Volumetric brain differences between the Roman rat strains: neonatal handling effects, sensorimotor gating and working memory

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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), in the elevated zero-maze test (ZM) of anxiety and in a PPI test, as well as measuring the volume of limbic and cortical brain regions (amygdala -Am-, hippocampus -Hc-, striatum -St-, medial prefrontal cortex -mPFc-, anterior cingulate cortex -ACC-, nucleus accumbens -NAc-, 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, lowered anxiety in the ZM 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, Am 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 Am 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 Am volume in parallel to the decrease of anxiety and the impairment of sensorimotor gating in RLA rats.

Key words: anxiety, sensorimotor gating, spatial learning, MRI, neonatal handling, Roman rat strains.

1. Introduction

Psychogenetic selection for their very good vs. extremely poor acquisition of the two-way active avoidance response in the shuttle box has led to the Roman High (RHA) – and Low-Avoidance (RLA) strains of rats [1-8].

As a consequence of such a bidirectional selection, anxiety/fearfulness and sensitivity to stress are among the principal behavioural traits that differentiate these two strains of rats. RLA rats are more anxious and/or fearful in several unconditioned and conditioned tasks/tests [9-12], showing also higher stress-induced HPA-axis responses than RHA rats [9,13–15] and a passive/reactive coping style compared with the proactive style of their RHA counterparts [3,9,14–19]

Moreover, RHA vs. RLA (and also RHAs compared with other rat strains) phenotypical profiles suggests that the former might be a potential animal model for studying some schizophrenia-relevant symptoms [20-23]. In this context, RHA rats show enhanced impulsive behavior [24,25], deficits in latent inhibition [26,27], impaired PPI [21–23,28], worse working memory and spatial reference learning/memory [20,21,29–32], enhanced locomotor activity as well as mesolimbic dopaminergic sensitization to repeated administration of dopaminergic psychostimulants [19,33,34], among other schizophrenia-related phenotypes.

Neonatal handling (NH; 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 [18,35–38], 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 [18,12,28] and decreases HPA-axis and prolactin post-stress responses [37,28,39,40]. 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 [18,37,38,41–44], showing even protective effects on spatial learning deficits in a mouse model of Alzheimer disease [41]. 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 [45]. 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 [46–48], are also increased in rat hippocampus after both NH and environmental enrichment treatment [49–51].

We present here two studies. In Experiment 1, control and NH-treated RLA and RHA rats were submitted to tests of anxiety-related behaviour under novelty (i.e. the novel object exploration test -NOE- and the elevated zero-maze -ZM- test), to a sensorimotor gating test (prepulse inhibition -PPI) and to magnetic resonance image measures of the volumes (sMRI) of specific brain regions. In experiment 2, which was focused on the effects of NH on attentional/cognitive processes in RLA and RHA rats, control and NH-treated rats of both strains and 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 anxiety and on the changes induced by NH on anxiety-related behavioural parameters and brain regions such as the hippocampus and the amygdala [28]. For reasons of integrity and coherence of the present paper we present here the most relevant of these published results 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) [28].

2.1. Material and methods

2.1.1. Animals and neonatal handling (NH) treatment

Sixty-four male RHA and RLA rats (32 from each strain) were used. 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 [31,12,28,52]. Each handling session consisted of first removing the mother from the litter and then placing the pups gently and individually in plastic cages (35 x 15 x 25 cm) lined with paper towel for a total period of 8 min. Each pup was gently 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. Each of the four experimental group (RLA and RHA control and NH-treated), was composed of 16 male rats representing 5-6 different litters, with 3-4 pups coming from each litter. For MRI studies, each experimental group was formed by 8 male rats pseudorandomly selected from the corresponding group of 16 animals in a manner that the original 5-6 different litters (1-2 rats per litter) were still represented.

The experiment was conducted 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 Council Directive (86/609/EEC) on this subject.

2.1.2 Novel Object Exploration (NOE) test

To assess novelty-induced behavioral inhibition/disinhibition, and to make 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. We have previously shown that the NOE test is a good index/marker of the anxiolytic-like effects of NH treatment, as NH-treated rats show increased levels of exploration of the novel object which are positively correlated (as shown by correlational and factorial analyses) with anti-anxiety NH effects in other tests [12,28].

Rats were 60 days old at the beginning of the NOE 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).

2.1.3 Elevated zero-maze (ZM) test

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. The main measure (the one included here) was the time spent into open sections (ZM-Time) (but see also Río-Alamos et al., 2015, 2017 [12,28].

2.1.4 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 placed on 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), namely “baseline acoustic startle (BAS)”, 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 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 [21].

2.1.5 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 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 [53]. 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 absence 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) [28].

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.

2.1.6. 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 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 $p \leq 0.05$.

