

Conservation of phenotypes and possible pre- and/or post-natal environmental influences in the Roman High- and Low-avoidance rat strains after embryo transfer.

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Running head: Conserved traits and pre-/postnatal effects in Roman rats after embryo transfer

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1. ABSTRACT

Roman High- (RHA-I) and Low-Avoidance (RLA-I) rat strains are bi-directionally bred for their good vs. non-acquisition of two-way active avoidance, respectively. They have recently 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-G4, respectively), and compared with generation 60 from our non-SPF colony. Phenotyping included two-way avoidance acquisition, context-conditioned fear, open-field behaviour, novelty-seeking, baseline startle, pre-pulse inhibition (PPI) and stress-induced corticosterone. Post-ET between-strain differences in avoidance acquisition, context-conditioned freezing and novelty-induced self-grooming are conserved. Other behavioural traits (i.e. hole-board head-dipping, novel object exploration, open-field activity, startle, PPI) differentiate the strains at G3-G5 but not at G1, suggesting that pre-/post-natal environment may have influenced these co-selected traits at G1, though further selection pressure (G1-G5) rescues the typical strain-related differences.

Key words: Roman rat strains; embryo transfer; behavioural phenotyping; two-way avoidance; stress-induced corticosterone

2. INTRODUCTION

Selection of *Rattus Norvegicus* for good vs. poor two-way active –shuttle box- avoidance acquisition was originally carried out in Rome and led to the outbred Roman high- (RHA) and low-avoidance (RLA) rat lines (Bignami 1965). From these original lines, the Swiss sublines, RHA/Verh and RLA/Verh (respectively), were derived in Switzerland since 1972 (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 through “brother x sister” mating, are maintained at the Autonomous University of Barcelona since 1997 (Spain; Dr. A. Fernández-Teruel; Escorihuela et al., 1999; Driscoll et al., 2009), while colonies of the outbred RHA/RLA rat lines are maintained at Geneva (Switzerland; Dr. T. Steimer; e.g., Steimer and Driscoll, 2003) and Cagliari (Italy; Dr. Giorgi and Corda; e.g., Giorgi et al., 2007).

Two-way active -shuttle box- avoidance acquisition, the criteria of bidirectional breeding/selection of RHA and RLA strains/lines, has been shown to be inversely related to anxiety, i.e. the higher the anxiety levels the poorer the acquisition of avoidance responses (e.g., Fernández-Teruel et al., 1991; López-Aumatell et al., 2009a; Díaz-Morán et al., 2012, and references therein). Not surprisingly, therefore, the research conducted with RLA and RHA (both from the outbred lines and from the inbred strains) rats over four decades have revealed that anxiety/fearfulness and sensitivity to stress are among the most relevant traits differentiating the two lines/strains. Thus, RLAs are more anxious and/or fearful than their RHA counterparts in both conditioned and unconditioned tests/tasks (e.g., Escorihuela et al., 1999; Steimer and Driscoll, 2003; López- Aumatell et al., 2009a, b; Díaz-Morán et al., 2012). Moreover, RLA rats display enhanced frustration responses following reward

loss (e.g., Rosas et al. 2007) and higher stress-induced HPA-axis and prolactin responses than RHAs (e.g., Steimer and Driscoll, 2003; Carrasco et al., 2008; Díaz-Morán et al., 2012). In summary, compared with RHAs, RLAs rats display increased anxiety, fearfulness, stress sensitivity and a predominantly passive (reactive) coping style when facing situations involving conflict or stress (e.g., Steimer and Driscoll, 2003; Díaz-Morán et al., 2012).

These between-strain differences in coping styles extend also to differential consumption of palatable tastes (e.g. Fernández-Teruel et al., 2002a), vulnerability to addiction (Giorgi et al., 2007), sexual behavior (Sanna et al., 2015) and novelty seeking (Driscoll et al., 2009; Escorihuela et al., 1999). In this regard, both outbred and inbred RHA rats exhibit more novelty seeking responses than their RLA counterparts in several behavioral tests/measures, including head-dipping in the hole-board test, preference for a novel arm in the Y-maze, and preference for novelty introduced in a familiar environment, among others (e.g. Steimer et al., 1998; Escorihuela et al., 1999; Fernández-Teruel et al., 2002a; Estanislau et al. 2013; Manzo et al., 2014; Rio-Álamos et al. 2015).

Del Rio et al. (2014) and Oliveras et al. (2015) have recently proposed that the Roman High- and Low-avoidance rat lines/strains may constitute a valid model of differential schizophrenia-related features. Thus, RHA-I rats present a number of schizophrenia-relevant phenotypes, such as enhanced impulsive behavior in the 5-choice serial reaction time test (5-CSRTT; Moreno et al., 2010; Klein et al., 2014). RHA-I rats also present prepulse inhibition (PPI) and latent inhibition deficits (Oliveras et al. 2015; Esnal et al. 2016), as well as spatial working and reference memory impairments (Oliveras et al., 2015).

At the neurochemical level, the Roman rat strains/lines are known to differ in several systems related to the above mentioned behavioral traits, such as mesocortical DAergic function, glutamate, GABA, opioids, and serotonin systems (Guitart-Masip et al. 2006, 2008; Giorgi et al. 2007; Klein et al. 2014). Similarly, strain-related anatomical and functional differences in several brain structures (relevant for anxiety, stress, addiction, novelty seeking and schizophrenia) have been reported in both outbred and inbred Roman rats, including the amygdala, hippocampus, nucleus accumbens, paraventricular hypothalamus and prefrontal cortex (e.g. Giorgi et al. 2007; Gomez et al. 2009; Meyza et al. 2009; Garcia-Falgueras et al. 2012). Finally, recent studies have identified some genetic loci and molecular pathways that may play a role in the behavioral traits differentiating the Roman lines/strains (Fernandez-Teruel et al. 2002; Sabariego et al. 2011).

The need to move the RHA-I and RLA-I rat strains to a barrier facility prompted us to set up a re-derivation through embryo transfer (ET), in order to eliminate the possible pathogens that were not allowed in the new barrier facility and to establish a specific pathogen-free (SPF) colony of Roman rat strains. Following embryo transfer we evaluated whether some of the most characteristic between-strain phenotypical differences (e.g. in two-way active avoidance acquisition, conditioned fear, anxiety, novelty seeking, sensorimotor gating and stress hormone responses) are preserved in the embryo-transferred generation (G1) and in the 3rd and 5th generations (G3 and G5, already derived from crossing, respectively, the 2nd generation -G2- and the 4th -G4- generations of RHA-I and RLA-I females and males post-embryo transfer), as compared to the non-SPF RHA-I/RLA-I rats (G60) from our regular breeding colony.

The aim of the present study was to evaluate whether the first (G1), third (G3) and fifth (G5) generations post-ET present (compared to G60) the typical between-strain differences in several representative phenotypes, such as 1) two-way -shuttle box- active avoidance acquisition (the criteria of bidirectional selection of the strains), inter-trial crossings and context-conditioned freezing/fear (related to conditioned anxiety and fear; e.g. Escorihuela et al. 1999; Lopez-Aumatell et al. 2009a,b), 2) novel object exploration (behavioral inhibition/disinhibition, curiosity toward a novel/unknown object, related to unlearned anxiety and novelty seeking; e.g. Río-Álamos et al. 2015), 3) open field activity and self-grooming (related to behavioral inhibition/fearfulness in face of a novel/aversive situation; e.g. Escorihuela et al. 1999; Estanislau et al. 2013), 4) hole-board head-dipping (related to novelty seeking; Escorihuela et al. 1999; Estanislau et al. 2013), 5) baseline startle (unconditioned emotional reflex/reactivity) and prepulse inhibition (PPI; sensorimotor gating, a pre-attentional process that is impaired in schizophrenic patients; e.g. Oliveras et al. 2015), and 6) stress-induced corticosterone response (differences in corticosterone levels in baseline conditions and after an acute stress situation in both rat strains; e.g. Díaz-Morán et al. 2012).

We hypothesized that, if genetic factors are the main determinants of the RHA-I vs. RLA-I phenotypic differences in two-way avoidance acquisition (RHA-I > RLA-I), context-conditioned fear (RHA-I < RLA-I), behavioural inhibition/fearfulness under novelty (RHA-I < RLA-I), novelty-induced self-grooming (RHA-I < RLA-I), novelty seeking (RHA-I > RLA-I), startle response (RHA-I < RLA-I), sensorimotor gating (RHA-I < RLA-I) and stress hormone response (RHA-I < RLA-I), then these trait differences should be observed already in the embryo-transferred generation (G1) of RHA-I and RLA-I rats.

3. MATERIAL AND METHODS

3.1. *Embryo Transfer (ET)*

OFA (Sprague Dawley) rats from a colony in our facility were used to set-up the rat ET procedure. Eight to 16 weeks old females naturally mated, after determination of their oestrus cycle phase by measure of vaginal impedance with an estrogenic monitor (model MK-11, Bionic Iberica SA, Spain), with 2 to 6 month old males were used as embryo donors. One day later, pseudo-pregnant females were obtained after mating with 2, 5 to 6 month old vasectomized males. Positive matings were detected by checking the presence of spermatozoa in the vaginal smear. In the preliminary study, embryos were collected the day after mating and incubated overnight in KSOM medium (EmbryoMax® KSOM Medium (1X) w/ 1/2 Amino Acids & Phenol Red, Merck Chemicals and Life Science SA, Spain). Only 2-cell embryos were transferred to pseudopregnant females. During the real transfers, embryos were collected 48 hours after mating. Females were euthanatized by cervical dislocation after anesthetizing with isoflurane. The abdominal cavity was opened by a transverse incision, the uterine horn was located and a cut was made between the oviduct and ovary and then through the uterus near the oviduct. The oviduct, with some adjacent uterine and ovarian tissue was then transferred into a 35-mm dish with M2 (EmbryoMax M2 Medium kit with phenol red, Merck Chemicals and Life Science SA, Spain) where the embryos were released tearing the oviduct. Obtained embryos were washed in EmbryoMax M2 medium 10 times before and another 10 times after introducing the embryos into the barrier.

