs ("2 Strain" x "2 treatment conditions" x "2 sex") were applied to measures of the novel object exploration. Appropriate repeated measures factorial ANOVAs, with "prepulse intensities" as within subject factor were applied for total percentage of PPI ("2 strain" x "2 treatment conditions" x "2 sex" x "4 prepulse intensity"). Whenever a "prepulse intensity x strain" interaction was found, factorial 2 x 2 x 2 ANOVAs ("2 strain" x "2 treatment condition" x "2 sex") were applied to each prepulse intensity.

Appropriate repeated measures factorial ANOVAs were applied to measures from the place task and reversal task in the Morris water maze, with "training day" as within subject factor ("2 strain" x "2 treatment conditions" x "4 or 5 training days"). Factorial 2 x 2 ANOVAs ("2 Strain" x "2 treatment condition") were applied to variables from the transfer test, first day of reversal task and delayed matching-to-place task.

1.3.6 Results

In the "NOE" test, RLA rats showed higher latency to explore the novel object and less time exploring it compared to RHAs ["Strain" effects, both Fs(7, 257) $\geq$ 39.38, and p $\leq$ 0,001]. NH significantly reduced latency and increased time of exploration ["NH" effects, both Fs (7, 257) $\geq$ 52.63, p $\leq$ 0,001]. As males explore more time the novel object than female rats a sex

effect was also observed ["Sex" effect, $F(7,257) = 16.06$, $p \leq 0{,}000$]. There were also "Strain x NH" interaction effects on both NOE variables, as NH effects were stronger in RLA rats ["Strain x NH" effects, both $Fs(7,257) \geq 13.74$, $p \leq 0{,}001$] (Figure 6A-B).

Results of baseline acoustic startle response (the average startle response to the 25 pulse-alone trials), measured in the PPI test, revealed an overall higher acoustic startle response in the RLA rat strain compared to RHA rats ["Strain" effect, $F(7,257) = 10.27$, $p = 0{,}002$; mean $\pm$ sem = 1135,24 (72.92) for RLA rats, and 877,06 (80.46) for RHA rats, respectively]. No NH effects or interactions were observed for baseline startle. In addition, male rats showed higher baseline acoustic startle response than female rats ["Sex" effect, $F(7,257) = 21.35$, $p \leq 0{,}001$].

The RLA rat strain showed overall higher total %PPI than RHA rats ["Strain" effect, $F(7,257) = 120.93$, $p \leq 0{,}001$] with between-strain differences in all prepulses [All $Fs(7, 257) \geq 36.72$, $p \leq 0{,}001$]. Male rats also showed higher levels of %PPI compared to female rats ["Sex" effect, $F(7,257) = 12.15$, $p \leq 0.001$] with differences in all prepulses except in 65dB [All $Fs(7, 257) \geq 6.04$, $p \leq 0{,}05$]. A "Strain x NH" interaction was observed on %PPI, as NH treatment induced a decrease of PPI levels in the RLA rat strain in all prepulses ["Strain x NH" effects, $F(7,257) = 20.66$, $p \leq 0.001$], while in RHA rats the opposite effect was observed at 70 and 75dB prepulses (see Duncan's tests in Figure 6C).

Regarding the Morris water maze, overall RLA rats travelled less distance than RHAs in both, the place task and the reversal task ["Strain" effect, both $Fs(3,44) \geq 11.39$, $p \leq 0{,}005$; Figure 7A and 7C; see Figure 8A-B for typical trajectories of both rat strains in the place task]. In the transfer test RLA rats performed more annulus crossings than RHAs ["Strain" effect, $F(3,44) = 26.21$, $p \leq 0{,}001$; Figure 7B]. A "NH" effect was observed in the first training day of the reversal task as NH-treated rats travelled less distance (i.e. showed higher distance savings between the second and the third trial) than control animals ["Strain" effect, $F(3,44) = 5.04$, $p \leq 0{,}030$; Figure 7D], thus suggesting an increase in cognitive flexibility induced by NH. In the delayed matching-to-place spatial task, NH-treated rats overall showed better working memory than controls ["NH" effect, $F(1,63) = 4.37$, $p \leq 0.05$; Figure 7E]. No "strain" or "strain x treatment" interaction effects were found. No main effects or interactions were observed in the cued task (see Figure 7F).

Figure 6

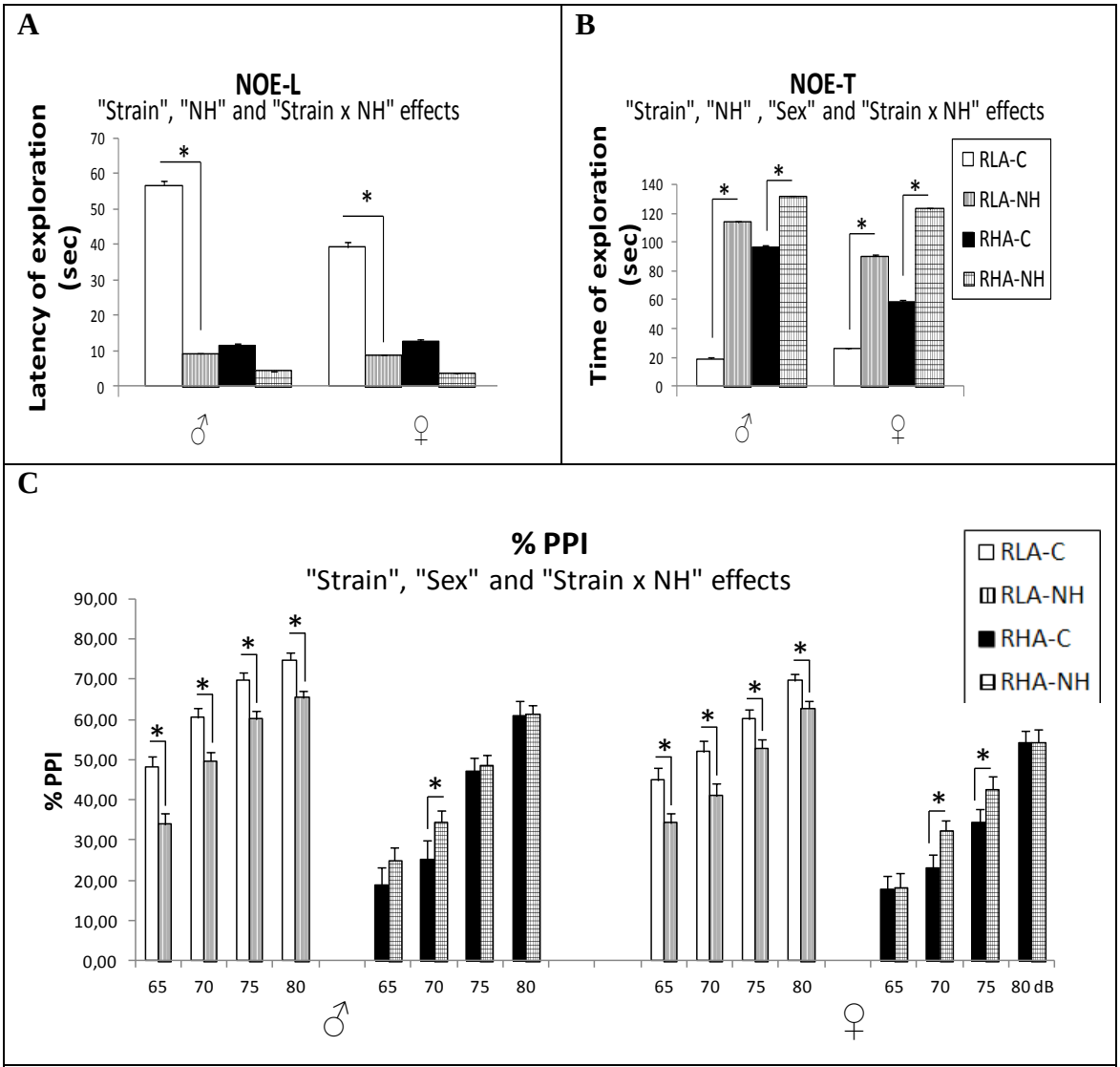

Figure 6.- Latency elapsed to begin the exploration of the novel object **(A)** and total time of exploration of the novel object in "NOE" test **(B).** Total percentage of prepulse inhibition (%PPI) in the "PPI" test **(C).** (A), (B) and (C) refers to animals from Batch 1. Means ± sem are shown for each variable.

Figure 7

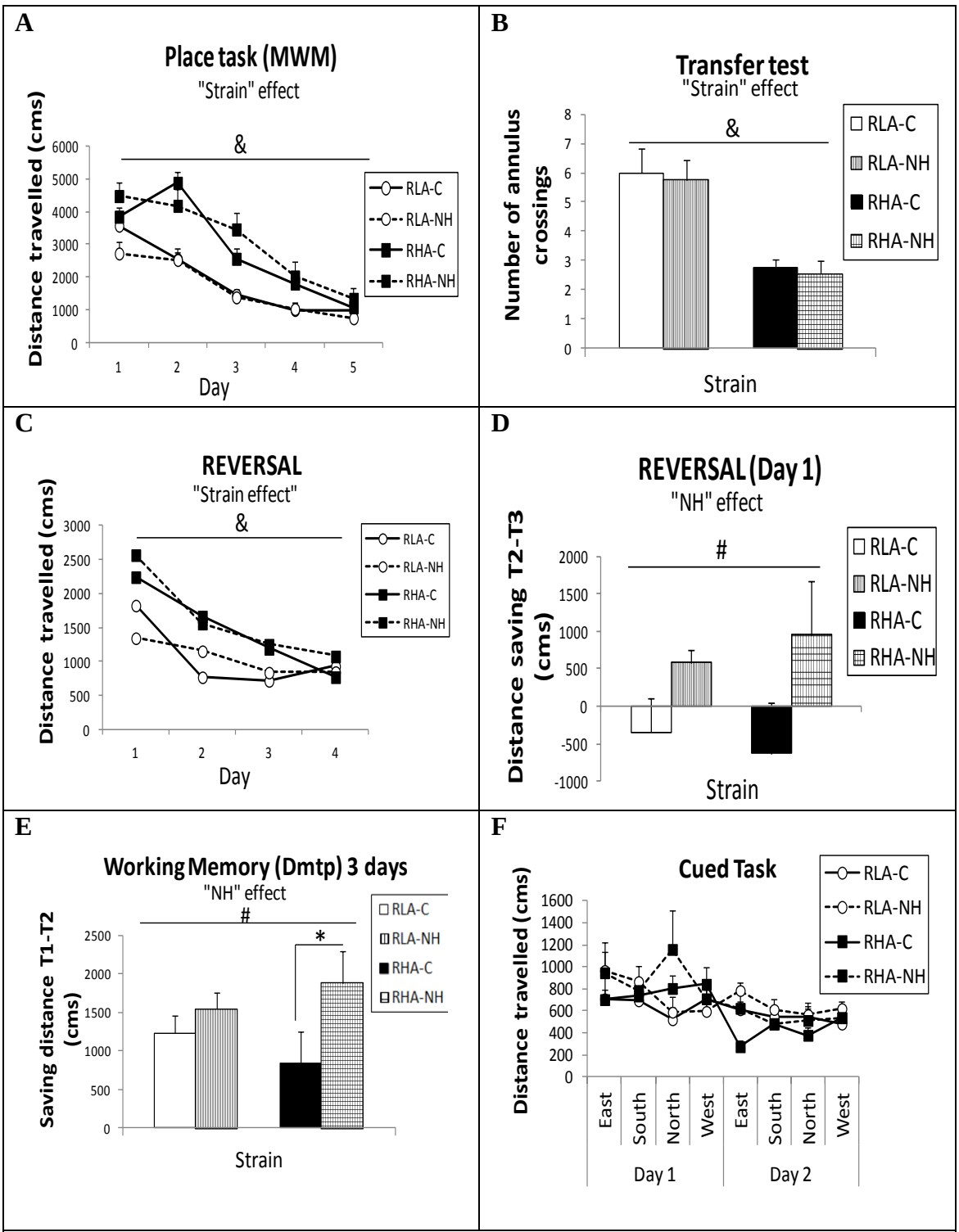

Figure 7.- Mean ± sem of performance measures of the Roman rats in several tasks of the Morris water maze test. **(A)** Distance travelled in the place task. **(B)** Number of annulus crossings in the transfer test. **(C)** Distance travelled in the reversal task. **(D)** Distance savings between the second and the third trial of the first training-day in the reversal task (cognitive flexibility). **(E)** Working memory, i.e. averaged distance savings between first and second trials in the delayed matching-to-place task. **(F)** Distance travelled in the cued task. **(A-D:** Batch 2); (**E:** Batch 3) and (**F**: Batch 4).

Figure 8

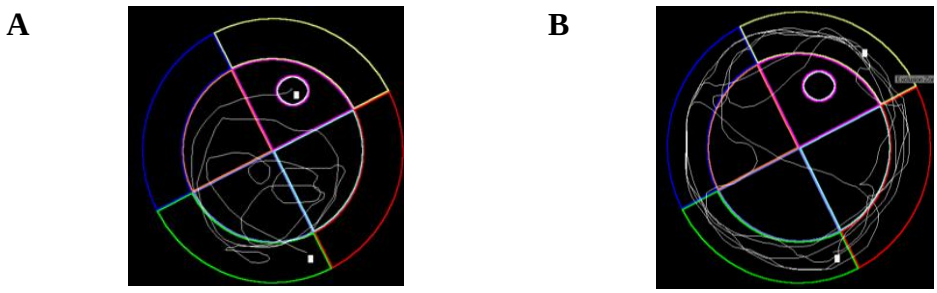

Figure 8.- Typical trajectories, performed by **(A)** RLA and **(B)** RHA, in the same trial at the beginning of the spatial place task in the Morris water maze (Batch 2). The distance travelled to find the platform by RLA rats is much shorter than that of their RHA counterparts.

3.3.7. DISCUSSION

Neonatal handling has been shown to induce enduring effects on processes involving neural plasticity. In particular, in connection with the present work, NH has been found to increase the magnitude of hippocampal long-term potentiation in young rats (Wilson et al., 1986), to enhance hippocampal nerve growth factor (NGF) mRNA levels (Mohammed et al., 1993; Pham et al., 1997) and the amount of NADPH-diaphorase-positive neurons (a potential marker of nitric oxide-producing neurons) (Vaid et al., 1997), to increase cortical dendritic spines (reviewed by Pham et al., 1999) and to prevent age-related hippocampal neurodegeneration (Fernández-Teruel et al., 1997; Meaney et al., 1988). In keeping with these findings, NH appears to generally improve cognitive abilities/performance in rodents, in particular in tasks dependent on (or sensitive to) hippocampal regulation (e.g. Escorihuela et al., 1994a-b; Fernández-Teruel et al., 1997, 2002b; Raineki et al., 2014; see also avoidance results from Study 1 and Study 2). Accordingly, we expected that NH would lead to improvements of PPI and spatial learning/memory in the *Roman* rat strains.

Partly in agreement with our hypotheses, the results from Study 3 revealed that NH induced some positive effects on spatial-cognitive flexibility (as indicated by the first training day of the reversal place task in the Morris water maze), as well as an overall performance improvement (also in both rat strains) of spatial working memory. In addition, NH treatment significantly improved PPI in RHA rats, whereas it impaired PPI in their RLA counterparts. The latter finding, which appears to be consistent and reliable, as it is present in both sexes

and across the four prepulse intensities, might seem paradoxical at first glance. However, we could hypothesize that, since PPI is modulated by the hippocampus (among other limbic and cortical brain regions; see "General discussion" of this Thesis), such an impairment of sensorimotor gating in NH-treated RLA rats might be related to (or mediated by) the decreased hippocampal volume observed in these animals. Moreover, and related to these findings, a decrease of "alertness" (i.e. arousal, or vigilance) as consequence of NH might also be involved in the impairment of PPI in NH-treated RLA rats (Grillon & Davis, 1997).

We will further and more extensively elaborate the discussion of the above issues in the "General discussion" section of the present Thesis.

4.- SUMMARY OF RESULTS

4.1.- Study 1:

"Neonatal handling decreases unconditioned anxiety, conditioned fear, and improves two-way avoidance acquisition: a study with the inbred Roman High (RHA-I) and Low-Avoidance (RLA-I) rats of both sexes".

The present study evaluated, for the first time in inbred RHA and RLA rats of both sexes, the potential long-lasting anti-anxiety effects of NH treatment on unconditioned (unlearned) and conditioned (learned) anxiety/fear responses.

Regarding the unconditioned anxiety/fear responses, NH decreased the latency to begin exploration and increased the time spent exploring a novel object (i.e. a graphite pencil) introduced into the rats' home-cage ("NOE" test). In addition, NH increased the time spent exploring the open sections and the number of head-dips in the elevated zero-maze test (ZM). In both the NOE and ZM tests the effects of NH were relatively more marked in the RLA strain. As concerns to conditioned fear/anxiety responses, a relevant novel finding, showed for the first time with inbred *Roman* rats, is that NH treatment improved two-way avoidance acquisition in RLA rats of both sexes (more markedly in female rats), while also decreased context-conditioned fear in both strains of rats.

Our results confirmed our hypothesis, as NH treatment overall decreased unconditioned and conditioned anxiety/fear responses, in both *Roman* rat strains and in both sexes, being these effects more evident in the RLA strain. These findings are mostly consistent with the results from previous studies of NH effects on unlearned (novelty-based) anxiety responses, carried out with the outbred *Roman* rat lines (e.g. Fernández-Teruel et al., 1991d, 1992a-b, 1997, 2002b, Steimer et al., 1998). However, the present is the first report of a long-lasting NH-improving effect on two-way avoidance acquisition in inbred RLA rats of both sexes (see Escorihuela et al., 1995b).

Our results suggests that the enduring anxiety/fear-reducing effect of NH treatment might partially counteract the genetically-based two-way avoidance acquisition deficit of the RLA strain, likely because NH is able to promote a change from a passive/reactive toward a more active/proactive coping style in front of stressors in the RLA strain. This idea was also supported by the increase of inter-trial crossings (ITCs) in NH-treated rats, since ITCs have been considered as "pseudo-avoidance" responses which indicate that animals are developing

active coping strategies to solve the approach/avoidance conflict involved in the task (for reviews see Castanon et al., 1995; Aguilar et al., 2004). Moreover, ITCs show high positive associations with avoidances and are a good positive predictor of two-way avoidance acquisition (Aguilar et al., 2004; López-Aumatell et al., 2011; Vicens-Costa et al., 2011; Díaz-Morán et al., 2012). In that context, it is known that learning the two-way avoidance response entails a "passive avoidance/active avoidance" conflict during the initial stages of acquisition (i.e. a tendency to freeze in the current compartment -thus receiving the electric shock- runs against a tendency to actively cross to the opposite compartment -thus escaping from or avoiding the insult-) which is known to be mediated by anxiety (Fernández-Teruel et al., 1991a; Gray, 1982; Gray & McNaughton, 2000; Vicens-Costa et al., 2011). In this regard, a more active/functional hippocampus has been related to increased anxiety in front of "approach/avoidance" conflicts -of which two-way avoidance acquisition is a particular case- (Gray & McNaughton, 2000) and, notably, RLA rats have been shown to have a more functional hippocampus than RHA rats (Meyza et al., 2009; García-Falgueras et al., 2012). Hippocampus and amygdala volume have been negatively correlated with the anxiety trait in humans (Barrós-Loscertales et al., 2006). Therefore, we considered interesting to measure hippocampal and amygdala volume in the RLA and RHA strains and to evaluate whether NH treatment may influence these brain regions in a manner consistent with its enduring anxiolytic effects.

4.2.- Study 2:

"Neonatal handling enduringly decreases anxiety and stress responses and reduces hippocampus and amygdala volume in a genetic model of differential anxiety: behavioral-volumetric associations in the Roman rat strains".

The main objective of this study was to evaluate whether the behavioral changes induced by NH treatment (observed in Study 1) were also consistent with physiological responses to stress and with the volume of specific brain regions related to anxiety and stress, such as the hippocampus and the amygdala.

Rats from both *Roman* strains were evaluated for unconditioned and conditioned anxiety/fear behavioral phenotypes and for pre/post-stress hormonal levels (corticosterone and prolactin).

We also evaluated, for the first time in the *Roman* rats, the volume of brain regions (Magnetic Resonance Image, MRI) related to the neurobiology of anxiety (e.g. total brain volume, amygdala, ventral/dorsal and total hippocampus and striatum volume). As NH decreased unconditioned and conditioned anxiety/fear responses (Study 1), we expected that such anxiolytic-like effects would be also paralleled by reductions in post-stress corticosterone and prolactin levels, as well as by reductions in the volume of brain regions linked to anxiety/stress responses or traits (i.e. hippocampus and amygdala).

Similar to findings from Study 1, the results of Study 2 again revealed enduring anti-anxiety effects of NH on the NOE and ZM tests, as well as in two-way avoidance acquisition, particularly in RLA rats. In addition, NH-treated rats of both strains exhibited lower post-stress corticosterone and prolactin responses than control animals.

The most relevant and novel finding of this study was, however, that NH was able to induce a reduction of amygdala volume, especially in the RLA strain. Moreover, regarding the hippocampus, a "strain x NH" interaction was observed as the treatment decreased hippocampus volume in RLA rats, while NH-treated RHA rats almost reached significance for hippocampus enlargement (compared to control RHA rats, $p = 0.05$ one-tailed Student's t-test). No differences were observed in striatum, which was included as a control region, because it is not involved (at least directly) in the anxiety/stress responses under study. Interestingly, positive associations were found between hippocampus and amygdala volume and anxiety responses, while hippocampus volume (in particular, ventral hippocampus volume) emerged as predictor of performance in the NOE and ZM test (assessed by multiple lineal regressions).

These results are overall consistent with Gray´s theory of the neuropsychology of anxiety, in which the septo-hipocampal system, in close interplay with the amygdala, are proposed as key neural structures underlying the behavioral inhibition system (BIS), which activity would mediate anxiety (Gray, 1982; Gray & McNaughton, 2000). Gray's theory has been confirmed by lesion and pharmacological studies (Gray & McNaughton, 2000, McNaughton and Corr, 2004), but studies in untreated rats have been inconclusive (Kalisch et al., 2006). Hence, our findings, of positive associations between anxiety-related responses (NOE and ZM tests) and hippocampus and amygdala volume in the inbred *Roman* rats, can be considered as further supporting Gray and McNaughton's model. Studies in healthy humans also lend support to this neurobiological model, by revealing associations between negative fear-related emotions

and neuroticism (a personality trait that encompasses increased anxiety and negative emotional responses) and hippocampus and amygdala activity and connectivity (Cremers et al., 2010; Hahn et al., 2010; Harenski et al., 2009; Hooker et al., 2008; Ormel et al., 2013; Tzschoppe et al., 2014). In addition, studies aimed to a more specific measure of anxiety levels (or behavioral inhibition) in humans, have reported that trait anxiety is positively associated with hippocampal and amygdala volume (Barrós-Loscertales et al., 2006; Cherbuin et al., 2008; Levita et al., 2014; Machado-de-Sousa et al., 2014; Rusch et al., 2001), and anxious temperament in rhesus monkeys is also positively associated with activation of both structures (Oler et al., 2010).

On the other hand, in Study 2, two relevant findings were observed with regard to PPI. First, a positive association between hippocampal volume and PPI and, second, a suggestive trend for a reduction of PPI levels induced by NH in RLA rats.

Considering that NH has shown to induce beneficial effects on cognition (i.e. NH- improving effects on hippocampal-dependent cognitive tasks; e.g. see Fernández-Teruel et al., 2002b; Raineki et al., 2014), and taking into account unpublished previous results (showing a slight tendency of NH to improve PPI in RHA rats), we conducted a third study aimed at clarifying the potential effects of NH on PPI and executive functions (i.e. spatial learning/memory, cognitive flexibility and working memory).

4.3.- Study 3 (see Annex 1):

"Neonatal handling induces strain-dependent effects on limbic brain areas and prepulse inhibition, and improves working memory in the Roman rat strains".

The main objective of this study was to evaluate potential effects of NH on attentional (PPI) and executive functions in the inbred *Roman* rat strains. To this aim NOE and PPI were evaluated in male and female RLA and RHA rats at the same age of those animals evaluated in Study 2. Executive functions (i.e. spatial learning/memory, cognitive flexibility and working memory) were also evaluated in males of both strains under several different tasks using the Morris water maze (e.g. place task, transfer test, reversal task, delayed matching-to-place and cued task). In addition, we report the volumes of brain regions that were not

included in Study 2 (medial prefrontal cortex, mPFc; anterior cingulated cortex, ACC; nucleus accumbens, NAc; lateral ventricles, LV), some of which have been proposed to be associated with the above mentioned attentional/cognitive tasks.

We expected that the RLA strain would show higher PPI levels and a better performance in the Morris water maze tasks (i.e better spatial/learning memory, cognitive flexibility and working memory) than RHA rats. We also hypothesized that NH should improve PPI levels and executive functions in both rat strains, but especially in the RHA strain.

As expected, RLA rats showed better performance in both, PPI and spatial learning and memory, confirming the better pre-attentive and cognitive ability compared to RHA rats. Regarding the NH effects, a "Strain x NH" interaction was observed on PPI measures, as NH treatment decreased PPI levels (in all prepulses) in both sexes of RLA rats, whereas in the RHA strain, NH improved PPI at 70dB and 75dB prepulses. This result confirmed some tendency which was observed in Study 2 (i.e. the decreasing effect of NH on PPI levels in the RLA strain).

With respect to spatial learning/memory, as measured in the Morris water maze, RLA rats outperformed their RHA counterparts in the place task, in the transfer (memory) test and in the reversal place task, thus confirming previous reports on the superiority of the RLA line/strain in several spatial cognitive abilities (e.g. Aguilar et al., 2002; Escorihuela et al., 1995a; Martínez-Membrives et al., 2015; Nil & Bättig, 1981; Oliveras et al., 2016). On the other hand, NH treatment improved working memory (in the delayed matching-to-place task) in both rat strains, but more clearly in RHA rats, and also improved cognitive flexibility (as measured in the first training day of the reversal place task) of both rat strains. No differences were observed in the cued task, thus ruling out perceptive, motivational or motor function impairments.

Interestingly, the novel MRI findings included in Study 3 revealed a "strain" effect on medial prefrontal cortex volume (RHA<RLA) and dramatic differences in the volume of the lateral ventricles (RHA>RLA). While there were NH effects on hippocampus and amygdala volumes (see Study 2 and Study 3), no effects of NH were observed in the volumes of the medial prefrontal cortex (mPFc), lateral ventricles (LV), anterior cingulated cortex (ACC) or nucleus accumbens (NAc).

With regard to the impairing NH effect on PPI in the RLA strain, evidence from human and rodent studies provides support for the "hypervigilance" hypothesis, which proposes that fear/anxiety or stress could facilitate the processing of sensory information due to an increase in the attention to the environment which would be mediated by cortical arousal and hippocampal activity (Foote et al., 1987; Grillon & Davis, 1997; Whalen et al., 1994; Chen et al., 2010; Hattori et al., 2007; Varty et al., 2000; Hendershott et al., 2016).

A more in depth and integrated discussion of the findings from the three studies of the present Thesis will be presented in the following "General discussion" section.

5.- GENERAL DISCUSSION

The main objective of this Doctoral Dissertation was to evaluate the potential effects of neonatal handling treatment (NH) on the differential anxiety/stress-related and the attentional/cognitive phenotypes/endophenotypes of the *Roman* strains of rats. We hypothesized that NH should be able to induce long-lasting anxiolytic-like effects, especially in the RLA strain, as well as improving effects on sensorimotor gating (PPI) and executive functions especially in the RHA strain. We also hypothesized that these NH-induced behavioral changes should be paralleled by different biomarkers such as the hormonal responses to stress exposure and, for the first time evaluated on *Roman* rats, the volumes of specific brain regions related to those phenotypes.

In order to facilitate a general and integrated discussion of the main findings, we can summarize them as follows:

i) RLA rats showed an enhanced anxiety/fear and stress-like profile compared with the hypoemotional RHA rats, thus confirming the face validity of the former for the study of anxiety/fear and stress sensitivity.

ii) RHA rats showed impairments in pre-attentive (sensorimotor gating) and executive functions, in line with what is commonly observed in schizophrenic patients, thus suggesting that RHA rats may be a useful tool for the study of some schizophrenia-relevant symptoms or phenotypes.

iii) Between-strain effects were observed in the volumes of specific brain regions, in a manner consistent with the observed differential phenotypes in anxiety/stress- and schizophrenia-related traits/responses, i.e. RLA rats showed larger amygdala, hippocampus and medial prefrontal cortex volumes, and decreased volume of the lateral ventricles, compared to RHA rats.

iv) NH induced long-lasting beneficial effects in both strains of rats, that is, anxiolytic effects in RLA rats (and to a lesser extent in RHA rats), and improving effects in sensorimotor gating and working memory performance in their RHA counterparts.

v) The behavioral changes induced by NH were paralleled by an overall reduction in post-stress hormonal responses (corticosterone and prolactin).

vi) NH caused enduring impairing effects on PPI in RLA rats.

vii) NH treatment decreased amygdala volume in both strains of rats, while also decreased hippocampus volume in the RLA strain.

From these results, we would like to highlight some relevant novel findings. First, that NH was able to partially counteract the genetically-based, two-way active avoidance acquisition deficit of the RLA strain, being this effect more pronounced in females. It is worth to point out that such a NH effect on avoidance acquisition in inbred RLA rats has been observed for the first time in the present work (Study 1; Escorihuela et al., 1995b). This means that the profile of the inbred RLA rats in such genetically-linked trait (i.e. their avoidance deficit in the shuttle-box, which constitutes their selection criteria), was susceptible to be improved by an environmental treatment (i.e. NH) on a long-lasting basis (see Study 1 and Study 2). This result leads to the conclusion that NH has been able to induce a change from a passive/reactive towards a more active/proactive coping style in the RLA strain of rats. This conclusion is also supported by the effects of NH on RLA's behavioral profiles in the remaining anxiety/fear-related measures of the present studies, such as the novel object exploration test (Study 1, Study 2, Study 3), the elevated zero-maze test (Study 1, Study 2), the measure of context-conditioned freezing/fear (Study 1) and the number of inter-trial crossings (Study 1) in the shuttle box session, which are overall consistent with previous studies of NH effects on unconditioned anxiety responses in the swiss outbred *Roman* rat sub-lines (i.e. RLA/Verh and RLA/Verh sub-lines) (Aguilar et al., 2002; Fernández-Teruel et al., 1991d; 1992a-b, 1993, 1997, 2002b; Steimer et al., 1998). The results of our recent embryo-transfer study with the *Roman* rat strains (Río-Álamos et al., 2017b *in press*) add still more relevance to the NH effects on anxiety/fear and avoidance acquisition. Thus, *Roman* rats re-derived through embryo-transfer to Sprague-Dawley receptor female rats, showed that the main phenotypic traits that constitute (or are closely linked to) the selection criteria (i.e. avoidances, inter-trial crossings, conditioned freezing) are conserved after embryo-transfer from the very first generation (i.e. in the F1 of *Roman* rats born from Sprague-Dawley mothers post embryo-transfer), pointing to the relevance of genetic factors in these phenotypes (Río-Álamos et al., 2017b *in press*; see also Castanon et al., 1995). Therefore, the fact that NH has been able to induce a significant improvement of these genetically-based phenotypes/traits in RLA rats is an interesting indicator of the potentiality of the treatment.

Second, behavioral changes induced by NH were paralleled by overall decreases of post-stress corticosterone and prolactin responses (Study 2). These results are also in line with previous studies from our laboratory showing reductions of post-stress HPA-axis and prolactin responses in the outbred RLA/Verh vs RHA/Verh rat sub-lines (Fernández-Teruel et al., 2002b; Steimer et al., 1998) as well as in other commonly used laboratory rat strains

(e.g. Levine, 1962; Levine et al., 1967; Meaney et al., 1988, 2013; Núñez et al., 1996). As suggested by several studies, NH treatment facilitates the maturation and regulation of the HPA-axis (e.g. Levine, 1962; Levine at al., 1967; Meaney et al., 1988, 2013), which might be promoted by the joint action of the repeated exposure to novelty, the maternal-siblings isolation and the maternal response after returning isolated pups to the home-cage (Catalani et al., 2011; Dinces et al., 2014; Meaney et al., 2013). Thus, the repeated low-mild stress involved in the NH procedure seems to provide proper conditions that enable a better regulation of the HPA-axis, which in turn would allow the rat to perform better adaptive responses to environmental challenges.

Third, RLA rats showed higher volumes of amygdala, hippocampus and prefrontal cortex, in addition to decreased volume of the lateral ventricles compared to their RHA counterparts. These results appear to fit well with the strain differential behavioral profiles -i.e. the anxiety/stress-prone profile of RLA rats would hypothetically fit with bigger amygdala and hippocampus, and the impaired attentional/cognitive profile of RHAs would cohere with smaller hippocampus and prefrontal cortex, and maybe with enlarged lateral ventricles. A most important finding is that NH was able to reduce amygdala volume in both strains, although more markedly in the RLA rats, while hippocampus volume was also reduced by NH in the RLA strain, with a non-significant trend ($p = 0.05$, one tailed) to the opposite effect being observed in NH-treated RHA rats (see Study 2). These NH effects on the volume of specific brain regions appear to be mostly consistent with the behavioral effects of NH, as treated RLA rats exhibit decreased anxious responses in parallel to reduced amygdala and hippocampus volume.

As discussed in Study 1 and Study 2, the observed strain and NH effects on anxiety-related behaviors (i.e. NOE, ZM, context-conditioned freezing/fear, two way avoidance acquisition), hippocampus and amygdala volume are in line with Gray´s theory of anxiety, in which the septo-hipocampal system, in close interplay with the amygdala, have been proposed as key neural regions underlying the behavioral inhibition system (BIS), which activity would mediate anxiety (e.g. Gray, 1982; Gray & McNaughton, 2000; McNaughton & Corr, 2004). Gray´s theory has received wide support from lesion and pharmacological studies, as well as by work with rhesus monkeys and humans leading to the conclusion that the anxiety trait is positively associated with hippocampal and amygdala volume and function (Bach et al., 2014; Barrós-Loscertales et al., 2006; Cherbuin et al., 2008; Levita et al., 2014; Machado-de-Sousa et al., 2014; Oler et al., 2010).