3. Results (experiment 1)

In the NOE 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 of the novel object ["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 $p < 0{,}001$]. In the ZM test, "Strain" and NH significant effects [both $Fs(3, 60) \geq 5.12$ and $p < 0{,}05$] were observed, since RHA rats spent longer time in the open sections and NH treatment increased this parameter in both rat strains (Table 2).

No significant effects were observed in "baseline startle" measured in the PPI session. 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 Experiment 2).

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 and Figure 2) compared to their RLA counterparts ["Strain" effects in all parameters, $Fs(3, 28) \geq 5.34$ and $p \leq 0{,}05$]. 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 volumes of the striatum, anterior cingulate or nucleus accumbens (Table 2 and Figure 2).

4. Experiment 2

Since some suggestive trend for effects of NH on PPI was observed at some prepulse intensities in experiment 1 (data not shown; but see total %PPI in Table 2), we conducted Experiment 2 in order to clarify potential NH effects on sensorimotor gating (PPI) and cognition. To this aim we included NH-treated and non-NH-treated RHA and RLA rats of both sexes, and we used a much larger rat sample to ensure that even subtle effects could arise. Animals were tested for PPI as well as in several spatial learning/memory tasks in the Morris water maze.

4.1. Materials and methods

Two-hundred fifty-eight male and female RHA and RLA rats were used. The number of rats for each strain and sex, as well as the ages of testing in each procedure/task are shown in Table 3.

Neonatal handling treatment, novel object exploration (NOE) test and prepulse inhibition (PPI) test (see Table 3, Batch 1) were administered using the same procedures as in Experiment 1 (see above).

The experiment was conducted 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 Council Directive (86/609/EEC) on this subject.

4.1.1. Morris water maze tasks

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: 28 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 behaviour 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 [32].

4.1.2. 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 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 [22] (see Figure 3A)

4.1.3. 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 [22] (see Figure 3A).

4.1.4. Reversal task (RT): Batch 2

Twenty four hours after the transfer test 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 3A).

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

Animals were allowed to swim for 90 s or until they located the hidden platform. This rat sample was randomly selected from the "Batch 1" sample, and they were naïve to the Morris water maze (i.e. they were not previously tested in any task in the Morris water maze). Each rat went through 2 trials per day during 3 testing days: 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 3B). 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 were 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 [21] (see Table 3, Batch 3).

4.1.6. Cued task: Batch 4

After four days of rest, animals were tested on a cued task. It consisted of 4 trials performed in a one-day test. A pseudorandom sample of rats from Batch 2 and Batch 3 (half from each) was selected for this 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 3C; Table 3, Batch 4).

4.1.7. 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 %PPI measures (“2 strain” x “2 treatment conditions” x “2 sex” x “prepulse intensities”). 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 days”). 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.

5. Results (experiment 2)

In the "NOE" test, RLA rats showed higher latency to explore the novel object and less time exploring than RHAs ["Strain" effects, both Fs(7, 257) ≥ 39.38, and p ≤ 0,001]. NH significantly reduced latency and increased time of exploration ["NH" effects, both Fs (7, 257) ≥ 52.63, p ≤ 0,001]. As males explored longer the novel object than female rats a sex effect was also observed ["Sex" effect, F(7,257) = 16.06, p ≤ 0,001]. There were also "Strain x NH" interactions, as NH effects were more pronounced in RLA rats ["Strain x NH" effects, both Fs(7,257) ≥13.74, p ≤ 0,001] (see Fig 4A-B).

Results of acoustic startle response (the average startle response of the 25 pulse-alone trials), measured in the PPI test (Figure 4D), 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 ± sem = 1135,24 (72.92) for RLA rats, and 877,06 (80.46) for RHA rats]. In the first 10-trials of pulse alone (BAS), RLA rats showed higher acoustic startle response than their RHA counterparts ["Strain" effect, F(7,257) = 22.50, p ≤ 0,001] and male rats showed higher acoustic startle response than female rats ["Sex" effect, F(7,257) = 21.14, p ≤ 0,001]. This "sex" effect was also observed regarding the pulse-alone trials used to calculate the %PPI ["Sex" effect, F(7,257) = 22.47, p ≤ 0,001] and in the last 5 pulse-alone trials ["Sex" effect, F(7,257) = 7.28, p ≤ 0,05]. No NH or interaction effects were observed for baseline startle (Figure 4D).

The RLA rat strain displayed higher total %PPI (Figure 4C) than RHA rats ["Strain" effect, F(7,257) = 120.93, p ≤ 0,001], with significant between-strain differences being observed in the four prepulse intensities [all Fs(7, 257) ≥ 36.72, p ≤ 0,001]. Male rats also showed higher levels of %PPI than female rats ["Sex" effect, F(7,257) = 12.15, p ≤ 0.001], with differences in all prepulse intensities [all Fs(7, 257) ≥6.04, p ≤ 0,05] except 65dB (Figure 4C). A "Strain x NH" interaction was observed on %PPI, as NH treatment elicited a decrease of %PPI in the RLA rat strain at all prepulse intensities ["Strain x NH" effects, F(7,257) = 20.66, p ≤ 0.001], while an improvement of %PPI was observed at 70 and 75dB prepulses in RHA rats (see Duncan's tests in Figure 4C).