To transfer RHA-I and RLA-I embryos, natural matings were set-up in the original colony. Oviducts in M2 were transported to the barrier facility in less than 20 minutes. There, embryos were recovered and washed 5 to 10 times in M2, introduced through the barrier in a Petri dish in 100µl drops covered by mineral oil. Once inside, embryos were washed another 5 to 10 times and transferred to a pseudo-pregnant female. During the surgical procedure inhalatory anaesthesia was used. A dorsal incision was done in each rat to reach the left ovary. A drop of epinefrine was used to minimize bleeding when opening the bursa ovarica and embryos were placed in the oviduct through the infundibulum of the Fallopian tube. The three layers of the surgical wound (muscular, subcutaneous and skin) were closed separately. The recipient females were maintained in pairs during the first two weeks after the embryo transfer procedure.

3.2. Experimental subjects

The animals used were males and females of the inbred Roman High- (RHA-I) and Low-Avoidance (RLA-I) rat strains (n= 112 and n=123, respectively; see “n” for each generation and sex below and in the “Results” section) from our colonies at the Autonomous University of Barcelona (Medical Psychology Unit, Department of Psychiatry and Forensic Medicine) since 1997. They were housed in same-sexed pairs and were maintained with food and water freely available (*ad libitum*), with a 12:12h light-dark cycle (lights on at 0800h) and controlled temperature ($22 \pm 2^{\circ}\text{C}$) and humidity (50-70%). Four generations of RHA-I and RLA-I were used in the experiments: G1, generation 1 after ET, born from

Sprague-Dawley mothers (RHA-I, n=14; RLA-I, n=16; both sexes); G3, generation 3 after ET, born from crossing ♀RHA-I x ♂RHA-I and ♀RLA-I x ♂RLA-I from generation 2 (RHA-I, n=46; RLA-I, n=46, both sexes); G5, generation 5 after ET, born from crossing ♀RHA-I x ♂RHA-I and ♀RLA-I x ♂RLA-I from generation 4 (RHA-I, n=10; RLA-I, n=11; males); G60, generation 60 from the regular non-SPF RHA-I/RLA-I strains maintained at our facility (without barrier) since 1997 (RHA-I, n=42; RLA-I, n=50; both sexes) (see below the exact “n” for each group –strain and sex- in the respective “Results” tables).

Within each generation, each experimental group was composed of rats from 7-10 different litters. Rats were approximately 2 months old at the beginning of testing in the “novel object exploration test” (see below) and 3 months old at the beginning of the remaining behavioral tests (i.e. open field, hole board, prepulse inhibition of startle, two-way –shuttle box- avoidance; see below). Interval between consecutive behavioral tests was 2-4 days.

Baseline and stress-induced corticosterone (see below) were measured in naive 5-month-old males from G3 (RHA-I, n=10; RLA-I, n=10) and G60 (RHA-I, n=12; RLA-I, n=12).

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

3.3. Tests

In this study we carried out a battery of tests to all the different groups of RLA-I and RHA-I in order to characterize the main phenotypes of the strains after ET. Each animal from G1, G3 and G5 was submitted to all behavioral tests in the order described below, whereas one sample of RHA-I/RLA-I rats from G60 performed (consecutively) NOE, PPI and shuttle box tests/tasks and two samples (RHA-I/RLA-I) from G60 were used separately for the open field and hole-board tests.

The different generations of animals (G1, G3, G5, G60) were not tested in parallel (i.e. the present is not a transversal design with “generation” as an independent factor) but in different periods from middle 2015 to the end of 2016. The only exception was corticosterone measurements, for which samples of G3 and G60 RHA-I and RLA-I male rats were simultaneously submitted to the procedure.

Experimental procedures within each generation were conducted counterbalancing strain and sex across time of the day and across days of testing.

Test 1: Novel object exploration test (NOE)

The test evaluated the exploratory response when a novel/unknown object was introduced in their home cage. The food was removed from the home cage before the test was started. One hour later, the unknown object (a graphite pencil Staendtl Noris, HB n°2) was perpendicularly

introduced in their cage through the grid cover, until the made contact with the cage bedding. To facilitate observation of the test each cage was pulled from the rack about 15cm, allow to score the latency of the first exploration and the total time exploring the novel object.

Test 2: Open Field Test (OF)

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

Test 3: Hole-Board Test (HT)

The apparatus was square box made of a white wood (66 x 66 x 47 cm) and containing four equidistant holes (diameter: 3.7 cm) on the floor. A novel object (rubber sphere, diameter: 3.5 cm) was placed in a container bellow each hole. The total number of head dips and the time spent head-dipping were measured during the first five minutes for each rat.

OFT and HBT were conducted in a black room which was illuminated with white fluorescent lights. Previously to the session and between trials, the apparatus was always cleaned with ethanol solution (5%) and dried with paper towel.

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22 *Test 4: Baseline startle and pre-pulse inhibition (PPI) of the acoustic startle response*
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26 Four sound-attenuated boxes (SR-Lab Startle Response System, San Diego Instruments, San Diego, USA) illuminated (10W) were used (90 x 55
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28 x 60 cm). Each box consists in a transparent Plexiglas cylinder (25 cm length and 8.2 cm diameter) on the platform with a sensor that detects the
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30 strength made in each trial of the test by the rat. The startle response was transduced by a piezoelectric accelerometer (Cibertec S.A. Madrid) into
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32 a voltage which was amplified, digitized and saved for analysis. Two speakers mounted 15 cm from each side of the cylinder provide the
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34 acoustic stimuli and a white noise generator provided background noise (55 dB). The session starts with 5 min of habituation in the apparatus.
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36 Then 10 “pulse-alone” trials (105 dB, 40 ms) are delivered in order to obtain a basal measure of the acoustic startle response (ASR). After this,
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38 each one of the 6 different types of trials are randomly administered 10 times (60 trials in total):
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42 • Pulse-alone trial (105 dB, 40 ms),
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45 • Prepulse 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
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48 • Non stimulus trial (only with the background noise 55 dB)
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52 The intervals between the trails were 10-20 s with a mean of 15 s. The startle response amplitude was defined as the maximum accelerometer
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54 voltage during the first 200 ms after the onset of the pulse.
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The degree of PPI (in percentage) is calculated according to the formula: $\%PPI = 100 - [(startle\ amplitude\ or\ prepulse\ trials / startle\ amplitude\ on\ pulse\ trials) \times 100]$

Test 5: Two-Way Active (Shuttle box) Avoidance Acquisition (SHAV)

The experiment was carried out with three shuttle boxes (Leticia, 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 box. The experimental room was kept dark. The shuttle boxes consisted of two equal sized compartments (25 x 25 x 28 cm), connected by an opening (8 x 10 cm). Training consisted of a single 40-trial sessions. A 2400-Hz, 63 dB tone pulse 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 stimulus) elapsed before training commenced. Each of the 40 trials consisted of a 10 s CS, followed by a 20 s US. The CS or US was terminated when the animal crossed to the other compartment, with crossing during the CS being considered as an avoidance response and 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 crossing (ITC) were scored within each block of trials. Freezing behavior, defined as the complete absence of movements except for breathing, was also scored during the 60 s ITI of trials 2-5 as an index of context-conditioned fear.

Test 6: Corticosterone extraction in basal and post-stress conditions

Both rat strains, from G3 and G60, were evaluated for their pre- and post-stress hormone responses at 5 months of age. Blood samples were taken (between 9:30 and 12:30 h) 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 x 40 cm x 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 cage in the animal room after each blood sampling. The tail-nick consisted of gently wrapping the animals with a cloth, making a 2 mm incision at the end of the tail arteries and then massaging the tail while collecting approximately 200 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 immune sorbent assay (competitive ELISA), determined plasma corticosterone levels. EMS Reader MF V.2.9-0 was the reader make used. Corticosterone EIA (Immunodiagnostic System Ltd, IDS Ltd; Boldon, Unnited Kingdom) was the corticosterone reagent used. Finally, the Immute® 1000 (Siemens) was the apparatus used to obtain the results.

3.4. Statistical analyses

ANOVAs “2 strains x 2 sexes” were applied to all behavioral variables within (separately for) each generation, except those where there was only one sex, in which case Student’s t-test was used. For corticosterone analysis, a repeated measures factorial ANOVA (“2 strains x 2 generations x pre-/post-stress”) was applied, followed by independent Student’s t-tests within each generation because we had a directed hypothesis that within each generation RLA-I rats should display higher stress-induced corticosterone levels than RHA-I rats.