From a more specific perspective, studies on functional hippocampus subdivisions have suggested that dorsal hippocampus (DHc) would be preferably involved in mnemic functions and spatial information, while the ventral hippocampus (VHc) would be more related to emotional processes (Bannerman et al., 2004, 2014). In that context, our findings from Study 2 showed greater VHc volume in the RLA compared with RHA rats, while NH reduced dorsal (but not ventral) hippocampus volume only in the RLA. As NH induced a long-lasting reduction of anxiety responses in the RLA strain, we would have expected a reduction of VHc volume after the treatment, but this was not the case. In an attempt to explain this apparently paradoxical result, some considerations should be taken into account. First, anxiolytic drugs administered in the DHc reduced anxiety/fear responses in the elevated plus maze test, whereas anxiogenic drugs elicit the opposite effects in the same test (Engin & Treit, 2008; Rezayat et al., 2005) and, second, temporary inactivation of the DHc by tetodrotoxin induced anxiolytic-like effects in the shock-probe burying test (Degroot & Treit, 2004), and the administration of the anxiolytic GABA-A agonist muscimol in both DHc and VHc disrupted fear conditioning (Zhang et al., 2014). The above evidence suggests that at least in some conditions, dorsal hippocampus might have a role on anxiety/fear processing. Our finding that NH reduces DHc volume (in parallel to anxiety-related responses) in RLA rats would be in line with that evidence. On the other hand, it is known that dorsal and ventral hippocampus have different connectivity with subcortical structures such as the amygdala. Thus, the ventral hippocampus is known to have more dense connections with amygdala and hypothalamus than dorsal hippocampus, although the latter has also connections lateral amygdaloid regions (Bannerman et al., 2004, 2014; Grigoryan & Segal, 2016). Such differential region-specific hippocampus-amygdala connectivity may partly explain the divergent involvement of DHc and VHc in different anxiety/fear-related tests or tasks (Bannerman et al., 2004, 2014).

Previous studies relating hippocampus volume and anxiety in untreated rats had been inconclusive (Kalisch et al., 2006), but our present findings lend support to the contention that hippocampus and amygdala volume (and, supposedly, their function) are positively related to anxiety (e.g. Bach et al., 2014; Bannerman et al., 2004, 2014; Barrós-Loscertales et al., 2006; Gray & McNaughton, 2000; McNaughton & Corr, 2004), by showing positive associations between the volume of both structures and anxiety/fear responses in the inbred *Roman* rats.

Of note, previous studies on the effects of environmental manipulations on hippocampus or amygdala volume and anxiety have essentially focused on chronic stress treatments (Bourgin et al., 2015; Delgado y Palacios et al., 2011; Lee et al., 2009; Schubert et al., 2009), which often lead to hippocampal atrophy and amygdala hypertrophy (Bourgin et al., 2015; Lee et al., 2009; Salm et al., 2004), supposedly as a consequence of the stress-induced hypercortisolemia which overstimulates these limbic areas (Bourgin et al., 2015; McEwen et al., 2015). However, the present is, to our knowledge, the first study evaluating and demonstrating very long-lasting changes (i.e. reductions) of hippocampus and amygdala volume after an infantile stress/anxiety-reducing treatment in a genetically-based rat model of anxiety. For this reason, we believe the present findings give further relevant support to the involvement of both brain regions in anxiety.

In Study 2 regression analysis revealed a positive association between hippocampal volume and PPI (Kumari et al., 2005; Schubert et al., 2009) but, quite unexpectedly, we also observed a slight trend to a reduction of PPI levels by NH in RLA rats. This unexpected (though non-significant) result of NH on sensorimotor gating, in addition to unpublished results from our laboratory showing a trend for a PPI improvement by NH in RHA rats, as well as report by Weiner et al. (1985) revealing that NH improved latent inhibition in rats, encouraged us to address the NH effects on PPI and executive functions (spatial learning and memory) in both rat strains by using much larger samples. Since NH (and other similar early-life treatments) has shown to rather systematically enhance cognitive abilities (Aguilar et al., 2002; Daskalakis et al., 2009; Escorihuela et al., 1994a, 1995a; Fernández-Teruel et al., 2002b; Meaney et al., 1988; Raineki et al., 2014; Reeb-Sutherland & Tang, 2011; Stamatakis et al., 2008; Tang, 2001; Zaharia et al., 1996), we expected to find improving NH effects on PPI as well as on different forms of spatial learning/memory (particularly in RHA rats, as they are relatively impaired in these tasks).

The results from Study 3 confirmed the better pre-attentional (PPI) and spatial learning/memory performance of RLA vs. RHA rats. This is in line with previous results in which RLA rats showed improved PPI and better spatial reference and working memory compared with RHA rats (Esnal et al., 2016; Martínez-Membrives et al., 2015; Oliveras et al., 2015, 2016), with PPI levels being a predictor of working memory performance in the Morris water maze (Oliveras et al., 2015).

Medial prefrontal cortex (mPFc) and hippocampus (Hc) are known to be involved in the modulation of PPI and executive functions, such as spatial reference and working memory (Bakshi & Geyer, 1998; Koch, 1999; Lacroix et al., 2000; Swerdlow et al., 2016). In the present studies, the rats having greater volumes of these two regions (i.e. RLA rats) exhibited better PPI and spatial learning/memory in some tasks. Neonatal hippocampal or prefrontal lesions have shown to systematically impair both PPI and executive functions (Alam et al., 2015; Bertrand et al., 2010; Black et al., 1998; Chambers et al., 1996; Day-Wilson et al., 2006; Déziel et al., 2015; Grecksch et al., 1999; Ishikawa et al., 2015; Labbate et al., 2014; Lipska et al., 2002; Sandner et al., 2010; Schneider & Koch, 2005; Schubert et al., 2009). Thus, it is not surprising that RHA rats showed worse performance in these tasks. In this context, structural volumetric studies related to schizophrenia, in both humans and rodents, lend support to the notion that decreased mPFc and Hc volumes, and, most importantly, enlarged lateral ventricles, are strongly related to the cognitive impairments associated to schizophrenia (see review Gilabert-Juan et al., 2013; Horga et al., 2011; Piontkewitz et al., 2011; Shenton et al., 2001). The extremely evident enlargement of the lateral ventricles observed in RHA rats, in addition to decreased mPFc and Hc volumes, strengths the possibility for this strain to be considered as a useful tool to study those cognitive impairments observed in schizophrenia.

Regarding treatment effects on attention (sensorimotor gating) and cognition, we report that NH elicited differential effects on PPI depending on the strain (see Study 3). Thus, NH increased PPI levels in RHA rats (more markedly in females), while PPI levels of RLAs were systematically decreased by NH in both sexes and for all prepulses (65, 70, 75 80dB). Interestingly, although RLA rats showed better spatial reference learning/memory than RHAs (i.e. better performance in the place task and the transfer test in the Morris water maze), the treatment improved the performance of both strains in both the reversal task and the delayed matching-to-place task (i.e. working memory), suggesting that NH induced an improvement in cognitive flexibility (reversal task) and working memory, which was relatively more marked in the RHA animals (see Duncan's test in Fig. 2H, Study 3).

The unexpected impairing effects of NH on PPI levels in RLA rats might be tentatively explained (at least in part) by a decrease of "alertness" or environmental monitoring induced by NH. Actually, in support of that it has been shown, in both humans and rodents, that fear, anxiety or stress facilitate information processing and PPI due to increased attention to the environment, a process mediated by cortical arousal in which hippocampus is also involved

(Chen et al., 2010; Gray & McNaughton, 2000; Grillon & Davis, 1997; Lindema et al., 2008; Whalen et al., 1994). In line with this, the anxious and hypervigilant inbred Wistar-Kyoto rats showed higher PPI levels compared to Sprague-Dawley rats (McAuley et al., 2009). Moreover, a transient increase of PPI was observed in mice after a two week-training with environmental enrichment (EE), or "EE + stressor", compared to mice reared in standard environment (SE) or "SE + stressor". But after a four week-training only the "SE + stressor" treatment increased PPI levels (Chen et al., 2010). The authors postulate that the frequent introduction of novel objects in EE is comparable to mild stress, as EE is known to activate the HPA-axis (Fox et al., 2006; Makatsori et al., 2003). Probably due to habituation processes the alertness induced by novel objects declines after two more weeks, in parallel to a decrease in PPI, but four-week stress in the SE group leads to improved PPI. Thus, when environmental conditions lead to a certain degree of arousal, attentional/cognitive processes and PPI are improved (Chen et al., 2010; Grillon & Davis, 1997). As anxiety/fear and hippocampus volume are reduced by NH in the RLA rats, it seems reasonable to suggest that the NH-induced impairment of PPI in RLA rats may be due to a decrease of alertness/arousal induced by the treatment (see Study 3, Annex 1).

On the other hand, the PPI improvement induced by NH in RHA rats was observed at 70dB (both sexes) and 75dB (only females). NH also induced a better cognitive flexibility (i.e. reversal task) and working memory performance in both strains, the latter being more clear in RHA rats. Since these are (to different extents) hippocampus-dependent tasks, as far as volume is thought to be related with function (Taubert et al., 2012; Woollett & Maguire, 2011), an increase in hippocampus volume by NH might have been expected in RHA rats. Although this was not the case, we would like to remark that NH-treated RHA rats showed a trend for enlarged hippocampus ($p = 0.05$ one-tailed Student's t-test) as compared with control RHA animals (Study 2, Study 3). Further studies, including larger rat samples could help to clarify this issue. In any case, it cannot be ruled out at present that the beneficial effects of NH on attentional/cognitive performance of RHA rats could be mediated by some NH effects on hippocampal function.

From a molecular point of view, Garoflos et al. (2007) carried an experiment in order to explain the cognitive enhancement induced by NH in rats. Handled and non-handled rats were evaluated with the aim to determine NH effects on neurotrophin-3 (NT-3) levels in the rat hippocampus and also to evaluate maternal care behavior (i.e. licking and grooming). They observed that a single exposure of NH increased the number of NT-3 cells in the

hippocampus (CA1) and maternal care was increased in handled rats compared to non-handled animals (Garoflos et al., 2007). Interestingly, positive associations were found between NT-3 levels and maternal care.

NT-3 along with brain-derived neurotrophic factor (BDNF) are part of the neurotrophins family, which have been proposed as key molecules in brain development (Lu et al., 2005). It has been shown that neurotrophins are involved in brain development by controlling cell proliferation, survival, differentiation, axonal growth, migration and synaptogenesis and also are involved in plasticity-related processes (Huang & Reichardt, 2003; Lu et al., 2005; McAllister et al., 1999). NH has been shown to increase the levels of nerve growth factor (NGF) in the hippocampus (Pham et al., 1997). Moreover, increased hippocampal BDNF in rat hippocampus has been reported after a single exposure to NH on PND1 (Garoflos et al., 2005).

It has been proposed that enriched environment (EE) shares some neurobehavioral similarities with NH treatment (regardless the markedly different procedures) as both elicit similar long-lasting effects on anxiety and cognition (i.e. increased exploratory behavior, improved acquisition of two-way active avoidance, improved memory in several learning tasks, improved spatial orientation and working memory performance in the Morris water maze task; see Escorihuela et al., 1994b; Fernández-Teruel et al., 1997, 2002b; Garoflos et al., 2007). In this regard, it has also been shown that EE also induced increases of NT-3 and BDNF expression in the rat hippocampus in addition to better spatial memory (Ickes et al., 2000; Torasdotter et al., 1996). Moreover, NH also increased the expression of glucocorticoid receptors, which are involved in the consolidation of learned information and their activation is necessary for optimal memory (de Kloet et al., 1998). To sum up, it seems safe to conclude that the increase of NT-3 and BDNF could be related to the brain plasticity processes induced by NH (Norrholm & Ouimet, 2001; Akaysha et al., 2002), which in turn may underlie the enduring improvement of cognitive abilities observed in neonatally-handled rats (Fenoglio et al., 2005; Fernández-Teruel et al., 1997, 2002b; Garoflos et al., 2007; Meaney et al., 1988; Pryce et al., 2003). Thus, the hippocampus seems to be a key target affected by NH treatment (and other environmental treatments which share some effects with NH, such as EE), as molecular changes are reported in this region after NH and behavioral performance in hippocampal-dependent tasks is also enhanced by NH. Moreover, maternal care provided by the dam to the offspring is a key feature that is able to trigger the molecular downstream of

neurotrophins, which in turn appear to be involved in the cognitive enhancement of NH (Garoflos et al., 2007, 2008).

Current and future work from our laboratory is aimed at evaluating whether NH induces molecular/neurochemical brain changes as these mentioned above (and others) in the *Roman* rat strains.

5.1.- Neonatal handling: possible explanations for its effects.

As mentioned above (see "Introduction"), one major challenge of research on several types of early-life manipulations/treatments is the identification of factors responsible for their effects. Negative consequences of early-life stress in rodent or child development have been widely described in the literature (e.g. Bowlby, 1969; Bruce et al., 2013; Lupien et al., 2009, Oliveras et al., 2016; Suomi, 1997), whereas relatively less is known regarding the positive effects of early-life interventions. Despite the well-described long-lasting positive consequences of early-life stimulation procedures –such as neonatal handling, NH- on cognitive, social, emotional and neuroendocrine responses (e.g. Benetti et al., 2007; Cañete et al., 2015; Fernández-Teruel et al., 1997, 2002b; Levine, 1956, 1962; Levine et al., 1967; Stamatakis et al., 2009; Stamatakis et al., 2015; Tang, 2001; Tang et al., 2012), controversies on their effectiveness and direction of effects have also been raised. With regard to NH treatment, these controversies and inconsistent findings might be due to differences in the NH procedures used (e.g. Raineki et al., 2014). To mention but a few relevant methodological differences in the neonatal handling procedures used in the different studies:

i) NH duration, ranging from 1 to 21 days of treatment (*1 day:* Garoflos et al., 2008. *10 days*: Benetti et al., 2007; Padoin et al., 2001. *15 days*: Meaney et al., 1985; Weinberg et al., 1978. *21 days*: e.g. Dinces et al., 2014; Escorihuela et al. 1994a, 1995a-b; Levine, 1956; Núñez et al., 1995, 1996).

ii) Tactile stimulation, provided by the experimenter with "naked hands" (e.g. Cañete et al., 2015; Escorihuela et al., 1994a, 1995a-b; Fernández-Teruel et al., 1992a-b), wearing fine latex gloves (Benetti et al., 2007), with a brush (Daskalakis et al., 2009) or in absence of tactile stimulation (Tang et al., 2012).

iii) The isolation time ranging from 1 to 15 min (*1 min:* Padoin et al., 2001. *3 min:* Denenberg & Karas, 1959; Levine, 1956. *8 min:* Escorihuela et al., 1994a-

b, 1995a-b; Núñez et al., 1995, 1996; Fernández-Teruel et al. 2002a-b. *15 min:* Daskalakis et al., 2009; Meaney et al., 1993; Pryce et al., 2001)

iv) Total time for tactile stimulation or strokes ranging from 10 strokes/10 sec to 3 strokes/5 sec (e.g. Daskalakis et al., 2009; Ecorihuela et al., 1994a-b, 1995a-b; Núñez et al., 1995, 1996; Fernández-Teruel et al., 2002a-b; present studies)

v) Experimental designs, usually with between-litter (e.g. Cañete et al., 2015; Meaney et al., 2013) vs split-litter design (e.g. Dinces et al., 2014; Tang, 2001)

However, as we advanced in the "Introduction", and despite the above procedural differences, there is agreement that at least three factors are critically involved in the effects neonatal handling. They are: i) isolation/maternal separation, ii) the exposure to a novel situation/environment and iii) tactile stimulation.

Supporting the relevance of each of the factors mentioned above, it has been proved that neonatal tactile stimulation reversed the detrimental effects of neonatal isolation (from PND 2-9) in conditioned and unconditioned anxiety tasks (Imanaka et al., 2008). Furthermore, 10 back-strokes delivered with a brush and combined with novelty exposure, enhanced spatial recognition memory in a Y-maze task. However, a total of 20 back-strokes, combined with novelty, reduced Y-maze performance, whereas the same treatment (i.e. 20 strokes) enhanced the performance of the siblings that stayed in the home-cage (so, these were not exposed to novelty) (Daskalakis et al., 2009; Zhang & Cai, 2008).

In addition, the licking delivered by the dams to the pups may also be considered as a "second handling", similarly to the tactile stimulation provided by the experimenter to the pups (or by the mothers or nurses to premature infants in Tiffany Field's studies; Field 1986, 2016). The idea that the effects of NH are mediated by the increase of maternal licking received by the pups is supported by many studies (e.g. Fenoglio et al., 2006; Lee & Williams, 1974; Levine, 1994; Liu et al., 1997; Pryce et al., 2001). It was demonstrated that 8-hours after a single exposure to neonatal handling on PND 1 (following Levine´s classical procedure of NH) *c-fos* positive cells were increased in hippocampus, parietal and occipital cortex compared to control rats, and during the four hours following the single NH treatment handled pups received more maternal lickings than non-handled rats (Garoflos et al., 2008). The delayed induction of *c-fos* probably indicates that its expression is secondary to "other handling" (i.e. the "second handling", as mentioned above) provided by the dam through licking behavior. Interestingly, positive associations between the number of *c-fos* cells in

hippocampus, parietal and occipital cortex and maternal licking during the 8-hours following handling and return to the home-cage, were also observed (Garoflos et al., 2008). Further supporting these findings, a single exposure to NH on PND 9 also led to increased maternal care and *c-fos* expression in the central nucleus of the amygdala and the bed nucleus of the stria terminalis (Fenoglio et al., 2006; Stamatakis et al., 2015).

Moreover, besides these mother reactions and changes in maternal care, the stimulation that constituted by repeated (daily) short periods of isolation (from their mother and littermates, being exposed to a novel environment for a few minutes) is by itself an important factor for the positive and long-term effects of the NH treatment (Macrì et al., 2008; Tang et al., 2006). The repeated (mild) stress involved in being isolated from their mother and littermates, provides a useful condition for the pups to enhance their ability to modulate and regulate the production of glucocorticoids (Meaney et al., 2013). In this regard, Levine´s studies showed that handled animals exhibit faster return to baseline corticosterone levels than non-handled animals, implicating a handling effect on the more adaptive development of pituitary-adrenal negative feedback in the rat (Ader, 1970; Levine, 1962; Levine et al., 1967). Thus, the mix of compounds involved in NH treatment seems to enhance the resilience that allows to cope better with other stressful situation later in life. In conclusion, although studies suggest that each of the above mentioned factors individually contributes to the positive effects of the NH treatment, it is likely that each one influences the other/s. Thus, pup isolation promotes enhanced maternal care, which in turns might provide a "second handling" trough their licking behavior, in addition to the handling delivered by the experimenter. Moreover, the novelty provided by the new environment in which pups are isolated, and the low-mild stress levels involved in the (usually repeated) isolation time, might also contribute to a better maturation and regulation of the HPA axis, thus facilitating the emergence of better coping strategies when confronting future stressors in life (e.g. Cañete, 2011; Escorihuela et al., 1994b; Fernández-Teruel et al., 2002b).

However, there are other interesting approaches aiming at clarifying whether other factors might be involved in the effectiveness of NH-like interventions. Thus, in a series of studies using a split-litter design, rats were isolated from their mother for 3 minutes, with half-litter being placed in a novel environment (i.e. individual cage) while the other half-litter stayed in group at their home-cage. Overall, novelty-pups showed increased disinhibition when tested under novelty situations, enhanced spatial memory, reduced aggression and better social memory than home-cage-pups (Dinces et al., 2014; Tang, 2001). However, in some litters the

novelty-induced effect was absent. A "maternal context" hypothesis was proposed, in which dams with lower basal levels and greater reactivity of corticosterone after acute stress (1-min in a swimming test at PND 21) set the context (i.e. maternal context) that allows treatment effectiveness in their pups (Catalani et al., 2011; Dinces et al., 2014; Tang, 2001; Tang et al., 2012).

Along these lines, maternal self-stress regulation might induce effects on HPA axis of the offspring by direct hormonal signaling via the placenta prenatally (Glover et al., 2010), and via maternal milk postnatally (Catalani et al., 2011). Low levels of maternal corticosterone offer both, a low level from which to mount its own stress response to other environmental challenges and a low baseline toward which the stress response can recover after stress (Tang et al., 2012). This argument is in line with studies in which mothers whose drinks were supplemented with low-moderate doses of corticosterone during lactation (i.e. corticosterone present in milk pierced through lactation to their pups), give birth to offspring that are better able to cope with the environment, with reduced fear, higher learning capabilities and higher expression of glucocorticoid receptors in the hippocampus, in comparison with the detrimental effects if mothers drinks were supplemented with high doses of corticosterone (Catalani et al, 2011).

As maternal care is one of the main sources of environmental stimuli for the progeny and a major determinant of behavior in adulthood (Bowlby, 1988; McEwen, 2003), it is of great relevance to understand the mechanism involved in this behavior. From a molecular point of view, it has been proposed that the estrogens are key factors involved in eliciting maternal behavior, with higher levels of estrogens being observed prior to parturition, so that maternal behavior could be expressed following a surge of oxytocin (Stamatakis et al., 2015; Young et al., 1997). Interestingly, both types of estrogen receptors (i.e. ERα and ERβ) participate in the regulation of oxytocinergic system, with ERβ involved in the regulation of the oxytocin levels (Patisaul et al., 2003) and ERα in the increase of oxytocin receptors (Vasudevan et al., 2001). Accordingly, mothers exhibiting high levels of licking and grooming, as well as nursing in the arched-back position, showed high levels of estrogen receptors and oxytocin receptors in the hypothalamus (Champagne et al., 2000, 2001, 2003). Administration of oxytocin into cerebral ventricles elicits maternal behavior in virgin rats (Pedersen et al., 1982), and blocking oxytocin actions disrupted maternal behavior (Pedersen & Boccia, 2003; Sabihi et al., 2014).

On the other hand, the serotonergic system also plays a role in regulating maternal behavior. Neurotoxic lesions of serotonergic median raphe nuclei or reduction in serotonergic input into the hypothalamus disrupt lactation and maternal behavior (Barofsky et al., 1983; Rowland et al., 1978), and administration of fluoxetine (i.e. a selective serotonin reuptake inhibitor) enhances maternal behavior (Johns et al., 2005; Pawluski et al., 2012). Interestingly, both serotonin (especially 5-HT1A receptors) and oxytocin are also known to modulate anxiety (Arrant et al., 2013; Neumann, 2008). Anxiety has been shown to be reduced during lactation in both rats (Neumann, 2003) and humans (Lonstein, 2007). In addition, it has been shown that prefrontal cortex is activated when pups are suckling and this activation is inhibited by oxytocin antagonists (Febo et al., 2005), and medial prefrontal cortex is active (assessed by EEG recordings) when rat dams express maternal behavior (i.e. pup retrieval and licking) (Hernández-González et al., 2005).

Since NH treatment markedly reduced anxiety/stress responses in treated rats, and especially in the high anxious and stress-sensitive RLA strain (Study 1 and Study 2), it would be interesting to more specifically study maternal behavior in the *Roman* rats, as well as to evaluate whether NH treatment (or the 8-min isolation included in the procedure) is able to increase maternal care in parallel to the expression of estrogen/oxytocin receptors and central oxytocin levels.

5.2.- Anxiety/fear responses, behavioral inhibition and stress sensitivity in RLA rats: generalizability to humans?

Another major challenge in basic neuroscience research is the translational value of animal models with respect to the understanding of human disease. Regarding anxiety disorders, the concept of "behavioral inhibition" (BI) is gaining increasing relevance, as it is considered one of the strongest developmental temperamental predictors of later anxiety-related behaviors, symptoms or disorders (Chronis-Tuscano et al., 2009; Prior et al., 2000; White et al., 2017). Meta-analytic studies have revealed that nearly half of the individuals who express high levels of BI in childhood will develop an anxiety disorder later in life, a four-fold increase in risk over individuals with no history of BI (Clauss & Blackford, 2012).

Briefly, the BI construct refers to a particular temperament or style of reacting that is exhibited when confronted with novel situations (Fox et al., 2005). Infants with high BI

spontaneously try to avoid unfamiliar people, unknown objects and novel situations (Schwartz et al., 2015; Sylvester et al., 2016), presenting a consistent tendency to display fear reactions and withdrawal in unfamiliar situations (Kagan et al., 1984), higher arousal in limbic-sympathetic axes (Kagan et al., 1987), higher heart rate with slower decreases to baseline (Kagan et al., 1984) and higher basal levels of cortisol compared to uninhibited children (Kagan et al., 1987; Schmidt et al., 1997). A trait similar to BI (i.e. neophobia, emotionality and/or shyness) can also be found among many animal species including non-human primates and rats (Cavigelli et al., 2007; Gosling et al., 1999; Wilson et al., 1994), showing that BI has trait-like characteristics that are associated with human and non-human primate anxiety (Fox et al., 2005; Kalin & Shelton, 2003). Pharmacological studies have shown that diazepam (i.e. an anxiolytic drug) selectively decreases BI without affecting other behaviors such as locomotion (Duzzioni et al., 2008; Wilson et al., 2004), while the drug has no effects on non-anxious animals (do-Rego et al., 2006), a finding that resembles the effects of several anxiolytic drugs in RLA rats (i.e. Driscoll & Stübi 1985; Fernández-Teruel et al., 1991b; Martin et al. 1982; Steimer & Driscoll 2003; Torres et al., 2007).

In a study with rats in which the expression of the immediate early genes *homer1* and *fos* was evaluated, individual differences in hippocampal gene expression (in CA1-CA3 areas) were predictive of individual differences in BI (Qi et al., 2010). As the hippocampus is activated in front of anxiogenic/stressful conditions, and it has been proposed to function as a comparator device to solve anxiety-mediated conflicts (i.e. conflict between competing goals/responses; Gray, 1982; Gray & McNaughton, 2000; McNughton & Corr, 2004), its activation related to a high BI temperamental profile (i.e. a high anxiety trait) shouldn't be surprising (Bach et al., 2014; Barrós-Loscertales et al., 2006; Cherbuin et al., 2008; Gray & McNaughton, 2000; Hahn et al., 2010; Levita et al., 2014: McEwen, 1999; Oler et al., 2010). The hippocampus is interconnected with the amygdala (see discussion of Study 2), which also plays an important role in regulating BI (e.g. Gray & McNaughton, 2000, and references therein), especially under conditions in which the hippocampus does not participates because there is no conflict between competing goals/responses (e.g. Gray & McNaughton, 2000). Accordingly, in a human functional magnetic resonance (fMRI) study, subjects categorized as behaviorally inhibited showed greater response in the amygdala in front of novel faces than non-inhibited subjects, with no differences being observed between both groups in front of familiar faces (Schwartz et al., 2003). Other studies have reported positive associations between amygdala volume, function or connectivity with behavioral inhibition (i.e. anxiety) in both humans and

non-human primates (Barrós-Loscertales et al., 2006; Hahn et al., 2010; Oler et al., 2010, Rahman et al., 2014).

Although the anxiety/stress-like profile of the RLA rats is robust and well accepted, it would be of great value to further characterize the RLA strain/line of rats in social tasks, and to evaluate whether they present decreased social behavior, compatible with a high BI profile. It is known that children with relatively high BI are shy with strangers and timid in unfamiliar situations, thus displaying withdrawal behavior when confronting unfamiliar situations (Biederman et al., 2001). In rats (male Sprague-Dawley), a low anxiety-provoking novelty test, similar to those used in children, induced BI responses that mimic the ones observed in humans. The latencies to start social interaction were good positive predictors of short-term glucocorticoid reactivity (Cavigelli et al., 2007).

Despite the lack of social interaction studies in our *Roman* rat strains, some other features of the BI profile seem to be fulfilled by the RLA rats. In this regard, the use of the novel object exploration (NOE) test, in which a novel object (i.e. a pencil) is introduced in the rats' home-cage (thus, in the familiar environment), could be useful for measuring behavioral inhibition (i.e. freezing behavior, fear, withdrawal, latency to start exploring the novel object) in front of unfamiliar objects (see Study 1, Study 2, Study 3; Cuenya et al., 2016; Fernández-Teruel et al., 2002b). In our studies with the *Roman* rat strains, the latency elapsed to the first exploration and the time spent exploring the novel object have been shown to predict amygdala and hippocampus volume (following multiple lineal regression, see Study 2), and have also shown associations with other anxiety-related behavioral responses, such as behavior in the elevated zero-maze test of anxiety, the basal acoustic startle response, the number of avoidances and the number of inter-trial crossings in the two-way active avoidance task (see correlations in study 1 and 2). It should also be taken into account that the NOE test was performed during adolescence (i.e. PND 60) and still correlated with amygdala and hippocampus volume evaluated when the rats were 6 months old, fitting with the idea that withdrawal response (i.e. emotionality or shyness) in front of unknown objects could be a good predictive trait for future anxiety-like responses or symptoms.

As NH treatment has shown to decrease anxiety/fear and stress sensitivity responses (especially in the RLA rats), it might be considered as a valid environmental treatment for the long-term study of anxiety and stress responses related to BI, as well as for studying whether the BI trait is sensitive to environmental-induced changes and the neurobiology underlying

these changes. In this context, some of the first early stimulation studies in humans took advantage of Levine's and Meaney's NH studies and developed a tactile/kinestesic stimulation (later on called "massage therapy") procedure which was applied to preterm infants with considerably positive neurodevelopmental results (e.g. Escorihuela et al., 1994b; Field, 2016). Massage therapy studies in humans (which could represent at least the tactile stimulation of our NH procedure) lend support to the NH effects observed in our studies and in other rodent studies. Massage therapy has been shown to exert beneficial effects on a series of conditions including prenatal depression, preterm infants weight gain, hypertension, autism and autoimmune conditions among others (Field, 2016). Specifically, massage therapy was shown to increase the mother-baby attachment (which could be considered as a form of maternal care in the case of rodents studies) (Gürol & Polat, 2012). Based on tactile/kinestecic stimulation during the first 12 days of life delivered to preterm infants, Field and colleagues (Field, 1986; Field et al., 2010) showed that: i) the daily weight gain for the stimulated infants was 47% more than non-stimulated preterm infants, ii) stimulated infants stayed longer time awake compared to non-stimulated ones, iii) based on Brazelton scale, stimulated infants showed higher levels of habituation, orientation and motor/behavioral maturity than unstimulated infants and, iv) hospitalization was reduced in 6 days compared to non-stimulated infants (Denenberg, 1975; Field, 1986; Escorihuela et al., 1994b). Moreover, this effects showed to persist at least during until the eight month of age, and facilitate the mother-baby attachment which is known to be an important factor for future develompent (Bolwby et al., 1969; Field, 1986; Field et al., 2010).

In conclusion, BI has been accepted as one of the main risk factor for future prevalence of anxiety/stress disorders among others. RLA rats seem to fit well with a high BI profile, providing a useful tool for the study of the mechanisms involved in BI and later anxiety/fear and stress sensitivity/symptoms. In this regard, NH might be also taken into account as a relevant environmental treatment in this field of research, as it is able to reverse in a robust manner the anxiety/stress profile of the RLA rats.

5.3.- The Roman High-avoidance rat: relevance as an animal model of schizophrenia-relevant features.

As described before, RHA rats appear to meet several key features related to schizophrenia. In summary, compared with the RLA strain/line, RHA rats show: i)

attentional/cognitive impairments, such as PPI, latent inhibition and spatial learning/memory deficits (Del Rio et al., 2014; Esnal et al., 2016; Fernández-Teruel et al., 1997, 2006; Moreno et al., 2010; Oliveras et al., 2015, 2016; see also Study 3); ii) improvements of PPI by the typical antipsychotic haloperidol and PPI impairment by the dopamine receptor agonist apomorphine (Oliveras et al., 2017); iii) higher expression of 5-HT2A receptor and a dramatic decrease of mGlu2 receptors in prefrontal cortex (Klein et al., 2014), similar to the pattern observed in untreated schizophrenic patients (González-Maeso et al., 2008); iv) decreased prefrontal cortex, hippocampus and amygdala volume, while also showing a robust enlargement of the lateral ventricles (Study 2, Study 3); v) mesocortical and mesolimbic dopaminergic hyperfunction and sensitization to repeated psychostimulant treatment (Corda et al., 2005; Giorgi et al., 2007; Guitart-Masip et al., 2008; Tournier et al., 2013), and vi) and higher impulsivity (Del Rio et al., 2014; Klein et al., 2014; Moreno et al., 2010).

As mentioned before, the present thesis is partially focused on the effects of NH on attentional/cognitive processes. With regard to attentional processes, deficits in PPI have been considered as an endophenotype with relevance in schizophrenia research. PPI could be briefly described as a normal reduction of the startle response to a stimulus induced by a weaker pre-stimulus. Two responses could be described in the processing of the acoustic stimulus in the PPI paradigm. First, the acoustic startle reflex as an uncontrolled and spontaneous response that can be induced by an acoustic stimulus. It is proposed that the acoustic input enters the cochlear nuclei (primary auditory pathway), and if a certain threshold is exceed, the info goes on towards the ventro tegmental nucleus (VTN) and then to the caudal pontine reticular nucleus (PnC), which is known to have direct projections to motor neurons eliciting the physical response (Bosch & Schmid, 2006; Kohl et al., 2013). This acoustic startle reflex response has been used as a valid measure of emotionality/reactivity trait in humans and rodents (Grillon et al., 1992; Swerdlow et al., 1992, 2016). Second, the prepulse inhibition response, which is proposed as a form of plasticity of the startle reflex. The mechanism of its inhibition requires forebrain structures such as nucleus accumbens, hippocampus, amygdala and prefrontal cortex (Bakshi & Geyer, 1998; Koch, 1999; Lacroix et al., 2000; Swerdlow et al., 2016). Briefly, the idea is that these structures inhibit the function of the pedunculopontine tegmental nucleus which in turns inhibits the PnC, thus reducing the motor response (i.e. the startle response) (Kohl et al., 2013; Swerdlow et al., 1992). The brain seems to need a period of 30-500 ms to process

correctly the information given by a first stimulus and therefore "gates out" or filters any new sensory event that might occur within this interval. This pre-attentive inhibitory process protects the brain against an overflow of information. Thus, the capacity of the prepulse stimulus to inhibit the startle response is an operational measure (usually presented as percentage of PPI) of the amount of sensorimotor gating, and this is the process that is proposed to be impaired in schizophrenia among other disorders (e.g. obsessive-compulsive disorder, Giles de la Tourette´s syndrome, autism, enuresis and Huntington´s disease) (Kohl et al., 2013; Swerdlow et al., 2016).

The acceptance of PPI as a robust and stable paradigm in the study of schizophrenia-related symptoms is supported by several human and rodent studies. For example, it has been shown that PPI is impaired in schizophrenic human patients (Grillon et al., 1992; Parwani et al., 2000; Swerdlow et al., 2016), in rodents submitted to pharmacological challenges with dopamine agonists (Swerdlow et al., 1992; Varty & Higgins, 1995), and in non-psychiatric controls after injections of amphetamine (Hutchison & Swift, 1999). As PPI procedure is almost identical in rodent and humans, and because it is considered as a reliable measure, many authors consider PPI as a paradigm with face, predictive and construct validity for the study of some attentional/cognitive schizophrenia-related symptoms (Geyer & Braff, 1987; Swerdlow et al., 1992; Swerdlow & Geyer, 1998; Swerdlow & Light, 2015).

In that context, rodent studies focused on the effects of regional brain lesions on PPI (i.e. mostly mPFc, hippocampus and amygdala), provide evidence for the PPI-impairing effects induced by these lesions compared with control (sham-lesioned) animals. Moreover, the lesions also induce a decrease of volume in the correspondent lesioned brain structure (Alam et al., 2015; Chin et al., 2011; Déziel et al., 2015; Labbate et al., 2014). Interestingly, hippocampus and prefrontal cortex lesions in rats have led, in some cases, to an increase in the volume of lateral ventricles (Bertrand et al., 2010; Sandner et al., 2010; Gilabert-Juan et al., 2013; Schneider and Koch, 2005). The enhanced volume of the lateral ventricles has been related to schizophrenia (Shenton et al., 2001). In keeping with this, rats reared in isolation (a model of schizophrenia related-features) showed reduced mPFc and hippocampus volume, increased volume of the lateral ventricles and impaired PPI (Schubert et al., 2009; Day-Wilson et al., 2006).