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; Figure 5A]. In the transfer test RLA rats performed more annulus crossings than their RHA counterparts ["Strain" effect, F(3,44) =26.21, p ≤ 0,001; Figure 5B]. A "NH" effect was observed in the first training day of the reversal task, as NH-treated rats showed higher distance savings between the second and the third trial) than control animals ["Strain" effect, F(3,44) =5.04, p ≤ 0,030; Figure 5C-D], 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; Figure 5E]. No "strain" or "strain x treatment" interaction effects were found. No main effects or interactions were observed in the cued task (Figure 5F).

6. Discussion

The main findings from experiment 1 were that: i) RLA rats showed higher behavioural inhibition (anxiety-like behaviour) in the NOE and ZM tests, and higher levels of PPI than RHAs; ii) NH reduced the latency to explore the novel object and increased the time spent exploring it (NOE test), especially in the RLA rats, and increased the time spent in the open sections of the elevated zero-maze; 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 this strain showed a two-fold increase in the volume of the lateral ventricles, with no strain differences being observed in striatum, nucleus accumbens or cingulate cortex volume; v) NH reduced amygdala volume in both strain/lines of rats, but more markedly in RLAs, and vi) a "strain x treatment" effect was observed on hippocampus, as NH-treatment decreased hippocampal volume in RLA rats while showing an opposite trend in RHA rats.

In the present paper, using the same rat sample of Río-Álamos et al. (2017) [28], we report the volumes of new specific brain regions which complete that volumetric MRI study. Thus because of their proposed involvement in attentional and cognitive processes, the volumes of the medial prefrontal cortex (mPFc), anterior cingulate cortex

(ACC), nucleus accumbens (NAc) and lateral ventricles (LV) of both rat strains are reported here. As summarized above, two outstanding and novel 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, especially those concerning hippocampus and amygdala, may be seen as supporting Gray´s theory on the neurobiology of anxiety, in which the septo-hipocampal system, in close interplay with the amygdala, has been proposed as a key neural circuitry underlying (and regulating) the behavioral inhibition system (BIS), which activity would mediate anxiety [5,6]. 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 [54–59]. Hence our results showing that, compared to RHA rats, in the more anxious RLA strain the hippocampus and amygdala are larger, and that NH treatment (which reduces anxiety in the NOE and elevated zero-maze tests, among others; see Río-Alamos et al. 2015, 2017 [12,28]) leads to a decrease in the volume of both regions in RLA rats, are in line with Gray and McNaughton's anxiety model (for a detailed discussion of these results in relation with Gray and McNaughton's model on the neuropsychology of anxiety see Río-Álamos et al. 2017 [28]).

On the other hand, the deficits in PPI (see also [21]), the decreased volume of prefrontal cortex and hippocampus and the markedly enlarged lateral ventricles of RHA rats are reminiscent of a schizophrenia-like phenotypical profile. The PPI and cognitive impairment of this strain [21], as well as the effects of neonatal handling, were further explored in experiment 2. The main findings of this study were the following: (i) Between-strain differences in the NOE test were confirmed, as well as the effects of neonatal handling, particularly in RLA rats of both sexes (Batch 1). (ii) Both sexes of RLA rats showed higher %PPI levels than their RHA counterparts. (iii) Particularly outstanding was that a "strain x NH" effect was observed on %PPI, as NH consistently reduced PPI levels in all the prepulses in RLA rats (both sexes), while the treatment improved %PPI at 70 and/or 75 dB in RHA rats of both sexes (see Batch 1). (iv) RLA rats displayed better spatial reference learning and memory than their RHA

counterparts, i.e. better performance in the place task and the transfer test measured in animals from Batch 2. (v) RHA rats show worse performance in the reversal learning task than their RLA counterparts, which suggests impaired cognitive flexibility of the former strain. (vi) A 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 (see Batch 2). (vii) NH improved working memory (delayed matching-to-place -DMTP- task) overall in both rat strains, with the effect being significant in the RHA rat strain (see Batch 3). (viii) No "strain", NH or "strain x NH" effects were observed in the cued task (Batch 4).

Thus, the between-strain differences and NH effects on the NOE test are in line with results from experiment 1 (see also Río-Alamos et al. 2017 [28]). The superiority of RLA rats regarding PPI, place learning, reversal learning (i.e. cognitive flexibility) and reference memory (i.e. transfer test) replicate previous findings [21,22,29–32]. Sex effects on PPI, showing that males are overall better in this test than females, have also been reported in the Roman rat strains [28].

