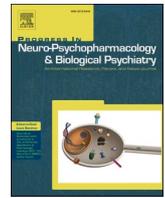




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## Neonatal handling enhances behavioural and attentional domains, and frontocortical synaptic maturation in rat models of schizophrenia-like behaviour and anxiety-related responses

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### ABSTRACT

The Roman inbred rat strains are a neurodevelopmental model, with the Roman High Avoidance (RHA) presenting specific behaviours and frontal cortex (FC) gene expression changes relevant to schizophrenia symptoms. We wanted to assess the potentially positive modulatory and enduring effects of neonatal handling (NH) on the innate traits associated with both the RHA and their counterpart Roman Low Avoidance (RLA). Male rats received NH or were left untreated (controls). Two different age groups were considered: adolescent and adults. The assessment encompassed exploratory behaviour, social behaviour, anxiety-related behaviour (self-grooming), sensorimotor gating (prepulse inhibition; PPI), and the analysis of gene expression associated with synaptic processes, cortical maturation, and neuroplasticity in the FC. In adolescent rats, NH increased novelty exploration and activity, and reduced novelty-induced self-grooming in RLAs, whereas it improved PPI in RHAs. In adult rats, NH increased novelty-induced activity in both strains, reduced self-grooming in RLA rats, and enhanced social interaction and PPI in RHAs. NH produced significant effects on gene expression in adolescent RHA rats. These effects were observed at the presynaptic level by a reduction of *Snap25* and increases of *Cables1* and *Cdk5*, and at the postsynaptic level by increases of *Grin2b*, *Homer1* and *Nrg1*, as well as by a NH-induced enhancement of *Bdnf*. NH also increased *Nrg1* and *Bdnf* expression in adult RLA rats. These findings show for the first time that NH is able to modulate several genetically linked synaptic/neuroplasticity alterations in RHA vs. RLA rats, which are paralleled by NH-induced improvements in novelty exploration, social behaviour and sensorimotor gating (PPI).

### 1. Introduction

Schizophrenia is a complex and severe mental disorder which is believed to originate during neurodevelopment. Genome-wide association studies (GWAS) have uncovered multiple genes related to schizophrenia (e.g., [Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014](#)), while epigenetic evidence points to gene-environment interactions across development in shaping the risk for psychosis and schizophrenia later in life (e.g., [Jaffe et al., 2016](#)).

Identifying the risk-associated genetic background is crucial for implementing preventive programs. In this context, animal models exhibiting phenotypes related to the disorder are of particular translational value to identify underlying genetic, epigenetic and neurodevelopmental features associated with increased vulnerability, or resilience towards schizophrenia-related behaviours.

One such models is the inbred Roman high-avoidance (RHA) rats, which compared with their Roman low-avoidance (RLA) counterparts and other rat strains exhibit impaired executive/cognitive functions,

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attentional processes and social behaviour, as well as novelty- and psychostimulant-induced hyperactivity (Esnañal et al., 2016; Martínez-Membrives et al., 2015; Moreno et al., 2010; Oliveras et al., 2015, 2016, 2023; Río-Álamos et al., 2017a, 2019; Sánchez-González et al., 2020; reviewed by Giorgi et al., 2019; Fernández-Teruel et al., 2021, 2023). Underlying these behavioural phenotypes, RHA rats display alterations in dopamine, serotonin, GABA and glutamate neurotransmitter systems (Fernández-Teruel et al., 2021; Giorgi et al., 2019; Fomsgaard et al., 2018; Klein et al., 2014; Tapias-Espinosa et al., 2023), grey and white matter structures (Río-Álamos et al., 2017b, 2019) and prefrontal cortex (PFC) activation (Meyza et al., 2009; Tapias-Espinosa et al., 2019, 2023). These neurobehavioral phenotypes lend face and construct validity to RHA rats as a model of schizophrenia-relevant features, while psychopharmacological findings add predictive validity to the RHA rat model (Oliveras et al., 2017; Sampedro-Viana et al., 2023b, 2024; Tapias-Espinosa et al., 2021).

We have recently reported differential expression of synaptic markers during neurodevelopment and increased density of thin (immature) dendritic spines in frontal cortex (FC) of RHA vs. RLA rats, pointing to an immature FC of RHA rats (Elfvig et al., 2019; Sánchez-González et al., 2021). A failure to reach a final stage of cortical maturation in at-risk individuals has been proposed to be at the core of schizophrenia (Catts et al., 2013; Gao et al., 2022). According to this neurodevelopmental hypothesis we recently mapped the FC transcriptome in adult Roman (RHA vs. RLA) rats and investigated whether there is a shift in the RHA compared to the RLA strain in functional gene networks differentially expressed during early neurodevelopment (Sønderstrup et al., 2023). We found a different maturational expression profile of synaptic components in the RHA strain, supporting a shift in FC maturation underlying schizophrenia-relevant behavioural traits and adding construct validity to this strain as a neurodevelopmental model (Sønderstrup et al., 2023). The altered expression of several synaptic markers indicated that aberrant cortical maturation during adolescence in RHA rats may lead to permanent neurodevelopmental anomalies present during adulthood in this strain, which may underlie their cognitive/attentional dysfunctions (Elfvig et al., 2019; Esnañal et al., 2016; Fernández-Teruel et al., 2021; Oliveras et al., 2015; Peralta-Vallejo et al., 2024; Río-Álamos et al., 2019; Sønderstrup et al., 2023).