4. RESULTS

4.1 Results of Embryo Transfer

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

4.2. G60 generation: typical differences between RHA-I and RLA-I rats

Test 1: “Novel object exploration” test (NOE)

The results of the NOE test are shown in **Table II**. ANOVA (“2 x 2”) showed a “strain” effect on “latency” to explore the novel object [“strain” effect; $F_{(1,30)} = 9.1$, $p < 0.01$], indicating that RLA-I rats lasted longer to initiate exploration of the object than RHA-I rats. “Strain”, “gender” and “strain x gender” effects were observed on “total time” exploring the novel object [“strain” effect, $F_{(1,30)} = 110.7$, $p < 0.001$; “gender” effect; $F_{(1,30)} = 16.7$, $p < 0.001$; “strain x gender” interaction; $F_{(1,30)} = 21.6$, $p < 0.001$] showing that RHA-I rats (i.e. “strain” effect) and males (i.e. “gender” effect) spent more time exploring the novel object than RLA-I rats and females respectively.

Test 2: “Open field” test (OF)

The results of the OF test are shown in **Table II**. Student’s t-test showed significant differences between both strains in number of “crossings” and total “time spent grooming” [$t_{(13)} = 6.8$, $p < 0.001$; $t_{(13)} = 8.6$, $p < 0.001$, respectively], indicating that RLA-I rats performed less crossings and spent longer time grooming than RHA-I rats.

Test 3: “Hole-board” test (HB)

Table III shows the results from the hole-board test. Student’s t-test showed significant between-strain differences in the total number of “head dips” and total “time spent head dipping” [$t_{(17)} = 5.8$, $p < 0.001$; $t_{(17)} = 8.2$, $p < 0.001$, respectively], as RHA-I rats displayed overall higher number of head dips and spent more time head-dipping than the RLA-I strain.

Test 4: “Prepulse inhibition” test (PPI)

ANOVA (“2 x 2”) showed “strain” effect in almost all the prepulse intensities [“strain” effect; PPI65, $F_{(1,30)} = 10.5$, $p < 0.01$, PPI70, $F_{(1,30)} = 28.7$, $p < 0.001$; PPI75, $F_{(1,30)} = 9.7$, $p < 0.01$] (see **Table IV**), indicating that RLA-I rats display significantly more prepulse inhibition than RHA-Is.

“Gender” effects were observed in all the prepulse intensities of the PPI test [“Gender” effect; PPI65, $F_{(1,30)} = 8.0$, $p < 0.01$; PPI70, $F_{(1,30)} = 18.3$, $p < 0.001$; PPI75, $F_{(1,30)} = 13.8$, $p < 0.001$; PPI80, $F_{(1,30)} = 11.6$, $p < 0.01$], as males showed higher PPI levels than female rats.

Results of the “baseline startle” response and “%PPI total” are shown in **Table V**. ANOVA showed a “strain” effect on “baseline startle” [“Strain” effect; $F_{(1,30)} = 6.6$, $p < 0.05$] indicating higher basal acoustic startle response in the RLA-I compared to RHA-I rats. “Strain” and “gender” effects were also observed on “%PPI total” [“Strain” effect; $F_{(1,30)} = 14.1$, $p < 0.001$; “Gender” effect, $F_{(1,30)} = 18.9$, $p < 0.001$], with RLA-I (i.e. “strain” effects) and males (i.e. “gender” effect) showing higher %PPI levels than RHA-I rats and females, respectively.

Test 5: “Two-way active (shuttle box) avoidance acquisition” (SHAV)

Table VI shows SHAV results. 2 x 2 ANOVA showed that RHA-Is performed more avoidances and inter-trial crossings than RLA-I rats [“strain” effect; $F_{(1,30)} = 220.7$, $p < 0.001$; $F_{(1,30)} = 50.1$, $p < 0.001$, respectively]. A Student t-test was used to assess between-strain differences in “time spent freezing” [Student t-test, $t_{(13)} = 6.48$, $p < 0.001$], showing that RLA-I rats spent significantly longer time freezing (an index of context-conditioned fear) than RHA-I rats.

4.3. Evolution of between-strain differences across G1, G3 and G5

Test 1: “Novel object exploration” test (NOE)

Results on “latency” to explore for the first time the novel object showed a “strain” effect in all generations [“strain” effect, G1, $F_{(1,26)}=5.4$, $p<0.01$; G3, $F_{(1,67)}=13.3$, $p<0.05$; G5, $t_{(19)}=2.3$, $p<0.035$], as RLA-I animals presented higher “latency” to explore for the first time the novel object compared to RHA-I rats. “Gender” effect was also observed in G3 (“Gender” effect, $F_{(1,67)}=4.2$, $p<0.05$), indicating that females spent overall less time exploring the novel object than males. Results on “total time” exploring the novel object showed a “strain” effect in G3 [“strain” effect, G3, $F_{(1,67)}=24.9$, $p<0.001$], as RHA-I rats spent longer time than RLA-Is exploring the object, but no significant effects were observed in G1 nor G5 (Table II).

Test 2: “Open field” test (OF)

The results of the OF test (Table II) showed a “strain” effect in G3 and G5 on the number of “crossings” [“strain” effect, G3, $F_{(1,67)}=8.1$, $p<0.01$; G5, $t_{(19)}=5.5$, $p<0.001$], as RHA-I showed higher ambulation than RLA-I. No differences were observed in G1. “Gender” effects in G1 and G3 [“gender” effect, $F_{(1,26)}=20.3$, $p<0.001$; $F_{(1,67)}=4.5$, $p<0.05$; respectively] indicate that females display higher number of “crossings” than male rats.

Regarding self-grooming behavior, results showed “strain” effects on “latency to start grooming” in all generations [“strain” effect; G1, $F_{(1,26)}=7.0$, $p<0.05$; G3, $F_{(1,68)}=41.9$, $p<0.001$; G5, $t_{(19)}=3.9$, $p<0.001$], as well as on “time spent grooming” [“strain” effect; G1, $F_{(1,26)}=11.8$, $p<0.01$; G3, $F_{(1,68)}=34.4$, $p<0.001$; G5, $t_{(19)}=5.8$, $p<0.001$], indicating that RLA-I rats started sooner and spent more time self-grooming than RHA-I rats. A “gender” effect was also observed in the “time spent grooming” in G1 [“gender” effect; $F_{(1,26)}=6.7$, $p<0.05$], as males showed higher grooming time than female rats.

In summary, in the OF test there were clear “strain” effects on “latency to start grooming” and on “time spent grooming” already in G1, G3 and G5, with the RLA-I rats groups performing more time grooming and shorter latency to start it than the RHA-I rats. Nevertheless, there was no significant “strain” effect on “crossings” in G1 (after embryo transfer), but such a “strain” effect is rescued in G3 and continues on G5.

Test 3: “Hole-board” test (HB)

Results of the HB test are shown in **Table III**. Two-way ANOVA showed “strain” effects in G3 and G5, with RHA-I rats groups performing more “head dips” than their RLA-I counterparts [“strain” effect; G3, $F_{(1,68)}=6.1$, $p<0.05$; G5, $t_{(19)}=3.3$, $p<0.005$]. “Gender” effect was also observed in G3 [“Gender” effect; $F_{(1,68)}=5.1$, $p<0.05$], as females showed more head-dips than male rats. No “strain” effect was observed in G1 on this variable.

A “strain” effect was observed on “time spent head dipping” in G5, indicating that RHA-I spent more time head-dipping than RLA-I rats [“strain” effect; G5, $t_{(19)}=3.2$, $p<0.008$]. “Gender” effects were also observed on “time spent head dipping” in G1 and G3, overall showing that females spent more time head-dipping than the males in both generations [“gender” effect; G1, $F_{(1,28)}=7.3$, $p<0.05$; G3, $F_{(1,68)}=15.4$, $p<0.001$].

Test 4: “Prepulse inhibition” test (PPI)

The %PPI for each experimental group and prepulse intensity is represented in **Table IV**. The two-way ANOVA revealed a significant “strain” effect in G1 only for the “%PPI65” intensity [“strain” effect; $F_{(1,28)}=5.8$, $p<0.05$]. “Strain” effects on all the prepulse intensities were observed in G3 [65, 70, 75 and 80 dB, “strain” effect; $F_{(1,68)}=11.1$, $p<0.001$; $F_{(1,68)}=15.6$, $p<0.001$; $F_{(1,68)}=13.6$, $p<0.001$; $F_{(1,68)}=8.0$, $p<0.01$; respectively], while in G5 “strain” effects were observed in prepulses 65,70 and 75 dB [“strain” effect; $t_{(19)}=3.1$, $p<0.005$; $t_{(19)}=3.0$, $p<0.006$; $t_{(19)}=3.0$, $p<0.006$; respectively], in all cases indicating that RLA-I showed higher PPI levels than RHA-I rats.

A significant “strain” effect was also observed for “baseline startle” (**Table V**) in G3 [“strain” effect; $F_{(1,68)}=14.2$, $p<0.001$], as RLA-I showed higher “baseline startle” response than RHA-I rats. “Gender” effect on “baseline startle” was observed in G1 and G3 [“Gender” effect; G1, $F_{(1,28)}=4.6$, $p<0.05$; G3, $F_{(1,68)}=11.3$, $p<0.001$], with females showing decreased “baseline startle” response compared to male rats.

ANOVA for the “total %PPI” revealed a significant “strain” effect in G3 and G5 but not in G1 [“Strain” effect; G3, $F_{(1,68)} = 15.9$, $p < 0.001$; G5, $t_{(19)} = 3.3$, $p < 0.004$].

In summary, there were “strain” effects on “total %PPI” in G3 and G5 indicating that RHA-I rats display impaired PPI as compared to RLA-I rats, but such a “strain” effect was not observed in G1.