With regard to NH effects, one possible explanation for the NH-induced PPI impairment in RLA rats in experiment 2 is that the anxiety/fear-reducing effects of NH may have also led to a decrease of "alertness" in RLA-treated rats, which could be related to the decreased hippocampal volume observed after NH in this rat strain in experiment 1 (see for example [6]). In this connection, it has been reported that fear or anxiety facilitate the processing of sensory information due to an increase in the attention to the environment (i.e. alertness), which would be mediated by cortical arousal, a process in which hippocampus is also involved [60,61]. The above contention is supported by several studies. In a human threat-of-shock experiment [62] it was demonstrated that PPI was increased by shock anticipation (i.e. the threat of shock, alertness), suggesting that an increase in the general level of alertness might facilitate processing of the prepulse. In another study, repeated administration of a stressor (restrain stress) induced an increase of PPI in mice, which was attributed to increased alertness [63]. In addition, prenatal stress of pregnant mothers increased PPI of the offspring [64]. On the other hand, the hypervigilant inbred Wistar-Kyoto rats (proposed as a model of vulnerability to anxiety and behavioral inhibition) showed heightened signs of anxiety and improved PPI levels compared to Sprague-Dawley rats [65]. Finally, we have found a positive association between behavioural inhibition and

increased anxiety-related responses (in the open field and elevated zero-maze tests) and PPI levels [66]. Thus it seems safe to conclude that, at least to some extent, higher levels of anxiety/fear might in some conditions facilitate PPI, due to increased alertness.

On the other hand, to the best of our knowledge this is the first time that a positive effect of NH on PPI is reported. NH induced an improvement of PPI in the (PPI-impaired) RHA rat strain, which was evident at two prepulse intensities and in both sexes. This seems a consistent and remarkable finding, given that RHA rats are markedly impaired in this test of sensorimotor gating [20–22], as well as in other attentional (schizophrenia-relevant) processes such as latent inhibition in different paradigms [26,27]. The fact that NH improves cognitive function/performance in rodents (for references see "Introduction"), as has been even shown in RHA and RLA rats [29,31,12,28] and in the DMPT (working memory) task in the present study, might explain the positive effect of the treatment on PPI in RHA rats.

PPI impairments, i.e. sensorimotor gating deficits, are considered an endophenotype of schizophrenia, among other psychiatric disorders [67–70]. RHA rats have shown to display consistent and reliable sensorimotor gating deficits [20,21,23,26], as well as impairments in latent inhibition (another attentional phenotype that has been related to schizophrenia symptoms; see [26,27]. At the neuroanatomical level there is growing evidence that decreased volumes of mPFc and Hc, and enlarged lateral ventricles, are endophenotypes that may be critically involved in schizophrenia [e.g. 46,71–80]. In this context, in rodent models of the disorder hippocampal and prefrontal cortex lesions have been shown to reduce the volume of these structures, and lead to PPI deficits and impairments of executive functions [71,74,81–84] that are also found in schizophrenic patients. Likewise, isolation-reared rats, considered an environmental/neurodevelopmental model of schizophrenia-relevant features, have also been shown to display volume reductions in prefrontal cortex and hippocampus, in addition to enlarged lateral ventricles and deficits in PPI and other cognitive processes [e.g. 22,46,71–74,83,85,86]. Moreover, prefrontal lesions 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 PPI and spatial learning/memory deficits observed in our RHA rats [20,21,87 and references therein].

Of note, compared to their RLA counterparts RHA rats show decreased medial prefrontal cortex and hippocampus volume, as well as markedly enlarged lateral ventricles and PPI and cognitive impairments. This profile of behavioural (attentional, cognitive) and volumetric/neuroanatomical findings in RHA rats fits well with volumetric and cognitive features observed in schizophrenic patients and in various rodent models of the disorder [72,74,75,83,88,89].

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 model (i.e. a MK-801 injection at postnatal day 7 combined with post-weaning isolation) of schizophrenia-related symptoms [83]. In this study, reductions of mPFc, but not in cingulate cortices, were observed in the "double hit" murine model. The reductions in mPFc volume have been associated to a loss of neuropil, consistent with observations of reduced density of both cortical dendritic spines/synapses and hippocampal pyramidal neurons [90,91]. This resembles the reduction of dorsolateral prefrontal cortex volume observed in schizophrenic patients, which is attributed to reduced spine density [92,93].

The volumetric reductions in mPFc and hippocampus observed in our RHA rats, compared to their RLA counterparts, may seem to cohere with the impairment of spatial reference learning/memory and working memory that is usually observed in the former strain [e.g. 21,22,29,31,32]. However, no effect of NH on medial prefrontal cortex was found in the present study, although in the spatial tasks performed with the Morris water maze (in which hippocampus and also mPFc are known to be involved [e.g. 94–96], NH-treated animals showed improvements of working memory and cognitive flexibility (i.e. reversal task). In a previous report we have demonstrated that prepulse inhibition predicts working memory performance in these strains of rats and in genetically heterogeneous rats, showing positive associations between both phenotypes [21]. Here, in Batch 3 we only included a delayed matching-to-place task (DMPT) 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. The finding that NH did not affect mPFc volume in any rat strain (experiment 1), in spite of the cognitive improvements observed (in working memory and in the transfer memory test, in experiment 2), may be connected with the fact that NH treatment is delivered in a developmental time point in which the mPFC region is in a "resting time", contrary to

what happens when rats are submitted to the isolation rearing procedure, which starts after weaning (i.e. when rats are about four weeks old), and in most cases leads to reductions in mPFc volume [85,86]. Thus, further studies are needed to better understand whether changes in mPFC function (perhaps not involving volume alterations) may mediate NH-induced cognitive improvements. Likewise, it remains to be established whether possible NH-induced changes in hippocampal function might be related to these NH positive cognitive effects.