In the human side, a recent prospective longitudinal neuroimaging study on clinical high risk (CHR) individuals who later developed full-blown psychosis, showed an accelerated rate of

gray matter thinning in mPFc and expansion of the ventricular system compared with subjects who did not develop psychosis and with healthy controls (Cannon et al., 2015; Chung et al., 2017). The ventricular enlargement showed to be linked to cortical (but not subcortical) gray matter reduction, and it was suggested that enlargement of the lateral ventricles might be due to reductions in the mechanical pressure that extra-ventricular tissues exert against the ventricular wall, allowing the CSF-filled compartments to expand. In this regard, ventricular enlargement may be related to the cortical thinning during the psychosis prodrome (Chung et al., 2017).

In conclusion, the main stream of evidence points to the fact that decreased volumes of the mPFc and hippocampus, and the enlargement of lateral ventricles are associated with PPI impairments, deficits in latent inhibition, higher locomotion and increased impulsivity (Alam et al., 2015; Chin et al., 2011; Déziel et al., 2015; Labbate et al., 2014; Piontkewitz et al., 2012; Schneider & Koch, 2005).

In our case, no NH effects were observed on either the medial prefrontal cortex or the volume of the lateral ventricles. However, NH improved cognitive plasticity in the reversal task and spatial working memory in both strains, but especially in the RHA- treated rats (see Study 3). One would expect that if NH improved executive functions and working memory, then this improvement should be also paralleled by increases in the volume of brain areas involved in these tasks, such as mPFc and hippocampus, but this was not the case. Several possible explanations for this apparent inconsistency could be proposed. First, those cognitive tasks performed in the Morris water maze are considered to be hippocampal-dependent tasks (Morris et al., 1986a-b; Wishaw 1985). NH-treated RHA rats showed a non-significant trend for enlarged hippocampus ($p = 0.05$ one-tailed Student's t-test) as compared with control RHA animals (Study 2, Study 3). Although this non-significant result should be further explored in future studies, it may suggest that some slight or subtle changes in the hippocampus of NH-treated RHA rats might have occurred, maybe in some aspects of the hippocampal function which do not have a direct expression as volume changes. Thus, it cannot be ruled out at present that the beneficial effects of NH on attentional/cognitive performance of RHA rats could be mediated by some NH effects on hippocampal function.

Second, NH effects on mPFc volume might be less expected (than those on the hippocampus or amygdala volume, for instance), because the treatment is delivered during a "resting time" period for the development of mPFc. Third, rearing rats in isolation decreased mPFc volume,

mainly because of reductions in the neuropil but not because of reductions in the number of neurons, compared to socially-reared rats (Day-Wilson et al., 2006). This suggests that connectivity between this region and other areas relevant to the disease (e.g. hippocampus, cingulated cortex, amygdala, nucleus accumbens) should also be taken into account as mPFc is a highly interconnected brain region. Thus, we cannot rule out the possibility of NH effects on mPFc connectivity as some phenotypes related to prefrontal cortex functions (such as executive functions) are improved in NH-treated rats.

In this regard, the current vision of schizophrenia points to the relevance of brain-region connectivity studies as a key approach. Many researchers have come to understand the disease as fundamentally a disorder of disconnection within and between certain functional networks in the brain (Cannon, 2015). Challenges still remain concerning the identification of which are the networks critically affected and which level of disconnection may be necessary for the expression of the schizophrenia-related symptoms. Disconnectivity in schizophrenia is supported by analyses using diffusion magnetic resonance imaging (dMRI) revealing fractional anisotropy (FA) deficits in white matter tracts (Patel et al., 2011). Available evidence supports that such disconnectivity might be particularly evident for the prefrontal cortex (Pettersson-Yeo et al., 2011; Wheeler et al., 2014). The streamline counts (SC) is a complementary measure that quantifies the number of streamlines that connect pair of regions, and its value is associated to the number of axons directly linking these regions (Jones et al., 2015). Among the regions strongly linked to PFC are anterior cingulate (Baiano et al., 2007), hippocampus (Harrison, 2004), thalamus (Pergola et al., 2015) and caudate (Simpson et al., 2010). In this regard, alterations in FA have been found to be more widespread in schizophrenic patients, suggesting myelination deficits in the hippocampal-prefrontal pathway compared to control subjects (Molina et al., 2017). So, it would be of great interest to perform connectivity studies with our *Roman* rats, in order to further characterize these schizophrenia-related phenotypes in both strains.

One relevant advantage of the RHA schizophrenia-like profile is that they display the schizophrenia-relevant symptoms without any intervention (other than selective breeding). Thus, the RHA strain/line of rats seems to spontaneously posses the schizophrenia-like profile, allowing the study of disease-related features in a more naturalistic way, which in turn offers the possibility to understand the mechanisms involved in schizophrenia without confounding effects of a given procedure (treatment or manipulation). In this regard, connectivity studies, MRI volumetry and *c-fos* early gene expression would be of great

interest to validate even more the potentiality of the RHA to mimic schizophrenia-like processes. Some of these studies are currently under way at our laboratory.

6- CONCLUSIONS

The main conclusions of the present doctoral thesis are:

1. Inbred RLA rats showed higher unconditioned and conditioned anxiety/fear responses than RHA rats, as well as higher post-stress corticosterone and prolactin hormone level responses compared to RHA rats.

2. These behavioral phenotypes were paralleled by between-strain differences in the volumes of specific brain-regions related to anxiety/stress responses, with RLA rats showing higher amygdala and hippocampus volume than their RHA counterparts. These results lend support to Gray´s anxiety theory, thus supporting the proposal of RLA rats as a valid animal model for the study of anxiety/stress-related mechanisms.

3. Inbred RHA rats showed impairments in both PPI and spatial cognitive functions compared to RLA animals. Such a profile is in line with some attentional (sensorimotor gating) and cognitive deficits observed in schizophrenic patients.

4. Compared to their RLA counterparts, RHA rats exhibit significant reductions of medial prefrontal cortex and hippocampus volume, as well as a two-fold enlargement of the lateral ventricles. Such a neuroanatomical volumetric profile is consistent with the attentional (PPI) and cognitive deficits observed in RHA rats, thus providing support to the proposal of this rat strain as a valid animal model of some schizophrenia-related features.

5. Neonatal handling (NH) treatment reduced unconditioned and conditioned anxiety/fear, as well as stress hormone responses in both rat strains, although in most cases NH effects were more marked in RLA rats. Of note, NH was even able to significantly improve two-way avoidance acquisition in this strain. Thus, NH seems to induce a change from a passive/reactive towards a more active/proactive coping style particularly in RLA rats.

6. For the first time we also demonstrate that NH was able to enduringly reduce amygdala volume in both rat strains, but especially in the RLA strain, while the treatment also reduced hippocampus volume in RLA rats. Positive associations

(correlations) between amygdala or hippocampus and anxiety-related measures were observed.

7. NH increased PPI levels (in both sexes) as well as working memory in RHA rats, whereas it consistently impaired PPI in RLA rats, which could be consistent with the reduction of hippocampus volume induced by NH in this strain.

REFERENCES

Ader, R. (1970). The effects of early experience on the adrenocortical response to different magnitudes of stimulation. *Physiology & Behavior*, *5*(8), 837–9. https://doi.org/10.1016/0031-9384(70)90168-X

Adib-Hajbaghery, M., Abasi, A., & Rajabi-Beheshtabad, R. (2014). Whole body massage for reducing anxiety and stabilizing vital signs of patients in cardiac care unit. *Medical Journal of the Islamic Republic of Iran*, *28*, 47. http://www.ncbi.nlm.nih.gov/pubmed/25405113

Adib-Hajbaghery, M., Rajabi-Beheshtabad, R., & Ardjmand, A. (2015). Comparing the effect of whole body massage by a specialist nurse and patients' relatives on blood cortisol level in coronary patients. *ARYA Atherosclerosis*, *11*(2), 126–32. http://www.ncbi.nlm.nih.gov/pubmed/26405441

Aguilar, R. (2002). Fearfulness in a large F2 progeny of the Roman Rats: paving the way for QTL's genes localization. (Doctoral Thesis). Department of Psychiatry and Forensic Medicine, Autonomous University of Barcelona, Spain. http://hdl.handle.net/10803/5562

Aguilar, R., Escorihuela, R. M., Gil, L., Tobeña, A., & Fernández-Teruel, A. (2002). Differences between two psychogenetically selected lines of rats in a swimming pool matching-to-place task: long-term effects of infantile stimulation. *Behavior Genetics*, *32*(2), 127–34. https://doi.org/10.1023/A:1015253807488

Aguilar, R., Gil, L., Fernández-Teruel, A., & Tobeña, A. (2004). Genetically-based behavioral traits influence the effects of Shuttle Box avoidance overtraining and extinction upon intertrial responding: a study with the Roman rat strains. *Behavioral Processes*, 66, 63-72. https://doi.org/10.1016/j.beproc.2004.01.002.

Alam, M., Angelov, S., Stemmler, M., von Wrangel, C., Krauss, J. K., & Schwabe, K. (2015). Neuronal activity of the prefrontal cortex is reduced in rats selectively bred for deficient sensorimotor gating. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *56*, 174–184. https://doi.org/10.1016/j.pnpbp.2014.08.017

Armario, A., Daviu, N., Muñoz-Abellán, C., Rabasa, C., Fuentes, S., Belda, X., … Nadal, R. (2012). What can We Know from Pituitary–Adrenal Hormones About the Nature and Consequences of Exposure to Emotional Stressors? *Cellular and Molecular Neurobiology*, *32*(5), 749–758. https://doi.org/10.1007/s10571-012-9814-6

Arrant, A. E., Coburn, E., Jacobsen, J., & Kuhn, C. M. (2013). Lower anxiogenic effects of serotonin agonists are associated with lower activation of amygdala and lateral orbital cortex in adolescent male rats. *Neuropharmacology*, *73*, 359–367. https://doi.org/10.1016/j.neuropharm.2013.05.030

Baiano, M., David, A., Versace, A., Churchill, R., Balestrieri, M., & Brambilla, P. (2007). Anterior cingulate volumes in schizophrenia: a systematic review and a meta-analysis of MRI studies. *Schizophrenia Research*, *93*(1–3), 1–12. https://doi.org/10.1016/j.schres.2007.02.012

Bach, D.R., Guitart-Masip, M., Packard, P.A., Miró, J., Falip, M., Fuentemilla, L., Dolan, R.J. (2014). Human hippocampus arbitrates approach-avoidance conflict. *Current Biology*, 24, 541-547. https://doi.org/10.1016/j.cub.2014.01.046.

Bakshi, V. P., & Geyer, M. A. (1998). Multiple limbic regions mediate the disruption of prepulse inhibition produced in rats by the noncompetitive NMDA antagonist dizocilpine. *The Journal of Neuroscience, 18*(20), 8394–401. http://www.ncbi.nlm.nih.gov/pubmed/9763482

Bakshi V, K. N. (2002). Animal models and endophenotypes of anxiety and stress disorders. In book: Neuropsychopharmacology:The fifth generation of progress. *American college of Neuropsychopharmacology. Ed. Lippincott Williams and Wilkins. Philadelphia, USA.*

Bannerman, D.M., Rawlins, J.N., McHugh, S.B., Deacon, R.M., Yee, B.K., Bast, T., …& Feldon, J. (2004). Regional dissociations within the hippocampus-memory and anxiety. *Neuroscience & Biobehavioral Reviews*, 28, 273-283. https://doi.org/10.1016/j.neubiorev.2004.03.004

Bannerman, D.M., Sprengel, R., Sandeerson, D.J., McHugh, S.B., Rawlins, J.N., Monyer, H., … & Seeburg, H. (2014). Hippocampal synaptic plasticity, spatial memory and anxiety. *Nature Reviews Neuroscience*, 15, 181-192. https://doi.org/10.1038/nrn3677.

Barofsky, A. L., Taylor, J., & Massari, V. J. (1983). Dorsal raphe-hypothalamic projections provide the stimulatory serotonergic input to suckling-induced prolactin release. *Endocrinology, 113*(5), 1894–1903. https://doi.org/10.1210/endo-113-5-1894

Barrós-Loscertales, A., Meseguer, V., Sanjuán, A., Belloch, V., Parcet, M. A., Torrubia, R., & Ávila, C. (2006). Behavioral Inhibition System activity is associated with increased amygdala and hippocampal gray matter volume: A voxel-based morphometry study. *NeuroImage, 33*(3), 1011–1015. https://doi.org/10.1016/j.neuroimage.2006.07.025

Benetti, F., Andrade de Araujo, P., Luiz Sanvitto, G., & Lucion, A. B. (2007). Effects of neonatal novelty exposure on sexual behavior, fear, and stress-response in adult rats. *Developmental Psychobiology, 49*(3), 258–264. https://doi.org/10.1002/dev.20181

Bentareha, R., Araujo, F., Ruano, D., Driscoll, P., Escorihuela, R. M., Tobeña, A., … Vitorica, J. (1998). Pharmacological properties of the GABA(A) receptor complex from brain regions of (hypoemotional) Roman high- and (hyperemotional) low-avoidance rats. *European Journal of Pharmacology, 354*(1), 91–7. https://doi.org/10.1016/S0014-2999(98)00428-2

Bertrand, J.-B., Langlois, J.-B., Bégou, M., Volle, J., Brun, P., d'Amato, T., … Suaud-Chagny, M.-F. (2010). Longitudinal MRI monitoring of brain damage in the neonatal ventral hippocampal lesion rat model of schizophrenia. *Hippocampus, 20*(2), 264–78. https://doi.org/10.1002/hipo.20628

Biederman, J., Hirshfeld-Becker, D. R., Rosenbaum, J. F., Hérot, C., Friedman, D., Snidman, N., … Faraone, S. V. (2001). Further Evidence of Association Between Behavioral Inhibition and Social Anxiety in Children. *American Journal of Psychiatry, 158*(10), 1673–1679. https://doi.org/10.1176/appi.ajp.158.10.1673

Bignami, G. (1965). Selection for high rates and low rates of avoidance conditioning in the rat. *Animal Behaviour*, *13*(2–3), 221–227. http://www.ncbi.nlm.nih.gov/pubmed/5835838

Black, M.D., Lister, S., Hitchcock, M.J., Van Giersbergen., & Sorensen, S.M. (1998). Neonatal hippocampal lesion model of schizophrenia in rats: Sex differences and persistence of effects into maturity. *Drug Development Research*, *43*, 206–213.

Blanchard, R. J., Yudko, E. B., Rodgers, R. J., & Blanchard, D. C. (1993). Defense system psychopharmacology: an ethological approach to the pharmacology of fear and anxiety. *Behavioural Brain Research*, *58*(1–2), 155–65. https://doi.org/10.1016/0166-4328(93)90100-5

Bodnoff, S. R., Suranyi-Cadotte, B., Quirion, R., & Meaney, M. J. (1987). Postnatal handling reduces novelty-induced fear and increases [3H]flunitrazepam binding in rat brain. *European Journal of Pharmacology*, *144*(1), 105–7. https://doi.org/10.1016/0014-2999(87)90016-1

Bosch, D., & Schmid, S. (2006). Activation of muscarinic cholinergic receptors inhibits giant neurones in the caudal pontine reticular nucleus. *European Journal of Neuroscience*, *24*(7), 1967–1975. https://doi.org/10.1111/j.1460-9568.2006.05085.x

Bourgin, J., Cachia, A., Boumezbeur, F., Djemaï, B., Bottlaender, M., Duchesnay, E., … Jay, T. M. (2015). Hyper-responsivity to stress in rats is associated with a large increase in amygdala volume. A 7T MRI study. *European Neuropsychopharmacology*, *25*(6), 828–835. https://doi.org/10.1016/j.euroneuro.2015.02.010

Bowlby, J. (1969). Attachment and loss. *Ed. Basic Books*. *New York, USA.*

Bowlby, J. (1988). Developmental psychiatry comes of age. *The American Journal of Psychiatry*, *145*(1), 1–10. https://doi.org/10.1176/ajp.145.1.1

Broadhurst, P. L. (1969). Psychogenetics of emotionality in the rat. *Annals of the New York Academy of Sciences*, *159*(3), 806–24. https://doi.org/ 10.1111/j.1749-6632.1969.tb12980.x

Bruce, J., Gunnar, M. R., Pears, K. C., & Fisher, P. A. (2013). Early Adverse Care, Stress Neurobiology, and Prevention Science: Lessons Learned. *Prevention Science*, *14*(3), 247–256. https://doi.org/10.1007/s11121-012-0354-6

Brunelli, S. A., Keating, C. C., Hamilton, N. A., & Hofer, M. A. (1996). Development of ultrasonic vocalization responses in genetically heterogeneous National Institute of Health (N:NIH) rats. I. Influence of age, testing experience, and associated factors. *Developmental Psychobiology*, *29*(6), 507–16. https://doi.org/ 10.1111/j.1749-6632.1969.tb12980.x

Buckley, P. F. (1998). Structural brain imaging in schizophrenia. *The Psychiatric Clinics of North America*, *21*(1), 77–92. http://www.ncbi.nlm.nih.gov/pubmed/9551492

Cannon, T. D. (2015). How Schizophrenia Develops: Cognitive and Brain Mechanisms Underlying Onset of Psychosis. *Trends in Cognitive Sciences*, *19*(12), 744–756. https://doi.org/10.1016/j.tics.2015.09.009

Cannon, T. D., Chung, Y., He, G., Sun, D., Jacobson, A., van Erp, T. G. M., … North American Prodrome Longitudinal Study Consortium. (2015). Progressive Reduction in Cortical Thickness as Psychosis Develops: A Multisite Longitudinal Neuroimaging Study of Youth at Elevated Clinical Risk. *Biological Psychiatry*, *77*(2), 147–157. https://doi.org/10.1016/j.biopsych.2014.05.023

Cañete, T. (2011). Efectos de la estimulación postnatal en el perfil cognitivo y emocional del modelo de ratón 3xTgAD para la enfermedad de Alzheimer. (Tesis Doctoral). Department de Biologia Cel·lular i de Fisiologia. http://hdl.handle.net/10803/83954

Cañete, T., Blázquez, G., Tobeña, A., Giménez-Llort, L., & Fernández-Teruel, A. (2015). Cognitive and emotional alterations in young Alzheimer's disease (3xTgAD) mice: Effects of neonatal handling stimulation and sexual dimorphism. *Behavioural Brain Research*, *281*, 156–171. https://doi.org/10.1016/j.bbr.2014.11.004

Carrasco, J., Márquez, C., Nadal, R., Tobeña, A., Fernández-Teruel, A., & Armario, A. (2008). Characterization of central and peripheral components of the hypothalamus-pituitary-adrenal axis in the inbred Roman rat strains. *Psychoneuroendocrinology*, *33*(4), 437–45. https://doi.org/10.1016/j.psyneuen.2008.01.001

Catalani, A., Alemà, G. S., Cinque, C., Zuena, A. R., & Casolini, P. (2011). Maternal corticosterone effects on hypothalamus–pituitary–adrenal axis regulation and behavior of the offspring in rodents. *Neuroscience & Biobehavioral Reviews*, *35*(7), 1502–1517. https://doi.org/10.1016/j.neubiorev.2010.10.017

Castanon, N., Perez-Diaz, F., & Mormède, P. (1995). Genetic analysis of the relationship between behavioral and neuroendocrine traits in roman high and low avoidances rat lines. *Behavior Genetics* 25: 371-384. https://doi.org/10.1007/BF02197288.

Cavigelli, S. A., Stine, M. M., Kovacsics, C., Jefferson, A., Diep, M. N., & Barrett, C. E. (2007). Behavioral inhibition and glucocorticoid dynamics in a rodent model. *Physiology & Behavior*, *92*(5), 897–905. https://doi.org/10.1016/j.physbeh.2007.06.016

Chambers, R., Moore, J., McEvoy, J. P., & Levin, E. D. (1996). Cognitive Effects of Neonatal Hippocampal Lesions in a Rat Model of Schizophrenia. *Neuropsychopharmacology*, *15*(6), 587–594. https://doi.org/10.1016/S0893-133X(96)00132-7

Champagne, F., Diorio, J., Sharma, S., & Meaney, M. J. (2001). Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. *Proceedings of the National Academy of Sciences*, *98*(22), 12736–12741. https://doi.org/10.1073/pnas.221224598

Champagne, F. A., Weaver, I. C. G., Diorio, J., Sharma, S., & Meaney, M. J. (2003). Natural Variations in Maternal Care Are Associated with Estrogen Receptor α Expression and Estrogen Sensitivity in the Medial Preoptic Area. *Endocrinology*, *144*(11), 4720–4724. https://doi.org/10.1210/en.2003-0564

Chen, Y., Mao, Y., Zhou, D., Hu, X., Wang, J., & Ma, Y. (2010). Environmental enrichment and chronic restraint stress in ICR mice: effects on prepulse inhibition of startle and Y-maze spatial recognition memory. *Behavioural Brain Research*, *212*(1), 49–55. https://doi.org/10.1016/j.bbr.2010.03.033

Cherbuin, N., Windsor, T. D., Anstey, K. J., Maller, J. J., Meslin, C., & Sachdev, P. S. (2008). Hippocampal volume is positively associated with behavioural inhibition (BIS) in a large community-based sample of mid-life adults: the PATH through life study. *Social Cognitive and Affective Neuroscience*, *3*(3), 262–269. https://doi.org/10.1093/scan/nsn018

Chin, C.-L., Curzon, P., Schwartz, A. J., O'Connor, E. M., Rueter, L. E., Fox, G. B., … Basso, A. M. (2011). Structural abnormalities revealed by magnetic resonance imaging in rats prenatally exposed to methylazoxymethanol acetate parallel cerebral pathology in schizophrenia. *Synapse*, *65*(5), 393–403. https://doi.org/10.1002/syn.20857

Chronis-Tuscano, A., Degnan, K. A., Pine, D. S., Perez-Edgar, K., Henderson, H. A., Diaz, Y., … Fox, N. A. (2009). Stable Early Maternal Report of Behavioral Inhibition Predicts Lifetime Social Anxiety Disorder in Adolescence. *Journal of the American Academy of Child & Adolescent Psychiatry*, *48*(9), 928–935. https://doi.org/10.1097/CHI.0b013e3181ae09df

Chung, Y., Haut, K. M., He, G., van Erp, T. G. M., McEwen, S., Addington, J., … North American Prodrome Longitudinal Study (NAPLS) Consortium. (2017). Ventricular enlargement and progressive reduction of cortical gray matter are linked in prodromal youth who develop psychosis. *Schizophrenia Research*. https://doi.org/10.1016/j.schres.2017.02.014

Clauss, J. A., & Blackford, J. U. (2012). Behavioral inhibition and risk for developing social anxiety disorder: a meta-analytic study. *Journal of the American Academy of Child & Adolescent Psychiatry*, *51*(10), 1066–1075.e1. https://doi.org/10.1016/j.jaac.2012.08.002

Corda, M. G., Piras, G., Lecca, D., Fernández-Teruel, A., Driscoll, P., & Giorgi, O. (2005). The psychogenetically selected Roman rat lines differ in the susceptibility to develop amphetamine sensitization. *Behavioural Brain Research*, *157*(1), 147–156. https://doi.org/10.1016/j.bbr.2004.06.016

Cremers, H. R., Demenescu, L. R., Aleman, A., Renken, R., van Tol, M.-J., van der Wee, N. J. A., … Roelofs, K. (2010). Neuroticism modulates amygdala—prefrontal connectivity in response to negative emotional facial expressions. *NeuroImage*, *49*(1), 963–970. https://doi.org/10.1016/j.neuroimage.2009.08.023

Cuenya, L., Sabariego, M., Donaire, R., Callejas-Aguilera, J.E., Torres, C., & Fernández-Teruel, A. (2016). Exploration of a novel object in late adolescence predicts novelty-seeking behavior in adulthood: Associations among behavioral responses in four novelty-seeking tests. *Behavioral Processes*, 125, 34-42. https://doi.org/10.1016/j.beproc.2016.02.003.

Daskalakis, N. P., Kaperoni, M., Koros, C., de Kloet, E. R., & Kitraki, E. (2009). Environmental and tactile stimulation modulates the neonatal handling effect on adult rat spatial memory. *International Journal of Developmental Neuroscience*, *27*(8), 747–755. https://doi.org/10.1016/j.ijdevneu.2009.08.013

Day-Wilson, K. M., Jones, D. N. C., Southam, E., Cilia, J., & Totterdell, S. (2006). Medial prefrontal cortex volume loss in rats with isolation rearing-induced deficits in prepulse inhibition of acoustic startle. *Neuroscience*, *141*(3), 1113–21. https://doi.org/10.1016/j.neuroscience.2006.04.048

de Kloet, E. R., Vreugdenhil, E., Oitzl, M. S., & Joëls, M. (1998). Brain Corticosteroid Receptor Balance in Health and Disease. *Endocrine Reviews*, *19*(3), 269–301. https://doi.org/10.1210/edrv.19.3.0331

de Kloet, E. R., Joëls, M., & Holsboer, F. (2005). Stress and the brain: from adaptation to disease. *Nature Reviews Neuroscience*, *6*(6), 463–475. https://doi.org/10.1038/nrn1683

Degroot, A., & Treit, D. (2002). Dorsal and ventral hippocampal cholinergic systems modulate anxiety in the plus-maze and shock probe tests. *Brain Research*, 1001, 60-71. https://doi.org/10.1016/j.brainres.2003.10.065.

Del Rio, C., Ignasi, O., Cañete, T., Blázquez, G., Tobeña, A., Fernández-teruel, A. (2014). Genetic Rat Models of Schizophrenia-Relevant Symptoms. *World Journal of Neuroscience*, *4*, 261–278. http://dx.doi.org/10.4236/wjns.2014.43030

Delgado y Palacios, R., campos, A., Henningsen, K., Verhoye, M., Dijkstra, J., & Van, Auderkeke. (2011). Magnetic resonance imaging and spectroscopy reveal differential hippocampal changes in anhedonic and resilient subtypes of the chronic mild stress rat model. *Biological Psychiatry*, 70, 449-457. https://doi.org/10.1016/j.biopsych.2011.05.014.

Denenberg, V. H., & Karas, G. G. (1959). Effects of differential infantile handling upon weight gain and mortality in the rat and mouse. *Science (New York, N.Y.)*, *130*(3376), 629–30. https://doi.org/10.1126/science.130.3376.629-a

Denenberg, V., Morton, J., Kline, N., & Grota, L. (1962). Effects of duration of infantile stimulation upon emotionality. *Canadian Journal of Psychology*, *16*(1), 72–76. http://www.ncbi.nlm.nih.gov/pubmed/13885407

Denenberg, V. H. (1975). Effects of exposure to stressors in early life upon late behavioral and biological processes. *Society, stress and disease: childhood and adolescence.*Vol. 2 New York, Oxford University Press, pp 269-28.1

Denenberg, V. H. (1999). Commentary: is maternal stimulation the mediator of the handling effect in infancy? *Developmental Psychobiology*, *34*(1), 1–3. https://doi.org/10.1002/(sici)1098-2302(199901)34:1<1::aid-dev2>3.0.co;2-u

Déziel, R. A., Ryan, C. L., & Tasker, R. A. (2015). Ischemic lesions localized to the medial prefrontal cortex produce selective deficits in measures of executive function in rats. *Behavioural Brain Research*, *293*, 54–61. https://doi.org/10.1016/j.bbr.2015.07.003

Díaz-Morán, S., Palència, M., Mont-Cardona, C., Cañete, T., Blázquez, G., Martínez-Membrives, E., … Fernández-Teruel, A. (2012). Coping style and stress hormone responses in genetically heterogeneous rats: Comparison with the Roman rat strains. *Behavioural Brain Research*, *228*(1), 203–210. https://doi.org/10.1016/j.bbr.2011.12.002

Dinces, S. M., Romeo, R. D., McEwen, B. S., & Tang, A. C. (2014). Enhancing offspring hypothalamic-pituitary-adrenal (HPA) regulation via systematic novelty exposure: the influence of maternal HPA function. *Frontiers in Behavioral Neuroscience*, *8*, 204. https://doi.org/10.3389/fnbeh.2014.00204

do-Rego, J.-C., Viana, A. F., Le Maître, E., Deniel, A., Rates, S. M. K., Leroux-Nicollet, I., & Costentin, J. (2006). Comparisons between anxiety tests for selection of anxious and non anxious mice. *Behavioural Brain Research*, *169*(2), 282–288. https://doi.org/10.1016/j.bbr.2006.01.018

Driscoll, P. & Bättig, K. (1982). Behavioral, emotional and neurochemical profiles of rats selected for extreme differences in active, two-way avoidance performance. In *Genetics of the Brain* , Ed I. Lieblich. Amsterdam: Elsevier, 95-123.

Driscoll, P. & Stübi, R. (1985). Dose-related effects of pentobarbital on the genetic differences seen between paired, Roman high- or low-avoidance rats in a shuttle box. *Pharmacology Biochemistry and Behavior*, 22, 435-9. https://doi.org/10.1016/0091-3057(85)90045-0

Driscoll, P., Ferré, P., Fernández-Teruel, A., Levi de Stein, M., Wolfman, C., Medina, J., … Escorihuela, R. M. (1995). Effects of prenatal diazepam on two-way avoidance behavior, swimming navigation and brain levels of benzodiazepine-like molecules in male Roman high- and low-avoidance rats. *Psychopharmacology*, *122*(1), 51–7. https://doi.org/10.1007/BF02246441

Driscoll, P., Escorihuela, R. M., Fernández-Teruel, A., Giorgi, O., Schwegler, H., Steimer, T., … Tobeña, A. (1998). Genetic selection and differential stress responses. The Roman lines/strains of rats. *Annals of the New York Academy of Sciences*, *851*, 501–10. https://doi.org/10.1111/j.1749-6632.1998.tb09029.x

Driscoll, P., Fernández-Teruel, A., Corda, M.G., Giorgi, O., & Steimer, T. (2009) Some guidelines for defining personality differences in rats. In *Handbook of Behavior Genetics*, Ed K. Young-Kyu. New York, NY, Springer, pp 281-300.

Duzzioni, M., Calixto, A. V., Duarte, F. S., & De Lima, T. C. M. (2008). Modulation of anxiety in rats evaluated in the elevated T-maze: Evidence of the relationship between substance P and diazepam. *Behavioural Brain Research*, *187*(1), 140–145. https://doi.org/10.1016/j.bbr.2007.09.007

Ellenbroek, B. A., & Cools, A. R. (2002). Apomorphine susceptibility and animal models for psychopathology: genes and environment. *Behavior Genetics*, *32*(5), 349–61. https://doi.org/10.1023/A:1020214322065

Engin, E., & Treit, D. (2008). Dissociation of the anxiolytic-like effects of Avpr1a and Avpr1b receptor antagonists in the dorsal and ventral hippocampus. *Neuropeptides*, 42, 411-412. https://doi.org/10.1016/j.npep.2008.04.004.

Escorihuela, R. M., Fernández-Teruel, A., Núñez, F. J., Zapata, A., & Tobeña, A. (1991). Beneficial effects of infantile stimulation on coping (avoidance) behavior in rats are prevented by perinatal blockade of benzodiazepine receptors with Ro 15-1788. *Neuroscience Letters*, *126*(1), 45–8. https://doi.org/10.1016/0304-3940(91)90367-3

Escorihuela, R. M., Fernández-Teruel, A., Núñez, F. J., Zapata, A., & Tobeña, A. (1992). Infantile stimulation and the role of the benzodiazepine receptor system in adult acquisition of two-way avoidance behavior. *Psychopharmacology*, *106*(2), 282–4. https://doi.org/10.1007/BF02801985

Escorihuela, R.M., Fernández-Teruel, A., & Tobeña, A. (1994a). Environmental enrichment reverses the detrimental action of early inconsistent stimulation and increases the beneficial effects of postnatal handling on shuttle-box learning in adult rats. *Behavioural Brain Research* 61, 169-73. https://doi.org/10.1101/gr.081497.108.

Escorihuela, R.M., Tobeña, A., & Fernández-Teruel, A. (1994b). L'estimulació infantil. Efectes de l'ambient primerenc i l'herència sobre l'emotivitat i l'aprenentatge. Ed. Servei de publicacions de la Universitat Autònoma de Barcelona.

Escorihuela, R. M., Tobena, A., & Fernández-Teruel, A. (1995a). Environmental enrichment and postnatal handling prevent spatial learning deficits in aged hypoemotional (Roman high-avoidance) and hyperemotional (Roman low-avoidance) rats. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *2*(1), 40–48. https://doi.org/10.1101/lm.2.1.40

Escorihuela, R.M., Tobeña, A., Driscoll, P., & Fernández-Teruel, A. (1995b). Effects of training, early handling, and perinatal flumazenil on shuttle box acquisition in Roman low-avoidance rats: towards overcoming a genetic deficit. *Neuroscience & Biobehavioral Reviews*, 19, 353-67. https://doi.org/10.1016/0149-7634(94)00051-2.

Escorihuela, R. M., Fernández-Teruel, A., Gil, L., Aguilar, R., Tobeña, A., & Driscoll, P. (1999). Inbred Roman high- and low-avoidance rats: differences in anxiety, novelty-seeking, and shuttlebox behaviors. *Physiology & Behavior*, *67*(1), 19–26. https://doi.org/10.1016/S0031-9384(99)00064-5

Esnal, A., Sánchez-González, A., Río-Álamos, C., Oliveras, I., Cañete, T., Blázquez, G., … Fernández-Teruel, A. (2016). Prepulse inhibition and latent inhibition deficits in Roman high-avoidance vs. Roman low-avoidance rats: Modeling schizophrenia-related features. *Physiology & Behavior*, *163*, 267–273. https://doi.org/10.1016/j.physbeh.2016.05.020

Fattore, L., Piras, G., Corda, M. G., & Giorgi, O. (2009). The Roman High- and Low-Avoidance Rat Lines Differ in the Acquisition, Maintenance, Extinction, and Reinstatement of Intravenous Cocaine Self-Administration. *Neuropsychopharmacology*, *34*(5), 1091–1101. https://doi.org/10.1038/npp.2008.43

Febo, M., Numan, M., & Ferris, C. F. (2005). Functional Magnetic Resonance Imaging Shows Oxytocin Activates Brain Regions Associated with Mother-Pup Bonding during Suckling. *The Journal of Neuroscience*, *25*(50), 11637–11644. https://doi.org/10.1523/JNEUROSCI.3604-05.2005

Fenoglio, K. A., Brunson, K. L., Avishai-Eliner, S., Stone, B. A., Kapadia, B. J., & Baram, T. Z. (2005). Enduring, handling-evoked enhancement of hippocampal memory function and glucocorticoid receptor expression involves activation of the corticotropin-releasing factor type 1 receptor. *Endocrinology*, *146*(9), 4090–6. https://doi.org/10.1210/en.2004-1285

Fenoglio, K. A., Chen, Y., & Baram, T. Z. (2006). Neuroplasticity of the Hypothalamic-Pituitary-Adrenal Axis Early in Life Requires Recurrent Recruitment of Stress-Regulating Brain Regions. *The Journal of Neuroscience*, *26*(9), 2434–2442. https://doi.org/10.1523/JNEUROSCI.4080-05.2006

Fernández-Teruel, A., Escorihuela, R. M., Jiménez, P., & Tobeña, A. (1990). Infantile stimulation and perinatal administration of Ro 15-1788: additive anxiety-reducing effects in rats. *European Journal of Pharmacology*, *191*(1), 111–4. https://doi.org/10.1016/0014-2999(90)94104-6

Fernández-Teruel, A., Escorihuela, R. M., Núñez, J. F., Zapata, A., Boix, F., Salazar, W., & Tobeña, A. (1991a). The early acquisition of two-way (shuttle-box) avoidance as an anxiety-mediated behavior: psychopharmacological validation. *Brain Research Bulletin*, *26*(1), 173–6. https://doi.org/10.1016/0361-9230(91)90205-X

Fernández-Teruel, A., Escorihuela, R.M., Tobeña, A., & Driscoll, P. (1991b). Stress and putative endogenous ligands for benzodiazepine receptors: the importance of characteristics of the aversive situation and of differential emotionality in experimental animals. *Experientia*. 47, 1051-6. https://doi.org/10.1007/BF01923340.