Test5: “Two-way active (Shuttle box) avoidance acquisition” (SHAV)

Table VI shows the results of two-way active (shuttle box) avoidance acquisition. The ANOVA applied to the results showed the expected “strain” effect on avoidances, as RHA-I rats performed significantly more “avoidance” responses than RLA-I rats in all generations –G1, G3 and G5- [“strain” effect; G1, $F_{(1,33)} = 133.4$, $p < 0.001$; G3, $F_{(1,68)} = 103.7$, $p < 0.001$; G5, $t_{(19)} = 8.9$, $p < 0.001$].

A “strain” effect on “time spent freezing” was observed in all generations, showing that RLA-Is spent longer time freezing than RHA-I rats [“strain” effect; G1, $F_{(1,33)} = 20.1$, $p < 0.001$; G3, $F_{(1,68)} = 118.2$, $p < 0.001$; G5, $t_{(19)} = 8.8$, $p < 0.001$]. There were also “gender” and “strain x gender” effects in G3 [“gender” effect; $F_{(1,68)} = 5.2$, $p < 0.001$; “strain x gender” interaction; $F_{(1,68)} = 6.1$, $p < 0.05$], with females spending more time freezing than male rats.

“Strain” effects were observed on “inter-trial crossing” in all generations [“strain” effect; G1, $F_{(1,31)} = 32.7$, $p < 0.001$; G3, $F_{(1,66)} = 31.4$, $p < 0.001$; G5, $t_{(19)} = 4.4$, $p < 0.005$], as RHA-Is showed more inter-trial crossings than RLA-I rats. There was also a “gender” effect on this parameter in G1 [“gender” effect; $F_{(1,31)} = 4.4$, $p < 0.05$].

In summary, in the two-way active avoidance task, and in all -G1, G3 and G5- generations post-embryo transfer and both sexes (as well as in G60, see above), we observed clear and consistent “strain” effects on all the variables that are related with the criteria of selection of the Roman strains: RLA-I rats show, compared to RHA-I rats, much impaired avoidance acquisition, less inter-trial crossing activity and more context-conditioned fear/freezing.

4.3. Corticosterone comparison in basal and post-stress condition between G3 and G60

Figure 1 shows corticosterone results during basal and post-stress conditions. Repeated measures factorial ANOVA revealed “Strain” [$F(1,40) = 44.3$, $p < 0.001$], “Strain x generation” [$F(1,40) = 8.7$, $p = 0.005$] and “Strain x generation x pre-/post-stress” [$F(1,40) = 13.9$, $p = 0.001$] effects on corticosterone levels. Further Student’s t-tests within each generation showed that post-stress corticosterone was higher in RLA-I rats than in their RHA-I counterparts both at G3 ($t(18) = 8.1$, $p < 0.001$) and G60 ($t(22) = 3.7$, $p < 0.002$). In summary, both rat strains are not different in basal corticosterone levels, but following stress RLA-I rats display higher levels of corticosterone than RHA-Is in both generations.

5. DISCUSSION

The present work shows a comparative study of some of the basic behavioral and hormonal profiles of three generations of RHA-I and RLA-I rats after ET, the G1 (born from Sprague Dawley after ET), the G3 (born from RHA-I and RLA-I of G2) and the G5 (born from RHA-I and RLA-I of G4), in relation with the typical differences between both strains as evaluated in a sample from the 60th generation (G60, from our regular non-SPF breeding colony).

First of all, it is important to say that the ET procedure was successful, because the rats of G1, G3 and G5, are, from the analytical/health point of view (data not shown), free of pathogens. This was the purpose of ET procedure, to establish a new, pathogen free RHA-I/RLA-I breeding colony into a barrier (SPF) facility.

5.1. G60: typical phenotypic differences between the Roman rat strains

Secondly, the RHA-I and RLA-I from G60 display the typical profiles of both strains, presenting clear cut differences in anxiety/fearfulness (RLA-I > RHA-I), behavioral disinhibition (RHA-I > RLA-I), novelty seeking (RHA-I > RLA-I), sensorimotor gating (RLA-I > RHA-I) and stress-induced corticosterone response (RLA-I > RHA-I). Thus, with regard to conditioned anxiety/fear-related responses, we observed that RHA-I rats made many more avoidances and inter-trial crossings (ITCs) than RLA-Is, whereas the former showed less context-conditioned

freezing responses -i.e. less context-conditioned fear- than the latter. These were expected results, and are in complete agreement with previous studies from our laboratory and others which consistently indicate that, other than showing very good two-way avoidance acquisition (the selection criterion for RHA-I rats) the RHA-I strain also displays less conditioned fear (as evidenced by conditioned freezing) than RLA-I rats (Díaz-Moran et al. 2012; Driscoll et al. 1998, 2009; Escorihuela et al. 1999; López-Aumatell et al. 2009a; Río-Álamos et al. 2015; Steimer and Driscoll 2003). As concerns to responses related to unconditioned anxiety (or emotional reactivity, as in the case of baseline startle), the present findings are also consistent with previous reports, indicating that RHA-I rats (compared to their RLA-I counterparts) show reduced self-grooming behavior and are more active -i.e. less behaviorally inhibited by novelty- in the open field, whereas they also present lower baseline startle responses than RLA-I rats (Escorihuela et al. 1999; Estanislau et al. 2013; López-Aumatell et al. 2009a,b; Río-Álamos et al. 2015; Steimer and Driscoll 2003).

Furthermore, regarding novelty seeking, the typical differences between the strains are also observed, as reflected by increased head-dipping and exploration of the novel object by RHA-I rats in the HB and NOE tests, respectively (Escorihuela et al. 1999, Estanislau et al. 2012; Manzo et al. 2014; Río-Álamos et al. 2015). The differences in PPI between both rat strains (RLA>RHA) in G60 are consistent with previous reports, indicating that RHA-I rats present a deficit in sensorimotor gating and other attentional processes, and thus this strain may be a model of some schizophrenia-relevant symptoms (Esnaol et al. 2016; Del Río et al. 2014; Oliveras et al. 2015).

Finally, the between-strain differences in post-stress corticosterone levels (RLA-I > RHA-I) also replicate previous works, which consistently indicate that RLA-I rats are more sensitive to stress and show greater endocrine responses to stressful conditions (corticosterone, ACTH, prolactin) than their RHA-I counterparts (Carrasco et al. 2008; Díaz-Moran et al. 2012; Steimer and Driscoll 2003; Steimer et al. 1998; for reviews see Driscoll and Bättig 1982; Driscoll et al. 1998, 2009).

5.2. Comparison of results from G1 and G3 and G5, and possible pre- and/or postnatal environmental influences

Already in G1 (the embryo transferred generation) we observed typical strain-related phenotypic characteristics related with the selection trait, i.e. two-way active avoidance responses, inter-trial crossings and conditioned fear (freezing) behavior (e.g. Castanon et al. 1995; Driscoll and Bättig 1982; Diaz-Morán et al. 2012; Río-Álamos et al. 2015). Behavioral and behavioral genetic studies have indicated that these three behavioral responses are related and have high heritability, i.e. they are strongly linked to strain-related genetic factors (e.g. Castanon et al. 1995; see also Driscoll and Bättig 1982, Díaz-Morán et al. 2012, and references therein). Therefore, the present results agree with these studies, by showing that in spite of the different pre- and postnatal maternal environment in G1 (with respect to G3, G5 or G60), RHA-I and RLA-I rats born from Sprague-Dawley mothers already show the typical between-strain differences in the three above mentioned phenotypes, thus suggesting that such characteristic (genetically-based) traits are not sensitive to changes in maternal environment (see also Castanon et al. 1995; Driscoll and

Bättig 1982). This is also consistent with findings from Driscoll and Bättig (1982), who carried out a cross-fostering study with the swiss sublines of Roman rats (from which the present RHA-I and RLA-I strains were derived) and reported no foster-mother influences on two-way avoidance behavior nor on freezing responses.

However, several other phenotypes -i.e. open field activity, NOE test behavior, baseline startle, PPI- which have been found to be co-selected in (and divergent between) the Roman strains (as also shown by the present results from the G60; see above), seem to be more influenced by the pre- and/or postnatal environment. In fact, in G1 there are no significant between-strain differences in measures related with anxiety/fearfulness and emotional reactivity, such as the number of crossings in the OF test and baseline startle, nor in measures related with novelty seeking like head-dipping in the HB test and the time exploring the novel object in the NOE test. Likewise, PPI levels are not different between the strains at G1. The absence of between-strain differences in these phenotypes in G1 suggests that these traits may be less linked/correlated to genetic factors (compared to the two-way avoidance, inter-trial crossings and conditioned freezing phenotypes), and that there may be some pre- and/or postnatal (possibly maternal) influences that directly affect or modulate such behaviors/responses at least in the first generation post-embryo transfer (G1). Importantly, however, between-strain differences in near all the above mentioned co-selected phenotypes are observed in G3 and G5 (with similar magnitudes as those seen in G60), thus suggesting that the possible environmental influences that are responsible for the absence of differences at G1 are in some way overcome through further generations of selective breeding (i.e. through further selection pressure). Remarkably also, the stress-induced corticosterone response in G3 was much higher in RLA-I than in RHA-I rats (this measure was not taken in

G1 nor G5 because of breeding limitations), in agreement with the typical difference observed between the strains in previous studies and with the results from G60 (e.g. see Carrasco et al. 2008; Díaz-Morán et al. 2012; Steimer et al. 1998; Steimer and Driscoll 2003).