Intrestingly, Schneider & Koch (2005) [74] 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 [71,72,75,89,97]. RHA rats showed a marked increase of lateral ventricle volume compared to RLA animals, in line with the above studies.

In summary, the observed 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 [20,22,23], appears to be consistent with the prevalent vision of the structural abnormalities related to this disease, as this rat strain shows a decrease in the volume of the hippocampus and medial prefrontal cortex, and a marked enlargement of the lateral ventricles, which are paralleled by deficits in sensorimotor gating and spatial learning/memory and cognitive flexibility (i.e. reversal task). Remarkably, NH treatment is able to induce long-lasting changes in hippocampal and amygdala volumes (i.e. reductions in RLA rats), but not in the prefrontal cortex, as well as strain-dependent effects on PPI and on some cognitive (spatial working memory) processes. Whether these NH-induced effects on attentional/cognitive processes are paralleled by (or consequence of) functional changes in some of the studied areas should be matter of further research.

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FIGURE LEGENDS

Figure 1.- Magnetic Resonance Images 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 [53]. The second row shows the images for the RLA strain, and the third row for the RHA strain.

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).

Figure 3.- A) 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). **B)** 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). **C)** 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).

Figure 4.- (A) Latency of time elapsed to begin the exploration of the novel object and **(B)** total time of exploration of the novel object in "NOE" test **(C)** Total percentage of prepulse inhibition in "PPI" test **(D)** Baseline acoustic startle response in the "PPI" test.

Figure 5.- 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).

TABLE LEGENDS

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.

Table 2.- Mean, S.E.M. and Fs (ANOVA) of behavioral and volumetric measures of the *Roman* high -and low-avoidance rats from Experiment 1 are shown. **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. **"ZM-Time",** time spent in open sections in the elevated zero-maze test. **"% PPI"**, total percentage of prepulse inhibition averaged for the four prepulse 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) [28] with permission.

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.