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

Neonatal handling (NH; during the first 2-3 weeks of life) has been shown to long-lastingly improve emotional profiles and stress coping ability, and to enhance cognitive and attentional performance in laboratory rodents (e.g., Fernández-Teruel et al., 2002; Raineki et al., 2014; Río-Álamos et al., 2015, 2017b, 2019). In addition, NH treatment enduringly reduces stress hormone responses and influences neuroplasticity and many neurotransmitter systems (e.g., Fernández-Teruel, 2022; Fernández-Teruel et al., 2002; Fu and Depue, 2019; Katsouli et al., 2014; Meaney et al., 1988, 1989; Panagiotaropoulos et al., 2004; Pondiki et al., 2006; Stamatakis et al., 2009; Taschetto Vey et al., 2020). Many of these positive NH effects have also been demonstrated in the Roman rat strains (e.g., Fernández-Teruel et al., 1997, 2002; Río-Álamos et al., 2017b, 2019; Sampedro-Viana et al., 2021, 2023a; Steimer et al., 1998).

Our aim was therefore to investigate whether NH influences adolescent and adult exploratory behaviour, social behaviour, sensorimotor gating (prepulse inhibition of the startle response –PPI), and further, whether this is associated with changes in gene expression of target genes in RHA vs. RLA rats in FC, according to the genes of interest from our previous studies (Elfvig et al., 2019; Sønderstrup et al., 2023).

## 2. Materials and methods

### 2.1. Animals

The animals used were 112 males of the inbred Roman high- (RHA,  $n = 56$ ) and low-avoidance (RLA,  $n = 56$ ) rats, from the permanent colonies maintained at the laboratory of the Medical Psychology Unit, Dept. Psychiatry and Forensic Medicine (Autonomous University of Barcelona, Spain) since 1996. Each experimental group (see below) consisted of rats from 9 different litters (1-2 rats per litter). Litters were culled to 10 pups (approximately half of each sex) one day after birth (postnatal day 1, PND1), the day in which the neonatal handling (NH) treatment started (see NH procedure below). Rats were maintained under a 12:12 h light-dark cycle (lights on at 08:00 a.m.), with controlled temperature ( $22 \pm 2$  °C) and humidity (50-70 %). They had food and water available ad libitum.

It should be noted that female rats were not included in the present study, due to the narrow time window of the adolescent period, which precluded the inclusion of females (of both strains, both ages, and two treatment conditions) to be studied in parallel with the males in the various behavioural tests and gene expression. While the model has primarily been validated using male rats, recent research has demonstrated sex-specific effects on social interaction and sensorimotor gating (Oliveras et al., 2022a, 2022b). Therefore, it is important to consider including females of both rat strains in future studies to provide a more comprehensive understanding of the processes studied here.

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

### 2.2. Neonatal handling (NH) treatment

NH was administered twice daily (at 9.30 and 17.00 h) between PND1-21, in a room with a temperature of  $22 \pm 2$  °C (see Río-Álamos et al., 2015; Río-Álamos et al., 2017b; Río-Álamos et al., 2019; Peralta-Vallejo et al., 2024). Each handling session consisted of first removing the mother from the litter and then placing the pups individually in plastic cages ( $35 \times 15 \times 25$  cm) lined with paper towel for a total period of 8 min. Each pup was gently stroked for 3–4 s at 0, 4 and 8 min with bare hands, after which they were returned to their home-cage with their mother and litter. Non-handled (Control, C) rats were left undisturbed except for the weekly cleaning of their home cages. Rats were weaned at PND 21, and were housed in same-sexed pairs of the same experimental group in regular ( $50 \times 25 \times 14$  cm) macrolon cages.

### 2.3. Novel object exploration (NOE) test

This test was used to test anxiety-related exploratory behaviour of novelty. Food was removed from the home cage (except 4 food pellets left in each cage) and the cage was pulled away from the rack 20 cm to allow direct observation of the rats' behaviour. One hour later, the novel object (graphite pencil Staedtler Noris, HB n°2) was introduced vertically in the cage through the grid cover until it reached the cage bedding, and then the “Latency to the first exploration” (NOE latency time) and the “total time of exploration” (NOE exploration time) were measured for 3 min by a well-trained researcher standing approximately 40-50 cm away from the cage front. Exploration of the novel object was defined as approaching the nose to it at 1 cm distance (or less) or touching/manipulating the object.