Fernández-Teruel, A., Escorihuela, R.M., Boix, F., & Tobeña, A. (1991c). Effects of different handling-stimulation procedures and benzodiazepines on two-way active avoidance acquisition in rats. *Pharmacological Research*, 24, 273-82. https://doi.org/10.1016/1043-6618(91)90091-B

Fernández-Teruel, A., Escorihuela, R.M., Driscoll, P., Tobeña, A., & Bättig, K. (1991d). Infantile (handling) stimulation and behavior in young Roman high- and low-avoidance rats. *Physiology & Behavior*, 50, 563-5. https://doi.org/10.1007/s10519-008-9234-z.

Fernández-Teruel, A., Escorihuela, R.M., Driscoll, P., Tobeña, A., & Bättig, K. (1992a). Differential effects of early stimulation and/or perinatal flumazenil treatment in young Roman low- and high-avoidance rats. *Psychopharmacology (Berl)*, 108, 170-6. https://doi.org/10.1007/BF02245303.

Fernández-Teruel, A., Escorihuela, R.M., Nuñez, J.F., Gomà, M., Driscoll, P., & Tobeña, A. (1992b). Early stimulation effects on novelty-induced behavior in two psychogenetically-selected rat lines with divergent emotionality profiles. *Neuroscience Letters*, 137, 185-8. https://doi.org/10.1016/0304-3940(92)90400-2.

Fernández-Teruel, A., Escorihuela, R. M., Castellano, B., González, B., & Tobeña, A. (1997). Neonatal handling and environmental enrichment effects on emotionality, novelty/reward seeking, and age-related cognitive and hippocampal impairments: focus on the Roman rat lines. *Behavior Genetics*, *27*(6), 513–26. https://doi.org/10.1023/A:1021400830503

Fernández-Teruel, A., Driscoll, P., Escorihuela, R.M., Tobeña, A., & Bättig, K. (1993). Postnatal handling, perinatal flumazenil, and adult behavior of the Roman rat lines. *Pharmacology Biochemistry and Behavior*, 44, 783-9. https://doi.org/10.1016/0091-3057(93)90006-F.

Fernández-Teruel, A., Escorihuela, R. M., Gray, J.A., Aguilar, R., Gil, L., Giménez-Llort, L., … Flint, J. (2002a) A quantitative trait locus influencing anxiety in the laboratory rat. *Genome Research*, 12, 618-26. https://doi.org/10.1101/gr.203402.

Fernández-Teruel, A., Giménez-Llort, L., Escorihuela, R. M., Gil, L., Aguilar, R., Steimer, T., & Tobeña, A. (2002b). Early-life handling stimulation and environmental enrichment: are some of their effects mediated by similar neural mechanisms? *Pharmacology, Biochemistry, and Behavior*, *73*(1), 233–45. https://doi.org/10.1016/S0091-3057(02)00787-6

Fernández-Teruel, A., Driscoll, P., Gil, L., Aguilar, R., Tobeña, A., & Escorihuela, R.M. (2002c) Enduring effects of environmental enrichment on novelty seeking, saccharin and ethanol intake in two rat lines (RHA/Verh and RLA/Verh) differing in incentive-seeking behavior. *Pharmacology Biochemistry and Behavior*, 73, 225-31. https://doi.org/10.1016/S0091-3057(02)0078-0

Fernández-Teruel, A., Blázquez, G., Pérez, M., Aguilar, R., Cañete, T., Guitart, M., … Tobeña, A. (2006). Latent inhibition threshold in Roman high-avoidance rats: A psychogenetic model of abnormalities in attentional filter? *Actas Españolas de Psiquiatría*, *34*(4), 257–263. http://www.ncbi.nlm.nih.gov/pubmed/16823687

Fernández-Teruel & Estanislau, C. (2016) Meanings of self-grooming depend on an inverted U-shaped function with aversiveness. *Nature Reviews Neuroscience*, 17, 591. https://doi.org/10.1038/nrn.2016.102.

Ferré, P., Fernández-Teruel, A., Escorihuela, R. M., Driscoll, P., Corda, M. G., Giorgi, O., & Tobeña, A. (1995). Behavior of the Roman/Verh high- and low-avoidance rat lines in anxiety tests: relationship with defecation and self-grooming. *Physiology & Behavior*, *58*(6), 1209–13. https://doi.org/10.1016/0031-9384(95)02068-3

Ferré, P., Nuñez, J.F., García, E., Tobeña, A., Escorihuela, R.M., & Fernández-Teruel, A. (1995b) Postnatal handling reduces anxiety as measured by emotionality rating and hyponeophagia tests in female rats. *Pharmacology Biochemistry and Behavior*, 51, 199-203. https://doi.org/10.1016/0091-3057(94)00419-J.

Field, T. (1986). Interventions for premature infants. *Journal for Pediatrics,* vol 109 pp183-191.

Field, T., Diego, M., & Hernandez-Reif, M. (2010). Preterm infant massage therapy research: a review. *Infant Behavioral Development*, 33, 115-24. https://doi.org/10.1016/j.infbeh.2009.12.004.

Field, T. (2016). Massage therapy research review. *Complementary Therapies in Clinical Practice*, *24*, 19–31. https://doi.org/10.1016/j.ctcp.2016.04.005

Foote, S. L., Bloom, F. E., & Aston-Jones, G. (1983). Nucleus locus ceruleus: new evidence of anatomical and physiological specificity. *Physiological Reviews*, *63*(3), 844–914. http://www.ncbi.nlm.nih.gov/pubmed/6308694

Fox, N. A., Henderson, H. A., Marshall, P. J., Nichols, K. E., & Ghera, M. M. (2005). Behavioral Inhibition: Linking Biology and Behavior within a Developmental Framework. *Annual Review of Psychology*, *56*(1), 235–262. https://doi.org/10.1146/annurev.psych.55.090902.141532

Fox, C., Merali, Z., & Harrison, C. (2006). Therapeutic and protective effect of environmental enrichment against psychogenic and neurogenic stress. *Behavioural Brain Research*, *175*(1), 1–8. https://doi.org/10.1016/j.bbr.2006.08.016

Francis, D. D., Champagne, F. C., & Meaney, M. J. (2000). Variations in maternal behaviour are associated with differences in oxytocin receptor levels in the rat. *Journal of Neuroendocrinology*, *12*(12), 1145–8. https://doi.org/10.1046/j.1365-2826.2000.00599.x

Fujita, O., Annen, Y., & Kitaoka, A. (1994). Tsukuba high- and low-emotional strains of rats (Rattus norvegicus): an overview. *Behavior Genetics*, *24*(4), 389–415. https://doi.org/10.1007/BF01067540

Garcia-Falgueras, A., Castillo-Ruiz, M. M., Put, T., Tobeña, A., & Fernández-Teruel, A. (2012). Differential hippocampal neuron density between inbred Roman high- (low anxious) and low-avoidance (high anxious) rats. *Neuroscience Letters*, *522*(1), 41–6. https://doi.org/10.1016/j.neulet.2012.06.011

Garoflos, E., Panagiotaropoulos, T., Pondiki, S., Stamatakis, A., Philippidis, E., & Stylianopoulou, F. (2005). Cellular mechanisms underlying the effects of an early experience on cognitive abilities and affective states. *Annals of General Psychiatry*, *4*(1), 8. https://doi.org/10.1186/1744-859X-4-8

Garoflos, E., Stamatakis, A., Pondiki, S., Apostolou, A., Philippidis, H., & Stylianopoulou, F. (2007). Cellular mechanisms underlying the effect of a single exposure to neonatal handling on neurotrophin-3 in the brain of 1-day-old rats. *Neuroscience*, *148*(2), 349–58. https://doi.org/10.1016/j.neuroscience.2007.06.020

Garoflos, E., Stamatakis, A., Rafrogianni, A., Pondiki, S., & Stylianopoulou, F. (2008). Neonatal handling on the first postnatal day leads to increased maternal behavior and fos levels in the brain of the newborn rat. *Developmental Psychobiology*, *50*(7), 704–13. https://doi.org/10.1002/dev.20332

Geyer, M. A., & Braff, D. L. (1987). Startle habituation and sensorimotor gating in schizophrenia and related animal models. *Schizophrenia Bulletin*, *13*(4), 643–68. https://doi.org/10.1093/schbul/13.4.643

Gilabert-Juan, J., Belles, M., Saez, A. R., Carceller, H., Zamarbide-Fores, S., Moltó, M. D., & Nacher, J. (2013). A "double hit" murine model for schizophrenia shows alterations in the structure and neurochemistry of the medial prefrontal cortex and the hippocampus. *Neurobiology of Disease*, *59*, 126–140. https://doi.org/10.1016/j.nbd.2013.07.008

Giorgi, O., Lecca, D., Piras, G., Driscoll, P., & Corda, M.G. (2003a). Dissociation between mesocortical dopamine release and fear-related behaviours in two psychogenetically selected lines of rats that differ in coping strategies to aversive conditions. *European Journal of Neuroscience*, 17, 2116-26. https://doi.org/10.1046/j.1460-9568./j.1460-9568.2003.02689.

Giorgi, O., Piras, G., Lecca, D., Hansson, S., Driscoll, P., & Corda, M. G. (2003b). Differential neurochemical properties of central serotonergic transmission in Roman high- and low-avoidance rats. *Journal of Neurochemistry*, *86*(2), 422–31. https://doi.org/10.1046/j.1471-4159.2003.01845.x

Giorgi, O., Piras, G., Lecca, D., & Corda, M.G. (2005) Behavioural effects of acute and repeated cocaine treatments: a comparative study in sensitisation-prone RHA rats and their sensitisation-resistant RLA counterparts. *Psychopharmacology (Berl)*, 180, 530-8. https://doi.org/10.1007/s00213-005-2177-7

Giorgi, O., Piras, G., & Corda, M. G. (2007). The psychogenetically selected Roman high- and low-avoidance rat lines: A model to study the individual vulnerability to drug addiction. *Neuroscience & Biobehavioral Reviews*, *31*(1), 148–163. https://doi.org/10.1016/j.neubiorev.2006.07.008

Glover, V., O'Connor, T. G., & O'Donnell, K. (2010). Prenatal stress and the programming of the HPA axis. *Neuroscience & Biobehavioral Reviews*, *35*(1), 17–22. https://doi.org/10.1016/j.neubiorev.2009.11.008

Gomes, V. de C., Hassan, W., Maisonnette, S., Johnson, L. R., Ramos, A., & Landeira-Fernandez, J. (2013). Behavioral evaluation of eight rat lines selected for high and low anxiety-related responses. *Behavioural Brain Research*, *257*, 39–48. https://doi.org/10.1016/j.bbr.2013.09.028

González-Maeso, J., Weisstaub, N. V, Zhou, M., Chan, P., Ivic, L., Ang, R., … Gingrich, J. A. (2007). Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling pathways to affect behavior. *Neuron*, *53*(3), 439–52. https://doi.org/10.1016/j.neuron.2007.01.008

González-Maeso, J., Ang, R. L., Yuen, T., Chan, P., Weisstaub, N. V., López-Giménez, J. F., … Sealfon, S. C. (2008). Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature*, *452*(7183), 93–97. https://doi.org/10.1038/nature06612

González-Maeso, J., & Sealfon, S. C. (2009). Psychedelics and schizophrenia. *Trends in Neurosciences*, *32*(4), 225–232. https://doi.org/10.1016/j.tins.2008.12.005

Gosling, S.D., John, O. P. (1999). Personality dimensions in non-humans animals: a cross-species review. *Current Directions in Psychological Science*, *8*, 69–75.

Grahame, N. J., Rodd-Henricks, K., Li, T. K., & Lumeng, L. (2000). Ethanol locomotor sensitization, but not tolerance correlates with selection for alcohol preference in high- and low-alcohol preferring mice. *Psychopharmacology*, *151*(2–3), 252–60. https://doi.org/10.1007/s002130000388

Gray, J. A. (1982). The Neuropsychology of Anxiety: An INquiry into the Functions into the septo-hippocampal System. *New York, NY: Ofxord University Press*.

Gray, J.A. & McNaughton, N. (2000). The Neuropsychology of Anxiety: an Enquiry into the funtions of the septo-hippocampal System. *Oxford University Press*, *2nd Edition*.

Grecksch, G., Bernstein, H. G., Becker, A., Höllt, V., & Bogerts, B. (1999). Disruption of Latent Inhibition in Rats with Postnatal Hippocampal Lesions. *Neuropsychopharmacology*, *20*(6), 525–532. https://doi.org/10.1016/S0893-133X(98)00081-5

Grigoryan, S., & Segal, M. (2016). Lasting differential effects on plasticity induced by prenatal stress in dorsal and ventral hippocampus. *Neural Plasticity*, https://doi.org/10.1155/2016/2540462.

Grillon, C., Ameli, R., Charney, D. S., Krystal, J., & Braff, D. (1992). Startle gating deficits occur across prepulse intensities in schizophrenic patients. *Biological Psychiatry*, *32*(10), 939–43. https://doi.org/10.1016/0006-3223(92)90183-Z

Grillon, C., & Davis, M. (1997). Effects of stress and shock anticipation on prepulse inhibition of the startle reflex. *Psychophysiology*, *34*(5), 511–7. https://doi.org/10.1111/j.1469-8986.1997.tb01737.x

Gur, R. E., & Pearlson, G. D. (1993). Neuroimaging in schizophrenia research. *Schizophrenia Bulletin*, *19*(2), 337–53. https://doi.org/10.1093/schbul/19.2.337

Guitart-Masip, M., Johansson, B., Cañete, T., Fernández-Teruel, A., Tobeña, A., Terenius, L., & Giménez-Llort, L. (2008) Regional adaptations in PSD-95, NGFI-A and secretogranin gene transcripts related to vulnerability to behavioral sensitization to amphetamine in the Roman rat strains. *Neuroscience*, 151, 195-208. https://doi.org/10.1016/j.neuriscience.2007.09.072

Gürol, A., & Polat, S. (2012). The Effects of Baby Massage on Attachment between Mother and their Infants. *Asian Nursing Research*, *6*(1), 35–41. https://doi.org/10.1016/j.anr.2012.02.006

Hahn, T., Dresler, T., Plichta, M. M., Ehlis, A.-C., Ernst, L. H., Markulin, F., … Fallgatter, A. J. (2010). Functional Amygdala-Hippocampus Connectivity During Anticipation of Aversive Events is Associated with Gray's Trait "Sensitivity to Punishment." *Biological Psychiatry*, *68*(5), 459–464. https://doi.org/10.1016/j.biopsych.2010.04.033

Harenski, C. L., Kim, S. H., & Hamann, S. (2009). Neuroticism and psychopathy predict brain activation during moral and nonmoral emotion regulation. *Cognitive, Affective, & Behavioral Neuroscience*, *9*(1), 1–15. https://doi.org/10.3758/CABN.9.1.1

Harrison, P. (2004). The hippocampus in schizophrenia: a review of the neuropathological evidence and its pathophysiological implications. *Psychopharmacology*, *174*(1), 151–62. https://doi.org/10.1007/s00213-003-1761-y

Haug, J. O. (1962). Pneumoencephalographic studies in mental disease. *Acta Psychiatrica et Neurologica (KjøBenhavn)*, *38*(Suppl. No. 165), 104. http://www.ncbi.nlm.nih.gov/pubmed/13960992

Hernández-González, M., Navarro-Meza, M., Prieto-Beracoechea, C. A., & Guevara, M. A. (2005). Electrical activity of prefrontal cortex and ventral tegmental area during rat maternal behavior. *Behavioural Processes*, *70*(2), 132–143. https://doi.org/10.1016/j.beproc.2005.06.002

Hooker, C. I., Verosky, S. C., Miyakawa, A., Knight, R. T., & D'Esposito, M. (2008). The influence of personality on neural mechanisms of observational fear and reward learning. *Neuropsychologia*, *46*(11), 2709–2724. https://doi.org/10.1016/j.neuropsychologia.2008.05.005

Horga, G., Bernacer, J., Dusi, N., Entis, J., Chu, K., Hazlett, E. A., … Buchsbaum, M. S. (2011). Correlations between ventricular enlargement and gray and white matter volumes of cortex, thalamus, striatum, and internal capsule in schizophrenia. *European Archives of Psychiatry and Clinical Neuroscience*, *261*(7), 467–476. https://doi.org/10.1007/s00406-011-0202-x

Huang, E. J., & Reichardt, L. F. (2003). Trk Receptors: Roles in Neuronal Signal Transduction. *Annual Review of Biochemistry*, *72*(1), 609–642. https://doi.org/10.1146/annurev.biochem.72.121801.161629

Hutchison, K. E., & Swift, R. (1999). Effect of d-amphetamine on prepulse inhibition of the startle reflex in humans. *Psychopharmacology*, *143*(4), 394–400. https://doi.org/10.1007/s002130050964

Ickes, B. R., Pham, T. M., Sanders, L. A., Albeck, D. S., Mohammed, A. H., & Granholm, A.-C. (2000). Long-Term Environmental Enrichment Leads to Regional Increases in Neurotrophin Levels in Rat Brain. *Experimental Neurology*, *164*(1), 45–52. https://doi.org/10.1006/exnr.2000.7415

Imanaka, A., Morinobu, S., Toki, S., Yamamoto, S., Matsuki, A., Kozuru, T., & Yamawaki, S. (2008). Neonatal tactile stimulation reverses the effect of neonatal isolation on open-field and anxiety-like behavior, and pain sensitivity in male and female adult Sprague–Dawley rats. *Behavioural Brain Research*, *186*(1), 91–97. https://doi.org/10.1016/j.bbr.2007.07.039

Ishikawa, J., Nishimura, R., & Ishikawa, A. (2015). Early-life stress induces anxiety-like behaviors and activity imbalances in the medial prefrontal cortex and amygdala in adult rats. *European Journal of Neuroscience*, *41*(4), 442–453. https://doi.org/10.1111/ejn.12825

Johannesson, M., López-Aumatell, R., Stridh, P., Diez, M., Blázquez, G., Martínez-Membrives, E., … Flint, J. (2009). A resource for the simultaneous high-resolution mapping of multiple quantitative trait loci in rats: the NIH heterogeneous stock. *Genome Research,* 19, 150-8. https://doi.org/10.1101/gr.081497.108.

Johns, J. M., Joyner, P. W., McMurray, M. S., Elliott, D. L., Hofler, V. E., Middleton, C. L., … Walker, C. H. (2005). The effects of dopaminergic/serotonergic reuptake inhibition on maternal behavior, maternal aggression, and oxytocin in the rat. *Pharmacology Biochemistry and Behavior*, *81*(4), 769–785. https://doi.org/10.1016/j.pbb.2005.06.001

Johnstone, E. C., Crow, T. J., Frith, C. D., Husband, J., & Kreel, L. (1976). Cerebral ventricular size and cognitive impairment in chronic schizophrenia. *Lancet (London, England)*, *2*(7992), 924–6. https://doi.org/10.1016/S0140-6736(76)90890-4

Jones, R. B., Mills, A. D., & Faure, J. M. (1991). Genetic and experiential manipulation of fear-related behavior in Japanese quail chicks (Coturnix coturnix japonica). *Journal of Comparative Psychology (Washington, D.C.: 1983)*, *105*(1), 15–24. https://doi.org/10.1037/0735-7036.105.1.15

Jones, J. T., DiFrancesco, M., Zaal, A. I., Klein-Gitelman, M. S., Gitelman, D., Ying, J., & Brunner, H. I. (2015). Childhood-onset lupus with clinical neurocognitive dysfunction shows lower streamline density and pairwise connectivity on diffusion tensor imaging. *Lupus*, *24*(10), 1081–6. https://doi.org/10.1177/0961203315572718

Kagan, J., Reznick, J.S., Clarke, C., Snidman, N., Garcia-Coll, C. (1984). Behavioral inhibition to the unfamiliar. *Child Development, 55*, 2212–2225.

Kagan, J., Reznick, J. S., & Snidman, N. (1987). The physiology and psychology of behavioral inhibition in children. *Child Development*, *58*(6), 1459–73. https://doi.org/10.2307/1130685

Kalin, N. H., Shelton, S. E., Davidson, R. J., & Kelley, A. E. (2001). The primate amygdala mediates acute fear but not the behavioral and physiological components of anxious temperament. *The Journal of Neuroscience*, *21*(6), 2067–74. http://www.ncbi.nlm.nih.gov/pubmed/11245690

Kalin, N. H., & Shelton, S. E. (2003). Nonhuman primate models to study anxiety, emotion regulation, and psychopathology. *Annals of the New York Academy of Sciences*, *1008*, 189–200. https://doi.org/10.1196/annals.1301.021

Kalisch, R., Schubert, M., Jacob, W., Keßler, M. S., Hemauer, R., Wigger, A., … Auer, D. P. (2006). Anxiety and Hippocampus Volume in the Rat. *Neuropsychopharmacology*, *31*(5), 925–932. https://doi.org/10.1038/sj.npp.1300910

Kalueff, A.V., Stewart, A.M., Song, C., Berridge, K.C., Graybiel, A.M., & Fentress, J.C. (2016) Neurobiology of rodent self-grooming and its value for translational neuroscience. *Nature Reviews Neuroscience*, 17, 45-59. https://doi.org/10.1038/nrn.2015.8.

Klein, A. B., Ultved, L., Adamsen, D., Santini, M. A., Tobeña, A., Fernández-Teruel, A., … Mikkelsen, J. D. (2014). 5-HT(2A) and mGlu2 receptor binding levels are related to differences in impulsive behavior in the Roman Low- (RLA) and High- (RHA) avoidance rat strains. *Neuroscience*, *263*, 36–45. https://doi.org/10.1016/j.neuroscience.2013.12.063

Koch, M. (1999). The neurobiology of startle. *Progress in Neurobiology*, *59*(2), 107–28. https://doi.org/10.1016/S0301-0082(98)00098-7

Kohl, S., Heekeren, K., Klosterkötter, J., & Kuhn, J. (2013). Prepulse inhibition in psychiatric disorders--apart from schizophrenia. *Journal of Psychiatric Research*, *47*(4), 445–52. https://doi.org/10.1016/j.jpsychires.2012.11.01

Koolhaas, J. M., Bartolomucci, A., Buwalda, B., de Boer, S. F., Flügge, G., Korte, S. M., … Fuchs, E. (2011). Stress revisited: A critical evaluation of the stress concept. *Neuroscience & Biobehavioral Reviews*, *35*(5), 1291–1301. https://doi.org/10.1016/j.neubiorev.2011.02.003

Kuhn, C. M., Schanberg, S. M., Field, T., Symanski, R., Zimmerman, E., Scafidi, F., & Roberts, J. (1991). Tactile-kinesthetic stimulation effects on sympathetic and adrenocortical function in preterm infants. *The Journal of Pediatrics*, *119*(3), 434–40. https://doi.org/10.1016/S0022-3476(05)82059-1

Kumari, V., Das, M., Zachariah, E., Ettinger, U., & Sharma, T. (2005). Reduced prepulse inhibition in unaffected siblings of schizophrenia patients. *Psycophysiology*, 42, 588-94. https://doi.org/10.1111/j.0048-5772.2005.00346.x.

Labbate, G. P., da Silva, A. V., & Barbosa-Silva, R. C. (2014). Effect of severe neonatal seizures on prepulse inhibition and hippocampal volume of rats tested in early adulthood. *Neuroscience Letters*, *568*, 62–66. https://doi.org/10.1016/j.neulet.2014.03.019

Lacroix, L., Broersen, L. M., Feldon, J., & Weiner, I. (2000). Effects of local infusions of dopaminergic drugs into the medial prefrontal cortex of rats on latent inhibition, prepulse inhibition and amphetamine induced activity. *Behavioural Brain Research*, *107*(1–2), 111–21. https://doi.org/10.1016/S0166-4328(99)00118-7

Lee, M.H.S, Williams, D. I. (1974). Changes in licking behaviour of rat mother following handling of young. *Animal Behaviour*, *22*, 679–681.

Lee, T., Jarome, t., Li, S.J., Kim, J.J., & Helmstetter, F.J. (2009). Chronic stress selectively reduces hippocampal volume in rats: a longitudinal magnetic resonance imaging study. *Neuroreport*, 20, 1554-1558. https://doi.org/10.1097/WNR.0b013e328332bb09.

Levine, S. (1956). A Further Study of Infantile Handling and Adult Avoidance Learning. *Journal of Personality*, *25*(1), 70–80. https://doi.org/10.1111/j.1467-6494.1956.tb01289.x

Levine, S. (1962). Plasma-free corticosteroid response to electrick schock in rat stimulated in infancy. *Science*, *135*, 795–796.

Levine, S; Haltmeyer, G.C., Karas, G.G., Denenberg, V. H. (1967). Physiological and behavioral effects of infantile stimulation. *Physiology & Behavior*, *2*(55–63).

Levine, S. (1994). Maternal behavior as a mediator of pup adrenocortical function. *Annals of the New York Academy of Sciences*, *746*(260–275).

Levita, L., Bois, C., Healey, A., Smyllie, E., Papakonstantinou, E., Hartley, T., & Lever, C. (2014). The Behavioural Inhibition System, anxiety and hippocampal volume in a non-clinical population. *Biology of Mood & Anxiety Disorders*, *4*(1), 4. https://doi.org/10.1186/2045-5380-4-4

Lindema, S., Gernet, M., Bennay, M., Koch, M., & Löscher, W. (2008). Comparative analysis of anxiety behaviors and sensorimotor functions in two rat mutants, ci2 and ci3, with lateralized rotational behavior. *Physiology and Behavior*, 93, 417-26. https://doi.org/10.1016/j.physbeh.2007.04.008

Liebsch, G., Montkowski, A., Holsboer, F., & Landgraf, R. (1998). Behavioural profiles of two Wistar rat lines selectively bred for high or low anxiety-related behaviour. *Behavioural Brain Research*, *94*(2), 301–10. https://doi.org/10.1016/S0166-4328(97)00198-8

Lipska, B., Aultman, J. M., Verma, A., Weinberger, D. R., & Moghaddam, B. (2002). Neonatal Damage of the Ventral Hippocampus Impairs Working Memory in the Rat. *Neuropsychopharmacology*, *27*(1), 47–54. https://doi.org/10.1016/S0893-133X(02)00282-8

Lipska, B. K. (2004). Using animal models to test a neurodevelopmental hypothesis of schizophrenia. *Journal of Psychiatry & Neuroscience: JPN*, *29*(4), 282–6. http://www.ncbi.nlm.nih.gov/pubmed/15309044

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., … Meaney, M. J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science (New York, N.Y.)*, *277*(5332), 1659–62. https://doi.org/10.1016/0006-8993(94)90533-9

Lonstein, J. S. (2007). Regulation of anxiety during the postpartum period. *Frontiers in Neuroendocrinology*, *28*(2–3), 115–141. https://doi.org/10.1016/j.yfrne.2007.05.002

López-Aumatell, R., Blázquez, G., Gil, L., Aguilar, R., Cañete, T., Giménez-Llort, L., … Fernández-Teruel, A. (2009a). The Roman High- and Low-Avoidance rat strains differ in fear-potentiated startle and classical aversive conditioning. *Psicothema*, *21*(1), 27–32. https://doi.org/10.1016/j.bbr.2009.03.024

López-Aumatell, R., Vicens-Costa, E., Guitart-Masip, M., Martínez-Membrives, E., Valdar, W., Johannesson, M., … Fernández-Teruel, A. (2009b). Unlearned anxiety predicts learned fear: A comparison among heterogeneous rats and the Roman rat strains. *Behavioural Brain Research*, *202*(1), 92–101. https://doi.org/10.1016/j.bbr.2009.03.024

López-Aumatell, R., Martínez-Membrives, E., Vicens-Costa, E., Cañete, T., Blázquez, G., Mont-Cardona, C., Johannesson, M., Flint, J., Tobeña, A., & Fernández-Teruel, A. (2011). Effects of environmental and physiological covariates on sex differences in unconditioned and conditioned anxiety and fear in a large sample of genetically heterogeneous (N/Nih-HS) rats. *Behavioural and Brain Functions,* 7:48. https://doi.org/10.1186/1744-9081-7-48.

Lu, B., Pang, P. T., & Woo, N. H. (2005). The yin and yang of neurotrophin action. *Nature Reviews Neuroscience*, *6*(8), 603–614. https://doi.org/10.1038/nrn1726

Lupien, S.J., McEwen, B.S, Gunnar, M.R., & Heim, C. (2009). Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nature Neuroscience Reviews*, 10, 434-45. https://doi.org/10.1038/nrn2639.

Machado-de-Sousa, J. P., Osório, F. de L., Jackowski, A. P., Bressan, R. A., Chagas, M. H. N., Torro-Alves, N., … Hallak, J. E. C. (2014). Increased Amygdalar and Hippocampal Volumes in Young Adults with Social Anxiety. *PLoS ONE*, *9*(2), e88523. https://doi.org/10.1371/journal.pone.0088523

Macrì, S., Chiarotti, F., & Würbel, H. (2008). Maternal separation and maternal care act independently on the development of HPA responses in male rats. *Behavioural Brain Research*, *191*(2), 227–234. https://doi.org/10.1016/j.bbr.2008.03.031

Makatsori, A., Duncko, R., Schwendt, M., Moncek, F., Johansson, B. B., & Jezova, D. (2003). Voluntary wheel running modulates glutamate receptor subunit gene expression and stress hormone release in Lewis rats. *Psychoneuroendocrinology*, *28*(5), 702–14. https://doi.org/10.1016/S0306-4530(02)00062-8

Manzo, L., Gómez, M. J., Callejas-Aguilera, J. E., Donaire, R., Sabariego, M., Fernández-Teruel, A., … Torres, C. (2014). Relationship between ethanol preference and sensation/novelty seeking. *Physiology & Behavior*, *133*, 53–60. https://doi.org/10.1016/j.physbeh.2014.05.003

Martin, J. R., Oettinger, R., Driscoll, P., Buzzi, R., & Bättig, K. (1982). Effects of chlordiazepoxide and imipramine on maze patrolling within two different maze configurations by psychogenetically selected lines of rats. *Psychopharmacology*, *78*(1), 58–62. https://doi.org/10.1007/BF00470589

Martínez-Membrives, E., López-Aumatell, R., Blázquez, G., Cañete, T., Tobeña, A., & Fernández-Teruel, A. (2015). Spatial learning in the genetically heterogeneous NIH-HS rat stock and RLA-I/RHA-I rats: Revisiting the relationship with unconditioned and conditioned anxiety. *Physiology & Behavior*, *144*, 15–25. https://doi.org/10.1016/j.physbeh.2015.03.003

McAllister, A. K., Katz, L. C., & Lo, D. C. (1999). Neurotrophins and synaptic plasticity. *Annual Review of Neuroscience*, *22*(1), 295–318. https://doi.org/10.1146/annurev.neuro.22.1.295

McAuley, J. D., Stewart, A. L., Webber, E. S., Cromwell, H. C., Servatius, R. J., & Pang, K. C. H. (2009). Wistar–Kyoto rats as an animal model of anxiety vulnerability: Support for a hypervigilance hypothesis. *Behavioural Brain Research*, *204*(1), 162–168. https://doi.org/10.1016/j.bbr.2009.05.036

McEwen, B. S. (1998). Stress, adaptation, and disease. Allostasis and allostatic load. *Annals of the New York Academy of Sciences*, *840*, 33–44. https://doi.org/10.1111/j.1749-6632.1998.tb09546.x

McEwen, B. S. (1999). Stress and hippocampal plasticity. *Annual Review of Neuroscience*, *22*(1), 105–122. https://doi.org/10.1146/annurev.neuro.22.1.105

McEwen, B. S. (2003). Early life influences on life-long patterns of behavior and health. *Mental Retardation and Developmental Disabilities Research Reviews*, *9*(3), 149–154. https://doi.org/10.1002/mrdd.10074

McEwen, B.S., Bowles, N.P., Gray, J.D., Hill, M.N., Hunter, R.G., & Karatsoreos, I.N. (2015). Mechanisms of stress in the brain. *Nature Neuroscience*, 18, 1353-1363. https://doi.org/10.1038/nn.4086.

McEwen, B. S. (2016). Stress-induced remodeling of hippocampal CA3 pyramidal neurons. *Brain Research*, *1645*, 50–54. https://doi.org/10.1016/j.brainres.2015.12.043

McIntosh, J., Anisman, H., & Merali, Z. (1999). Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: gender-dependent effects. *Brain Research. Developmental Brain Research*, *113*(1–2), 97–106. https://doi.org/10.1016/S0165-3806(99)00005-X

McNaughton, B.S., & Corr, P.J. (2004). A two-dimensional neuropsychology of defense: fear/anxiety and defensive distance. *Neuroscience & Biobehavioral Reviews*, 28, 285-305. https://doi.org/10.1016/j.neubiorev.2004.03.005.

Meaney, M. J., Aitken, D. H., Bodnoff, S. R., Iny, L. J., Tatarewicz, J. E., & Sapolsky, R. M. (1985). Early postnatal handling alters glucocorticoid receptor concentrations in selected brain regions. *Behavioral Neuroscience*, *99*(4), 765–70. https://doi.org/10.1037/0735-7044.99.4.765

Meaney, M. J., Aitken, D. H., van Berkel, C., Bhatnagar, S., & Sapolsky, R. M. (1988). Effect of neonatal handling on age-related impairments associated with the hippocampus. *Science (New York, N.Y.)*, *239*(4841 Pt 1), 766–8. https://doi.org/10.1126/science.3340858

Meaney, M. J., Bhatnagar, S., Larocque, S., McCormick, C., Shanks, N., Sharma, S., … Plotsky, P. M. (1993). Individual differences in the hypothalamic-pituitary-adrenal stress response and the hypothalamic CRF system. *Annals of the New York Academy of Sciences*, *697*, 70–85. http://www.ncbi.nlm.nih.gov/pubmed/8257024

Meaney, M. J., Aitken, D. H., Bodnoff, S. R., Iny, L. J., Tatarewicz, J. E., & Sapolsky, R. M. (2013). Early postnatal handling alters glucocorticoid receptor concentrations in selected brain regions. *Behavioral Neuroscience*, *127*(5), 637–641. https://doi.org/10.1037/a0034187

Meyza, K. Z., Boguszewski, P. M., Nikolaev, E., & Zagrodzka, J. (2009). Diverse Sensitivity of RHA/Verh and RLA/Verh Rats to Emotional and Spatial Aspects of a Novel Environment as a Result of a Distinct Pattern of Neuronal Activation in the Fear/Anxiety Circuit. *Behavior Genetics*, *39*(1), 48–61. https://doi.org/10.1007/s10519-008-9234-z

Mohammed, A.H., Henricksson, B.G., Söderström, S., Ebendal, S., Olsson, T., & Seckl, J.R. (1993). Environmental influences on the central nervous system and their implications for the aging rat. *Behavioural Brain Research*, 57, 183-191. https://doi.org/10.1007/10.1016/0166-4328(93)90134-C.