Figures and tables

Figure 1

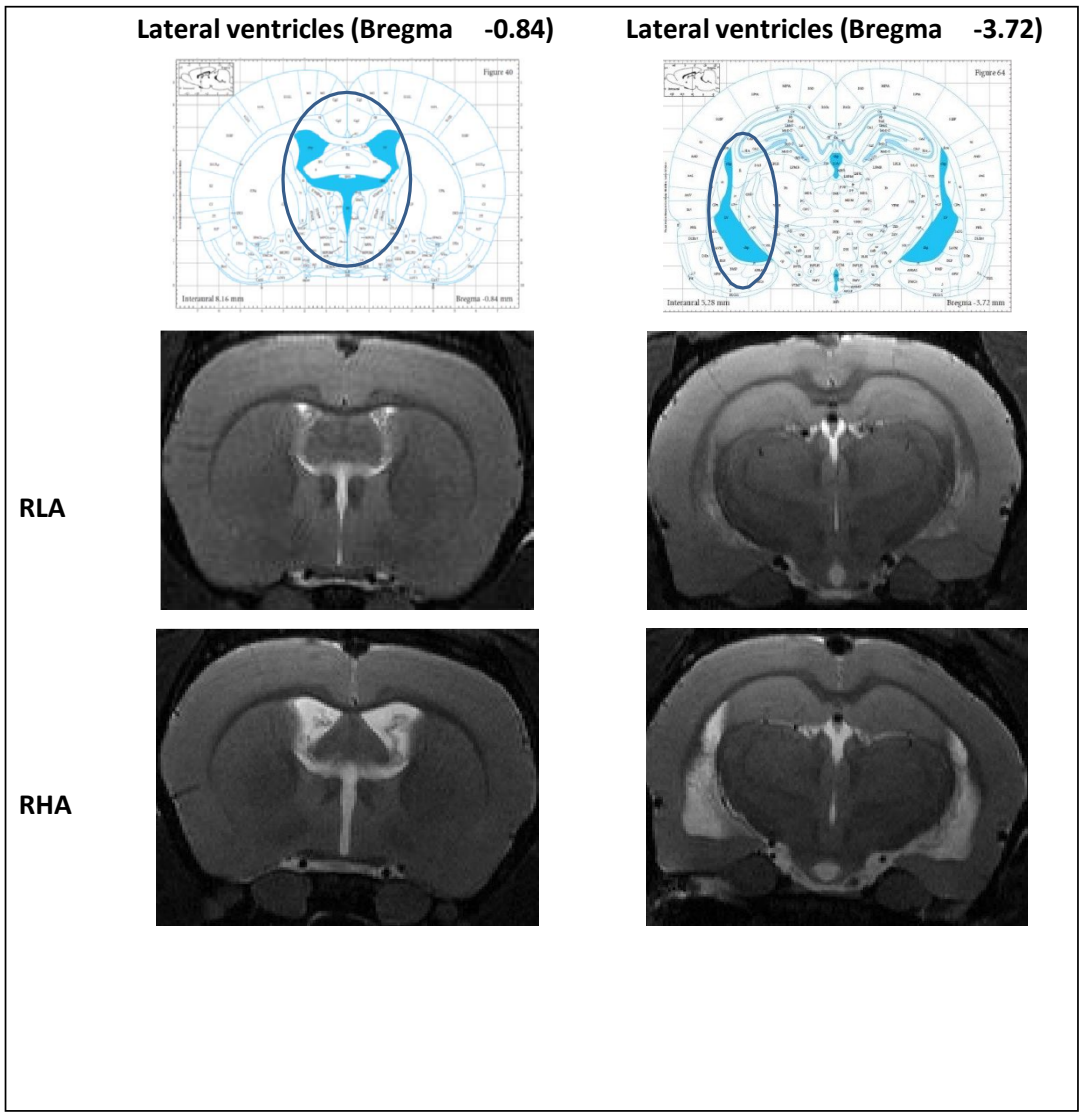
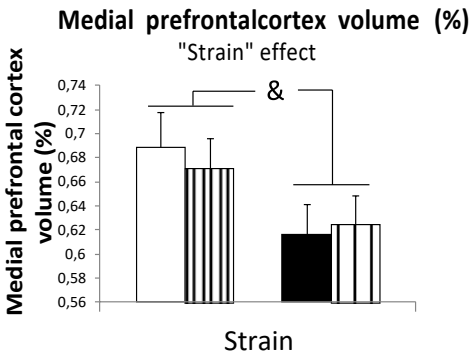


Figure 2

A



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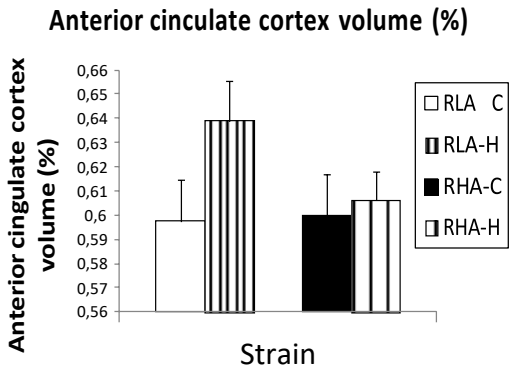

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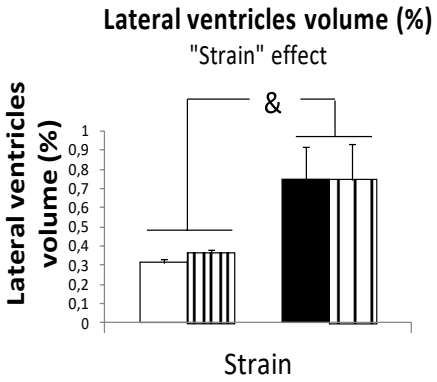

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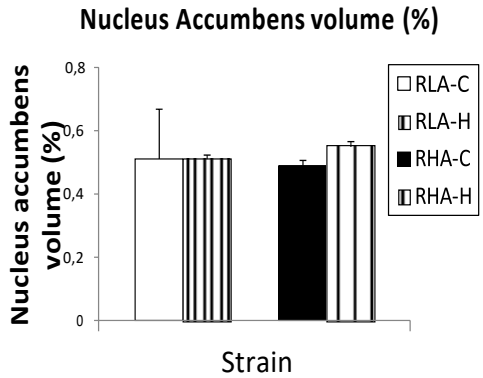
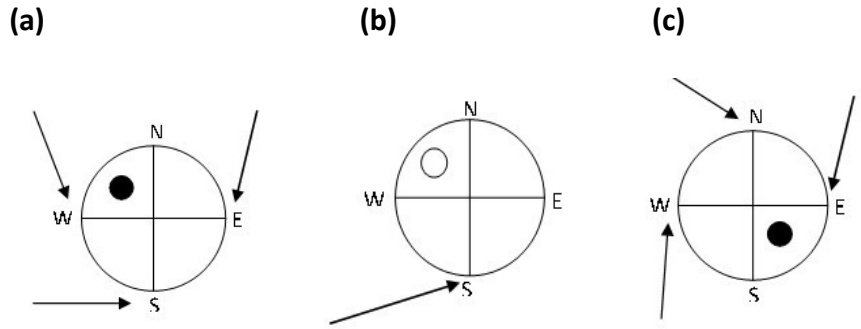