### 2.4. Social interaction (SI) test

For the social interaction (SI) test, two acrylic boxes ( $65 \times 23 \times 20$  cm) were placed in front of each other at 12 cm, to prevent physical

contact between the animals, in a red-lit room with a camera on the roof connected to a monitor outside the room to record the test. Each box had two 3 cm diameter holes at its ends. The hole facing to the room wall (distal hole) was named as “non-social hole”, while the one facing the other box was named as “social hole” (Sampedro-Viana et al., 2021).

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

For SI testing, performed 24 h later, the holes were opened and two weight-matched non-familiar rats (maximum weight difference 20 g) of the same strain were placed (one rat in each box) into the set-up for a 15-min test. The time spent exploring the social hole (social time) and the non-social hole (non-social time), time spent self-grooming (grooming time) and total number of crossings (crossings) were recorded (each box was divided into three equal squares by lines painted on the floor to measure horizontal activity –crossings-) by two well-trained observers (between-observer reliability  $r = 0.95$ ) watching the TV monitor outside the testing room. The SI boxes were cleaned with 70 % ethanol solution after each test and dried with paper towel.

### 2.5. Prepulse inhibition of the acoustic startle response (PPI)

The PPI test was conducted using two sound attenuated startle boxes (the boxes and software from SR-Lab Startle Response System, San Diego Instruments, US). Animals were individually placed in an acrylic cylinder, which was situated in a dimly illuminated box and on the top of a platform with a sensor that detects the strength made by the rat in each trial. Noise bursts were delivered via a speaker mounted 15 cm above the cylinder. The data are transduced by an accelerometer into a voltage which is amplified, digitized, and saved into a computer for further analysis.

The session consisted of 5 min of habituation, after which 10 “pulse-alone” trials (105 dB(A), SPL, 40 ms) were delivered in order to obtain a stable baseline of startle responses (BL1). Next, six types of trials were randomly administered 10 times (60 trials): (i) Pulse-alone trials (105 dB(A), SPL, 40 ms; BL2; used to calculate the percentage of PPI); (ii) prepulses of 65/70/75/80 dB (A), SPL (20 ms) followed by the pulse stimulus (105 dB (A), SPL, 40 ms) with an inter-stimulus interval of

100 ms; or (iii) no-stimulus trials (background noise of 55 dB). The inter-trial interval was 15 s (range 10–20 s). The %PPI for each prepulse intensity was calculated with the following formula: %PPI = [100 – (startle amplitude on prepulse trials/startle amplitude on “BL2” pulse-alone trials × 100)].

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

### 2.6. Experimental design

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

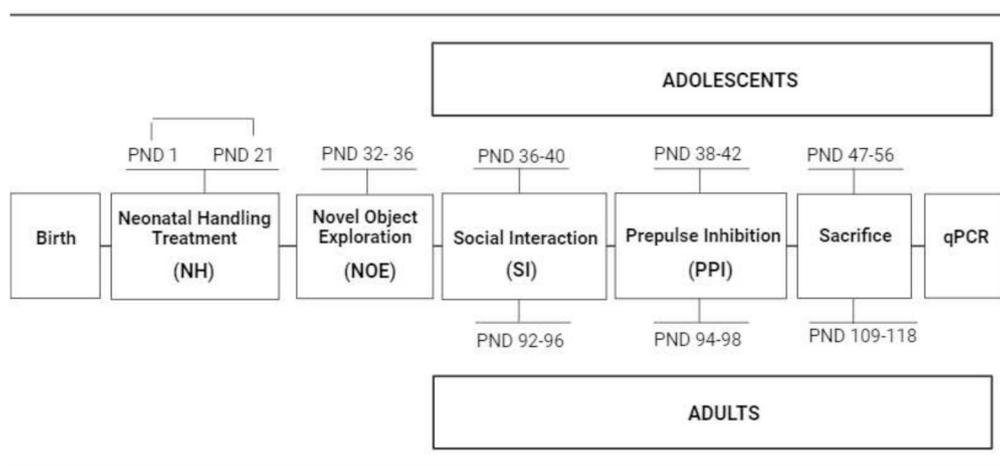
We calculated the sample size based on previous PPI experiments with adult rats where the mean difference between strains in “Total PPI” was 15 % (SD = 14.5). The study would require a sample size of 15 subjects for each strain (i.e., a total sample size of 30, assuming equal group sizes), to achieve a power of 80 % and a level of significance of 5 % (two sided), for detecting a true difference in means between strains. Since we have 4 experimental groups per strain (C and NH, and two ages), we designed the study to have at least 10 animals per experimental group (i.e., at least 40 rats per strain). The above calculations are based on the formula.

$$n = (Z\alpha/2 + Z\beta)^2 \cdot 2 \cdot \sigma^2 / d^2$$

where  $Z\alpha/2$  is the critical value of the Normal distribution at  $\alpha/2$  (e.g. for a confidence level of 95 %,  $\alpha$  is 0.05 and the critical value is 1.96),  $Z\beta$  is the critical value of the Normal distribution at  $\beta$  (e.g. for a power of 80 %,  $\beta$  is 0.2 and the critical value is 0.84),  $\sigma^2$  is the population variance, and  $d$  is the difference you would like to detect.