Molina, V., Lubeiro, A., Soto, O., Rodriguez, M., Álvarez, A., Hernández, R., & de Luis-García, R. (2017). Alterations in prefrontal connectivity in schizophrenia assessed using diffusion magnetic resonance imaging. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *76*, 107–115. https://doi.org/10.1016/j.pnpbp.2017.03.001

Moreno, M., Cardona, D., Gómez, M.J., Sánchez-Santed, F., Tobeña, A., Fernández-Teruel, A., … & Flores, P. (2010) Impulsivity characterization in the Roman high- and low-avoidance rat strains: behavioral and neurochemical differences. *Neuropsychopharmacology*, 35, 1198-208. https://doi.org/10.1038/npp.2009.224.

Morris, R.G.M., Anderson, E., Lynch, G.S., & Baudry, M. (1986a). Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartat receptor antagonist, AP5. *Nature*, 319, 774–776.

Morris, R.G.M., Hagan, J.J., & Rawlins, J.N.P. (1986b). Allocentric spatial learning by hippocampectomised rats: a further test of the "spatial mapping" and "working memory" theories of hippocampal function. *Quarterly Journal of Experimental Psychology*, 38B, 365–395. https://doi.org/

Mortazavi, S. H., Khaki, S., Moradi, R., Heidari, K., & Vasegh Rahimparvar, S. F. (2012). Effects of massage therapy and presence of attendant on pain, anxiety and satisfaction during labor. *Archives of Gynecology and Obstetrics*, *286*(1), 19–23. https://doi.org/10.1007/s00404-012-2227-4

Nestler, E.J. & Hyman, S.E. (2010). Animal models of neuropsychiatric disorders. *Nature Neuroscience,* 13, 1161-9.

Neumann, I. D. (2003). Brain mechanisms underlying emotional alterations in the peripartum period in rats. *Depression and Anxiety*, *17*(3), 111–121. https://doi.org/10.1002/da.10070

Neumann, I. D. (2008). Brain Oxytocin: A Key Regulator of Emotional and Social Behaviours in Both Females and Males. *Journal of Neuroendocrinology*, *20*(6), 858–865. https://doi.org/10.1111/j.1365-2826.2008.01726.x

Nil, R., & Bättig, K. (1981). Spontaneous maze ambulation and Hebb-Williams learning in Roman high-avoidance and Roman low-avoidance rats. *Behavioral and Neural Biology*, *33*(4), 465–75. https://doi.org/10.1016/S0163-1047(81)91833-1

Norrholm, S. D., & Ouimet, C. C. (2001). Altered dendritic spine density in animal models of depression and in response to antidepressant treatment. *Synapse*, *42*(3), 151–163. https://doi.org/10.1002/syn.10006

Núñez, J. F., Ferré, P., García, E., Escorihuela, R. M., Fernández-Teruel, A., & Tobeña, A. (1995). Postnatal handling reduces emotionality ratings and accelerates two-way active avoidance in female rats. *Physiology & Behavior*, *57*(5), 831–5. https://doi.org/10.1016/0031-9384(94)00308-R

Nuñez, J.F., Ferré, P., Escorihuela, R.M., Tobeña, A., & Fernández-Teruel, A. (1996). Effects of postnatal handling of rats on emotional, HPA-axis, and prolactin reactivity to novelty and conflict. *Physiology and Behavior*, 60, 1355-9. https://doi.org/10.1016/S0031-9384(96)00225-9.

Oler, J. A., Fox, A. S., Shelton, S. E., Rogers, J., Dyer, T. D., Davidson, R. J., … Kalin, N. H. (2010). Amygdalar and hippocampal substrates of anxious temperament differ in their heritability. *Nature*, *466*(7308), 864–868. https://doi.org/10.1038/nature09282

Oliveras, I., Río-Álamos, C., Cañete, T., Blázquez, G., Martínez-Membrives, E., Giorgi, O., … Fernández-Teruel, A. (2015). Prepulse inhibition predicts spatial working memory performance in the inbred Roman high- and low-avoidance rats and in genetically heterogeneous NIH-HS rats: relevance for studying pre-attentive and cognitive anomalies in schizophrenia. *Frontiers in Behavioral Neuroscience*, *9*, 213. https://doi.org/10.3389/fnbeh.2015.00213

Oliveras, I., Sánchez-González, A., Piludu, M. A., Gerboles, C., Río-Álamos, C., Tobeña, A., & Fernández-Teruel, A. (2016). Divergent effects of isolation rearing on prepulse inhibition, activity, anxiety and hippocampal-dependent memory in Roman high- and low-avoidance rats: A putative model of schizophrenia-relevant features. *Behavioural Brain Research*, *314*, 6–15. https://doi.org/10.1016/j.bbr.2016.07.047

Oliveras, I., Sánchez-González, A., Sampedro-Viana, D., Piludu, M. A., Río-Alamos, C., Giorgi, O., … Fernández-Teruel, A. (2017). Differential effects of antipsychotic and propsychotic drugs on prepulse inhibition and locomotor activity in Roman high- (RHA) and low-avoidance (RLA) rats. *Psychopharmacology*, *234*(6), 957–975. https://doi.org/10.1007/s00213-017-4534-8

Ormel, J., Bastiaansen, A., Riese, H., Bos, E. H., Servaas, M., Ellenbogen, M., … Aleman, A. (2013). The biological and psychological basis of neuroticism: Current status and future directions. *Neuroscience & Biobehavioral Reviews*, *37*(1), 59–72. https://doi.org/10.1016/j.neubiorev.2012.09.004

Padoin, M. J., Cadore, L. P., Gomes, C. M., Barros, H. M., & Lucion, A. B. (2001). Long-lasting effects of neonatal stimulation on the behavior of rats. *Behavioral Neuroscience*, *115*(6), 1332–40. https://doi.org/10.1037//0735-7044.115.6.1332

Papini, M.R., Fuchs, P.N., & Torres, C. (2015) Behavioral neuroscience of psychological pain. *Neuroscience & Biobehavioral Reviews*, 48, 53-69. https://doi.org/10.1016/jneubiorev.2014.11.012.

Parwani, A., Duncan, E. J., Bartlett, E., Madonick, S. H., Efferen, T. R., Rajan, R., … Rotrosen, J. P. (2000). Impaired prepulse inhibition of acoustic startle in schizophrenia. *Biological Psychiatry*, *47*(7), 662–9. https://doi.org/10.1016/S0006-3223(99)00148-1

Patel, S., Mahon, K., Wellington, R., Zhang, J., Chaplin, W., & Szeszko, P. R. (2011). A meta-analysis of diffusion tensor imaging studies of the corpus callosum in schizophrenia. *Schizophrenia Research*, *129*(2–3), 149–155. https://doi.org/10.1016/j.schres.2011.03.014

Patil, S. T., Zhang, L., Martenyi, F., Lowe, S. L., Jackson, K. A., Andreev, B. V, … Schoepp, D. D. (2007). Activation of mGlu2/3 receptors as a new approach to treat schizophrenia: a randomized Phase 2 clinical trial. *Nature Medicine*, *13*(9), 1102–1107. https://doi.org/10.1038/nm1632

Patisaul, H. B., Scordalakes, E. M., Young, L. J., & Rissman, E. F. (2003). Oxytocin, but not oxytocin receptor, is rRegulated by oestrogen receptor beta in the female mouse hypothalamus. *Journal of Neuroendocrinology*, *15*(8), 787–93. https://doi.org/10.1046/j.1365-2826.2003.01061.x

Paxinos, G., & Watson, C. (1998). The rat brain in Stereotaxic Coordinates. Compact 6th Edition, Ed. CA. Academic Press, San Diego.

Pawluski, J. L., Charlier, T. D., Fillet, M., Houbart, V., Crispin, H. T., Steinbusch, H. W., & van den Hove, D. L. (2012). Chronic fluoxetine treatment and maternal adversity differentially alter neurobehavioral outcomes in the rat dam. *Behavioural Brain Research*, *228*(1), 159–168. https://doi.org/10.1016/j.bbr.2011.11.043

Pedersen, C. A., Ascher, J. A., Monroe, Y. L., & Prange, A. J. (1982). Oxytocin induces maternal behavior in virgin female rats. *Science (New York, N.Y.)*, *216*(4546), 648–50. https://doi.org/10.1126/science.7071605

Pedersen, C. A., & Boccia, M. L. (2003). Oxytocin antagonism alters rat dams' oral grooming and upright posturing over pups. *Physiology & Behavior*, *80*(2–3), 233–41. https://doi.org/10.1016/j.physbeh.2003.07.011

Pergola, G., Selvaggi, P., Trizio, S., Bertolino, A., & Blasi, G. (2015). The role of the thalamus in schizophrenia from a neuroimaging perspective. *Neuroscience & Biobehavioral Reviews*, *54*, 57–75. https://doi.org/10.1016/j.neubiorev.2015.01.013

Petrovszki, Z., Adam, G., Tuboly, G., Kekesi, G., Benedek, G., Keri, S., & Horvath, G. (2013). Characterization of gene-environment interactions by behavioral profiling of selectively bred rats: the effect of NMDA receptor inhibition and social isolation. *Behavioural Brain Research*, *240*, 134–45. https://doi.org/10.1016/j.bbr.2012.11.022

Pettersson-Yeo, W., Allen, P., Benetti, S., McGuire, P., & Mechelli, A. (2011). Dysconnectivity in schizophrenia: Where are we now? *Neuroscience & Biobehavioral Reviews*, *35*(5), 1110–1124. https://doi.org/10.1016/j.neubiorev.2010.11.004

Pham, T. M., Söderström, S., Henriksson, B. G., & Mohammed, A. H. (1997). Effects of neonatal stimulation on later cognitive function and hippocampal nerve growth factor. *Behavioural Brain Research*, *86*(1), 113–20. https://doi.org/10.1016/S0166-4328(96)02252-8

Pham, T.M., Söderström, S., Winblad, B., & Mohammed, A.H. (1999) Effects of environmental enrichment on cognitive function and hippocampal NGF in the non-handled rats. *Behavioural Brain Research*, 103, 63-70.

Piontkewitz, Y., Arad, M., & Weiner, I. (2011). Abnormal Trajectories of Neurodevelopment and Behavior Following In Utero Insult in the Rat. *Biological Psychiatry*, *70*(9), 842–851. https://doi.org/10.1016/j.biopsych.2011.06.007

Piontkewitz, Y., Arad, M., & Weiner, I. (2012). Tracing the development of psychosis and its prevention: What can be learned from animal models. *Neuropharmacology*, *62*(3), 1273–1289. https://doi.org/10.1016/j.neuropharm.2011.04.019

Piras, G., Giorgi, O., & Corda, M.G. (2010) Effects of antidepressants on the performance in the forced swim test of two psychogenetically selected lines of rats that differ in coping strategies to aversive conditions. *Psychopharmacology (Berl)*, 211, 403-14. https://doi.org/10.1007/s00213-010-1904-x.

Piras, G., Piludu, M.A., Giorgi, O, Corda, M.G. (2014) Effects of chronic antidepressant treatments in a putative genetic model of vulnerability (Roman low-avoidance rats) and resistance (Roman high-avoidance rats) to stress-induced depression. *Psychopharmacology (Berl)*, 231, 43-53. https://doi.org/10.1007/s00213-013-3205-7.

Powell, C. M., & Miyakawa, T. (2006). Schizophrenia-relevant behavioral testing in rodent models: a uniquely human disorder? *Biological Psychiatry*, *59*(12), 1198–207. https://doi.org/10.1016/j.biopsych.2006.05.008

Prior, M., T, D. S. W., Sanson, A., & Oberklaid, F. (2000). Does Shy-Inhibited Temperament in Childhood Lead to Anxiety Problems in Adolescence? *Journal of the American Academy of Child & Adolescent Psychiatry*, *39*(4), 461–468. https://doi.org/10.1097/00004583-200004000-00015

Pryce, C. R., Bettschen, D., & Feldon, J. (2001). Comparison of the effects of early handling and early deprivation on maternal care in the rat. *Developmental Psychobiology*, *38*(4), 239–51. https://doi.org/10.1002/dev.1018

Pryce, C. R., Bettschen, D., Nanz-Bahr, N. I., & Feldon, J. (2003). Comparison of the effects of early handling and early deprivation on conditioned stimulus, context, and spatial learning and memory in adult rats. *Behavioral Neuroscience*, *117*(5), 883–893. https://doi.org/10.1037/0735-7044.117.5.883

Qi, C., Roseboom, P. H., Nanda, S. A., Lane, J. C., Speers, J. M., & Kalin, N. H. (2010). Anxiety-related behavioral inhibition in rats: a model to examine mechanisms underlying the risk to develop stress-related psychopathology. *Genes, Brain, and Behavior*, *9*(8), 974–84. https://doi.org/10.1111/j.1601-183X.2010.00636.x

Rahman, A.S., Xu, J., & Potenza, M.N. (2014). Hippocampal and amygdalar volumetric differences in pathological gambling: a preliminary study of the associations with the behavioral inhibition system. *Neuropsychopharmacology*, 39, 738-745. https://doi.org/10.1038/npp.2013.260.

Raineki, C., Lucion, A. B., & Weinberg, J. (2014). Neonatal handling: An overview of the positive and negative effects. *Developmental Psychobiology*, *56*(8), 1613–1625. https://doi.org/10.1002/dev.21241

Rat Genome Sequencing and Mapping Consortium, A., Baud, A., Hermsen, R., Guryev, V., Stridh, P., Graham, D., … Flint, J. (2013). Combined sequence-based and genetic mapping analysis of complex traits in outbred rats. *Nature Genetics*, *45*(7), 767–75. https://doi.org/10.1038/ng.2644

Ratajczak, P., Wozniak, A., & Nowakowska, E. (2013). Animal models of schizophrenia: developmental preparation in rats. *Acta Neurobiologiae Experimentalis*, *73*(4), 472–84. http://www.ncbi.nlm.nih.gov/pubmed/24457639

Reeb-Sutherland, B. C., & Tang, A. C. (2011). Dissociation between neonatal novelty-induced preferential maternal care and enhancement in cognitive, social, and emotional functions. *Behavioural Brain Research*, *224*(2), 318–325. https://doi.org/10.1016/j.bbr.2011.06.010

Rezayat, M., Roohbakhsh, A., Zarrindast, M.R., Massoudi, R., & Djahanguiri, B. (2005). Cholecystokinin and GABA interaction in the dorsal hippocampus of rats in the elevated plus-maze test of anxiety. *Physiology & Behavior*, 84, 775-782. https://doi.org/10.1016/j.physbeh.2005.03.002.

Río-Álamos, C., Oliveras, I., Cañete, T., Blázquez, G., Martínez-Membrives, E., Tobeña, A., & Fernández-Teruel, A. (2015). Neonatal handling decreases unconditioned anxiety, conditioned fear, and improves two-way avoidance acquisition: a study with the inbred Roman high (RHA-I)- and low-avoidance (RLA-I) rats of both sexes. *Frontiers in Behavioral Neuroscience*, *9*. https://doi.org/10.3389/fnbeh.2015.00174

Río-Álamos, C., Oliveras, I., Piludu, M. A., Gerbolés, C., Cañete, T., Blázquez, G., … Fernández-Teruel, A. (2017). Neonatal handling enduringly decreases anxiety and stress responses and reduces hippocampus and amygdala volume in a genetic model of differential anxiety: Behavioral-volumetric associations in the Roman rat strains. *European Neuropsychopharmacology*, *27*(2), 146–158. https://doi.org/10.1016/j.euroneuro.2016.12.003

Río-Álamos, C., Gerbolés, C., Tapias-Espinosa, C., Sampedro-Viana, D., Oliveras, I., Sánchez-González, A., … & Fernández-Teruel, A. (2017b) Conservation of phenotypes and possible pre- and/or post-natal envioronmental influences in the Roman High- and Low-avoidance rat strains after embryo transfer. *Behavior Genetics, in Press.*

Rosas, J.M., Callejas-Aguilera, J.E., Escarbajal, M., Gómez, M.J., de la Torre, L., Agüero, A., … & Torres, C. (2007) Successive negative contrast effect in instrumental runway behaviour: a study with Roman high- (RHA) and Roman low- (RLA) avoidance rats. *Behavioural Brain Research*, 185, 1-8. https://doi.org/10.1016/j.bbr.2007.07.027.

Rowland, D., Steele, M., & Moltz, H. (1978). Serotonergic mediation of the suckling-induced release of prolactin in the lactating rat. *Neuroendocrinology*, *26*(1), 8–14. http://www.ncbi.nlm.nih.gov/pubmed/673144

Rusch, B. D., Abercrombie, H. C., Oakes, T. R., Schaefer, S. M., & Davidson, R. J. (2001). Hippocampal morphometry in depressed patients and control subjects: relations to anxiety symptoms. *Biological Psychiatry*, *50*(12), 960–4. https://doi.org/10.1016/S0006-3223(01)01248-3

Sabihi, S., Dong, S. M., Durosko, N. E., & Leuner, B. (2014). Oxytocin in the medial prefrontal cortex regulates maternal care, maternal aggression and anxiety during the postpartum period. *Frontiers in Behavioral Neuroscience*, *8*, 258. https://doi.org/10.3389/fnbeh.2014.00258

Sallés, J., López de Jesús, M., Goñi, O., Fernández-Teruel, A., Driscoll, P., Tobeña, A., & Escorihuela, R. M. (2001). Transmembrane signaling through phospholipase C in cortical and hippocampal membranes of psychogenetically selected rat lines. *Psychopharmacology*, *154*(2), 115–25. https://doi.org/10.1007/s002130000621

Salm, A.K., Pavelko, M., Krouse, E.M., Webster, W., Kraszpulski, M., & Birkle, D.L. (2004). Lateral amygdaloid nucleus expansion in adult rats is associated with exposure to prenatal stress. *Developmental Brain Research*, 148, 159-67. https://doi.org/10.1016/j.devbrainres.2003.11.005.

Sandner, G., Angst, M.-J., Guiberteau, T., Guignard, B., & Brasse, D. (2010). MRI and X-ray scanning images of the brain of 3-, 6- and 9-month-old rats with bilateral neonatal ventral hippocampus lesions. *NeuroImage*, *53*(1), 44–50. https://doi.org/10.1016/j.neuroimage.2010.06.007

Sawa, A., & Snyder, S. H. (2002). Schizophrenia: Diverse Approaches to a Complex Disease. *Science*, *296*(5568), 692–695. https://doi.org/10.1126/science.1070532

Schanberg, S. M., & Field, T. M. (1987). Sensory deprivation stress and supplemental stimulation in the rat pup and preterm human neonate. *Child Development*, *58*(6), 1431–47. https://doi.org/10.2307/1130683

Scheibel AB; Kovelman JA. (1979). Dendritic disarray in the hippocampus of paranoid schizophrenia. Abstract and poster session presented in *Annual Meeting of the Society of Biological Psychiatry*.

Scheibel AB; Kovelman JA. (1981). Disorientation of the hippocampal pyramidal cell and its processes in the schizophrenic patient. *Biological Psychiatry*, *16*, 101–102.

Schmidt, L. A., Fox, N. A., Rubin, K. H., Sternberg, E. M., Gold, P. W., Smith, C. C., & Schulkin, J. (1997). Behavioral and neuroendocrine responses in shy children. *Developmental Psychobiology*, *30*(2), 127–40. https://doi.org/10.1002/(SICI)1098-2302(199703)30:2<127::AID-DEV4>3.0.CO;2-S

Schneider, M., & Koch, M. (2005). Behavioral and morphological alterations following neonatal excitotoxic lesions of the medial prefrontal cortex in rats. *Experimental Neurology*, *195*(1), 185–198. https://doi.org/10.1016/j.expneurol.2005.04.014

Schubert, M. I., Porkess, M. V., Dashdorj, N., Fone, K. C. F., & Auer, D. P. (2009). Effects of social isolation rearing on the limbic brain: A combined behavioral and magnetic resonance imaging volumetry study in rats. *Neuroscience*, *159*(1), 21–30. https://doi.org/10.1016/j.neuroscience.2008.12.019

Schwabe, K., Freudenberg, F., & Koch, M. (2007). Selective Breeding of Reduced Sensorimotor Gating in Wistar Rats. *Behavior Genetics*, *37*(5), 706–712. https://doi.org/10.1007/s10519-007-9166-z

Schwartz, C. E., Wright, C. I., Shin, L. M., Kagan, J., & Rauch, S. L. (2003). Inhibited and Uninhibited Infants "Grown Up": Adult Amygdalar Response to Novelty. *Science*, *300*(5627), 1952–1953. https://doi.org/10.1126/science.1083703

Schwartz, C. E., Kunwar, P. S., Hirshfeld-Becker, D. R., Henin, A., Vangel, M. G., Rauch, S. L., … Rosenbaum, J. F. (2015). Behavioral inhibition in childhood predicts smaller hippocampal volume in adolescent offspring of parents with panic disorder. *Translational Psychiatry*, *5*(7), e605. https://doi.org/10.1038/tp.2015.95

Sharma, S., Powers, A., Bradley, B., & Ressler, K. J. (2016). Gene × Environment Determinants of Stress- and Anxiety-Related Disorders. *Annual Review of Psychology*, *67*(1), 239–261. https://doi.org/10.1146/annurev-psych-122414-033408

Shenton, M. E., Dickey, C. C., Frumin, M., & McCarley, R. W. (2001). A review of MRI findings in schizophrenia. *Schizophrenia Research*, *49*(1–2), 1–52. https://doi.org/10.1016/S0013-4686(03)00372-4

Simpson, E. H., Kellendonk, C., & Kandel, E. (2010). A possible role for the striatum in the pathogenesis of the cognitive symptoms of schizophrenia. *Neuron*, *65*(5), 585–96. https://doi.org/10.1016/j.neuron.2010.02.014

Sloan Wilson, D., Clark, A. B., Coleman, K., & Dearstyne, T. (1994). Shyness and boldness in humans and other animals. *Trends in Ecology & Evolution*, *9*(11), 442–446. https://doi.org/10.1016/0169-5347(94)90134-1

Smith, R.C., Calderon, M., Ravichandran, G.K., … Schoolar, J.C. (1984) Nuclear magnetic resonance in schizophrenia: a preliminary study. *Psychiatry Research,* 12, 137-147. https://doi.org/10.1016/0165-1781(84)90013-1

Snyder, S. H. (2008). Neuroscience: A complex in psychosis. *Nature*, *452*(7183), 38–39. https://doi.org/10.1038/452038a

Stamatakis, A., Stamatakis, A., Pondiki, S., Stamatakis, A., Pondiki, S., Kitraki, E., … Stylianopoulou, F. (2008). Effect of neonatal handling on adult rat spatial learning and memory following acute stress. *Stress*, *11*(2), 148–159. https://doi.org/10.1080/10253890701653039

Stamatakis, A., Toutountzi, E., Fragioudaki, K., Kouvelas, E. D., Stylianopoulou, F., & Mitsacos, A. (2009). Selective effects of neonatal handling on rat brain N-methyl-d-aspartate receptors. *Neuroscience*, *164*(4), 1457–1467. https://doi.org/10.1016/j.neuroscience.2009.09.032

Stamatakis, A., Kalpachidou, T., Raftogianni, A., Zografou, E., Tzanou, A., Pondiki, S., & Stylianopoulou, F. (2015). Rat dams exposed repeatedly to a daily brief separation from the pups exhibit increased maternal behavior, decreased anxiety and altered levels of receptors for estrogens (ERα, ERβ), oxytocin and serotonin (5-HT1A) in their brain. *Psychoneuroendocrinology*, *52*, 212–228. https://doi.org/10.1016/j.psyneuen.2014.11.016

Steimer, T., Escorihuela, R.M., Fernández-Teruel, A., & Driscoll, P. (1998). Long-term behavioural and neuroendocrine changes in Roman high- (RHA/Verh) and low-(RLA-Verh) avoidance rats following neonatal handling. *International Journal of Developmental Neuroscience*, 16, 165-74. https://doi.org/10.106/S0736-5748(98)00032-x.

Steimer, T., & Driscoll, P. (2003). Divergent stress responses and coping styles in psychogenetically selected Roman high-(RHA) and low-(RLA) avoidance rats: behavioural, neuroendocrine and developmental aspects. *Stress (Amsterdam, Netherlands)*, *6*(2), 87–100. https://doi.org/10.1080/1025389031000111320

Steimer, T., & Driscoll, P. (2005). Inter-individual vs line/strain differences in psychogenetically selected Roman High-(RHA) and Low-(RLA) Avoidance rats: neuroendocrine and behavioural aspects. *Neuroscience and Biobehavioral Reviews*, *29*(1), 99–112. https://doi.org/10.1016/j.neubiorev.2004.07.002

Suomi, S. J. (1997). Early determinants of behaviour: evidence from primate studies. *British Medical Bulletin*, *53*(1), 170–84. https://doi.org/10.1093/oxfordjournals.bmb.a011598

Swerdlow, N. R., Caine, S. B., Braff, D. L., & Geyer, M. A. (1992). The neural substrates of sensorimotor gating of the startle reflex: a review of recent findings and their implications. *Journal of Psychopharmacology*, *6*(2), 176–190. https://doi.org/10.1177/026988119200600210

Swerdlow, N. R., & Geyer, M. A. (1998). Using an animal model of deficient sensorimotor gating to study the pathophysiology and new treatments of schizophrenia. *Schizophrenia Bulletin*, *24*(2), 285–301. https://doi.org/10.1093/oxfordjournals.schbul.a033326

Swerdlow, N. R., & Light, G. A. (2015). Animal Models of Deficient Sensorimotor Gating in Schizophrenia: Are They Still Relevant? In *Current topics in Behavioral Neurosciences* (Vol. 28, pp. 305–325). https://doi.org/10.1007/7854_2015_5012

Swerdlow, N. R., Braff, D. L., & Geyer, M. A. (2016). Sensorimotor gating of the startle reflex: what we said 25 years ago, what has happened since then, and what comes next. *Journal of Psychopharmacology*, *30*(11), 1072–1081. https://doi.org/10.1177/0269881116661075

Sylvester, C. M., Barch, D. M., Harms, M. P., Belden, A. C., Oakberg, T. J., Gold, A. L., … Pine, D. S. (2016). Early Childhood Behavioral Inhibition Predicts Cortical Thickness in Adulthood. *Journal of the American Academy of Child & Adolescent Psychiatry*, *55*(2), 122–129.e1. https://doi.org/10.1016/j.jaac.2015.11.007

Tang, A. C. (2001). Neonatal Exposure to Novel Environment Enhances Hippocampal-Dependent Memory Function During Infancy and Adulthood. *Learning & Memory*, *8*(5), 257–264. https://doi.org/10.1101/lm.43101

Tang, A. C., & Zou, B. (2002). Neonatal exposure to novelty enhances long-term potentiation in CA1 of the rat hippocampus. *Hippocampus*, *12*(3), 398–404. https://doi.org/10.1002/hipo.10017

Tang, A. C., Akers, K. G., Reeb, B. C., Romeo, R. D., & McEwen, B. S. (2006). Programming social, cognitive, and neuroendocrine development by early exposure to novelty. *Proceedings of the National Academy of Sciences*, *103*(42), 15716–15721. https://doi.org/10.1073/pnas.0607374103

Tang, A. C., Reeb-Sutherland, B. C., Romeo, R. D., & McEwen, B. S. (2012). Reducing Behavioral Inhibition to Novelty via Systematic Neonatal Novelty Exposure: The Influence of Maternal Hypothalamic-Pituitary-Adrenal Regulation. *Biological Psychiatry*, *72*(2), 150–156. https://doi.org/10.1016/j.biopsych.2012.03.021

Taubert, M., Villringer, A., & Ragert, P. (2012). Learning-Related Gray and White Matter Changes in Humans. *The Neuroscientist*, *18*(4), 320–325. https://doi.org/10.1177/1073858411419048

Tobeña, A. & Fernández-Teruel, A. (2010). Modelos animales en psiquiatría. In: Tratado de psiquiatría. In: J. Vallejo Ruiloba y C. Leal Cercós (Eds.) Tratado de Psiquiatría vol. I. Psiquiatría Editores SL/Ars Médica, Barcelona. pp 414–426.

Torres, C., Cándido, A., Escarbajal, M.D., de la Torre, L., Maldonado, A., Tobeña, A., & Fernández-Teruel, A. (2005) Successive negative contrast in one-way avoidance learning in female Roman rats. *Physiology and Behavior*, 85, 377-82. https://doi.org/10.1016/jphysbeh.2005.02.023.

Torres, C., Escarbajal, M.D., Cándido, A., de la Torre, L., Gómez, M.J., & Maldonado, A. (2007). One-way avoidance learning and diazepam in female roman high-avoidance and low-avoidance rats. *Behavioural Pharmacology*, 18, 251-253. https://doi.org/10.1097/FBP.0b013e328157f43c.

Torasdotter, M., Metsis, M., Henriksson, B. G., Winblad, B., & Mohammed, A. H. (1996). Expression of neurotrophin-3 mRNA in the rat visual cortex and hippocampus is influenced by environmental conditions. *Neuroscience Letters*, *218*(2), 107–10. https://doi.org/10.1016/S0304-3940(96)13127-X

Tournier, B.B., Steimer, T., Millet, P., Moulin-Sallanon, M., Ibañez, V., & Ginovart, N. (2013). Innately low D2 receptor availability is associated with high novelty-seeking and enhanced behavioural sensitization to amphetamine. *International Journal of Neuropsychopharmacology*, 16, 1819-34

Tsuji, S., Yuhi, T., Furuhara, K., Ohta, S., Shimizu, Y., & Higashida, H. (2015). Salivary oxytocin concentrations in seven boys with autism spectrum disorder received massage from their mothers: a pilot study. *Frontiers in Psychiatry*, *6*, 58. https://doi.org/10.3389/fpsyt.2015.00058

Tzschoppe, J., Nees, F., Banaschewski, T., Barker, G. J., Büchel, C., Conrod, P. J., … IMAGEN consortium. (2014). Aversive learning in adolescents: modulation by amygdala-prefrontal and amygdala-hippocampal connectivity and neuroticism. *Neuropsychopharmacology□: Official Publication of the American College of Neuropsychopharmacology*, *39*(4), 875–84. https://doi.org/10.1038/npp.2013.287

Vaid, R.R., Yee, B.K., Shalev, U., Rawlins, J.N.P., Weiner, I., Feldon, J., & Totterdell, S. (1997). Neonatal nonhandling and in utero prenatal stress reduce the density of NADPH-diaphorase-reactive neurons in the fascia dentata and Ammon's horn of rats. *Journal of Neuroscience*, 17, 5599-609.

Van Haaren, F. (1993). Methods in behavioral pharmacology. Elsevier Science, Amsterdam.

Varty, G. B., & Higgins, G. A. (1995). Reversal of dizocilpine-induced disruption of prepulse inhibition of an acoustic startle response by the 5-HT2 receptor antagonist ketanserin. *European Journal of Pharmacology*, *287*(2), 201–5. https://doi.org/10.1016/0014-2999(95)00660-5

Vasudevan, N., Davidkova, G., Zhu, Y. S., Koibuchi, N., Chin, W. W., & Pfaff, D. (2001). Differential interaction of estrogen receptor and thyroid hormone receptor isoforms on the rat oxytocin receptor promoter leads to differences in transcriptional regulation. *Neuroendocrinology*, *74*(5), 309–24. https://doi.org/54698

Vicens-Costa, E., Martínez-Membrives, E., López-Aumatell, R., Guitart-Masip, M., Cañete, T., Blázquez, G., … Fernández-Teruel, A. (2011). Two-way avoidance acquisition is negatively related to conditioned freezing and positively associated with startle reactions: A dissection of anxiety and fear in genetically heterogeneous rats. *Physiology & Behavior*, *103*(2), 148–156. https://doi.org/10.1016/j.physbeh.2010.12.009

Walker, D. L., & Davis, M. (1997). Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle increases produced by conditioned versus unconditioned fear. *The Journal of Neuroscience*, *17*(23), 9375–83. http://www.ncbi.nlm.nih.gov/pubmed/9364083

Weinberg, J., Krahn, E. A., & Levine, S. (1978). Differential effects of handling on exploration in male and female rats. *Developmental Psychobiology*, *11*(3), 251–259. https://doi.org/10.1002/dev.420110309

Weiner, I., Schnabel, I., Lubow, R.E., & Feldon, J. (1985). The effects of early handling on latent inhibition in male and female rats. Developmental Psychobiology, 18(4), 291-7. https://doi.org/10.1002/dev.420130107.

Weiss, K. M. (1968). Some effects of the conditioned suppression paradigm on operant discrimination performance1. *Journal of the Experimental Analysis of Behavior*, *11*(6), 767–775. https://doi.org/10.1901/jeab.1968.11-767

Weiss, I. C., & Feldon, J. (2001). Environmental animal models for sensorimotor gating deficiencies in schizophrenia: a review. *Psychopharmacology*, *156*(2–3), 305–26. https://doi.org/10.1007/s002130100800

Whalen, P. J., Kapp, B. S., & Pascoe, J. P. (1994). Neuronal activity within the nucleus basalis and conditioned neocortical electroencephalographic activation. *The Journal of Neuroscience*, *14*(3 Pt 2), 1623–33. http://www.ncbi.nlm.nih.gov/pubmed/8126559

Wheeler, A. L., Chakravarty, M. M., Lerch, J. P., Pipitone, J., Daskalakis, Z. J., Rajji, T. K., … Voineskos, A. N. (2014). Disrupted Prefrontal Interhemispheric Structural Coupling in Schizophrenia Related to Working Memory Performance. *Schizophrenia Bulletin*, *40*(4), 914–924. https://doi.org/10.1093/schbul/sbt100

White, L. K., Degnan, K. A., Henderson, H. A., Pérez-Edgar, K., Walker, O. L., Shechner, T., … Fox, N. A. (2017). Developmental Relations Among Behavioral Inhibition, Anxiety, and Attention Biases to Threat and Positive Information. *Child Development*, *88*(1), 141–155. https://doi.org/10.1111/cdev.12696

Willig, F., M'Harzi, M., & Delacour, J. (1991). Contribution of the Roman strains of rats to the elaboration of animal models of memory. *Physiology & Behavior*, *50*(5), 913–9. https://doi.org/10.1016/0031-9384(91)90414-J

Willig, F., Van de Velde, D., Laurent, J., M'Harzi, M., & Delacour, J. (1992). The Roman strains of rats as a psychogenetic tool for pharmacological investigation of working memory: example with RU 41656. *Psychopharmacology*, *107*(2–3), 415–24. https://doi.org/10.1007/bf02245169

Willner, P. (1991). Behavioral models in psychpopharmacology. Cambridge University Press, Cambridge.