Figure 3

A)



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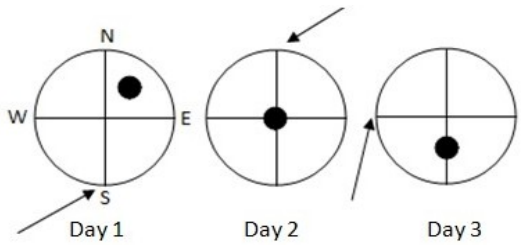

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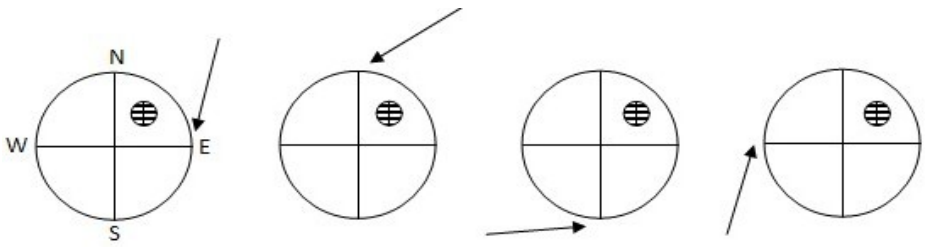
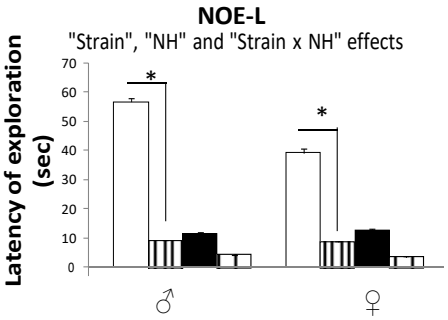


Figure 4

A



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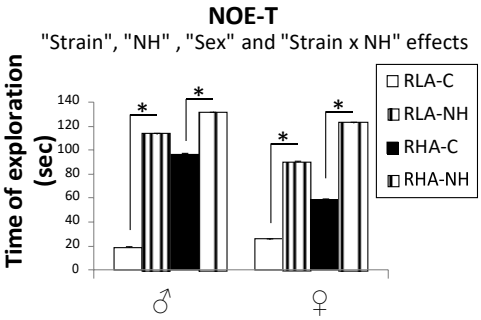

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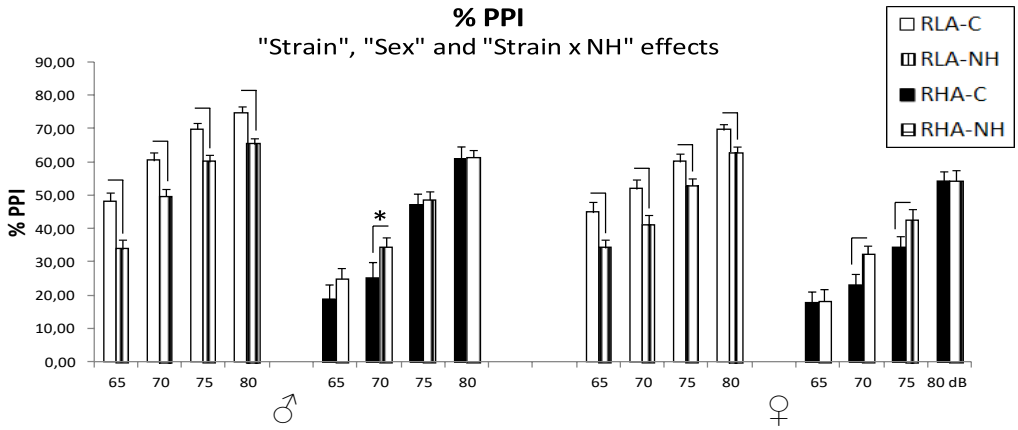