Behavioural evaluation started at PND32–36 with the NOE test, which was administered to all rats from C ( $n = 56$ , 28 per strain) and NH ( $n = 56$ , 28 per strain) groups (see timeline in Fig. 1). The NOE test was administered at this young age to leave enough time to perform the other two tests (SI and PPI) during the adolescence period, and because we know from several previous studies that when administered between

## Overview of the experimental and timeline design



**Fig. 1.** Overview and timeline of the experimental schedule (see “Materials and methods”). The study was carried out with male rats of the RHA and RLA strains, receiving neonatal handling (NH) or no treatment (controls, C), and including two ages, adolescents and adults. NOE test;  $n = 112$  (28 rats per group). SI test: adolescents,  $n = 10$  in each group; adults,  $n = 18$  in each. PPI test;  $n = 10$  rats/group. qPCR;  $n = 8$ /group (randomly selected rats from each experimental group). Groups/age: RLA-C, RLA-NH, RHA-C, RHA-NH. PND; postnatal day.

30 and 60 days of age the NOE test is a very reliable and consistent “behavioural marker” indicating that NH treatment is effective (e.g., Río-Álamos et al., 2015, 2017b, 2019; Sampedro-Viana et al., 2021, 2023a).

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

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

For both the NOE and the SI tests the observers were blinded to experimental groups. The PPI session was automated and controlled by a software (see above).

Nine to 15 days after finishing the PPI test (PND47-56, adolescent; PND109-118, adults), randomly selected rats ( $n = 8$ ) from each of the 8 groups were anesthetized under isoflurane and brain samples were taken and deep frozen for future gene expression studies (Fig. 1). Sacrifice was done between 9:00 a.m. and 13:00 p.m.

We established this period of 9-15 days between the last behavioural test (PPI) and the day of brain sampling because the objective of the study was to obtain baseline measures (i.e., “trait” measures) of gene expression, rather than gene expression measures that could be influenced by behavioural tests. Thus, the day of sacrifice was scheduled so that for animals that finished the PPI test earlier the brain sampling day was also earlier, whereas for rats that did the PPI test later the day of brain sampling was also among the last days. Thus, for example, those adolescent rats that did the PPI test at PND38 were sacrificed at PND47, and those adult rats that did the PPI at PND94 were sacrificed at PND109; whereas adolescent rats tested for PPI at PND42 were sacrificed at PND56, and adult rats tested for PPI at PND98 were sacrificed at PND118 (see Fig. 1).

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

### 2.7. RNA extraction and sample preparation

From each selected rat, the FC was dissected out as done in the previous studies (Elfving et al., 2019; Sønderstrup et al., 2023), immediately frozen in liquid nitrogen, and stored at  $-80$  °C. RNA was extracted from the FC tissue as described earlier in (Tapias-Espinosa et al., 2021) with minor modifications. Briefly, RNA from tissue samples (around 30  $\mu$ g) was extracted using the miRNeasy mini kit (Qiagen; cat. no. 217, 084) in a RNAase-free environment. RNA samples were subjected to DNase treatment using the RNAasy-Free DNase (Qiagen; cat. No. 79254). The RNA concentration and the purity were determined by a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific). The purified RNA (200 ng) was transcribed into complementary DNA (cDNA) using qScript cDNA SuperMix kit (Quanta Biosciences, cat. no. 95,048), according to the manufacturer instructions. The cDNA products were diluted 1:5 with RNase/DNase-free water, and stored at 80 °C.

### 2.8. Real-time PCR

Real-time qPCR was performed on individual samples in duplicate in 96-well PCR-plates using the Stratagene Mx3005p qPCR System (Agilent Technologies) and Fast SYBR Green Master Mix (Applied Biosystems, cat. no. 438,512). To compare the multiple samples between the assays, a positive control (a pool of cDNA from all samples used as a calibrator) and a negative control (RNase/DNase-free water) were included in each run. The expression of 2 reference genes (Gapdh, Rpl13A) and 8 target genes (Drd1, Grind2b, Snap25, Nrg1, Homer1, Bdnf, Cables1, Cdk5, Table 1) were investigated. Briefly, each SYBR Green reaction (10  $\mu$ l total volume) contained  $1 \times 5$   $\mu$ l Fast SYBR Green Master Mix (Applied Biosystems, cat. no. 438,512), 0.3  $\mu$ M primer pairs, and 2  $\mu$ l of diluted cDNA. The thermal conditions for the PCR were 20 s at 95 °C, followed by 40 cycles of 5 s denaturation at 95 °C, 30 s annealing at 60 °C, and 1 cycle of 60s at 95 °C, 30s at 55 °C and 60 s at 95 °C. Each run was completed by dissociation curve analysis to confirm the amplification specificity and absence of primer dimers. We generated a standard curve, performed in duplicate, on each plate. A comparative cycle of threshold fluorescence (Ct) method was used, and the relative transcription level (mRNA amount values) from each individual sample of the target genes was normalized with the geometric mean of the relative mRNA amount of the housekeeping reference genes (GAPDH and RPL13 A) and expressed as relative quantity to the calibrator sample using the Pfaffl method (Pfaffl, 2001).