It is worth to point out that, at variance with the above mentioned co-selected traits that may have been influenced by pre-/postnatal environment (and do not show between-strain differences at G1), self-grooming levels (in the OF test), another co-selected phenotype that typically differs between the Roman lines/strains (RHA<RLA, as also observed here in G60; e.g. Estanislau et al. 2013; Steimer et al. 1998; Steimer and Driscoll 2003), is well conserved after embryo transfer in G1. Thus, self-grooming behavior differs between RHA-I and RLA-I at G1 (RHA-I < RLA-I), indicating that this displacement or de-arousal activity (Fernandez-Teruel and Estanislau 2016; Kalueff et al. 2016), induced by the anxiety/neophobia (or arousal) involved in the stressful OF situation, is reduced in RHA-I as compared to RLA-I rats (Estanislau et al. 2013; Steimer et al. 1998; Steimer and Driscoll 2003). Thus, it is a striking finding that self-grooming responds to the selection without being influenced by the change in pre-/postnatal environment (i.e. embryo transfer, Sprague-Dawley mothers), which may suggest that this behavior is more strongly genetically linked/correlated to the phenotypic criteria of selection of the Roman strains (i.e. two-way avoidance responses, inter-trial crossings and freezing) than the other co-selected traits mentioned above.

Self-grooming in rodents is a very complex, innate, repetitive, self-directed and sequential (cephalocaudal progression) behavior which is evolutionarily well conserved. It has a variety of functions, such as hygiene, social communication, thermoregulation and de-arousal (Kalueff et al. 2016). In addition, there is evidence indicating high sensitivity of self-grooming (and self-grooming sequencing) to genetic influences

(Kalueff et al. 2016; see also Greer and Capecchi 2002), and it has been shown that self-grooming can be bidirectionally affected by psychological stress (Fernandez-Teruel and Estanislau 2016; Kalueff et al. 2016). These factors, jointly with the fact that grooming is modulated by limbic circuitry (including hypothalamus and amygdala) and that previous anatomical studies show clear differences in these regions between the Roman rat strains (e.g. Carrasco et al. 2008; Garcia-Falgueras et al. 2012; Gomez et al. 2009; Meyza et al. 2009), might suggest that novelty-induced self-grooming is genetically correlated with the selection traits in these strains, and this could be related to the conservation of self-grooming in spite of embryo transfer and changes in pre-/post-natal (maternal) environmental influences.

These remain, however, speculative suggestions, given that the present study design cannot provide definitive answers neither to the degree of maternal pre-/post-natal influences on some co-selected traits (i.e. OF crossings, exploration of novel object in the NOE test, baseline startle, PPI) nor to the possible genetic link between self-grooming and the selection traits (e.g. two-way avoidance) of the Roman strains. Appropriate answers on the “weight” of genetic and non-genetic factors influencing these co-selected traits can only be provided by studying the hybrids of non-segregating (RHA-I, RLA-I and F1) and segregating (F2 and the two backcrosses, “F1 x RHA-I” and “F1 x RLA-I”) populations (e.g. Castanon et al. 1995) and, thus, they are far from the objectives of the present study.

Turning back to the pre-/post-natal environmental influences that may be responsible for the absence of between-strain differences in some co-selected traits (as mentioned above) variations of maternal care may be of relevance here. In fact, previous studies demonstrate that variation in maternal care influences the development of behavioral traits of the offspring (Francis et al. 1999). This maternal effect leads to modifications of

the DNA methylation pattern of the offspring, thus affecting the regulation of the multiple regions of the genome that modulate behavioral expression (Francis et al. 1999). This hypothesis is supported by studies revealing that maternal care-induced methylation changes can be reversed by cross-fostering, thus suggesting a relation between DNA methylation and parental care (Zhang et al. 2013). According to the previous evidence, it might be suggested that there may be some epigenetic/molecular mechanism/s acting pre- and/or postnatally on the offspring (i.e. modifications of the methylation pattern of some DNA regions) that may lead to relevant changes in some (co-selected) phenotypes after embryo transfer and thus prevent the emergence of significant RHA-I vs. RLA-I differences in the G1 in the present study. In any case, although such mechanisms might potentially be involved in the discussed effects, it is actually obvious that further specific studies will be needed to confirm if epigenetic changes are involved in the phenotypic changes observed between the first generation (G1) post-embryo transfer (G1) and the successive generations of selective breeding.

5.3. *Conclusions*

To sum up and recapitulating the main findings from G1, G3 and G5, the present study shows the following main findings: **(i)** The main phenotypic characteristics of the Roman strains, those more closely linked to the selection criterion, were conserved in G1 after embryo transfer, i.e. between-strain differences in variables associated with two-way avoidance in the shuttle box (avoidance responses and inter-trial crossings – $RHA-I > RLA-I$, and context-conditioned freezing – $RHA-I < RLA-I$) were preserved after embryo transfer, indicating a strong genetic basis associated with the selected trait (see Castanon et al. 1995). **(ii)** One co-selected trait, namely self-grooming (which is related to anxiety, stress and arousal) presents also the characteristic between-strain differences at G1 ($RLA-I > RHA-I$), suggesting a possible genetic link/correlation between self-grooming and the selection traits. Conversely, typical differences between the strains in other co-selected traits (i.e. crossings in the OF test, exploration of novel object in the NOE test, 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. **(iii)** Stress-induced corticosterone response, an index of the rats' sensitivity to stress, is higher in $RLA-I$ than in $RHA-I$ rats at G3 (consistent with G60 and with many previous reports; see refs. above), thus indicating that G3 $RLA-I$ rats are more sensitive to stress than $RHA-I$ rats and that phenotypic strain-related differences in stress hormone responses are also present in the present $RHA-I$ and $RLA-I$ rats derived from embryo transfer. **(iv)** 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) on some co-selected phenotypes that would supposedly be less genetically correlated with the selection traits these that have the highest heritability (i.e. two-way avoidance responding, inter-trial crossings, conditioned freezing/fear).

Therefore, in spite of possible pre-/post-natal environmental influences which seem to affect several phenotypes at G1 (i.e. in the beginning of this new selection procedure of the RHA-I and RLA-I strains, through embryo transfer), it is worth to highlight that RHA-I and RLA-I rats from G3 and G5 present the typical differences in anxiety/fear (RLA-I > RHA-I), novelty seeking (RLA-I < RHA-I), startle response (RLA-I > RHA-I), prepulse inhibition/sensorimotor gating (RLA-I > RHA-I) and stress-induced corticosterone (RLA-I > RHA-I) that have characterized the RHA vs RLA (lines/strains) phenotypic profiles for decades (e.g. Carrasco et al. 2008; Castanon et al. 1995; Diaz-Moran et al. 2012; Driscoll and Bättig 1982; Driscoll et al. 1998, 2009; Escorihuela et al. 1999; Estanislau et al. 2013, López-Aumatell et al. 2009a,b; Oliveras et al. 2015; Río-Álamos et al. 2015, Steimer and Driscoll 2003).

These findings, by comparison with those from the G60 and from all the reviewed literature, make us confident to state that the newly generated (through embryo transfer) RHA-I and RLA-I rat strains continue to present phenotypic profiles that make them a good model of differential anxiety/fear and stress sensitivity, behavioural inhibition/disinhibition, novelty seeking and some attention-related (schizophrenia-relevant) symptoms or traits.

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Ethical approval.- All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All the procedures were in accordance with the Spanish Royal Decree (RD 53/2013) for the protection of experimental animals and with the European Communities Council Directive (2010/63/EU)

Informed consent.- Informed consent was obtained from all individual participants included in the study.

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FIGURE LEGEND

Figure 1.- Mean \pm SEM baseline (CORT_basal) and post-stress (CORT_POST_STRESS, 20 min exposure to a 50 x 50 x 60 cm novel cage) corticosterone levels in RHA-I and RLA-I rats from the 3rd generation post-embryo transfer (G3) and from the 60th generation of the original non-SPF strains (G60). ** $p \leq 0.001$, * $p \leq 0.002$ between the groups indicated (Student's t-test for independent samples, because there was a directed hypothesis that within each generation RLA-I rats would show higher corticosterone response to stress than RHA-I rats). G3, RLA-I n=10, RHA-I n=10; G60, RLA-I n=12, RHA-I n=12.

TABLE LEGENDS

Table II.- Latency (s); latency to explore the novel object, i.e. time elapsed until the rat's first exploration of the object. **Total time (s);** total time spent exploring the novel object. **Crossings (#);** total number of crossings in the OF test. **Latency grooming (s);** time elapsed until the rat's first grooming activity. **Time spent grooming (s);** total time spent self-grooming. Means \pm SEM are shown. **S=** Strain effect in ANOVA; **G=** Gender effect in ANOVA; **SxG=** Interaction between Strain and Gender in ANOVA. "nt", not tested.

Table III.- Head dips (#); total number of head dips in the HB test. **Time spent head dipping (s);** total time spent head-dipping in the HB test. Means \pm SEM are shown. **S=** Strain effect in ANOVA; **G=** Gender effect in ANOVA. "nt", not tested

Table IV.- %PPI65, %PPI70, %PI75, %PPI80; % prepulse inhibition at the 4 different prepulse intensities (65, 70, 75, 80 dB). Means \pm SEM are shown.

S= Strain effect in ANOVA; **G=** Gender effect in ANOVA.

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Table V.- Baseline startle (mV); amplitude of the baseline acoustic startle response. **%PPI Total;** total % prepulse inhibition (averaged across the four prepulse intensities). Means \pm SEM are shown. **S**= Strain effect in ANOVA; **G**= Gender effect in ANOVA.