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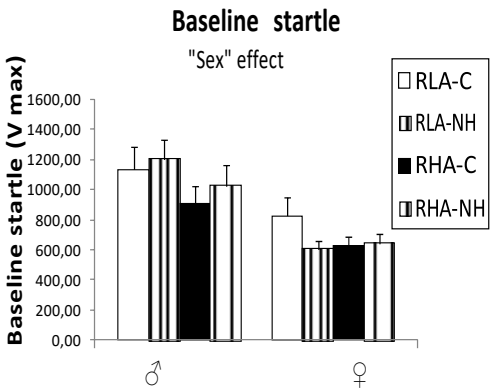
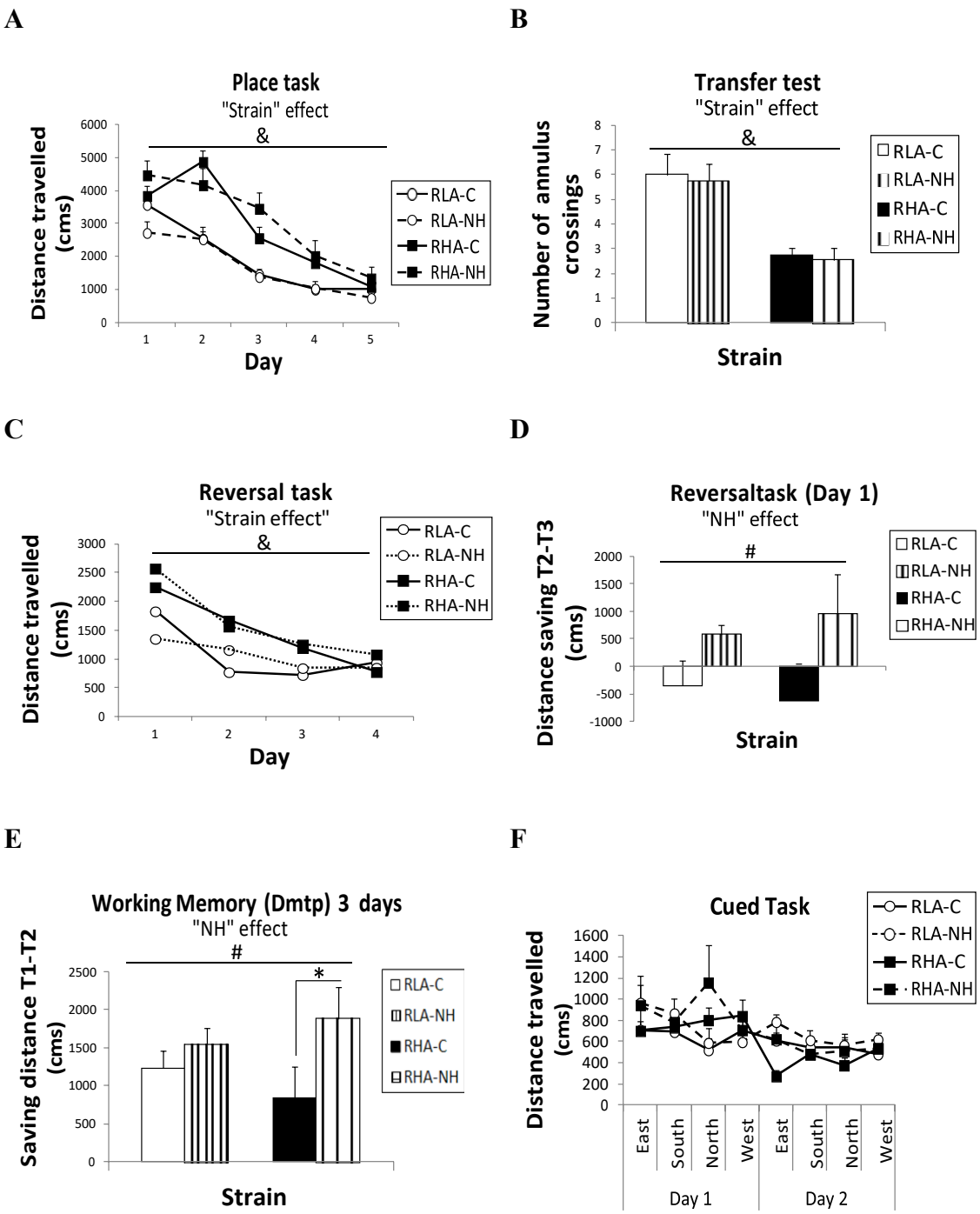


Figure 5


A
Place task
"Strain" effect
&
Distance travelled (cms)
Day
RLA-C
RLA-NH
RHA-C
RHA-NH
B
Transfer test
"Strain" effect
&
Number of annulus crossings
Strain
C
Reversal task
"Strain effect"
&
Distance travelled (cms)
Day
D
Reversaltask (Day 1)
"NH" effect
#
Distance saving T2-T3 (cms)
Strain
E
Working Memory (Dmtp) 3 days
"NH" effect
#
*
Saving distance T1-T2 (cms)
Strain
F
Cued Task
Distance travelled (cms)
East
South
North
West
Day 1
Day 2

Tables

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 2. Behavioral and volumetric 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"**
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
ZM-Time	63,81 (11,66)	92,56 (12,89)	96,19 (8,53)	116,44 (9,69)	**6.75***	**5.12***	0.15
%PPI	59,96 (2,93)	52,39 (4,03)	46,49 (4,83)[a]	45,70 (3,64)	**6.8***	1.1	1.7
BV (mm3)	1875,5 (25,1)	1830,2 (9,3)	1728,8 (45,2)[a]	1678,9 (25,2)	**26.08*** **	2.66	0.006
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

(*) Results taken and adapted from Rio-Alamos et al. (2017), with permission.

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	