Wilson, M. A., Burghardt, P. R., Ford, K. A., Wilkinson, M. B., & Primeaux, S. D. (2004). Anxiolytic effects of diazepam and ethanol in two behavioral models: comparison of males and females. *Pharmacology Biochemistry and Behavior*, *78*(3), 445–458. https://doi.org/10.1016/j.pbb.2004.04.017

Wilson, D.A., Willner, J., Kurz, E.M., & Nadel, L. (1996). Early handling increases hippocampal long-term potentiation in young rats. *Behavioural Brain Research*, 21, 223-227. https://doi.org/10.1016/0166-4328(86)90240-8.

Whishaw, I. Q. (1985). Formation of a place learning-set by the rat: a new paradigm for neurobehavioral studies. *Physiology & Behavior*, 35, 139–143.

Wood, C. M., Nicolas, C. S., Choi, S.-L., Roman, E., Nylander, I., Fernández-Teruel, A., … Lodge, D. (2017). Prevalence and influence of cys407* Grm2 mutation in Hannover-derived Wistar rats: mGlu2 receptor loss links to alcohol intake, risk taking and emotional behaviour. *Neuropharmacology*, *115*, 128–138. https://doi.org/10.1016/j.neuropharm.2016.03.020

Woollett, K., & Maguire, E. A. (2011). Acquiring "the Knowledge" of London's layout drives structural brain changes. *Current Biology : CB*, *21*(24), 2109–14. https://doi.org/10.1016/j.cub.2011.11.018

Young, L. J., Muns, S., Wang, Z., & Insel, T. R. (1997). Changes in oxytocin receptor mRNA in rat brain during pregnancy and the effects of estrogen and interleukin-6. *Journal of Neuroendocrinology*, *9*(11), 859–65. https://doi.org/10.1046/j.1365-2826.1997.00654.x

Yousefi, H., Mirzamohamadi, M., & Nazari, F. (2015). The effect of massage therapy on occupational stress of Intensive Care Unit nurses. *Iranian Journal of Nursing and Midwifery Research*, *20*(4), 508. https://doi.org/10.4103/1735-9066.161001

Zaharia, M. D., Kulczycki, J., Shanks, N., Meaney, M. J., & Anisman, H. (1996). The effects of early postnatal stimulation on Morris water-maze acquisition in adult mice: genetic and maternal factors. *Psychopharmacology*, *128*(3), 227–39. https://doi.org/10.1007/s002130050130

Zeier, H., Baettig, K., & Driscoll, P. (1978). Acquisition of DRL-20 behavior in male and female, Roman high- and low-avoidance rats. *Physiology & Behavior*, *20*(6), 791–3. https://doi.org/10.1016/0031-9384(78)90307-4

Zhang, M., & Cai, J.-X. (2008). Neonatal tactile stimulation enhances spatial working memory, prefrontal long-term potentiation, and D1 receptor activation in adult rats. *Neurobiology of Learning and Memory*, *89*(4), 397–406. https://doi.org/10.1016/j.nlm.2007.10.010

Zhang, W.N., Bast, T., Xu, Y., & Feldon, J. (2014). Temporary inhibition of dorsal or ventral hippocampus by muscimol: distinct effects on measures of innate anxiety on the elevated plus maze, but similar disruption of contextual fear conditioning. *Behavioral Brain Research*, 262, 47-56. https://doi.org/10.1016/j.bbr.2013.10.044.

Annex 1

(Manuscript to be submitted)

Annex 1

(Manuscript to be submitted)

NEONATAL HANDLING INDUCES STRAIN-DEPENDENT EFFECTS ON LIMBIC BRAIN AREAS AND PREPULSE INHIBITION, AND IMPROVES WORKING MEMORY IN THE ROMAN RAT STRAINS

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ABSTRACT

The present work was devoted to evaluate whether the differences between the Roman high- (RHA) and low-avoidance (RLA) rat strains in novelty-induced behavioural inhibition/disinhibition, sensorimotor gating (prepulse inhibition, PPI) and spatial learning/memory are paralleled by differences in the volume of relevant brain areas (measured through magnetic resonance image, MRI) related to these behavioural phenotypes. To that purpose, we conducted two experiments. Experiment 1 involved testing adult rats from both strains, either untreated (controls) or treated with neonatal handling (NH; administered during the first 21 days of life), in a novel object exploration test (NOE) and for PPI, as well as measuring the volume of limbic and cortical brain regions (amygdala -Amy-, hippocampus -Hc-, striatum -Str-, medial prefrontal cortex -mPFC-, anterior cingulate cortex -ACC-, nucleus accumbens -nAcc-, lateral ventricles -LV-). Experiment 2 consisted in submitting rats to NOE and PPI tests, and to several spatial learning/memory tasks using the Morris water maze. RHA rats show higher exploration of the novel object in the NOE test and impaired PPI compared to RLA rats. RLAs display better spatial reference learning and memory. The results revealed that the RLA strain shows greater Hc, Amy and mPFC volume than its RHA counterpart, whereas the latter presents dramatically enlarged lateral ventricles. NH treatment markedly enhanced NOE test exploration in RLA rats, improved PPI in RHA rats and impaired it in the RLA strain, and produced beneficial effects on spatial working memory mainly in RHA rats. NH treatment decreased Hc and Amy volume in the RLA strain. The results are discussed in terms of the possible relationships of strain-related brain volumetric differences and the behavioral (anxiety-related and schizophrenia-relevant) traits differentiating both rat strains, and highlighting the novel findings that NH, an anxiety/stress-reducing treatment, is for the first time shown to enduringly reduce Hc and Amy volume in parallel to the decrease of anxiety and the impairment of sensorimotor gating in RLA rats.

Key words: anxiety, sensorimotor gating, spatial learning, hippocampus, amygdala, medial prefrontal cortex, lateral ventricles, neonatal handling, Roman rat strains.

1. Introduction

The inbred *Roman* High (RHA-I) – and Low-Avoidance (RLA-I; from now on RHA and RLA respectively) strain/lines of rats have been psycho-genetically selected for their very good (RHA) v/s extremely poor (RLA) acquisition of the two-way active avoidance response in the shuttle box, a task involving a "passive avoidance/active avoidance" conflict during the initial stages of acquisition which is mediated by anxiety (Driscoll et al., 1998; Fernández-Teruel et al., 1991; Gray, 1982; Gray & McNaughton, 2000; Vicens-Costa et al., 2011; Wilcock & Fulker, 1973), being the number of avoidance responses inversely related to anxiety (Escorihuela et al., 1993; LópezAumatell et al., 2009, 2011; Weiss, 1968).

Over four decades of studies carried with the *Roman* rats, have led to the conclusion that anxiety/fearfulness and stress sensitivity are among the principal behavioral traits that differentiate these two strain/lines of rats. The RLA are more anxious and/or fearful than the RHA rats in several unconditioned and conditioned tasks/tests (Díaz-Morán et al., 2012; Ferré et al., 1995; Martínez-Membrives et al., 2015; Río-Álamos et al., 2015), showing higher stress-induced HPA-axis responses than their RHA counterparts (Carrasco et al., 2008; Díaz-Morán et al., 2012; Steimer & Driscoll, 2003, 2005). Thus, the RLA rats, which typically display passive/reactive coping style in front of conflict or stressful situations, may be considered as a valid animal model to study anxiety/fear and stress sensitivity and related underlying mechanisms (e.g. Díaz-Morán et al., 2012; Driscoll et al. 1998, 2009; Fernández-Teruel et al., 1997, 2002a; Giorgi et al. 2007; Steimer and Driscoll 2003, 2005).

On the other hand, the RHA profile suggests that they might be a potential animal model for studying some schizophrenia-relevant symptoms (Del Rio et al., 2014; Oliveras et al. 2015, 2016, 2017). Briefly, compared to RLAs, the RHA rats show enhanced impulsive behavior (Moreno et al., 2010; Zeier et al., 1978), deficits in latent inhibition (Esnal et al. 2016; Fernández-Teruel et al., 2006), impaired PPI (Oliveras et al., 2015, 2016, 2017; Río-Álamos et al., 2017), worse working memory and spatial reference learning/memory (Aguilar et al., 2002; Del Rio et al., 2014; Driscoll et al., 1995; Escorihuela et al., 1995; Martínez-Membrives et al., 2015; Oliveras et al. 2015), enhanced locomotor activity as well as mesolimbic dopaminergic sensitization to repeated administration of dopaminergic psychostimulants (Corda et al., 2005; Giorgi et

al., 2007; Guitart-Masip et al., 2008), and augmented mesocortical dopaminergic response to stress (Giorgi et al., 2007) among others features related to schizophrenia.

Neonatal handling (NH), which is an environmental stimulation treatment typically administered during the first three weeks of life, has shown to elicit long-lasting anxiolytic-like and anti-stress effects. Thus, NH enduringly reduces anxiety/stress responses in a variety of test/tasks in rodents (Anisman et al., 1998; Fernández-Teruel et al., 2002; Meaney et al., 1988; Raineki et al., 2014), improves acquisition of the conflict-/anxiety-mediated two-way active avoidance task in RLA rats of both sexes as well as in other commonly used rat strains (e.g. Fernández-Teruel et al. 2002; Río-Álamos et al., 2015, 2017) and decreases HPA-axis and prolactin post-stress responses (Meaney et al. 1988; Nuñez et al., 1996; Río-Álamos et al., 2017; Steimer et al., 1998). In summary, NH appears to improve the ability to efficiently cope with challenging/stressful situations, especially in the RLA rats.

Moreover, NH has been reported to improve cognitive abilities (i.e. executive functions) in rats and mice under different spatial learning/memory paradigms (Cañete et al., 2015; Cui et al., 2006; Fernández-Teruel et al., 2002; Meaney et al., 1988; Raineki et al., 2014; Stamatakis et al., 2008; Zaharia et al., 1996), showing also protective effects on spatial learning deficits in a mouse model of Alzheimer disease (Cañete et al., 2015). From a molecular point of view, NH has also shown to induce beneficial effects, as *c-fos* expression has been shown to be increased in rat hippocampus after a single exposure of NH (following Levine´s procedure) on PND1 (Garoflos et al., 2008). In this regard, neurotrophins (NT-3 and BDNF), which have been proposed as key molecules in brain development involved in cell proliferation, survival, differentiation, axonal growth, migration, synaptogenesis and also plasticity-related processes (Huang & Reichardt, 2003; Lu et al., 2005; McAllister et al., 1999), are also increased in rat hippocampus after both NH and environmental enrichment (EE) treatment (Garoflos et al., 2007; Ickes et al., 2000; Torasdotter et al., 1996).

We present here two studies. In Experiment 1, which includes here results (with permission) from a previous published study (Río-Álamos et al., 2017), control and NH-treated RLA and RHA rats were submitted to a test of behavioural inhibition under novelty (i.e. the novel object exploration test -NOE-), to a sensorimotor gating attentional test (prepulse inhibition -PPI-) and to magnetic resonance image (MRI)

measures of the volumes of specific brain regions. As said above the results were partially published in Río-Álamos et al. (2017), but we report here new additional data related to the volumes of brain regions associated to attentional/cognitive tasks (i.e. medial prefrontal cortex, mPFc; anterior cingulated cortex, ACC; nucleus accumbens, NAc; lateral ventricles, LV). In experiment 2, which was focused on attentional/cognitive processes, control and NH-treated RLA and RHA rats of both sexes were evaluated for PPI and for spatial learning/memory in several tasks in the Morris water maze, all of them measuring processes which are typically impaired in schizophrenia.

2. Experiment 1

The behavioral results and part of the MRI results (i.e. hippocampus, amygdala and striatum volumes) included in this section (Experiment 1) have already been published in a paper that was essentially focused on both anxiety and the changes induced by NH on behavioural parameters and on anxiety-related brain regions such as the hippocampus and the amygdala (Río-Álamos et al., 2017). For reasons of integrity and coherence of the present paper, which is mainly focused on attention and cognition (as we well see in Experiment 2), we present here again the most relevant of these published results (with permission) along with novel sMRI results regarding volumes of prefrontal cortex, anterior cingulate cortex, nucleus accumbens and lateral ventricles, evaluated in the same animals used by Río-Álamos et al. (2017).

The experiment was conducted between 09:00.19:00 in accordance with the Spanish legislation on “Protection of Animals Used for Experimental and Other Scientific Purposes” and the European Council Directive (86/609/EEC) on this subject.

Table 1.- Experimental sample of Experiment 1

Experimental group	NOE-PPI test (60-110 PND)		MRI (6 months)	
	♂	♀	♂	♀
RLA-C	16	-	8	-
RLA-NH	16	-	8	-
RHA-C	16	-	8	-
RHA-NH	16	-	8	-

Table 1.- Male rat sample of: C and NH, control (non-handled) and neonatally-handled groups, respectively, for RLA and RHA rat strains. "NOE", novel object exploration test. "PPI", prepulse inhibition test. "MRI", Magnetic resonance image. "PND", post-natal day in which animals were evaluated.

2.1. Material and methods

Animals and neonatal handling (NH) treatment

NH was given twice daily (at 9:30 and 17:00 h) between postnatal days 1-21 following the same method as in previous works (Escorihuela et al., 1995; Fernández-Teruel et al., 1992; Río-Álamos et al., 2015, 2017). 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. At minutes 0, 4 and 8 of this 8-min period. Each pup was gently handled/stroked for 3-4 s at 0, 4 and 8 minutes, after which they were returned to their home-cage with their mother and litter. Control (C) non-handled groups were left undisturbed, except for regular cage cleaning once a week, until weaning.

Novel Object Exploration test (NOE)

To assess novelty-induced behavioral inhibition/disinhibition, and to be sure that NH was present in treated animals, a novel object exploration (NOE) test was conducted, consisting in the evaluation of the rats' exploratory response when a novel object was introduced in their home-cage. Rats were 60 days old at the beginning of the test. The test started by removing the food from the home-cage (leaving only four pellets in each cage). One hour later, the novel object (graphite pencil Staedtler Noris, HB nº2) was perpendicularly introduced in their home cage through the grid cover, until it made contact with the cage bedding. To facilitate observation, each cage was pulled from the rack about 20 cm. Latency to the first exploration (NOE-L; time elapsed until the first exploration of the novel object) and the total time (NOE-T) spent exploring the pencil for each individual rat were scored in a 3-min test. The experimenter/observer was standing at 50 cm from the cage front (see Study 1 and 2).

Prepulse Inhibition of the Acoustic Startle Response (PPI)

Four sound attenuated boxes (SR-Lab Startle Response System, San Diego Instruments, USA) were used. Each box consists of a Plexiglas cylinder situated on the top of a platform with a sensor that detects the strength made by the rat in each trial. Two speakers situated 15 cm from each side of the cylinder deliver the acoustic stimuli and a white noise generator provides the background noise. Each box was constantly lit by a 10 W lamp. The data were transduced by an accelerometer into a voltage which is amplified, digitized and saved into a computer for analysis.

Subjects were 110 days old at the beginning of testing. The session started with a 5 min habituation period in the startle chambers. Then, 10 "pulse-alone" trials (105 dB, 40ms) were delivered in order to obtain a stable baseline of startle. After this, each one of the six different types of trials are randomly administered 10 times (60 trials in total):

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

At the end, in order to measure the habituation to the startle stimulus, five "pulse-alone" trials were delivered (105 dB, 40 ms).

The interval between trials was 10-20 s with a mean of 15s. The startle magnitude was recorded during 200 ms after the onset of the pulse.

The %PPI for each prepulse intensity was calculated by applying the following formula: %PPI = 100 - (startle amplitude on prepulse trial / startle amplitude on pulse trial) x 100 (Oliveras et al., 2015; see also Study 2).

Magnetic Resonance Image (MRI) volumetry

In vivo 1H-Magnetic resonance studies were performed, when rats (n=32, randomly selected from the original sample of experiment 1) were 6 months old, at the joint NMR facility of the Autonomous University of Barcelona and CIBER-BBN (Cerdanyola del Vallès, Spain), using a 7-Tesla horizontal magnet (*BioSpec 70/30*, Bruker BioSpin, Ettlingen, Germany) equipped with actively shielded gradients (B-GA20S) using a dedicated rat brain quadrature receive surface coil, actively decoupled from a transmit volume coil with 72mm inner. Rats were positioned in the scanner bed, which allowed localized delivery of anesthesia (isoflurane, 1.5-2.5% in O2 at 1 L/min; respiratory frequency monitored with a pressure probe and kept between 60–80 breaths/min). A recirculation water system, integrated in the animal bed, was used to control the body temperature as measured with a rectal probe (37ºC±1ºC).

T2-weighted fast spin-echo were initially obtained in axial, sagittal and coronal planes to be used as reference scout images for accurate slice selection of the axial planes through the measured areas of the brain. Imaging parameters for these images were: effective echo time (TEeff)=36 ms, repetition time (TR)=2 s, echo train length (ETL)=8, field of view (FOV)=3,5×3,5cm^2, matrix size (MTX)=256×256, slice thickness (ST)=0.5 mm. Using these scout images high resolution T2-weigthed images were acquired in the axial plane with the following parameter: TEeff=39 ms, TR=4.5 s, ETL=8, FOV=3.2×3.2cm^2, MTX=320×320 and ST=0.5 mm.

Using ImageJ software, brain volume (BV); total hippocampus (THc); dorsal hippocampus (DHc); ventral hippocampus (VHc), amygdala (Am), dorsal striatum (St), medial prefrontal cortex (mPFc), anterior cingulated cortex (ACC), lateral ventricles

(LV) and nucleus accumbnes (NAc) were manually outlined by two raters blinded to group status (between-rater reliability $r \geq 0.89$). Briefly, this software allows the user to outline the boundaries of the region of interest (ROI) on a MRI and afterward to calculate the corresponding area using the formula that is shown below. All ROI´s were delimitated from rostral to caudal.

All brain region borders were defined according to the rat brain atlas (Paxinos and Watson, 1998; Wolf et al., 2002). For the delineation of the brain volume (BV) the most anterior brain slice included was the first slice in which prefrontal cortex appears (approximately 5.16 from bregma). The most posterior brain slice included corresponded to a level of approximately -9.60 from bregma. The entire included brain tissue was distributed over 35 consecutive slices in each individual animal.

For total hippocampus, the starting rostral slice was defined by the cornu ammonis (CA) and dentate gyrus (DG) and coincided with the dorsal hippocampal commissure to a level of approximately -1.92mm from bregma. The caudal boundary was defined by the absent of DG and the clear separation of the two cerebral hemispheres. Moreover, the aqueduct opened up and became a clearly visible, large, round circle. The last hippocampal slice included corresponded to a level of approximately -6.72mm from bregma. 10 consecutive slices from each animal were scored. The rostral amygdala slice included corresponds to a level of approximately -1.20mm from bregma, were the postero-anterior commissure linked the striatum with the amygdala, which at this point presents a kind of triangular shape. The most caudal slice included corresponds to a level of approximately -5.04 mm from bregma where the ventral subiculum can still be differentiated from the Amygdala nuclei. 9 consecutive slices from each animal were scored. The first dorsal striatum slice included corresponds to a level of approximately 2.28 from bregma, where lateral ventricles begin to appear. In this slice, dorsal striatum is laterally surrounded by the external capsule and in the rostral region is surrounded by the genu of the corpus callosum. The ventral part of the dorsal striatum is traced by a diagonal imaginary line that connects the bottom of the lateral ventricle with the final point of the external capsule. The most caudal slice included corresponds to a level of approximately -0.96 from bregma, in which dorsal striatum can be clearly separated from the globus pallidus and the internal capsule. 6 consecutive slices from each animal were scored. (Total brain volume, hippocampus, amygdala and striatum were already published in Río-Álamos et al., 2017).

The medial prefrontal (mPFc) included the prelimbic and infralimcic cortex and 4 consecutive slices were scored for each animal. The starting rostral slice was at the level of 4.20 from bregma, at the first appearance of the forceps minor of the corpus callosum, and the caudal slice was at 2.52 from bregma just before the decusation of the corpus callosum. The anterior cingulate cortex (ACC) included the anterior cingulate cortex area 1 and 2 and was scored on 7 consecutive slices for each animal. The starting rostral slice was at the level of 2.28 from brgema, when corpus callosum already decussate and the caudal slice is at -1.56 from bregma just before the appearance of the retrospenial granular cortex. Lateral ventricles (LV) were scored on 11 consecutive slices for each animal, being the most rostral at the level of 0.12 and the caudal slice at 4.80 from bregma. Lateral ventricles can be easily recognized in T2-weighted images by the presence of a hypersignal. Nucleus accumbens (NAc) was socred on just 3 slices for each animal, with the rostral slice at 2.52 from bregma and the most caudal slice at 1.56 from bregma.

Volumes of delimitated area on each slice were calculated using the following formula:

[(Field of view (FOV) / Matrix size (MTX)) x Slice thickness] x number of pixels included in delimitated area.

3. Results and discussion (Experiment 1)

Statistical analyses were performed using the "Statistical package for social science" (SPSS, version 17). As ANOVA analysis revealed that total brain volume was greater in RLA than in RHA rats, the volume of every structure was corrected and expressed as a percentage (%) of brain volume, which was used for analysis. Factorial 2 x 2 ANOVAs ("2 Strain" x "2 treatment conditions") were applied to measures from the novel object exploration, prepulse inhibition and MRI. Post-hoc Duncan´s multiple range tests were applied to all dependent variables following significant ANOVA effects. Significance level was set at $p \leq 0.05$.

In the "novel object recognition" test, RLA rats showed higher latency to explore the novel object and less time exploring it than RHAs, ["Strain" effects, both $Fs(3, 60) \geq 69.74$ and $p < 0{,}001$]. NH significantly reduced latency and increased time of exploration ["NH" effects, both $Fs(3, 60) \geq 32.13$ and $p < 0{,}001$]. There were also

"Strain x NH" interactions, as NH effects were globally stronger in RLA rats in both parameters ["Strain x NH" effects, both $Fs(3, 60) \geq 71.48$ and $p < 0{,}001$].

No significant effects were observed in "baseline startle" measured in the PPI test. Moreover, compared to RHAs, RLA rats showed higher levels of prepulse inhibition ["Strain" effect, $F(3, 60) = 6.48$, $p = 0{,}013$] (see below Table 2, and also the Study 2).

Table 2.- Mean, S.E.M. and F (ANOVA), in behavioral and volumetric relevant measures of the *Roman* high -and low-avoidance rats from Experiment 1 (*).

	Means (± SEM)				F p-value		
	RLA-C	RLA-NH	RHA-C	RHA-NH	"Strain"	"NH"	"Strain x NH"
BV (mm3)	1875,5 (25,1)	1830,2 (9,3)	1728,8 (45,2)[a]	1678,9 (25,2)	**26.08*** **	2.66	.006
Hc (%)	5,43 (0,080)	5,26 (0,053)[a]	4,64 (0,039)[a]	4,79 (0,068)	**104.9*** **	0.1	**7.4***
Am (%)	1,85 (0,033)	1,71 (0,033)[a]	1,56 (0,029)[a]	1,50 (0,033)	**57.5*** **	**8.4***	2.0
St (%)	2,51(0,051)	2,40 (0,091)	2,50 (0,027)	2,50 (0,040)	0.30	1.32	0.57
Log NOE-L	3,70 (0,25)	1.68 (0,21)[a]	1,17 (0,15)[a]	1,03 (0,13)	**69.7*** **	**32.1*** **	**24.8*** **
NOE-T	12,69 (3,61)	114,38 (6,70)[a]	121,87 (3,79)[a]	145,94 (3,45)[b]	**235.0*** **	**187.5*** **	**71.4*** **
Baseline Startle	1299,2 (290,6)	1136,4 (147,9)	837,4 (135,7)	990,9 (48,8)	2.0	0.0	0.5
% PPI	59,96 (2,93)	52,39 (4,03)	46,69 (4,83)[a]	45,70 (3,64)	**6.8***	1.1	0.7

Table 2.- C and NH, control (non-handled) and neonatally-handled groups, respectively. **"BV"**, total brain volume. **"Hc"**, hippocampus volume. **"Am"**, amygdala volume. **"St"**, striatum volume. **"Log NOE-L"**, latency to explore (time elapsed to begin exploration of) the novel object (transformed into their logarithm values) in the NOE test. **"NOE-T"**, total time exploring the novel object in NOE test. **"Baseline Startle"**, averaged acoustic startle response (for the 25 pulse-alone trials) in the PPI test. **"% PPI"**, total percentage of prepulse inhibition averaged for the four prepuse intensities (i.e. 65 dB, 70 dB, 75 dB, 80 dB). Bold numbers mean significant ANOVA effects for "Strain", "Treatment" or "Strain x Treatment (NH)". *p < 0.05, *** p < 0.001. "**a",** differences vs. RLA-C; "**b**" differences vs. RHA-C (Duncan's multiple range tests). n=64 for behavioral variables (n=16/group); n=32 for MRI volumetry results (n=8/group). (*) The results included in this table have been published in Río-Álamos et al. (2017). Table adapted from Río-Álamos et al. (2017) with permission.

Regarding MRI measures, RLA rats showed larger brain volume than the RHAs ["Strain" effect, $F(3, 28) = 26.08$, $p < 0.001$]. RLA rats also showed greater hippocampus (Table 2), amygdala (Table 2) and medial prefrontal cortex (Figure 2A) volume than RHA rats, while RHAs showed a significant (two-fold) enlargement of the

lateral ventricles (see Figure 1) compared to their RLA counterparts ["Strain" effects in all parameters, *F*s(3, 28) ≥ 5.34 and p ≤ 0,05]. NH-treated animals also showed a global decrease of amygdala volume ["NH" effect, *F*(3, 28) = 8.43, p=0,007; Table 2], which was especially pronounced in RLA rats (see Duncan's test, Table 2). Finally, "NH x Strain" effects were observed on hippocampus volume ["Strain x NH" effects, *F*(3, 28) = 7.47 and p = 0,01; Table 2], as NH significantly reduced hippocampus volume in RLA rats, with a trend in the opposite direction being observed in RHA rats (Table 2). No strain or NH effects were observed on striatum, anterior cingulate or nucleus accumbens volume (Table 2 and Figure 2).

Figure 1

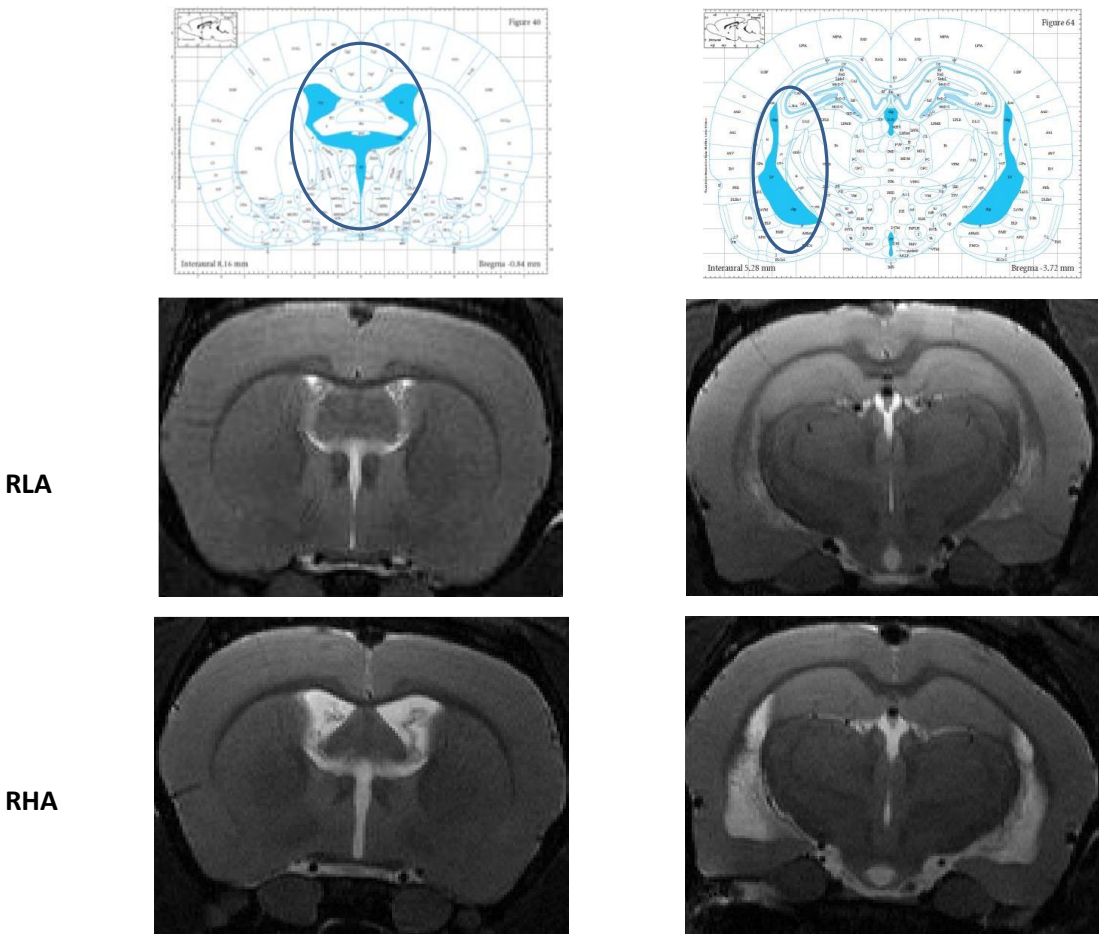

Figure 1 .- Magnetic Resonance Image s showing the differences between RLA and RHA rat strains in the volume of the lateral ventricles at two different bregma: at -0.84 (left column) and -3.72 (right column). The first row shows, within the open circle, the delimitation for the lateral ventricles (Paxinos & Watson, 1998). The second row shows the images for the RLA strain, and the third row for the RHA strain.

Figure 2

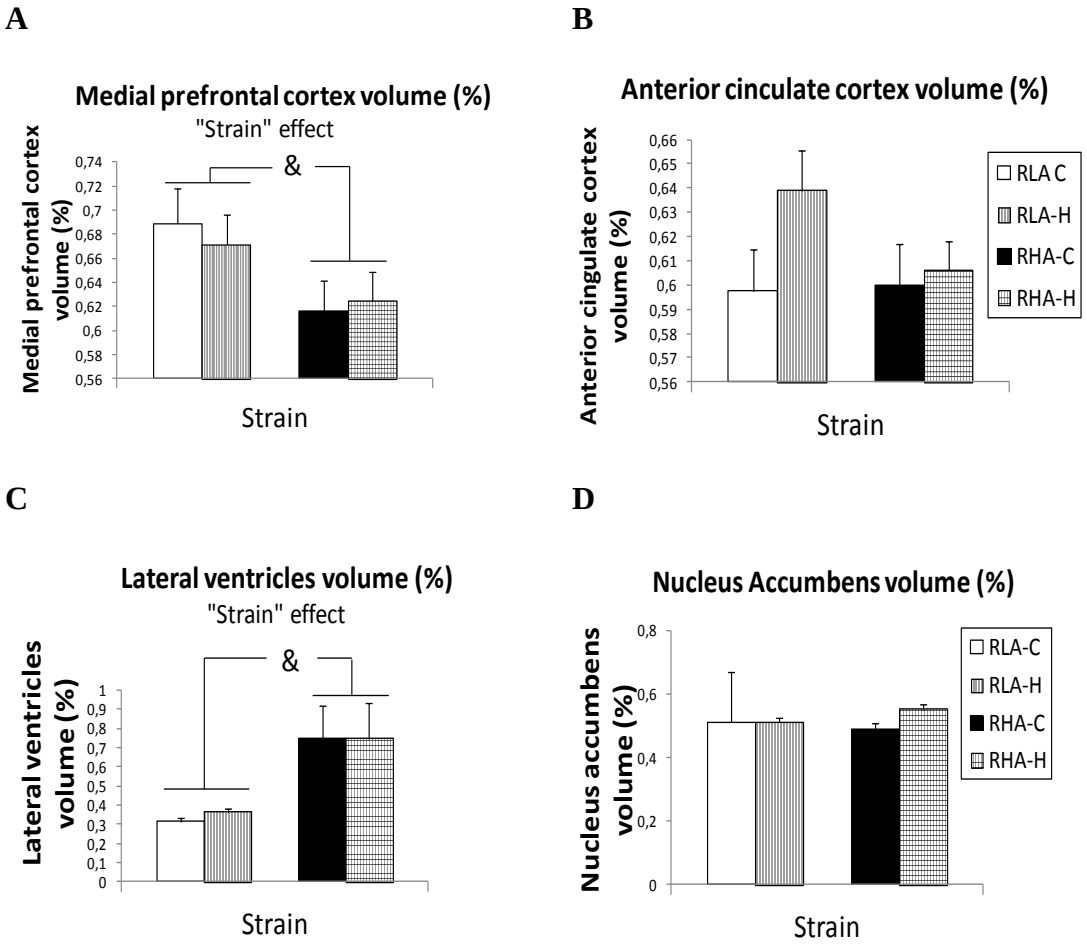

Figure 2.- Mean ±S.E.M of (A) medial prefrontal cortex volume (%); (B) anterior cingulated cortex volume (%); (C) lateral ventricles volume (%); (D) nucleus accumbens volume (%). "&" indicates "Strain" effect. Group symbols: C, control non-handled group; H, neonatally handled (NH) group. (n=32; 8/group, randomly taken from the total sample of animals from Experiment 1)

In summary, the main findings of Experiment 1 were that: i) RLA rats showed higher behavioural inhibition (anxiety) in the NOE test than RHAs, as well as higher levels of PPI; ii) NH reduced the latency to explore the novel object and increased the time spent exploring it, especially in the RLA rats; iii) PPI levels were not significantly affected by NH (although a non-significant trend for an impairment was observed in RLA rats); iv) RLA showed greater hippocampus, amygdala and medial prefrontal cortex volume than RHA rats, whereas the latter strain showed a two-fold increase in the volume of lateral ventricles, with no strain differences being observed in striatum volume, which was included as a brain control region (i.e. a brain area that is not involved in anxiety responses or PPI responses); v) NH reduced amygdala volume in both strain/lines of rats, especially in RLAs, and vi) a "strain x treatment" effect was observed on hippocampus, as NH-treatment decreased hippocampal volume in RLA rats.

In the present paper we report the volumes of new specific brain region with the aim of completing the volumetric MRI study of Río-Álamos et al. (2017) using the same rat sample used that study. The new brain areas included here, because of their proposed involvement in attentional and cognitive processes, are the medial prefrontal cortex (mPFc), anterior cingulate cortex (ACC), nucleus accumbens (NAc) and lateral ventricles (LV). As summarized above, two of the novel outstanding results are that RLA rats exhibit enlarged prefrontal cortex volume, while RHA rats show a two-fold enlargement of the volume of lateral ventricles compared with their RLA counterparts.