Table VI.- Avoidances (#); total number of avoidances in the SHAV task. **Time spent freezing (s);** total time spent freezing (to the context) in the SHAV test during inter-trial intervals 2-5. **Inter trial crossings (#);** total number of inter-trial crossings in SHAV task. Means \pm SEM are shown. **S**= Strain effect in ANOVA; **G**= Gender effect in ANOVA; **SxG**= Interaction between Strain and Gender in ANOVA.

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

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

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Table II.- Within-generation strain comparisons for behavioral measures in the Novel Object Exploration and Open Field tests

				NOVEL OBJECT EXPLORATION TEST				OPEN FIELD TEST					
Generation	Strain	Sex	N	LATENCY(s)	2-Way ANOVA F (p)	TOTAL TIME(s)	2-Way ANOVA F (p)	CROSSINGS (#)	2-Way ANOVA F (p)	LATENCY GROOMING (s)	2-Way ANOVA F(p)	TIME SPENT GROOMING(s)	2-Way ANOVA F (p)
G1	RLA	♂	9	63.3 (21.0)	S: 5.4 ($<.001$)	38.7 (12.4)	No effect	73.6 (6.3)	G: 20.3 ($<.001$)	103.7 (19.5)	S: 7.0 ($<.001$)	21.8 (4.7)	G: 6.7 ($<.001$)
	RLA	♀	7	25.7 (10.0)		28.7 (10.0)		107.3 (8.5)		161.7 (38.3)		7.6 (2.4)	S: 11.8
	RHA	♂	7	13.0 (4.7)		48.3 (15.1)		88.0 (6.3)		184.6 (38.2)		4.9 (1.4)	($<.01$)
	RHA	♀	7	11.3 (4.9)		56.0 (14.8)		124.0 (9.8)		249.6 (33.2)		2.4 (1.7)	
G3	RLA	♂	18	35.8 (8.1)	G: 13.3 ($<.001$) S: 4.2 ($<.05$)	38.8 (6.6)	S: 24.9 ($<.001$)	66.6 (6.1)	G: 4.5 ($<.05$) S: 8.1 ($<.01$)	105.2 (12.2)	S: 41.9 ($<.001$)	26.3 (3.6)	S: 34.4 ($<.001$)
	RLA	♀	18	19.6 (4.8)		52.3 (8.0)		94.9 (8.2)		114.7 (11.1)		24.6 (4.7)	
	RHA	♂	17-18	11.5 (3.0)		79.8 (6.0)		100.4 (9.2)		192.6 (17.8)		7.7 (1.8)	
	RHA	♀	18	6.9 (1.5)		95.0 (11.6)		104.1 (6.2)		222.6 (18.0)		5.6 (1.5)	
G5	RLA	♂	11	23.3 (8.2)	Student t-test $t_{(19)}=2.3$ ($<.035$)	38.1 (9.3)	No effect	67.2 (4.5)	Student t-test $t_{(19)}=-5.5$ ($<.001$)	108.5 (10.0)	Student t-test $t_{(19)}=-3.9$ ($<.001$)	18.2 (1.9)	Student t-test $t_{(19)}=5.8$ ($<.001$)
	RHA	♂	10	3.3 (1.7)		49.5 (8.0)		107.3 (5.8)		204.2 (22.7)		4.9 (1.3)	
G60	RLA	♂	9	38.4 (14.4)	S: 9.1 ($<.001$)	14.7 (5.1)	S: 110.7 ($<.001$) G: 16.7 ($<.001$) SxG: 21.6 ($<.001$)	47.3 (4.1)	Student t-test $t_{(13)}=0.8$ ($<.001$)	nt		35.4 (8.1)	Student t-test $t_{(13)}=8.6$ ($<.001$)
	RLA	♀	12	41.0 (10.3)		18.9 (4.9)		nt		nt		nt	
	RHA	♂	6	3.5 (0.6)		130.0 (6.3)		74.1 88.2)		nt		9.2 (2.3)	
	RHA	♀	7	8.9 (3.6)		63.6 (13.4)		nt		nt		nt	

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Table III.- Within-generation strain comparisons for behavioral measures obtained in the Hole Board test.

Generation	Strain	Sex	N	HEAD DIPS(#)	2-Way ANOVA F (p)	TIME SPENT HEAD DIPPING(s)	2-Way ANOVA F (p)
G1	RLA	♂	9	9.4 (1.6)	No effect	21.3 (4.2)	G: 7.3 (<.05)
	RLA	♀	7	11.3 (0.9)		27.3 (3.7)	
	RHA	♂	7	9.0 (1.4)		15.0 (2.3)	
	RHA	♀	7	11.6 (1.7)		36.7 (8.4)	
G3	RLA	♂	18	5.7 (0.6)	G: 5.1 (<.05)	10.3 (1.2)	G: 15.4 (<.001)
	RLA	♀	18	8.7 (0.7)		20.2 (2.5)	
	RHA	♂	18	8.8 (1.0)	S: 6.1 (<.05)	15.5 (2.1)	
	RHA	♀	18	9.7 (1.0)		22.0 (2.3)	
G5	RLA	♂	11	4.5 (0.6)	Student t-test $t_{(19)} = 3.3$ (<.005)	11.0 (1.9)	Student t-test $t_{(19)} = 3.2$ (<.008)
	RHA	♂	10	7.8 (0.8)		23.0 (3.5)	
G60	RLA	♂	9	6.3 (1.0)	Student t-test $t_{(17)} = 0.2$ (<.001)	9.7 (1.7)	Student t-test $t_{(17)} = 0.2$ (<.001)
	RLA	♀	-	nt		nt	
	RHA	♂	10	10.2 (1.7)		21.0 (3.7)	
	RHA	♀	-	nt		nt	

Table IV.- Within-generation strain comparisons for behavioral measures obtained in the different pre-pulse intensities of the Pre-Pulse Inhibition test (PPI).

Generation	Strain	Sex	N	%PPI65	2-Way ANOVA F (p)	%PPI70	2-Way ANOVA F (p)	%PPI75	2-Way ANOVA F (p)	%PP80	2-Way ANOVA F (p)
G1	RLA	♂	9	37.6 (7.4)	S: 5.8 (<.05)	55.0 (8.3)	No effect	63.4 (5.0)	No effect	74.8 (3.0)	No effect
	RLA	♀	7	53.0 (6.4)		57.2 (6.2)		64.7 (9.4)		73.4 (4.6)	
	RHA	♂	7	25.4 (6.8)		53.1 (5.6)		58.0 (6.0)		71.9 (7.1)	
	RHA	♀	7	25.6 (11.3)		46.5 (7.4)		53.7 (9.2)		67.5 (7.4)	
G3	RLA	♂	18	42.8 (5.1)	S: 11.1 (<.001)	58.5 (4.8)	S: 15.6 (<.001)	66.7 (4.6)	S: 13.6 (<.001)	70.2 (3.5)	S: 8.0 (<.01)
	RLA	♀	18	43.5 (4.5)		53.6 (3.4)		61.2 (3.7)		69.3 (3.3)	
	RHA	♂	18	22.6 (7.1)		38.1 (5.4)		48.5 (4.1)		60.5 (3.4)	
	RHA	♀	18	26.9 (5.1)		34.0 (6.1)		48.2 (4.39)		56.6 (5.3)	
G5	RLA	♂	11	51.2 (6.8)	Student t-test $t_{(19)}=3.1$ (<.005)	63.6 (4.8)	Student t-test $t_{(19)}=3.0$ (<.006)	72.5 (3.4)	Student t-test $t_{(19)}=3.0$ (<.006)	77.0 (3.3)	No effect
	RHA	♂	10	23.5 (5.3)		40.8 (5.7)		55.2 (4.6)		69.1 (4.2)	
G60	RLA	♂	9	57.7 (3.0)	G: 8.0 (<.01)	70.7 (2.9)	G: 18.3 (<.001)	76.0 (2.5)	G: 13.8 (<.001)	81.6 (2.5)	G: 11.6 (<.01)
	RLA	♀	12	37.3 (5.5)		49.5 (4.1)		59.7 (4.5)		63.0 (6.0)	
	RHA	♂	6	34.9 (5.4)		44.5 (4.0)		62.7 (3.4)		77.1 (3.2)	
	RHA	♀	7	24.1 (6.5)		25.9(6.6)		41.8 (7.7)		61.4 (3.3)	

Table V.- Within-generation strain comparisons for behavioral measures obtained in baseline startle and percentage of total pre-pulse inhibition (%PPI).

Generation	Strain	Sex	N	BASELINE STARTLE	2-Way ANOVA F(p)	%PPI TOTAL	2-Way ANOVA F(p)
G1	RLA	♂	9	2144.9 (453.4)	G: 4.6 (<.05)	57.7 (5.0)	No effect
	RLA	♀	7	1370.2 (92.6)		62.1 (5.9)	
	RHA	♂	7	1564.9 (422.65)		52.1 (5.8)	
	RHA	♀	7	852.2 (127.1)		48.3 (8.1)	
G3	RLA	♂	18	2684.1 (302.7)	G: 11.3 (<.001) S: 14.2 (<.001)	59.6 (3.8)	S: 15.9 (<.001)
	RLA	♀	18	1500.2 (224.7)		56.9 (3.4)	
	RHA	♂	18	1408.8 (211.7)		42.4 (4.4)	
	RHA	♀	18	1093.3 (109.0)		41.5 (4.7)	
G5	RLA	♂	11	1573.3 (385.5)	No effect	66.07 (3.8)	Student t-test $t_{(19)}=3.3$ (<.004)
	RHA	♂	10	1031.1 (138.6)		47.14 (4.3)	
G60	RLA	♂	9	2091.3 (501.6)	S: 6.6 (<.05)	71.5 (2.6)	G: 18.9 (<.001) S: 14.1 (<.001)
	RLA	♀	12	1510.4 (341.0)		52.4 (4.8)	
	RHA	♂	6	900.2 (193.3)		54.8 (2.9)	
	RHA	♀	7	696.1 (214.3)		38.3 (4.5)	

Table VI.- Within-generation strain comparisons for behavioral measures obtained in the two-way avoidance -Shuttle box- acquisition task.