### 2.9. Statistical analysis

Factorial ANOVA (2 strain  $\times$  2 treatments  $\times$  2 ages), in some cases with repeated measures (e.g., time intervals in the SI test, different prepulse intensities in the PPI test), were carried out. *Posthoc* Duncan’s multiple range tests were performed to explore differences between groups following significant ANOVA effects. Before analysis of gene expression differences, the Grubbs outlier test was run, and significant outliers removed. Pearson’s correlation coefficients were also performed among the behavioural variables as well as among these and gene expression values (Supplementary Tables 1-2).

## 3. Results

### 3.1. NOE test

The factorial ANOVA (2 strain  $\times$  2 NH) showed strain ( $F(1,108) = 170.7$   $p < 0.001$ ), NH ( $F(1,108) = 24.5$   $p < 0.001$ ) and “strain  $\times$  NH” ( $F(1,108) = 22.2$   $p < 0.001$ ) effects on the latency to the first exploration of the novel object. *Posthoc* Duncan’s test showed that RLA-Control group was different from both RLA-NH and RHA-Control (Fig. 2A). Regarding exploration time (of the novel object), ANOVA revealed strain ( $F(1,108) = 21.6$   $p < 0.001$ ) and NH ( $F(1,108) = 73.5$   $p < 0.001$ ) effects. *Posthoc* Duncan’s test revealed that RHA rats explored more the object and NH increased exploration time in both rat strains (see Fig. 2B).

### 3.2. Social Interaction (SI) test

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

Separate ANOVA for age did not reveal significant effects for social time (all  $F_s < 1.64$ , all  $p > 0.21$ ; Fig. 3A) or non-social time (all  $F_s < 2.98$ , all  $p > 0.09$ ; Fig. 4A) in adolescent rats. In adults, ANOVA showed significant NH ( $F(1,66) = 5.49$   $p = 0.022$ ), “Strain  $\times$  NH” ( $F(1,66) = 5.77$

**Table 1**  
Characteristics of gene-specific real-time qPCR primers.

Gene symbol <sup>a</sup>	Protein name	Accession no. <sup>b</sup>	Primer sequence	Amplicon size <sup>c</sup>
Reference genes				
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	NM_017008	(+) tcaccaccatggagaagge (-) gcta agcagttggtggtgca	168
Rpl13A	Ribosomal protein L13A	NM_173340	(+) acaagaaaagcggatggtg (-) ttccggtaatggatctttgc	167
Presynaptic markers				
Cables1	Cdk5 and Abl enzyme substrate 1	NP_001402620.1	(+) caggaatggcaggacactgaa (-) cgggttccgaagcatttgt	159
Cdk5	cyclin-dependent kinase 5	NP_543161.1	(+)cagtggtcctgacacgaccaa aaggctctctgccgagat	178
Snap25	Synaptosomal associated protein 25 kDa	NM_030991	(+)jagc agg tga gcg gca tca t (-)ctg gcg att ctg ggt gtc aat	190
Postsynaptic markers				
Nrg1	Neuregulin-1	NM_001271118.1	(+)agcgaaaggtatgtatcagcca (-)ggacacgggtggagacattt	111
Homer1	Homer scaffolding protein 1	NM_031707.1	(+)caccgatgtgacacagaaac (-)tgttgccttccactgcttcac	220
Bdnf	Brain-derived neurotrophic factor	NM_001270630	(+)gaaagtcccggatcaaaaag (-)cgccagccaattctcttttg	187
Receptors				
Drd1	Dopamine receptor D1	NM_012546	(+)ggaggacaccggatgta (-)atgaggacgatgaaatgg	69
Grin2b	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	NM_012574.1	(+)tccgaagctggtgataatcc (-)cttctggcacgggactgtat	238

<sup>a</sup> Following RGD nomenclature.

<sup>b</sup> Genbank accession number of cDNA and corresponding gene, available at <http://www.ncbi.nlm.nih.gov/>.

<sup>c</sup> Amplicon length in base pairs.

$p = 0.019$ ) and “strain x interval” (Huynh-Feldt (df 2134) = 3.15  $p = 0.047$ ) effects on social time (Fig. 3B), but no significant effects for non-social time (all Fs < 1.95, all  $p > 0.16$ ; Fig. 4B). *Posthoc* Duncan’s tests showed that adult RLA rats exhibited more social time than RHAs, whereas NH significantly increased this measure in the RHA strain (Fig. 3A-B).