Our findings can be seen as supporting Gray´s theory on the neurobiology of anxiety, in which the septo-hipocampal system, in close interplay with the amygdala and prefrontal (and cingulate) cortex, has been proposed as a key neural circuitry underlying (and regulating) the behavioral inhibition system (BIS), which activity would mediate anxiety (Gray, 1982; Gray & McNaughton, 2000). Gray´s theory has been confirmed by lesion and pharmacological studies, and also by studies in humans and rhesus monkeys leading to the conclusion that anxiety traits/responses are positively associated with hippocampal and amygdala volumes (Bach et al., 2014; Barrós-Loscertales et al., 2006; Cherbuin et al., 2008; Levita et al., 2014; Machado-de-Sousa et al., 2014; Oler et al., 2010). As previous studies in untreated rats have been inconclusive (Kalisch et al., 2006), our findings might be relevant as a further support to Gray and McNaughton's proposals, and to complement these by showing positive associations/correlations

among BIS-related structures (i.e. amygdala and hippocampus) and anxiety/fear responses in our inbred *Roman* rats (see Río-Álamos et al. 2017).

In parallel, the strain differences observed on the volumes of some of the structures measured, in particular hippocampus (RHA < RLA), medial prefrontal cortex (RHA < RLA) and lateral ventricles (RHA > RLA) may be seen as giving support for the RHA rat strain as a useful valid animal model for the study of some schizophrenia-relevant phentotypes/features. Several studies have shown that decreased volumes of mPFc, Hc and enlarged lateral ventricles are endophenotypes critically involved in schizophrenia (Shenton et al., 2001). Hippocampal and prefrontal cortex lesions shown to reduce the volume of these structures, leading to PPI and impairments of executive functions (Alam et al., 2015; Bertrand et al., 2010; Gilabert-Juan et al., 2013; Labbate et al., 2014; Schneider and Koch, 2005). Isolation-reared rats, considered a neurodevelopmental/environmental schizophrenia model, also have shown to display volume reductions in prefrontal cortex and hippocampus (Day-Wilson et al., 2006; Gilabert-Juan et al., 2013; Schubert et al., 2009), in addition to enlarged lateral ventricles, a phenotype that is considered one of the most relevant structural endophenotype markers of schizophrenia (Bertrand et al., 2010; Chin et al., 2011; Huang & Reichardt, 2003; Sanfilipo et al., 2000; Schneider & Koch, 2005).

The above described schizophrenic-like profile of the RHA rats encouraged us to further characterization as described in Experiment 2.

4. Experiment 2

The present experiment is aimed at evaluating potential NH effects on attention and cognition. In Experiment 1 two findings related to PPI drawn our attention: First, PPI showed positive associations with hippocampal volume (Río-Álamos et al., 2017), in line with the prevalent hypothesis on the neurobiological mechanisms involved in PPI modulation (Koch & Schnitzler, 1997; Swerdlow et al., 2001; Swerdlow et al., 2016). Second, although not significantly, neonatally-handled RLA rats showed a striking tendency to decrease PPI levels. In addition, unpublished data of our laboratory point to a clear tendency of NH to improve PPI in RHA rats.

Thus, we conducted Experiment 2 in order to clarify potential NH effects on attention (i.e. sensorimotor gating, PPI) and cognition. To that aim we included NH-treated and non-handled RHA and RLA rats of both sexes, and we used a much rat larger sample to ensure that even subtle effects could arise.

Table 3.- Experimental sample of experiment 2

Experimental group	NOE-PPI test (Batch 1) (PND 60-110)		PT, TT, RT (Batch 2) (PND 120)		DMTP (Batch 3) (PND 140)		Cued task (Batch 4) (PND 150)	
	♂	♀	♂	♀	♂	♀	♂	♀
RLA-C	35	32	12	-	16	-	12	
RLA-NH	38	35	12	-	18	-	12	
RHA-C	28	27	12	-	14	-	12	
RHA-NH	37	26	12	-	16	-	12	

Table 3.- C and NH, control (non-handled) and neonatally-handled groups, respectively, for RLA and RHA rat strains. **Batch 1**: "NOE", novel object exploration test. "PPI", prepulse inhibition test. **Batch 2**: "PT", place task in the Morris water maze test (MWM); (TT) transfer test in the MWM; (RT) reversal task in the MWM. **Batch 3**: "DMTP", delayed matching-to- place task in the MWM. **Batch 4**: Cued task in the MWM. "PND", post-natal day in which animals were evaluated. "♂", male rats. "♀", female rats.

7.1 Materials and methods

Neonatal handling treatment, novel object exploration (NOE) test and prepulse inhibition (PPI) test were performed using the same procedures as in Experiment 1 (see above). The number of rats for each strain and sex, as well as the ages of testing are depicted in Table 3.

Morris water maze test

The testing apparatus consisted of a circular pool (diameter: 150 cm, height: 60 cm), filled to a depth of 30 cm with 24 °C water. There were no local signals available within the swimming pool. Four points equally spaced around the perimeter of the tank were arbitrarily designed to serve as starting locations (N, S, E, and W). On this basis, the tank was divided into four equal quadrants. Located somewhere within one of these quadrants was a circular platform (diameter: 15 cm, height: 27 cm) whose upper surface was 2 cm below the water level. The parameters measured in all tasks were total distance travelled; latency elapsed in reached the platform and speed. Moreover, distance travelled in both, center and periphery was also scored. Animal behavior was monitored by a video camera mounted on the ceiling above the center of the pool and using a computerized tracking system (Smart v.2.5.14; PANLAB, Barcelona, Spain). Four different starting positions were equally spaced around the perimeter of the pool. (Martínez-Membrives et al., 2015).

Place task (PT): Batch 2

The training session consisted of 3 consecutive trials each day, during five days, starting from one of the four starting positions. The order of starting points (N, S, E or W) was randomly determined and the platform stayed always in the same place. A trial began by placing the rat into the water facing the wall of the pool at one of the starting points and if the rat failed to escape within 90 s (i.e. reach the platform), it was gently guided to the platform by the experimenter. Once the rat reached the platform, it was allowed to stay there for 15 s. Approximately 20 min elapsed between consecutive trials (Oliveras et al., 2016) (see Figure 3A)

Transfer test (TT): Batch 2

Recall of the platform location of the place task was tested 24 h later (just one trial) over 60 sec in a test in which the platform had been removed. The starting position for every animal was the “south”, as in the last day of place task it was not used. Since the platform in this task had been removed, the parameters measured were (a) latency to reach the platform position, (b) distance travelled until reaching the platform position, (c) number of entries into the platform position (namely, annulus crossings), (d) time spent in the platform quadrant and (e) distance travelled in platform quadrant (Oliveras et al., 2016) (see Figure 3B).

Reversal task (RT): Batch 2

After a day of rest, animals underwent a reversal task. The platform was located (and stayed fixed during the whole reversal phase) in the quadrant opposite to that used in the place task. Training consisted in 3 consecutive trials/day, during four consecutive days. Each of the 3 daily trials started from one of the four starting positions. A trial began by placing the rat into the water facing the wall of the pool at one of the starting points and if the rat failed to escape (i.e. to reach the platform) within 90 s, it was gently guided to the platform by the experimenter. Once the rat reached the platform, it was allowed to stay there for 15 s. Approximately 20 min elapsed between consecutive trials (see Figure 3C).

Figure 3

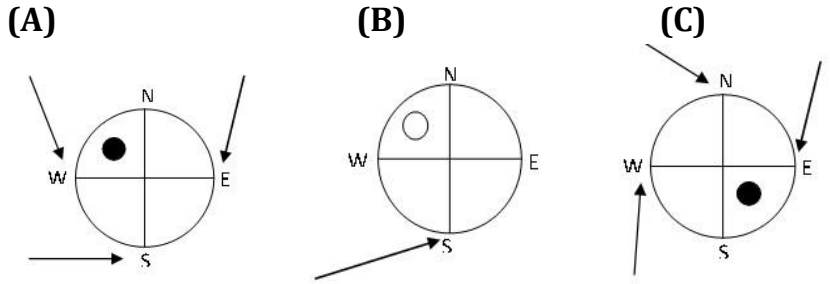

Figure 3.- The arrows indicate the starting position for **(A)** place task, **(B)** transfer test and **(C)** reversal task. Black circles indicate the platform location; open circle indicates that the platform had been removed in the transfer test. **(A)**, **(B)** and **(C)** were performed by rats from Batch 2 (n=12 per group).

Delayed matching-to-place (DMTP): Batch 3

Animals were allowed to swim for 90 s or until they located the hidden platform. Each rat went through 2 trials per day: a sample/acquisition trial (T1) and a retention trial (T2). The two trials were separated by 30 s because the rat was allowed to stay on the platform for 15 s and then spent another 15 s in an individual cage before the second trial started. The starting position and the location of the platform were pseudorandomly varied every day, but remained constant during the two trials of each day (see Figure 4). Several room cues were constantly visible from the pool. The parameters measured were, total distance travelled, time elapsed (latency) until the rat reached the platform and swimming speed. Moreover, the distance travelled in both, center and periphery was also scored. The index of spatial working memory was "Mean T1-T2," i.e. distance

savings in T2 vs. T1 (computed by the subtraction of T1- T2) averaged for the 3 training days (Oliveras et al., 2015).

Figure 4

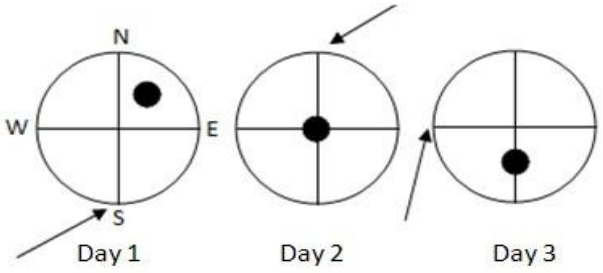

Figure 4.- The arrows indicate the starting position for the two trials of each training-day on the delayed matching-to-place task. Black circles indicate the platform location for each training-day. This task was performed by rats from Batch 3 (n= 14-18 animals per group)

Cued task: Batch 4

After four days of rest, animals were tested on a cued task. It consisted in 4 trials performed in a one-day test. The order of the starting positions was pseudo-randomly determined. For this test, the platform was about 1 cm above the water surface and it was cued with a small striped (black and white) flag. The platform stayed fixed for the whole training day. Approximately 15 min elapsed between consecutive trials. The parameter used in this task was the distance travelled to reach the platform in each trial (see Figure 5).

Figure 5

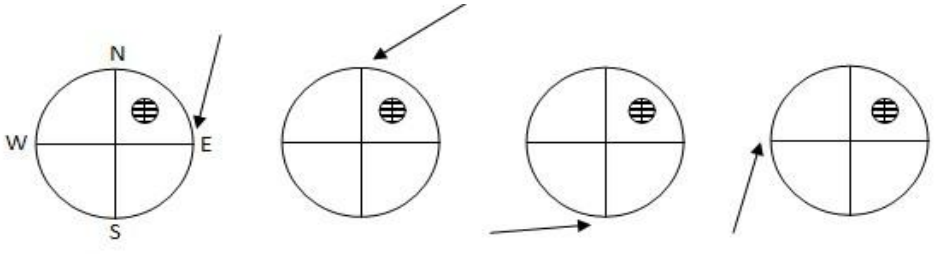

Figure 5.- The arrows indicate the four starting positions, one for each trial, in the cued task. Semi-open circles indicate that the platform was above the surface of the water and marked by a black and white flag. A pseudorandom sample of animals taken from both batches 2 and 3 were pooled to perform this task (n=12 animals per group)

8. Results and discussion (Experiment 2)

Statistical analyses were performed using the "Statistical package for social science" (SPSS, version 17). Factorial 2 x 2 x 2 ANOVAs ("2 Strain" x "2 treatment conditions" x "2 sex") were applied to measures of the novel object exploration. Appropriate repeated measures factorial ANOVAs, with "prepulse intensities" as within subject factor were applied for total percentage of PPI ("2 strain" x "2 treatment conditions" x "2 sex" x "prepulse intensity"). Whenever a "prepulse intensity x strain" interaction was found, factorial 2 x 2 x 2 ANOVAs ("2 strain" x "2 treatment condition" x "2 sex") were applied to each prepulse intensity.

Appropriate repeated measures factorial ANOVAs, were applied to measures from place task and reversal task in the Morris water maze, with "training day" as within subject factor ("2 strain" x "2 treatment conditions" x "4 or 5 training day"). Factorial 2 x 2 ANOVAs ("2 Strain" x "2 treatment condition") were applied to variables from transfer test, first day of reversal task and delayed matching-to-place task.

In the "NOE" test, RLA rats showed higher latency to explore the novel object and less time exploring it compared to RHAs ["Strain" effects, both $Fs(7, 257) \geq 39.38$, and $p \leq 0{,}001$]. NH significantly reduced latency and increased time of exploration ["NH" effects, both $Fs\ (7, 257) \geq 52.63$, $p \leq 0{,}001$]. As males longer the novel object than female rats a sex effect was also observed ["Sex" effect, $F(7,257) = 16.06$, $p \leq 0{,}000$]. There were also "Strain x NH" interactions, as NH effects were stronger in RLA rats ["Strain x NH" effects, both $Fs(7,257) \geq 13.74$, $p \leq 0{,}001$] (see Fig 6A-B).

Results of acoustic startle response (the average startle response of the 25 pulse-alone trials), measured in the PPI test, revealed an overall higher acoustic startle response in the RLA rat strain compared than in their RHA counterparts ["Strain" effect, $F(7,257) = 10.27$, $p = 0{,}002$; mean $\pm$ sem = 1135,24 (72.92) for RLA rats, and 877,06 (80.46) for RHA rats]. No NH or interaction effects were observed for baseline startle. In addition, male rats showed an overall higher acoustic startle response compared with female rats ["Sex" effect, $F(7,257) = 21.35$, $p \leq 0{,}001$].

The RLA rat strain displayed overall higher total %PPI than RHA rats ["Strain" effect,

F(7,257) = 120.93, p ≤ 0,001] with significant between-strain differences being observed in all prepulse intensities [all Fs(7, 257) ≥ 36.72, p ≤ 0,001]. Male rats also showed higher levels of %PPI compared to female rats ["Sex" effect, F(7,257) = 12.15, p ≤ 0.001], with differences in all prepulses except at 65dB [All Fs(7, 257) ≥6.04, p ≤ 0,05]. A "Strain x NH" interaction was observed on %PPI, as NH treatment elicited a decrease of PPI levels in the RLA rat strain in all prepulse intensities ["Strain x NH" effects, F(7,257) = 20.66, p ≤ 0.001], while in RHA rats the opposite effect was observed at 70 and 75dB prepulses (see Duncan's tests in Figure 6C).

Regarding the different spatial tasks in the Morris water maze, RLA rats travelled overall less distance than RHAs in both, the place task and the reversal task ["Strain" effect, both Fs(3,44) ≥11.39, p ≤ 0,005]. In the transfer test RLA rats performed more annulus crossings than their RHA counterparts ["Strain" effect, F(3,44) =26.21, p ≤ 0,001]. A "NH" effect was observed in the first training day of the reversal task, as NH-treated rats travelled less distance (i.e. showed higher distance savings between the second and the third trial) than control animals ["Strain" effect, F(3,44) =5.04, p ≤ 0,030], thus suggesting an improvement of cognitive flexibility induced by NH. In the delayed matching-to-place spatial task, NH-treated rats overall showed better working memory than controls ["NH" effect, F(1,63) = 4.37, p ≤ 0.05]. No "strain" or "strain x treatment" interaction effects were found. No main effects or interactions were observed in the cued task (see Fig. 7A-F and Fig. 8A-B).

Figure 6

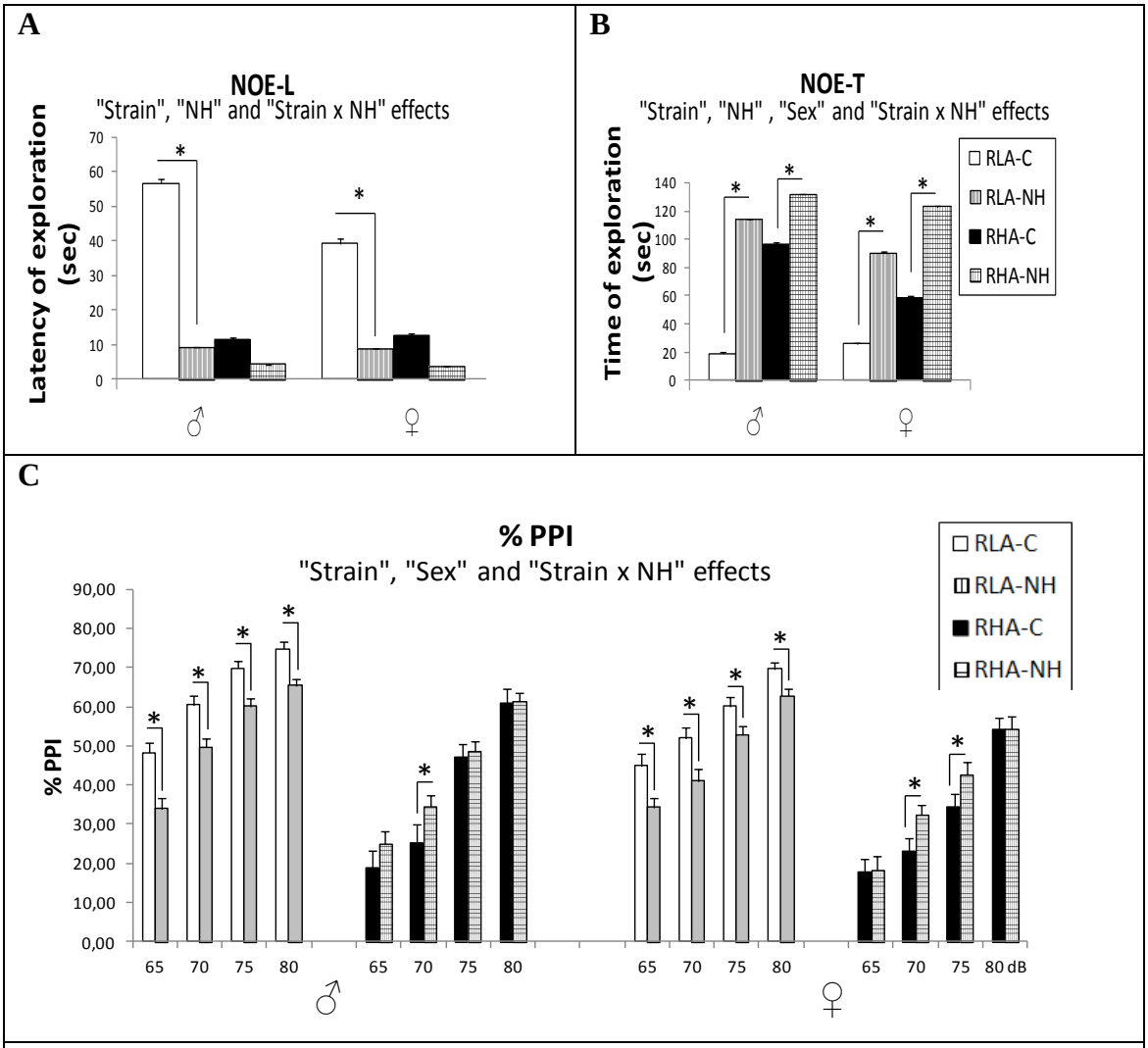

Figure 6A-C. Latency of time elapsed to begin the exploration of the novel object **(A)** and total time of exploration of the novel object in "NOE" test **(B)** Total percentage of prepulse inhibition in "PPI" test **(C)**

Figure 7

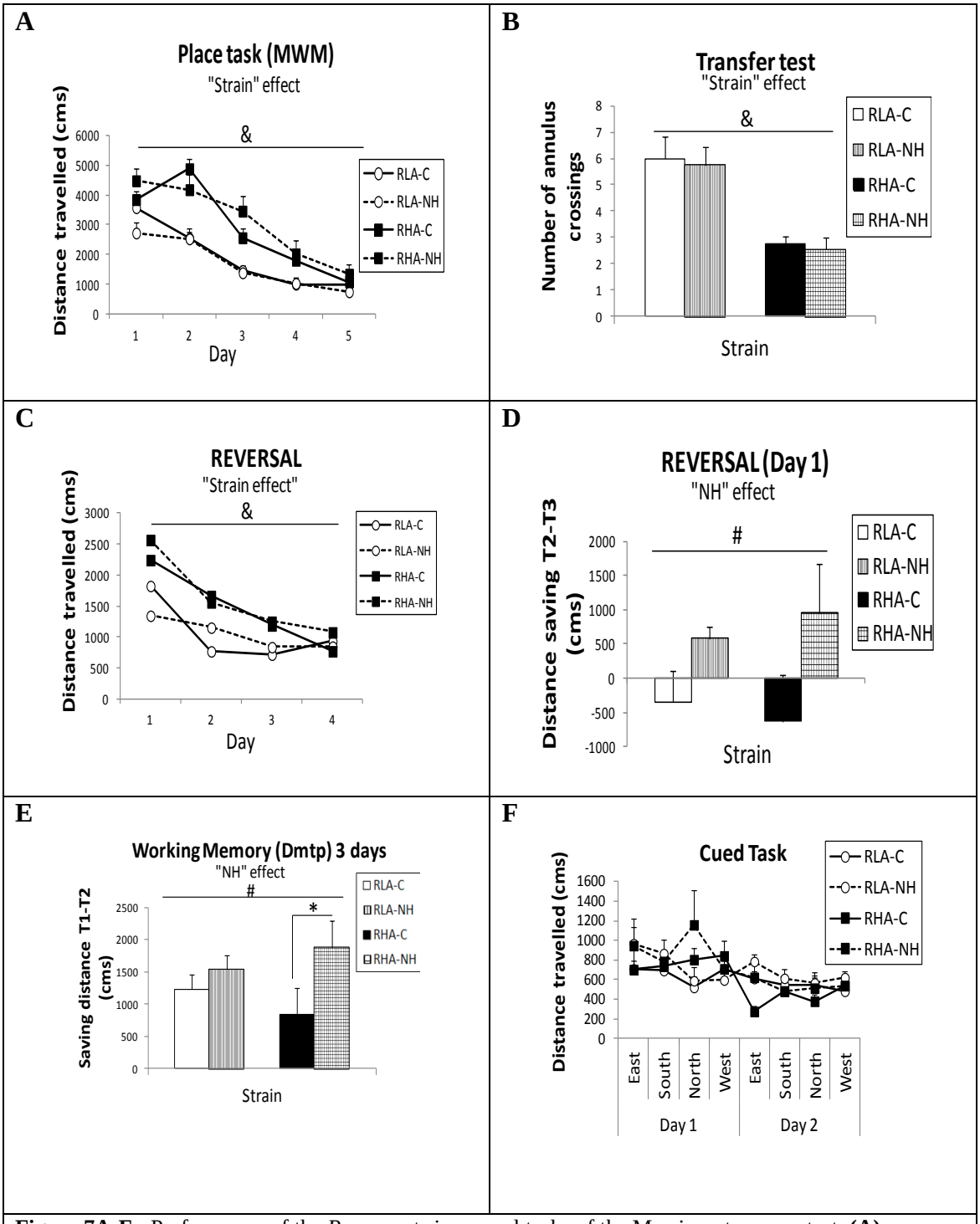

Figure 7A-F.- Performance of the *Roman* rats in several tasks of the Morris water maze test. **(A)** Distance travelled in the place task **(B)** Number of annulus crossings in the transfer test of the place task **(C)** Distance travelled in the reversal task, **(D)** distance savings between the second and the third trial of the first training-day in the reversal task (cognitive flexibility) **(E)** Working memory (i.e. saving distance between the first and the second trial) in the delayed matching-to-place task **(F)** Distance travelled in the cued task. **(A-D:** Batch 2); (**E:** Batch 3) and (**F**: Batch 4).

Figure 8

Figure 8.- Typical trajectories, performed by **(A)** RLA and **(B)** RHA, in the same trial at the beginning of the spatial place task in the Morris water maze (Batch 2). The distance travelled to find the platform by RLA rats is much shorter than that of their RHA counterparts.

A summary of the main findings of Experiment 2 would as follows:

i) We confirmed the behavioural disinhibition (i.e. anti-anxiety) effect of NH on unconditioned anxiety as measured in the NOE test, especially in the RLA rats of both sexes (Batch 1).

ii) Both sexes of RLA rats showed higher PPI levels than their RHA counterparts.

iii) Remarkably, a "strain x NH" effect was observed on PPI levels, as NH consistently reduced PPI levels in all the prepulses in RLA rats (both sexes), while the treatment improved PPI levels in at 70-75 dB in RHA rats of both sexes. Hence, the trend for detrimental NH effects on PPI levels from RLA rats (Experiment 1) was confirmed, as well as the expected improvement of PPI levels in RHA rats (Batch 1).

iv) RLA rats displayed better spatial reference memory than their RHA counterparts, i.e. better performance in the place task and the transfer test measured in animals from Batch 2.

v) An overall NH positive effect was observed on cognitive flexibility in the first training day of the reversal place task, particularly when taking into account distance savings between trial 2 and 3 of that training session (Batch 2).

vi) NH improved working memory (delayed matching-to-place task) overall in both rat strains, with the effect being significant in the RHA rat strain (Batch 3).

vii) No "strain", NH or "strain x NH" effects were observed in the cued task (Batch 4).

One possible explanation for the NH-induced PPI impairment in RLA rats is that the reducing anxiety/fear effects induced by NH also lead to a decrease of environment

monitoring or state of "alertness" in the RLA treated rats, which could be related to the decreased hippocampal volume after NH in that rat strain (see for example Gray and McNaughton 2000). In this connection, it has been reported that fear or anxiety might facilitate the processing of sensory information due to an increase in the attention to the environment, which would be mediated by cortical arousal, a process in which hippocampus is also involved (Foote et al., 1987; Whalen et al., 1994). The above contention is supported by several studies. In a human threat-of-shock experiment (Grillon & Davis, 1997) it was demonstrated that PPI was increased by shock anticipation (i.e. the threat of shock), suggesting that an increase in the general level of alertness might facilitate the processing of the prepulse. In another study, a two-week training within an enriched environment (EE) or EE + stressor (chronic restraint) induced a transient increase of PPI, compared to mice reared in standard environment (SE) or SE + the same stressor. But following a four-week training, only the SE + stressor showed an increase of PPI (Chen et al., 2010). The authors suggested that repeated introduction of novel objects in EE is comparable to mild stress exposure (Larsson et al., 2002), as EE is known to activate the HPA axis (Fox et al., 2006; Makatsori et al., 2003). Thus, the alertness induced by the novel objects in EE groups seems to decrease after two weeks due to habituation mechanisms, in parallel to a decrease of PPI levels, but four-week stress in the SE group led to increased PPI (Chen et al., 2010). In addition, prenatal stress (restraint) of pregnant mothers increased PPI of the offsprings (Lehmann et al., 2000). Finally, the hypervigilant inbred Wistar-Kyoto rats (proposed as a model of vulnerability to anxiety and behavioral inhibition) showed heightened signs of anxiety and PPI levels compared to Sprague-Dawley rats (McAuley et al., 2009). Thus it seems safe to conclude that higher levels of anxiety/fear and stress might in some conditions facilitate improvements of PPI, due to higher alertness.

Another support to the above hypothesis (i.e. the "hypervigilance" hypothesis) came from Gray´s model of anxiety (Gray, 1982; Gray & McNaughton, 2000), stating that the activation of the behavioral inhibition system (BIS), a process in which amygdala and hippocampus activity are key elements, results in behavioral inhibition, increased arousal and enhanced attention to the environment. As NH effects go in the opposite direction (i.e. supposedly decreasing activity of the BIS), lower PPI levels might be a consequence of the anti-anxiety/fear effect of the treatment, leading to decreased levels of alertness in RLA rats. This contention is also supported by results from the present

Experiment 1 (see Río-Álamos et al. 2017)), in which reductions of amygdala and hippocampus volume observed in NH-treated RLA rats fit with the above ideas.

On the other hand, it is worth noting that for the first time we show that NH induced the an improvement of PPI in the (PPI-impaired) RHA rat strain. This is a remarkable finding, given that RHA rats are markedly impaired in this test of sensorimotor gating (Del Río et al. 2014; Oliveras et al. 2015, 2016), as well as in other attentional (schizophrenia-relevant) processes such as latent inhibition in different paradigms (Fernandez-Teruel et al., 2006; Esnal et al., 2016). Whether the present NH positive effect might be related to other cognitive improvements elicited by NH in this strain (as seen in Experiment 2), or to NH-induced changes in the function of relevant specific brain regions would deserve further studies.

5. General discussion

PPI impairments, i.e. sensorimotor gating deficits, are considered an endophenotype of schizophrenia, among other psychiatric disorders (Kohl et al., 2013; Swerdlow et al., 2016; Swerdlow & Light, 2015; Weiss & Feldon, 2001). RHA rats have shown to display consistent and reliable sensorimotor gating deficits (Del Rio et al., 2014; Oliveras et al., 2015, 2017; Esnal et al., 2016), as well as impairments in latent inhibition (another attentional phenotype that has been related to schizophrenia symptoms; Esnal et al., 2016; Fernández-Teruel et al. 2006). To further characterize the schizophrenia-related profile of RHA rats, in the present study we evaluated MRI volumes of specific brain regions that have been proposed to be involved in this disorder (i.e. medial prefrontal cortex and lateral ventricles, besides the anterior cingulate cortex and nucleus accumbens; in addition to the inclusion of hippocampus, amygdala and striatum volume, which were already delimitated in Río-Álamos et al., 2017). As expected, we have found that, compared to their RLA counterparts, RHA rats showed decreased medial prefrontal cortex (mPFc) volume and markedly enlarged lateral ventricles, which add to the finding of a reduced hippocampal volume in this strain (Río-Álamos et al., 2017; Experiment 1). This profile of volumetric findings in RHA rats fits well with volumetric studies in schizophrenic patients and with rodent models of the disorder (Chin et al., 2011; Gilabert-Juan et al., 2013; Horga et al., 2011; Piontkewitz et al., 2011; Schneider & Koch, 2005; Shenton et al., 2001).

There is mounting evidence showing prefrontal cortex dysfunctions and morphological abnormalities related to schizophrenia (Benes et al., 2000; Callicott et al., 2003; Ishikawa et al., 2015). Neuronal activity of prefrontal cortex has been shown to be reduced in rats selectively bred for deficient sensorimotor gating (Alam et al., 2015). Lesions within the prefrontal cortex, which lead to reduced volume in this region, have also been shown to induce deficits in executive functions (Déziel et al., 2015). Moreover, prefrontal lesions showed to induce disruptions in the mesoacumbal dopaminergic system, leading to alterations of PPI and enhanced sensitivity to PPI-disruptive effects of apomorphine, in a manner similar to deficits observed in schizophrenia (Schneider & Koch, 2005) and compatible with the deficits observed in our RHA rats (Del Rio et al., 2014; Oliveras et al., 2015, 2017 and references therein). In addition, mPFc volume is reduced in rats reared in isolation, which also show deficits in PPI and other cognitive processes (Day-Wilson et al., 2006; Oliveras et al., 2016; Schubert et al., 2009).

In our study, we found no strain or NH effects in the volume of the anterior cingulate cortex (ACC). Differences in mPFc but not in ACC are in line with a study performed on a "double hit" murine (i.e. a MK-801 injection at PND 7 combined with post-weaning isolation) model of schizophrenia-related symptoms (Gilabert-Juan et al., 2013). In this study differences in mPFc (i.e. infralimbic and prelimbic), but not in cingulate cortices were observed in the "double hit" murine model.

Reductions in mPFc have been associated to a loss of neuropil, consistent with observations of reduced density of both dendritic spines/synapses and hippocampal pyramidal neurons (Silva-Gómez et al., 2003; Varty et al., 1999), resembling the reduction of dorsolateral prefrontal cortex volume observed in schizophrenic patients, which is attributed to reduced spine density (Garey et al., 1998; Glantz & Lewis, 2000). The volumetric reductions in mPFc and hippocampus observed in our RHA rats may be seen as important for the rat model, as they are very similar to those consistently found in schizophrenia (Levitt et al., 2010; Shenton et al., 2001; Yoshida et al., 2011). No effect of NH on medial prefrontal cortex was found in our work, although in the spatial tasks performed with the Morris water maze (in which hippocampus and also mPFc are known to be involved; e.g. Morris et al., 1986a-b; Wishaw 1985), treated animals showed an improvement of working memory.

That RHA showed a decreased mPFc volume compared to RLA rats, support previous findings in which the former presents worse spatial learning/memory navigation (Martínez-Membrives et al., 2015). In a previous report we demonstrate that prepulse inhibition predicts working memory performance in these strains of rats and in genetically heterogeneous rats, showing positive correlations between both phenotypes (Oliveras et al., 2015). Here, in Batch 3 we only included a delay matching-to-place task for the evaluation of working memory. No strain effect was observed, mainly because NH improved working memory in both strain/lines of rats, especially in the RHA rats. As mPFc is a highly interconnected brain region, we hypothesize that a NH effect is more unlikely to be observed in this region. Moreover, NH treatment is delivered in a time of development in which this region is in a "resting time", contrary to what happens in the isolation rearing procedure, in which treatment is delivered after weaning (i.e. when rats are about four weeks old), most of them showing reductions in mPFc volume (Day-Wilson et al., 2006; Schubert et al., 2009). However, to better understand how NH improves working memory, especially in the RHA rats, without affecting mPFc volume would, further studies will be needed. One possibility is that hippocampus (which is highly connected with mPFc) might be involved in the working memory improvement. It remains to be established whether possible NH-induced changes in hippocampal function -that are not evident at the structural/volumetric level- of RHA rats might be related to these positive cognitive effects.

Intrestingley, Schenider & Koch (2005) also found that cortical damage (PFc) induced lateral ventricular enlargement. Enlargement of lateral ventricles (LV) has been consistently observed in schizophrenia, and many studies lend support to this region as a robust biomarker of the disorder (Bertrand et al., 2010; Chin et al., 2011; Piontkewitz et al., 2011; Sandner et al., 2010; Shenton et al., 2001). RHA rats showed a marked increase of lateral ventricle volume compared to RLA animals, in line with the above studies.

In summary, the phenotypic profile of RHA rats, which have been proposed as a potential animal model for the study of some behavioural, attentional, cognitive and neural impairments observed in schizophrenia (Del Rio et al., 2014; Oliveras et al. 2016, 2017), appears to be consistent with the prevalent vision of the structural abnormalities related to this disease, showing a decrease in the volume of the

hippocampus and medial prefrontal cortex, with a marked enlargement of the lateral ventricles.

In conclusion, NH treatment seems to work as a modulator according to (and depending on) the phenotypes observed in each of the *Roman* strains/lines of rats. While on anxiety/fear responses and related neural structures (hippocampus, amygdala) NH approaches RLA rats to the profile or values observed in their RHA counterparts (e.g. inducing a decrease in anxiety/fear responses and hippocampus/amygdala volume and changing RLA's coping style towards a more active/proactive profile), NH treatment induces improvements in attentional/cognitive processes in RHA rats that approach their performance to that observed in RLA animals (see also Escorihuela et al. 1995; Fernández-Teruel et al., 2002). RLA rats continue to be considered as a valid animal model for the study of anxiety/fear and stress sensitivity mechanisms. RHA rats' profile is consistent with several phenotypes observed in schizophrenia, and might be a useful tool to understand some neural and neurobiological mechanisms involved in this disease without the need of further manipulations or treatments.