Generation	Strain	Sex	N	AVOIDANCES (#)	2-Way ANOVA F (p)	TIME SPENT FREEZING (s)	2-Way ANOVA F (p)	INTER TRIAL CROSSINGS (#)	2-Way ANOVA F (p)
G1	RLA	♂	9	2.6 (1.2)	S: 133.4 (<.001)	181.8 (9.4)	S: 20.1 (<.001)	8.8 (1.8)	G: 4.4 (<0.05)
	RLA	♀	9	4.7 (2.1)		151.7 (13.0)		26.8 (8.6)	
	RHA	♂	7	26.4 (2.4)		106.6 (20.2)		53.7 (10.5)	S: 32.7 (<.001)
	RHA	♀	12	27.3 (2.0)		110.3 (10.3)		66.8 (6.3)	
G3	RLA	♂	18	2.6 (0.9)	S: 103.7 (<.001)	192.6 (6.2)	G: 5.2 (<.01)	15.4 (2.8)	S: 31.4 (<.001)
	RLA	♀	18	4.5 (1.5)		191.2 (6.0)		26.3 (5.7)	
	RHA	♂	18	25.1 (2.2)		103.4 (7.6)	S: 118.2 (<.001)	63.9 (7.7)	
	RHA	♀	18	21.3 (2.7)		135.2 (6.3)	SxG: 6.1 (<.05)	53.3 (8.9)	
G5	RLA	♂	11	1.9 (0.8)	Student t-test t ₍₁₉₎ = 8.9 (<.001)	191.6 (6.9)	Student t-test t ₍₁₉₎ = 8.8 (<.001)	11 (2.6)	Student t-test t ₍₁₉₎ = 4.4 (<.005)
	RHA	♂	10	27.0 (2.8)		101.7 (7.6)		70 (14.5)	
G60	RLA	♂	9	1.4 (0.4)	S: 220.7 (<.001)	197.8 (9.3)	Student t-test t ₍₁₃₎ = 6.48 (<.001)	10.0 (2.1)	S: 50.1 (<.001)
	RLA	♀	12	1.4 (0.6)		-		11.3 (1.9)	
	RHA	♂	6	25.0 (1.9)		155.3 (12.7)		43.8 (5.4)	
	RHA	♀	7	20.4 (2.8)		-		59.1 (12.2)	

Figure 1

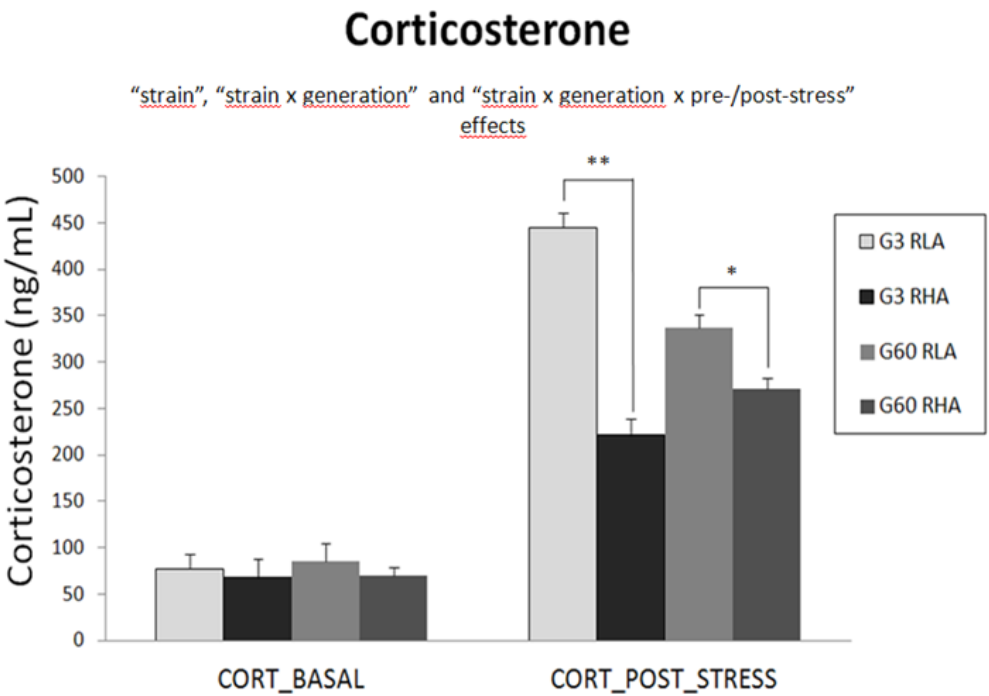


Figure 1

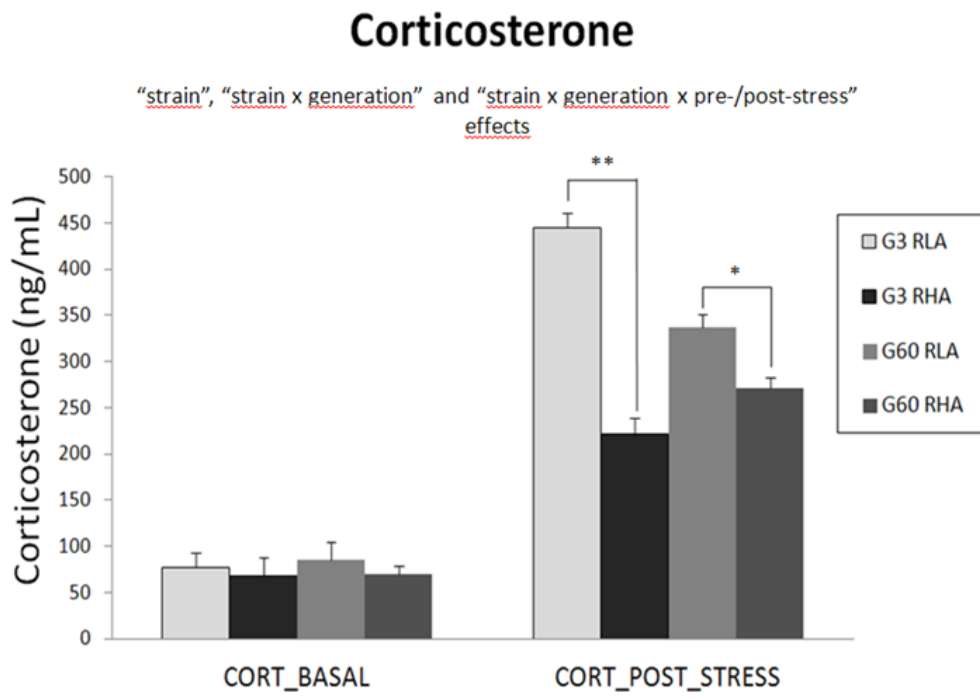


Table I.- Summary of the results obtained after the embryo transfer procedure, including the total number of pups born in generation 1 (G1, born from Sprague-Dawley –SD- rats)

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

Table II

Table II.- Within-generation strain comparisons for behavioral measures in the Novel Object Exploration and Open Field tests

				NOVEL OBJECT EXPLORATION TEST				OPEN FIELD TEST					
Generation	Strain	Sex	N	LATENCY(s)	2-Way ANOVA F (p)	TOTAL TIME(s)	2-Way ANOVA F (p)	CROSSINGS (#)	2-Way ANOVA F (p)	LATENCY GROOMING (s)	2-Way ANOVA F(p)	TIME SPENT GROOMING(s)	2-Way ANOVA F (p)
G1	RLA	♂	9	63.3 (21.0)	S: 5.4 (<.01)	38.7 (12.4)	No effect	73.6 (6.3)	G: 20.3 (<.001)	103.7 (19.5)	S: 7.0 (<.05)	21.8 (4.7)	G: 6.7 (<.05)
	RLA	♀	7	25.7 (10.0)		28.7 (10.0)		107.3 (8.5)		161.7 (38.3)		7.6 (2.4)	
	RHA	♂	7	13.0 (4.7)		48.3 (15.1)		88.0 (6.3)		184.6 (38.2)		4.9 (1.4)	
	RHA	♀	7	11.3 (4.9)		56.0 (14.8)		124.0 (9.8)		249.6 (33.2)		2.4 (1.7)	
G3	RLA	♂	18	35.8 (8.1)	G: 13.3 (<.001)	38.8 (6.6)	S: 24.9 (<.001)	66.6 (6.1)	G: 4.5 (<.05)	105.2 (12.2)	S: 41.9 (<.001)	26.3 (3.6)	S: 34.4 (<.001)
	RLA	♀	18	19.6 (4.8)		52.3 (8.0)		94.9 (8.2)		114.7 (11.1)		24.6 (4.7)	
	RHA	♂	17-18	11.5 (3.0)		79.8 (6.0)		100.4 (9.2)		192.6 (17.8)		7.7 (1.8)	
	RHA	♀	18	6.9 (1.5)		95.0 (11.6)		104.1 (6.2)		222.6 (18.0)		5.6 (1.5)	
G5	RLA	♂	11	23.3 (8.2)	Student t-test $t_{(19)}=2.3$ (<.035)	38.1 (9.3)	No effect	67.2 (4.5)	Student t-test $t_{(19)}=-5.5$ (<.001)	108.5 (10.0)	Student t-test $t_{(19)}=-3.9$ (<.001)	18.2 (1.9)	Student t-test $t_{(19)}=5.8$ (<.001)
	RHA	♂	10	3.3 (1.7)		49.5 (8.0)		107.3 (5.8)		204.2 (22.7)		4.9 (1.3)	
G60	RLA	♂	9	38.4 (14.4)	S: 9.1 (<.01)	14.7 (5.1)	S: 110.7 (<.001)	47.3 (4.1)	Student t-test $t_{(13)}=6.8$ (<.001)	nt		35.4 (8.1)	Student t-test $t_{(13)}=8.6$ (<.001)
	RLA	♀	12	41.0 (10.3)		18.9 (4.9)		nt		nt		nt	
	RHA	♂	6	3.5 (0.6)		130.0 (6.3)		74.1 88.2)		nt		9.2 (2.3)	
	RHA	♀	7	8.9 (3.6)		63.6 (13.4)		nt		nt		nt	

Table III.- Within-generation strain comparisons for behavioral measures obtained in the Hole Board test.