The factorial ANOVA revealed strain ( $F(1,102) = 17.95$   $p < 0.001$ ) and NH ( $F(1,102) = 7.0$   $p = 0.01$ ) effects on self-grooming, since RLA rats showed overall enhanced grooming and NH markedly reduced it (Fig. 5A). There also appeared “strain x NH” ( $F(1,102) = 14.31$   $p < 0.001$ ), “strain x age” ( $F(1,102) = 4.52$   $p = 0.036$ ) and “NH x age” ( $F(1,102) = 4.51$   $p = 0.036$ ) effects (Fig. 5A), since NH-reducing effects on grooming were particularly marked in adult RLA rats (see Duncan’s tests in Fig. 5A). Separate ANOVA for each age showed a “strain x NH” effect ( $F(1,36) = 5.40$   $p = 0.026$ ) in adolescent rats, since the NH-reducing effect was observed only in RLA rats (see Duncan’s test in Fig. 5A left). In adults, the ANOVA revealed strain ( $F(1,66) = 26.66$   $p < 0.001$ ), NH ( $F(1,66) = 14.94$   $p < 0.001$ ) and “strain x NH” ( $F(1,66) = 10.45$   $p = 0.002$ ), indicating a marked reduction of grooming by NH in RLA rats (see Duncan’s test in Fig. 5A right).

As for crossings in the SI test, ANOVA revealed strain ( $F(1,102) = 65.1$   $p < 0.001$ ), NH ( $F(1,102) = 29.99$   $p < 0.001$ ), age ( $F(1,102) = 16.72$   $p < 0.001$ ) and “strain x age” ( $F(1,102) = 6.89$   $p = 0.01$ ) effects (Fig. 5B). These indicate that RHAs are more active than RLA rats, NH increases activity, adults are more active than adolescent rats, but such an effect is particularly evident in RHA rats (Duncan’s tests in Fig. 5B). Separate ANOVA analyses for each age yielded strain and NH effects in adolescent rats ( $F(1,36) = 11.9$   $p < 0.001$ , and  $F(1,36) = 11.6$   $p = 0.002$ , respectively), as well as in adult rats ( $F(1,66) = 77.9$   $p < 0.001$ , and  $F(1,66) = 10.6$   $p < 0.002$ , respectively) (Fig. 5B), reflecting that RHA rats are more active at both ages, whereas NH globally increases activity also at both ages (Fig. 5B).

### 3.3. Prepulse inhibition

A factorial repeated measures ANOVA (2 strain  $\times$  2 NH  $\times$  2 age  $\times$  4 prepulse intensities as within-subject factor) revealed strain ( $F(1,70) = 28.57$   $p < 0.001$ ) and age ( $F(1,70) = 7.03$ ,  $p = 0.01$ ) effects, and a nearly significant “strain x NH” ( $F(1,7) = 3.95$   $p = 0.051$ ) effect (Fig. 6A-B). The analysis also yielded a significant “strain x NH x age x intensity” effect (Huynh-Feldt (2.6184.14) = 3.98  $p = 0.012$ ), indicating that strain and NH effects were dependent upon the other factors (Fig. 6A).

Separate ANOVA for each age revealed strain ( $F(1,36) = 12.04$   $p < 0.001$ ) and “strain x NH x intensity” ( $F(3,108) = 3.10$   $p = 0.03$ ) effects in adolescent rats. *Posthoc* comparisons indicate that RHA-C exhibited significantly lower PPI than RLA-C, whereas RHA-NH rats showed better %PPI at the 70 dB prepulse intensity (see Duncan’s tests in Fig. 6A-B).

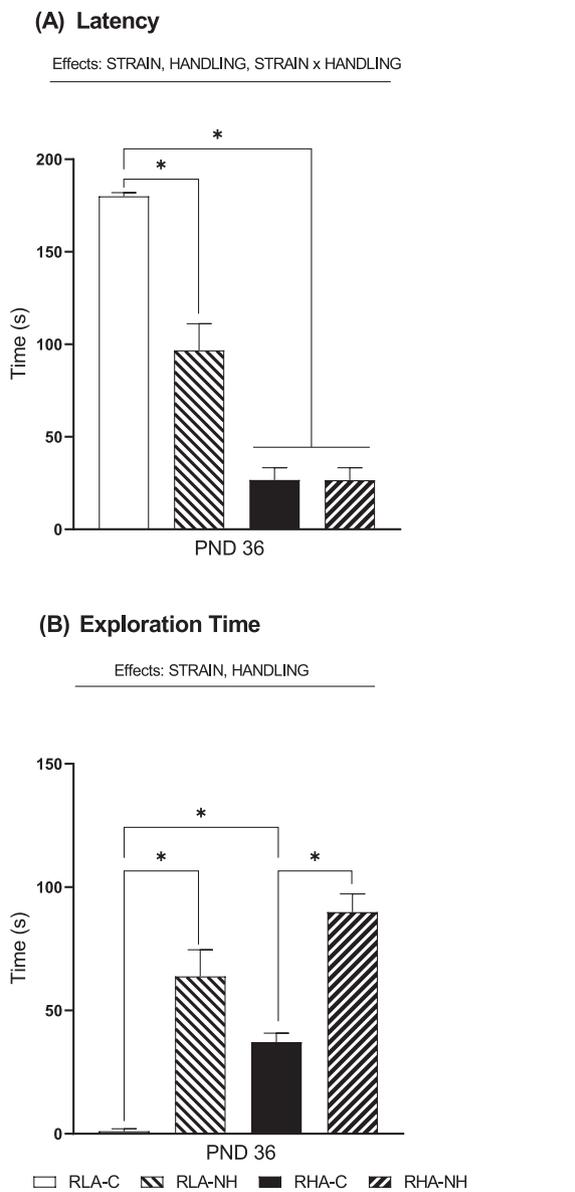
For adult rats, the ANOVA yielded strain ( $F(1,34) = 16.42$   $p < 0.001$ ), “strain x NH” ( $F(1,34) = 4.32$   $p = 0.045$ ) and “strain x intensity” (Huynh-Feldt(1.85,63.01) = 7.34  $p = 0.002$ ) effects on %PPI. *Posthoc* Duncan’s comparisons indicate that NH increased %PPI at 65-70 dB prepulse intensities, as well as Total-PPI, in RHA rats, while not affecting RLA rats (Fig. 6A-B).