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REFERENCES

Aguilar, R., Escorihuela, R. M., Gil, L., Tobeña, A., & Fernández-Teruel, A. (2002). Differences between two psychogenetically selected lines of rats in a swimming pool matching-to-place task: long-term effects of infantile stimulation. *Behavior Genetics*, *32*(2), 127–34. https://doi.org/10.1023/A:1015253807488

Alam, M., Angelov, S., Stemmler, M., von Wrangel, C., Krauss, J. K., & Schwabe, K. (2015). Neuronal activity of the prefrontal cortex is reduced in rats selectively bred for deficient sensorimotor gating. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *56*, 174–184. https://doi.org/10.1016/j.pnpbp.2014.08.017

Anisman, H., Zaharia, M. D., Meaney, M. J., & Merali, Z. (1998). Do early-life events permanently alter behavioral and hormonal responses to stressors? *International Journal of Developmental Neuroscience*, *16*(3–4), 149–164. https://doi.org/10.1016/S0736-5748(98)00025-2

Bach, D.R., Guitart-Masip, M., Packard, P.A., Miró, J., Falip, M., Fuentemilla, L., Dolan, R.J. (2014). Human hippocampus arbitrates approach-avoidance conflict. *Current Biology*, 24, 541-547. https://doi.org/10.1016/j.cub.2014.01.046.

Barrós-Loscertales, A., Meseguer, V., Sanjuán, A., Belloch, V., Parcet, M. A., Torrubia, R., & Ávila, C. (2006). Behavioral Inhibition System activity is associated with increased amygdala and hippocampal gray matter volume: A voxel-based morphometry study. *NeuroImage*, *33*(3), 1011–1015. https://doi.org/10.1016/j.neuroimage.2006.07.025

Benes, F. M., Todtenkopf, M. S., Logiotatos, P., & Williams, M. (2000). Glutamate decarboxylase(65)-immunoreactive terminals in cingulate and prefrontal cortices of schizophrenic and bipolar brain. *Journal of Chemical Neuroanatomy*, *20*(3–4), 259–69. https://doi.org/10.1016/S0891-0618(00)00105-8

Bertrand, J.-B., Langlois, J.-B., Bégou, M., Volle, J., Brun, P., d'Amato, T., … SuaudChagny, M.-F. (2010). Longitudinal MRI monitoring of brain damage in the neonatal ventral hippocampal lesion rat model of schizophrenia. *Hippocampus*, *20*(2), 264–78. https://doi.org/10.1002/hipo.20628

Callicott, J. H., Mattay, V. S., Verchinski, B. A., Marenco, S., Egan, M. F., & Weinberger, D. R. (2003). Complexity of Prefrontal Cortical Dysfunction in Schizophrenia: More Than Up or Down. *American Journal of Psychiatry*, *160*(12), 2209–2215. https://doi.org/10.1176/appi.ajp.160.12.2209

Cañete, T., Blázquez, G., Tobeña, A., Giménez-Llort, L., & Fernández-Teruel, A. (2015). Cognitive and emotional alterations in young Alzheimer's disease (3xTgAD) mice: Effects of neonatal handling stimulation and sexual dimorphism. *Behavioural Brain Research*, *281*, 156–171. https://doi.org/10.1016/j.bbr.2014.11.004

Carrasco, J., Márquez, C., Nadal, R., Tobeña, A., Fernández-Teruel, A., & Armario, A. (2008). Characterization of central and peripheral components of the hypothalamus-pituitary-adrenal axis in the inbred Roman rat strains. *Psychoneuroendocrinology*, *33*(4), 437–45. https://doi.org/10.1016/j.psyneuen.2008.01.001

Chen, Y., Mao, Y., Zhou, D., Hu, X., Wang, J., & Ma, Y. (2010). Environmental enrichment and chronic restraint stress in ICR mice: effects on prepulse inhibition of startle and Y-maze spatial recognition memory. *Behavioural Brain Research*, *212*(1), 49–55. https://doi.org/10.1016/j.bbr.2010.03.033

Cherbuin, N., Windsor, T. D., Anstey, K. J., Maller, J. J., Meslin, C., & Sachdev, P. S. (2008). Hippocampal volume is positively associated with behavioural inhibition (BIS) in a large community-based sample of mid-life adults: the PATH through life study. *Social Cognitive and Affective Neuroscience*, *3*(3), 262–269. https://doi.org/10.1093/scan/nsn018

Chin, C.-L., Curzon, P., Schwartz, A. J., O'Connor, E. M., Rueter, L. E., Fox, G. B., … Basso, A. M. (2011). Structural abnormalities revealed by magnetic resonance imaging in rats prenatally exposed to methylazoxymethanol acetate parallel cerebral pathology in schizophrenia. *Synapse*, *65*(5), 393–403. https://doi.org/10.1002/syn.20857

Corda, M. G., Piras, G., Lecca, D., Fernández-Teruel, A., Driscoll, P., & Giorgi, O. (2005). The psychogenetically selected Roman rat lines differ in the susceptibility to develop amphetamine sensitization. *Behavioural Brain Research*, *157*(1), 147–156. https://doi.org/10.1016/j.bbr.2004.06.016

Cui, M., Yang, Y., Yang, J., Zhang, J., Han, H., Ma, W., … Cao, J. (2006). Enriched environment experience overcomes the memory deficits and depressive-like behavior induced by early life stress. *Neuroscience Letters*, *404*(1–2), 208–212. https://doi.org/10.1016/j.neulet.2006.05.048

Day-Wilson, K. M., Jones, D. N. C., Southam, E., Cilia, J., & Totterdell, S. (2006). Medial prefrontal cortex volume loss in rats with isolation rearing-induced deficits in prepulse inhibition of acoustic startle. *Neuroscience*, *141*(3), 1113–21. https://doi.org/10.1016/j.neuroscience.2006.04.048

Del Rio, C., Ignasi, O., Cañete, T., Blázquez, G., Tobeña, A., Fernández-teruel, A. (2014). Genetic Rat Models of Schizophrenia-Relevant Symptoms. *Wourld Journal of Neuroscience*, *4*, 261–278. http://dx.doi.org/10.4236/wjns.2014.43030

Déziel, R. A., Ryan, C. L., & Tasker, R. A. (2015). Ischemic lesions localized to the medial prefrontal cortex produce selective deficits in measures of executive function in rats. *Behavioural Brain Research*, *293*, 54–61. https://doi.org/10.1016/j.bbr.2015.07.003

Díaz-Morán, S., Palència, M., Mont-Cardona, C., Cañete, T., Blázquez, G., Martínez-Membrives, E., … Fernández-Teruel, A. (2012). Coping style and stress hormone responses in genetically heterogeneous rats: Comparison with the Roman rat strains. *Behavioural Brain Research*, *228*(1), 203–210. https://doi.org/10.1016/j.bbr.2011.12.002

Driscoll, P., Ferré, P., Fernández-Teruel, A., Levi de Stein, M., Wolfman, C., Medina, J., … Escorihuela, R. M. (1995). Effects of prenatal diazepam on two-way avoidance behavior, swimming navigation and brain levels of benzodiazepine-like molecules in male Roman high- and low-avoidance rats. *Psychopharmacology*, *122*(1), 51–7. https://doi.org/10.1007/BF02246441

Driscoll, P., Escorihuela, R. M., Fernández-Teruel, A., Giorgi, O., Schwegler, H., Steimer, T., … Tobeña, A. (1998). Genetic selection and differential stress responses. The Roman lines/strains of rats. *Annals of the New York Academy of Sciences*, *851*, 501–10. https://doi.org/10.1111/j.1749-6632.1998.tb09029.x

Escorihuela, R. M., Fernández-Teruel, A., Zapata, A., Nüñez, J. F., & Tobeńa, A. (1993). Flumazenil Prevents the Anxiolytic Effects of Diazepam, Alprazolam and Adinazolam on the Early Acquisition of Two-Way Active Avoidance. *Pharmacological Research*, *28*(1), 53–58. https://doi.org/10.1006/phrs.1993.1109

Escorihuela, R. M., Tobena, A., & Fernández-Teruel, A. (1995). Environmental enrichment and postnatal handling prevent spatial learning deficits in aged hypoemotional (Roman high-avoidance) and hyperemotional (Roman lowavoidance) rats. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *2*(1), 40–48. https://doi.org/10.1101/lm.2.1.40

Esnal, A., Sánchez-González, A., Río-Álamos, C., Oliveras, I., Cañete, T., Blázquez, G., … Fernández-Teruel, A. (2016). Prepulse inhibition and latent inhibition deficits in Roman high-avoidance vs. Roman low-avoidance rats: Modeling schizophrenia-related features. *Physiology & Behavior*, *163*, 267–273. https://doi.org/10.1016/j.physbeh.2016.05.020

Fernández-Teruel, A., Escorihuela, R. M., Núñez, J. F., Zapata, A., Boix, F., Salazar, W., & Tobeña, A. (1991). The early acquisition of two-way (shuttle-box) avoidance as an anxiety-mediated behavior: psychopharmacological validation. *Brain Research Bulletin*, *26*(1), 173–6. https://doi.org/10.1016/03619230(91)90205-X

Fernández-Teruel, A., Escorihuela, R. M., Driscoll, P., Tobeña, A., & Bättig, K. (1992). Differential effects of early stimulation and/or perinatal flumazenil treatment in young Roman low- and high-avoidance rats. *Psychopharmacology*, *108*(1–2), 170–6. https://doi.org/10.1007/BF02245303

Fernández-Teruel, A., Giménez-Llort, L., Escorihuela, R. M., Gil, L., Aguilar, R., Steimer, T., & Tobeña, A. (2002). Early-life handling stimulation and environmental enrichment: are some of their effects mediated by similar neural mechanisms? *Pharmacology, Biochemistry, and Behavior*, *73*(1), 233–45. https://doi.org/10.1016/S0091-3057(02)00787-6

Fernández-Teruel, A., Blázquez, G., Pérez, M., Aguilar, R., Cañete, T., Guitart, M., … Tobeña, A. (2006). Latent inhibition threshold in Roman high-avoidance rats: A psychogenetic model of abnormalities in attentional filter? *Actas Espanolas de Psiquiatria*, *34*(4), 257–263. http://www.ncbi.nlm.nih.gov/pubmed/16823687

Ferré, P., Fernández-Teruel, A., Escorihuela, R. M., Driscoll, P., Corda, M. G., Giorgi, O., & Tobeña, A. (1995). Behavior of the Roman/Verh high- and low-avoidance rat lines in anxiety tests: relationship with defecation and self-grooming. *Physiology & Behavior*, *58*(6), 1209–13. https://doi.org/10.1016/00319384(95)02068-3

Foote, S. L., Bloom, F. E., & Aston-Jones, G. (1983). Nucleus locus ceruleus: new evidence of anatomical and physiological specificity. *Physiological Reviews*, *63*(3), 844–914. http://www.ncbi.nlm.nih.gov/pubmed/6308694

Fox, C., Merali, Z., & Harrison, C. (2006). Therapeutic and protective effect of environmental enrichment against psychogenic and neurogenic stress. *Behavioural Brain Research*, *175*(1), 1–8. https://doi.org/10.1016/j.bbr.2006.08.016

Garey, L. J., Ong, W. Y., Patel, T. S., Kanani, M., Davis, A., Mortimer, A. M., … Hirsch, S. R. (1998). Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia. *Journal of Neurology, Neurosurgery, and Psychiatry*, *65*(4), 446–53. https://doi.org/10.1136/jnnp.65.4.446

Garoflos, E., Stamatakis, A., Pondiki, S., Apostolou, A., Philippidis, H., & Stylianopoulou, F. (2007). Cellular mechanisms underlying the effect of a single exposure to neonatal handling on neurotrophin-3 in the brain of 1-day-old rats. *Neuroscience*, *148*(2), 349–58. https://doi.org/10.1016/j.neuroscience.2007.06.020

Garoflos, E., Stamatakis, A., Rafrogianni, A., Pondiki, S., & Stylianopoulou, F. (2008). Neonatal handling on the first postnatal day leads to increased maternal behavior and fos levels in the brain of the newborn rat. *Developmental Psychobiology*, *50*(7), 704–13. https://doi.org/10.1002/dev.20332

Gilabert-Juan, J., Belles, M., Saez, A. R., Carceller, H., Zamarbide-Fores, S., Moltó, M. D., & Nacher, J. (2013). A "double hit" murine model for schizophrenia shows alterations in the structure and neurochemistry of the medial prefrontal cortex and the hippocampus. *Neurobiology of Disease*, *59*, 126–140. https://doi.org/10.1016/j.nbd.2013.07.008

Giorgi, O., Piras, G., Lecca, D., Hansson, S., Driscoll, P., & Corda, M. G. (2003). Differential neurochemical properties of central serotonergic transmission in Roman high- and low-avoidance rats. *Journal of Neurochemistry*, *86*(2), 422–31. https://doi.org/10.1046/j.1471-4159.2003.01845.x

Giorgi, O., Piras, G., & Corda, M. G. (2007). The psychogenetically selected Roman high- and low-avoidance rat lines: A model to study the individual vulnerability to drug addiction. *Neuroscience & Biobehavioral Reviews*, *31*(1), 148–163. https://doi.org/10.1016/j.neubiorev.2006.07.008

Glantz, L. A., & Lewis, D. A. (2000). Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Archives of General Psychiatry*, *57*(1), 65–73. https://doi.org/10.1001/archpsyc.57.1.65

Gray, J. A. (1982). The Neuropsychology of Anxiety: An INquiry into the Functions into the septo-hippocampal System. *New York, NY: Ofxord University Press*.

Gray, J.A. & McNaughton, N. (2000). The Neuropsychology of Anxiety: an Enquiry into the funtions of the septo-hippocampal System. *Oxford University Press*, *2nd Editio*.

Grillon, C., & Davis, M. (1997). Effects of stress and shock anticipation on prepulse inhibition of the startle reflex. *Psychophysiology*, *34*(5), 511–7. https://doi.org/10.1111/j.1469-8986.1997.tb01737.x

Guitart-Masip, M., Johansson, B., Cañete, T., Fernández-Teruel, A., Tobeña, A., Terenius, L., & Giménez-Llort, L. (2008). Regional adaptations in PSD-95, NGFIA and secretogranin gene transcripts related to vulnerability to behavioral sensitization to amphetamine in the Roman rat strains. *Neuroscience*, *151*(1), 195–208. https://doi.org/10.1016/j.neuroscience.2007.09.072

Hattori, S., Hashimoto, R., Miyakawa, T., Yamanaka, H., Maeno, H., Wada, K., & Kunugi, H. (2007). Enriched environments influence depression-related behavior in adult mice and the survival of newborn cells in their hippocampi. *Behavioural Brain Research*, *180*(1), 69–76. https://doi.org/10.1016/j.bbr.2007.02.036

Hendershott, T. R., Cronin, M. E., Langella, S., McGuinness, P. S., & Basu, A. C. (2016). Effects of environmental enrichment on anxiety-like behavior, sociability, sensory gating, and spatial learning in male and female C57BL/6J mice. *Behavioural Brain Research*, *314*, 215–25. https://doi.org/10.1016/j.bbr.2016.08.004 Horga, G., Bernacer, J., Dusi, N., Entis,

J., Chu, K., Hazlett, E. A., … Buchsbaum, M. S. (2011). Correlations between ventricular enlargement and gray and white matter volumes of cortex, thalamus, striatum, and internal capsule in schizophrenia. *European Archives of Psychiatry and Clinical Neuroscience*, *261*(7), 467–476. https://doi.org/10.1007/s00406-011-0202-x

Huang, E. J., & Reichardt, L. F. (2003). Trk Receptors: Roles in Neuronal Signal Transduction. *Annual Review of Biochemistry*, *72*(1), 609–642. https://doi.org/10.1146/annurev.biochem.72.121801.161629

Ickes, B. R., Pham, T. M., Sanders, L. A., Albeck, D. S., Mohammed, A. H., & Granholm, A.-C. (2000). Long-Term Environmental Enrichment Leads to Regional Increases in Neurotrophin Levels in Rat Brain. *Experimental Neurology*, *164*(1), 45–52. https://doi.org/10.1006/exnr.2000.7415

Ishikawa, J., Nishimura, R., & Ishikawa, A. (2015). Early-life stress induces anxietylike behaviors and activity imbalances in the medial prefrontal cortex and amygdala in adult rats. *European Journal of Neuroscience*, *41*(4), 442–453. https://doi.org/10.1111/ejn.12825

Kalisch, R., Schubert, M., Jacob, W., Keßler, M. S., Hemauer, R., Wigger, A., … Auer, D. P. (2006). Anxiety and Hippocampus Volume in the Rat. *Neuropsychopharmacology*, *31*(5), 925–932. https://doi.org/10.1038/sj.npp.1300910

Koch, M., & Schnitzler, H. U. (1997). The acoustic startle response in rats--circuits mediating evocation, inhibition and potentiation. *Behavioural Brain Research*, *89*(1–2), 35–49. https://doi.org/10.1016/S0166-4328(97)02296-1

Kohl, S., Heekeren, K., Klosterkötter, J., & Kuhn, J. (2013). Prepulse inhibition in psychiatric disorders--apart from schizophrenia. *Journal of Psychiatric Research*, *47*(4), 445–52. https://doi.org/10.1016/j.jpsychires.2012.11.018

Labbate, G. P., da Silva, A. V., & Barbosa-Silva, R. C. (2014). Effect of severe neonatal seizures on prepulse inhibition and hippocampal volume of rats tested in early adulthood. *Neuroscience Letters*, *568*, 62–66. https://doi.org/10.1016/j.neulet.2014.03.019

Larsson, F., Winblad, B., & Mohammed, A. H. (2002). Psychological stress and environmental adaptation in enriched vs. impoverished housed rats. *Pharmacology, Biochemistry, and Behavior*, *73*(1), 193–207. https://doi.org/10.1016/S00913057(02)00782-7

Laviola, G., Hannan, A. J., Macrì, S., Solinas, M., & Jaber, M. (2008). Effects of enriched environment on animal models of neurodegenerative diseases and psychiatric disorders. *Neurobiology of Disease*, *31*(2), 159–168. https://doi.org/10.1016/j.nbd.2008.05.001

Lehmann, J., Stöhr, T., & Feldon, J. (2000). Long-term effects of prenatal stress experiences and postnatal maternal separation on emotionality and attentional processes. *Behavioural Brain Research*, *107*(1–2), 133–44. https://doi.org/10.1016/S0166-4328(99)00122-9

Levita, L., Bois, C., Healey, A., Smyllie, E., Papakonstantinou, E., Hartley, T., & Lever, C. (2014). The Behavioural Inhibition System, anxiety and hippocampal volume in a non-clinical population. *Biology of Mood & Anxiety Disorders*, *4*(1), 4. https://doi.org/10.1186/2045-5380-4-4

Levitt, J. J., Bobrow, L., Lucia, D., & Srinivasan, P. (2010). A selective review of volumetric and morphometric imaging in schizophrenia. *Current Topics in Behavioral Neurosciences*, *4*, 243–81. https://doi.org/10.1007/7854_2010_53

López-Aumatell, R., Vicens-Costa, E., Guitart-Masip, M., Martínez-Membrives, E., Valdar, W., Johannesson, M., … Fernández-Teruel, A. (2009). Unlearned anxiety predicts learned fear: A comparison among heterogeneous rats and the Roman rat strains. *Behavioural Brain Research*, *202*(1), 92–101. https://doi.org/10.1016/j.bbr.2009.03.024

López-Aumatell, R., Martínez-Membrives, E., Vicens-Costa, E., Cañete, T., Blázquez, G., Mont-Cardona, C., … Fernández-Teruel, A. (2011). Effects of environmental and physiological covariates on sex differences in unconditioned and conditioned anxiety and fear in a large sample of genetically heterogeneous (N/Nih-HS) rats. *Behavioral and Brain Functions*, *7*(1), 48. https://doi.org/10.1186/1744-9081-7-48

Lu, B., Pang, P. T., & Woo, N. H. (2005). The yin and yang of neurotrophin action. *Nature Reviews Neuroscience*, *6*(8), 603–614. https://doi.org/10.1038/nrn1726

Machado-de-Sousa, J. P., Osório, F. de L., Jackowski, A. P., Bressan, R. A., Chagas, M. H. N., Torro-Alves, N., … Hallak, J. E. C. (2014). Increased Amygdalar and Hippocampal Volumes in Young Adults with Social Anxiety. *PLoS ONE*, *9*(2), e88523. https://doi.org/10.1371/journal.pone.0088523

Makatsori, A., Duncko, R., Schwendt, M., Moncek, F., Johansson, B. B., & Jezova, D. (2003). Voluntary wheel running modulates glutamate receptor subunit gene expression and stress hormone release in Lewis rats. *Psychoneuroendocrinology*, *28*(5), 702–14. https://doi.org/10.1007/7854_2010_53

Martínez-Membrives, E., López-Aumatell, R., Blázquez, G., Cañete, T., Tobeña, A., & Fernández-Teruel, A. (2015). Spatial learning in the genetically heterogeneous NIH-HS rat stock and RLA-I/RHA-I rats: Revisiting the relationship with unconditioned and conditioned anxiety. *Physiology & Behavior*, *144*, 15–25. https://doi.org/10.1016/j.physbeh.2015.03.003

McAllister, A. K., Katz, L. C., & Lo, D. C. (1999). Neurotrophins and synaptic plasticity. *Annual Review of Neuroscience*, *22*(1), 295–318. https://doi.org/10.1146/annurev.neuro.22.1.295

McAuley, J. D., Stewart, A. L., Webber, E. S., Cromwell, H. C., Servatius, R. J., & Pang, K. C. H. (2009). Wistar–Kyoto rats as an animal model of anxiety vulnerability: Support for a hypervigilance hypothesis. *Behavioural Brain Research*, *204*(1), 162–168. https://doi.org/10.1016/j.bbr.2009.05.036

Meaney, M. J., Aitken, D. H., van Berkel, C., Bhatnagar, S., & Sapolsky, R. M. (1988). Effect of neonatal handling on age-related impairments associated with the hippocampus. *Science (New York, N.Y.)*, *239*(4841 Pt 1), 766–8. https://doi.org/10.1126/science.3340858

Moreno, M., Cardona, D., Gómez, M. J., Sánchez-Santed, F., Tobeña, A., FernándezTeruel, A., … Flores, P. (2010). Impulsivity Characterization in the Roman High- and Low-Avoidance Rat Strains: Behavioral and Neurochemical Differences. *Neuropsychopharmacology*, *35*(5), 1198–1208. https://doi.org/10.1038/npp.2009.224

Morris, R.G.M., Anderson, E., Lynch, G.S., & Baudry, M. (1986a). Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartat receptor antagonist, AP5. *Nature,* 319, 774–776.

Morris, R.G.M., Hagan, J.J., & Rawlins, J.N.P. (1986b). Allocentric spatial learning by hippocampectomised rats: a further test of the "spatial mapping" and "working memory" theories of hippocampal function. *Quarterly Journal of Experimental Psychology,* 38B, 365–395. https://doi.org/

Nuñez, J.F., Ferré, P., Escorihuela, R.M., Tobeña, A., & Fernández-Teruel, A. (1996). Effects of postnatal handling of rats on emotional, HPA-axis, and prolactin reactivity to novelty and conflict. *Physiology and Behavior*, 60, 1355-9. https://doi.org/10.1016/S0031-9384(96)00225-9.

Oler, J. A., Fox, A. S., Shelton, S. E., Rogers, J., Dyer, T. D., Davidson, R. J., … Kalin, N. H. (2010). Amygdalar and hippocampal substrates of anxious temperament differ in their heritability. *Nature*, *466*(7308), 864–868. https://doi.org/10.1038/nature09282

Oliveras, I., Río-Álamos, C., Cañete, T., Blázquez, G., Martínez-Membrives, E., Giorgi, O., … Fernández-Teruel, A. (2015). Prepulse inhibition predicts spatial working memory performance in the inbred Roman high- and low-avoidance rats and in genetically heterogeneous NIH-HS rats: relevance for studying pre-attentive and cognitive anomalies in schizophrenia. *Frontiers in Behavioral Neuroscience*, *9*, 213. https://doi.org/10.3389/fnbeh.2015.00213

Oliveras, I., Sánchez-González, A., Piludu, M. A., Gerboles, C., Río-Álamos, C., Tobeña, A., & Fernández-Teruel, A. (2016). Divergent effects of isolation rearing on prepulse inhibition, activity, anxiety and hippocampal-dependent memory in Roman high- and low-avoidance rats: A putative model of schizophrenia-relevant features. *Behavioural Brain Research*, *314*, 6–15. https://doi.org/10.1016/j.bbr.2016.07.047

Oliveras, I., Sánchez-González, A., Sampedro-Viana, D., Piludu, M. A., Río-Alamos, C., Giorgi, O., … Fernández-Teruel, A. (2017). Differential effects of antipsychotic and propsychotic drugs on prepulse inhibition and locomotor activity in Roman high- (RHA) and low-avoidance (RLA) rats. *Psychopharmacology*, *234*(6), 957–975. https://doi.org/10.1007/s00213-017-4534-8

Paxinos, G., & Watson, C. (1998). The rat brain in Stereotaxic Coordinates. Compact 6th Edition, Ed. CA. Academic Press, San Diego.

Piontkewitz, Y., Arad, M., & Weiner, I. (2011). Abnormal Trajectories of Neurodevelopment and Behavior Following In Utero Insult in the Rat. *Biological Psychiatry*, *70*(9), 842–851. https://doi.org/10.1016/j.biopsych.2011.06.007

Raineki, C., Lucion, A. B., & Weinberg, J. (2014). Neonatal handling: An overview of the positive and negative effects. *Developmental Psychobiology*, *56*(8), 1613–1625. https://doi.org/10.1002/dev.21241

Río-Álamos, C., Oliveras, I., Cañete, T., Blázquez, G., Martínez-Membrives, E., Tobeña, A., & Fernández-Teruel, A. (2015). Neonatal handling decreases unconditioned anxiety, conditioned fear, and improves two-way avoidance acquisition: a study with the inbred Roman high (RHA-I)- and low-avoidance (RLA-I) rats of both sexes. *Frontiers in Behavioral Neuroscience*, *9*. https://doi.org/10.3389/fnbeh.2015.00174

Río-Álamos, C., Oliveras, I., Piludu, M. A., Gerbolés, C., Cañete, T., Blázquez, G., … Fernández-Teruel, A. (2017). Neonatal handling enduringly decreases anxiety and stress responses and reduces hippocampus and amygdala volume in a genetic model of differential anxiety: Behavioral-volumetric associations in the Roman rat strains. *European Neuropsychopharmacology*, *27*(2), 146–158. https://doi.org/10.1016/j.euroneuro.2016.12.003

Sánchez-González, A., Esnal, A., Río-Álamos, C., Oliveras, I., Cañete, T., Blázquez, G., … Fernández-Teruel, A. (2016). Association between prepulse inhibition of the startle response and latent inhibition of two-way avoidance acquisition: A study with heterogeneous NIH-HS rats. *Physiology & Behavior*, *155*, 195–201. https://doi.org/10.1016/j.physbeh.2015.12.011

Sandner, G., Angst, M.-J., Guiberteau, T., Guignard, B., & Brasse, D. (2010). MRI and X-ray scanning images of the brain of 3-, 6- and 9-month-old rats with bilateral neonatal ventral hippocampus lesions. *NeuroImage*, *53*(1), 44–50. https://doi.org/10.1016/j.neuroimage.2010.06.007

Sanfilipo, M., Lafargue, T., Arena, L., Rusinek, H., Kushner, K., Lautin, A., … Wolkin, A. (2000). Fine volumetric analysis of the cerebral ventricular system in schizophrenia: further evidence for multifocal mild to moderate enlargement. *Schizophrenia Bulletin*, *26*(1), 201–16 http://www.ncbi.nlm.nih.gov/pubmed/10755682

Schneider, M., & Koch, M. (2005). Behavioral and morphological alterations following neonatal excitotoxic lesions of the medial prefrontal cortex in rats. *Experimental Neurology*, *195*(1), 185–198. https://doi.org/10.1016/j.expneurol.2005.04.014

Schubert, M. I., Porkess, M. V., Dashdorj, N., Fone, K. C. F., & Auer, D. P. (2009). Effects of social isolation rearing on the limbic brain: A combined behavioral and magnetic resonance imaging volumetry study in rats. *Neuroscience*, *159*(1), 21–30. https://doi.org/10.1016/j.neuroscience.2008.12.019

Shenton, M. E., Dickey, C. C., Frumin, M., & McCarley, R. W. (2001). A review of MRI findings in schizophrenia. *Schizophrenia Research*, *49*(1–2), 1–52. https://doi.org/10.1016/S0013-4686(03)00372-4

Silva-Gómez, A. B., Rojas, D., Juárez, I., & Flores, G. (2003). Decreased dendritic spine density on prefrontal cortical and hippocampal pyramidal neurons in postweaning social isolation rats. *Brain Research*, *983*(1–2), 128–36. https://doi.org/10.1016/S0006-8993(03)03042-7

Stamatakis, A., Stamatakis, A., Pondiki, S., Stamatakis, A., Pondiki, S., Kitraki, E., … Stylianopoulou, F. (2008). Effect of neonatal handling on adult rat spatial learning and memory following acute stress. *Stress*, *11*(2), 148–159. https://doi.org/10.1080/10253890701653039

Steimer, T., Escorihuela, R.M., Fernández-Teruel, A., & Driscoll, P. (1998). Long-term behavioural and neuroendocrine changes in Roman high- (RHA/Verh) and low- (RLA-Verh) avoidance rats following neonatal handling. *International Journal of Developmental Neuroscience*, 16, 165-74. https://doi.org/10.106/S0736-5748(98)00032-x.

Steimer, T., & Driscoll, P. (2003). Divergent stress responses and coping styles in psychogenetically selected Roman high-(RHA) and low-(RLA) avoidance rats: behavioural, neuroendocrine and developmental aspects. *Stress (Amsterdam, Netherlands)*, *6*(2), 87–100. https://doi.org/10.1080/1025389031000111320

Steimer, T., & Driscoll, P. (2005). Inter-individual vs line/strain differences in psychogenetically selected Roman High-(RHA) and Low-(RLA) Avoidance rats: neuroendocrine and behavioural aspects. *Neuroscience and Biobehavioral Reviews*, *29*(1), 99–112. https://doi.org/10.1016/j.neubiorev.2004.07.002

Svensson, T. H. (1987). Peripheral, autonomic regulation of locus coeruleus noradrenergic neurons in brain: putative implications for psychiatry and psychopharmacology. *Psychopharmacology*, *92*(1), 1–7. https://doi.org/10.1007/BF00215471

Swerdlow, N. R., Geyer, M. A., & Braff, D. L. (2001). Neural circuit regulation of prepulse inhibition of startle in the rat: current knowledge and future challenges. *Psychopharmacology*, *156*(2–3), 194–215. https://doi.org/10.1007/s002130100799

Swerdlow, N. R., & Light, G. A. (2015). Animal Models of Deficient Sensorimotor Gating in Schizophrenia: Are They Still Relevant? In *Current topics in behavioral neurosciences* (Vol. 28, pp. 305–325). https://doi.org/10.1007/7854_2015_5012

Swerdlow, N. R., Braff, D. L., & Geyer, M. A. (2016). Sensorimotor gating of the startle reflex: what we said 25 years ago, what has happened since then, and what comes next. *Journal of Psychopharmacology*, *30*(11), 1072–1081. https://doi.org/10.1177/0269881116661075

Torasdotter, M., Metsis, M., Henriksson, B. G., Winblad, B., & Mohammed, A. H. (1996). Expression of neurotrophin-3 mRNA in the rat visual cortex and hippocampus is influenced by environmental conditions. *Neuroscience Letters*, *218*(2), 107–10.). https://doi.org/10.1007/s002130100799

van Praag, H., Kempermann, G., & Gage, F. H. (2000). Neural consequences of environmental enrichment. *Nature Reviews Neuroscience*, *1*(3), 191–198. https://doi.org/10.1038/35044558

Varty, G. B., Marsden, C. A., & Higgins, G. A. (1999). Reduced synaptophysin immunoreactivity in the dentate gyrus of prepulse inhibition-impaired isolationreared rats. *Brain Research*, *824*(2), 197–203. https://doi.org/10.1016/S00068993(99)01173-7

Varty, G. B., Paulus, M. P., Braff, D. L., & Geyer, M. A. (2000). Environmental enrichment and isolation rearing in the rat: effects on locomotor behavior and startle response plasticity. *Biological Psychiatry*, *47*(10), 864–73. https://doi.org/10.1016/S0006-3223(99)00269-3

Vicens-Costa, E., Martínez-Membrives, E., López-Aumatell, R., Guitart-Masip, M., Cañete, T., Blázquez, G., … Fernández-Teruel, A. (2011). Two-way avoidance acquisition is negatively related to conditioned freezing and positively associated with startle reactions: A dissection of anxiety and fear in genetically heterogeneous rats. *Physiology & Behavior*, *103*(2), 148–156. https://doi.org/10.1016/j.physbeh.2010.12.009

Weiss, K. M. (1968). Some effects of the conditioned suppression paradigm on operant discrimination performance1. *Journal of the Experimental Analysis of Behavior*, *11*(6), 767–775. https://doi.org/10.1901/jeab.1968.11-767

Weiss, I. C., & Feldon, J. (2001). Environmental animal models for sensorimotor gating deficiencies in schizophrenia: a review. *Psychopharmacology*, *156*(2–3), 305–26. https://doi.org/10.1007/s002130100800

Whalen, P. J., Kapp, B. S., & Pascoe, J. P. (1994). Neuronal activity within the nucleus basalis and conditioned neocortical electroencephalographic activation. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *14*(3 Pt 2), 1623–33. http://www.ncbi.nlm.nih.gov/pubmed/8126559

Wilcock, J., & Fulker, D. W. (1973). Avoidance learning in rats: genetic evidence for two distinct behavioral processes in the shuttle box. *Journal of Comparative and Physiological Psychology*, *82*(2), 247–53. https://doi.org/10.1037/h0033914

Whishaw, I. Q. (1985). Formation of a place learning-set by the rat: a new paradigm for neurobehavioral studies. *Physiology & Behavior*, 35, 139–143.

Yoshida, T., McCarley, R. W., & Niznikiewicz, M. A. (2011). Letter to the Editor. *Schizophrenia Research*, *127*(1–3), 268–269. https://doi.org/10.1016/j.schres.2010.05.017

Zaharia, M. D., Kulczycki, J., Shanks, N., Meaney, M. J., & Anisman, H. (1996). The effects of early postnatal stimulation on Morris water-maze acquisition in adult mice: genetic and maternal factors. *Psychopharmacology*, *128*(3), 227–39. https://doi.org/10.1007/s002130050130

Zeier, H., Baettig, K., & Driscoll, P. (1978). Acquisition of DRL-20 behavior in male and female, Roman high- and low-avoidance rats. *Physiology & Behavior*, *20*(6), 791–3. https://doi.org/10.1016/0031-9384(78)90307-4