Generation	Strain	Sex	N	HEAD DIPS(#)	2-Way ANOVA F (p)	TIME SPENT HEAD DIPPING(s)	2-Way ANOVA F (p)
G1	RLA	♂	9	9.4 (1.6)	No effect	21.3 (4.2)	G: 7.3 (<.05)
	RLA	♀	7	11.3 (0.9)		27.3 (3.7)	
	RHA	♂	7	9.0 (1.4)		15.0 (2.3)	
	RHA	♀	7	11.6 (1.7)		36.7 (8.4)	
G3	RLA	♂	18	5.7 (0.6)	G: 5.1 (<.05)	10.3 (1.2)	G: 15.4 (<.001)
	RLA	♀	18	8.7 (0.7)		20.2 (2.5)	
	RHA	♂	18	8.8 (1.0)	S: 6.1 (<05)	15.5 (2.1)	
	RHA	♀	18	9.7 (1.0)		22.0 (2.3)	
G5	RLA	♂	11	4.5 (0.6)	Student t-test $t_{(19)}= 3.3$ (<.005)	11.0 (1.9)	Student t-test $t_{(19)}= 3.2$ (<.008)
	RHA	♂	10	7.8 (0.8)		23.0 (3.5)	
G60	RLA	♂	9	6.3 (1.0)	Student t-test $t_{(17)}= 3.2$ (<.001)	9.7 (1.7)	Student t-test $t_{(17)}= 3.2$ (<.001)
	RLA	♀	-	nt		nt	
	RHA	♂	10	10.2 (1.7)		21.0 (3.7)	
	RHA	♀	-	nt		nt	

Table IV.- Within-generation strain comparisons for behavioral measures obtained in the different pre-pulse intensities of the Pre-Pulse Inhibition test (PPI).

Generation	Strain	Sex	N	%PPI65	2-Way ANOVA F (p)	%PPI70	2-Way ANOVA F (p)	%PPI75	2-Way ANOVA F (p)	%PP80	2-Way ANOVA F (p)
G1	RLA	♂	9	37.6 (7.4)	S: 5.8 (<.05)	55.0 (8.3)	No effect	63.4 (5.0)	No effect	74.8 (3.0)	No effect
	RLA	♀	7	53.0 (6.4)		57.2 (6.2)		64.7 (9.4)		73.4 (4.6)	
	RHA	♂	7	25.4 (6.8)		53.1 (5.6)		58.0 (6.0)		71.9 (7.1)	
	RHA	♀	7	25.6 (11.3)		46.5 (7.4)		53.7 (9.2)		67.5 (7.4)	
G3	RLA	♂	18	42.8 (5.1)	S: 11.1 (<.001)	58.5 (4.8)	S: 15.6 (<.001)	66.7 (4.6)	S: 13.6 (<.001)	70.2 (3.5)	S: 8.0 (<.01)
	RLA	♀	18	43.5 (4.5)		53.6 (3.4)		61.2 (3.7)		69.3 (3.3)	
	RHA	♂	18	22.6 (7.1)		38.1 (5.4)		48.5 (4.1)		60.5 (3.4)	
	RHA	♀	18	26.9 (5.1)		34.0 (6.1)		48.2 (4.39)		56.6 (5.3)	
G5	RLA	♂	11	51.2 (6.8)	Student t-test $t_{(19)}=3.1$ (<.005)	63.6 (4.8)	Student t-test $t_{(19)}=3.0$ (<.006)	72.5 (3.4)	Student t-test $t_{(19)}=3.0$ (<.006)	77.0 (3.3)	No effect
	RHA	♂	10	23.5 (5.3)		40.8 (5.7)		55.2 (4.6)		69.1 (4.2)	
G60	RLA	♂	9	57.7 (3.0)	G: 8.0 (<.01)	70.7 (2.9)	G: 18.3 (<.001)	76.0 (2.5)	G: 13.8 (<.001)	81.6 (2.5)	G: 11.6 (<.01)
	RLA	♀	12	37.3 (5.5)		49.5 (4.1)		59.7 (4.5)		63.0 (6.0)	
	RHA	♂	6	34.9 (5.4)		44.5 (4.0)		62.7 (3.4)		77.1 (3.2)	
	RHA	♀	7	24.1 (6.5)		25.9(6.6)		41.8 (7.7)		61.4 (3.3)	

Table V.- Within-generation strain comparisons for behavioral measures obtained in baseline startle and percentage of total pre-pulse inhibition (%PPI).

Generation	Strain	Sex	N	BASELINE STARTLE	2-Way ANOVA F(p)	%PPI TOTAL	2-Way ANOVA F(p)
G1	RLA	♂	9	2144.9 (453.4)	G: 4.6 (<.05)	57.7 (5.0)	No effect
	RLA	♀	7	1370.2 (92.6)		62.1 (5.9)	
	RHA	♂	7	1564.9 (422.65)		52.1 (5.8)	
	RHA	♀	7	852.2 (127.1)		48.3 (8.1)	
G3	RLA	♂	18	2684.1 (302.7)	G: 11.3 (<.001) S: 14.2 (<.001)	59.6 (3.8)	S: 15.9 (<.001)
	RLA	♀	18	1500.2 (224.7)		56.9 (3.4)	
	RHA	♂	18	1408.8 (211.7)		42.4 (4.4)	
	RHA	♀	18	1093.3 (109.0)		41.5 (4.7)	
G5	RLA	♂	11	1573.3 (385.5)	No effect	66.07 (3.8)	Student t-test $t_{(19)}=3.3$ (<.004)
	RHA	♂	10	1031.1 (138.6)		47.14 (4.3)	
G60	RLA	♂	9	2091.3 (501.6)	S: 6.6 (<.05)	71.5 (2.6)	G: 18.9 (<.001) S: 14.1 (<.001)
	RLA	♀	12	1510.4 (341.0)		52.4 (4.8)	
	RHA	♂	6	900.2 (193.3)		54.8 (2.9)	
	RHA	♀	7	696.1 (214.3)		38.3 (4.5)	

Table VI

Table VI.- Within-generation strain comparisons for behavioral measures obtained in the two-way avoidance -Shuttle box- acquisition task.

Generation	Strain	Sex	N	AVOIDANCES (#)	2-Way ANOVA F (p)	TIME SPENT FREEZING (s)	2-Way ANOVA F (p)	INTER TRIAL CROSSINGS (#)	2-Way ANOVA F (p)
G1	RLA	♂	9	2.6 (1.2)	S: 133.4 (<.001)	181.8 (9.4)	S: 20.1 (<.001)	8.8 (1.8)	G: 4.4 (<0.05)
	RLA	♀	9	4.7 (2.1)		151.7 (13.0)		26.8 (8.6)	
	RHA	♂	7	26.4 (2.4)		106.6 (20.2)		53.7 (10.5)	
	RHA	♀	12	27.3 (2.0)		110.3 (10.3)		66.8 (6.3)	
G3	RLA	♂	18	2.6 (0.9)	S: 103.7 (<.001)	192.6 (6.2)	G: 5.2 (<.01)	15.4 (2.8)	S: 31.4 (<.001)
	RLA	♀	18	4.5 (1.5)		191.2 (6.0)		26.3 (5.7)	
	RHA	♂	18	25.1 (2.2)		103.4 (7.6)		63.9 (7.7)	
	RHA	♀	18	21.3 (2.7)		135.2 (6.3)		53.3 (8.9)	
G5	RLA	♂	11	1.9 (0.8)	Student t-test $t_{(19)} = 8.9$ (<.001)	191.6 (6.9)	Student t-test $t_{(19)} = 8.8$ (<.001)	11 (2.6)	Student t-test $t_{(19)} = 4.4$ (<.005)
	RHA	♂	10	27.0 (2.8)		101.7 (7.6)		70 (14.5)	
G60	RLA	♂	9	1.4 (0.4)	S: 220.7 (<.001)	197.8 (9.3)	Student t-test $t_{(13)} = 6.48$ (<.001)	10.0 (2.1)	S: 50.1 (<.001)
	RLA	♀	12	1.4 (0.6)		-		11.3 (1.9)	
	RHA	♂	6	25.0 (1.9)		155.3 (12.7)		43.8 (5.4)	
	RHA	♀	7	20.4 (2.8)		-		59.1 (12.2)	