Regarding baseline startle, factorial ANOVA only showed an age effect ( $F(1,102) = 86.3$   $p < 0.001$ ), as baseline startle was much higher in adult than adolescent rats (Fig. 6C).

### 3.4. Differentially expressed genes in the FC

The three-way factorial ANOVA for *Cables1* expression levels showed strain ( $F(1,58) = 21.41$   $p < 0.001$ ), NH ( $F(1,58) = 6.94$   $p < 0.011$ ) effects, and a nearly significant “NH x Age” ( $F(1,58) = 3.70$   $p = 0.06$ ) effect. *Posthoc* Duncan’s tests showed that adolescent RHA-NH rats exhibited higher expression levels than RHA-C, and increased expression levels in adult RHA-C compared to RLA-C rats (Fig. 7A).

ANOVA on the expression levels of *CDK5* revealed NH ( $F(1,54) = 8.23$   $p = 0.006$ ) and “strain x NH” ( $F(1,54) = 6.10$   $p = 0.017$ ) effects

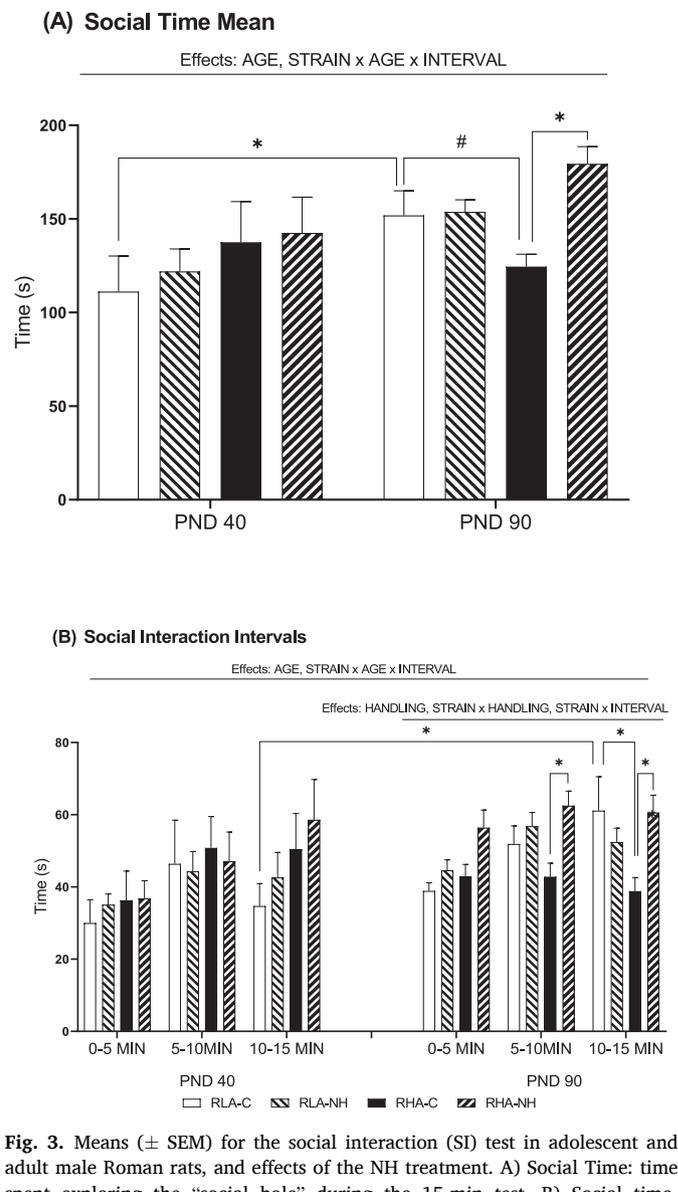


**Fig. 2.** Means ( $\pm$  SEM) for the novel object exploration (NOE) test. A) “Latency”; time elapsed until the first exploration of the novel object. B) “Exploration Time”; time spent exploring the novel object.  $n = 28$ /group. Group symbols: RLA-C, RHA-C; control, non-handled groups. RLA-NH, RHA-NH; groups receiving neonatal handling. “STRAIN”, “HANDLING” and “STRAIN x HANDLING” effects refer to statistically significant main or interaction effects from factorial ANOVA. \*  $p < 0.05$ , Duncan’s multiple range test following significant ANOVA effects.

(Fig. 7B). *Posthoc* Duncan’s tests showed that adolescent RHA-C rats exhibited lower expression levels than RLA-C and RHA-NH (Fig. 7B).

ANOVA for *Snap25* expression showed strain ( $F(1,55) = 13.22$   $p < 0.001$ ), NH ( $F(1,55) = 8.53$   $p = 0.005$ ), and “strain x NH” ( $F(1,55) = 13.87$   $p < 0.001$ ) effects (Fig. 7C). *Posthoc* Duncan’s tests showed that, at both ages the RHA-C group exhibited much higher expression levels than the respective RLA-C and RHA-NH groups (Fig. 7C).

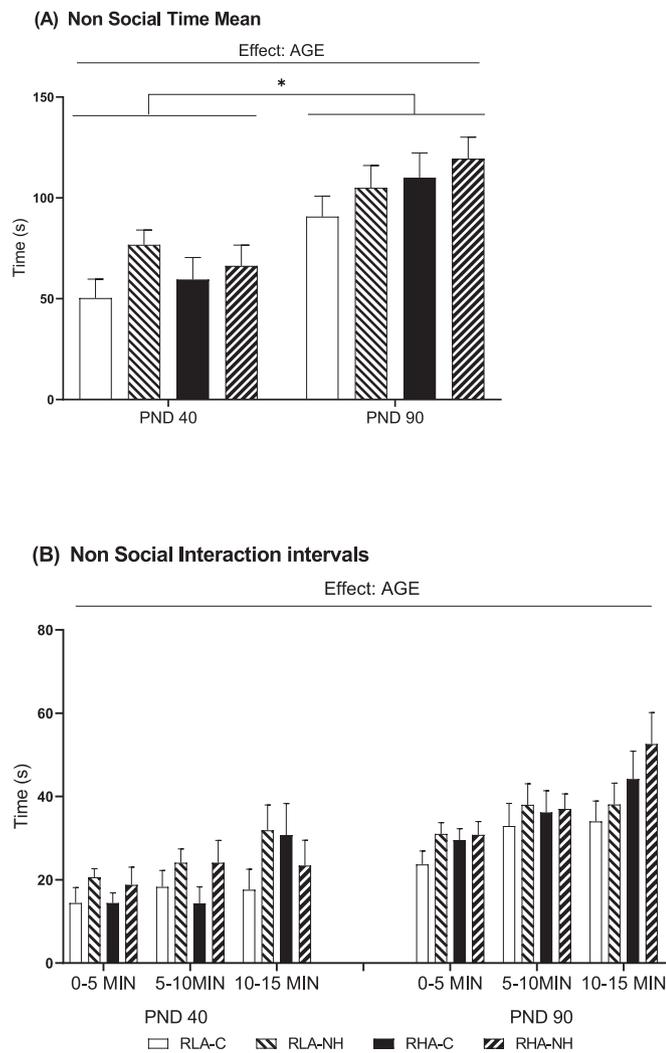
As for the expression levels of genes related to postsynaptic activity (*Nrg1*, *Homer1*), the ANOVA for *Nrg1* expression showed NH ( $F(1,59) = 14.69$   $p < 0.001$ ) and “strain x NH x age” ( $F(1,59) = 14.55$   $p < 0.001$ ) effects (Fig. 8A). *Posthoc* Duncan’s tests revealed a decreased expression of *Nrg1* in adolescent RHA-C vs. RLA-C and RHA-NH rats, as well as decreased expression in adult RLA-C relative to RLA-NH and RHA-C groups (Fig. 8A).



**Fig. 3.** Means ( $\pm$  SEM) for the social interaction (SI) test in adolescent and adult male Roman rats, and effects of the NH treatment. A) Social Time: time spent exploring the “social hole” during the 15-min test. B) Social time-intervals: time spent exploring the “social hole” during each 5-min interval of the test. Group symbols: RLA-C, RHA-C; control, non-handled groups. RLA-NH, RHA-NH; groups receiving neonatal handling. “0-5 MIN”, “5-10 MIN”, “10-15 MIN”, are the three 5-min intervals of the SI test. The number of rats per group was,  $n = 10$  for adolescents (PND40), and  $n = 18$  for adults (PND90). “AGE”, “HANDLING”, “STRAIN x AGE x INTERVAL”, “STRAIN x HANDLING” and “STRAIN x INTERVAL” effects refer to statistically significant main or interaction effects from factorial ANOVA. \*  $p < 0.05$ , Duncan’s multiple range test following significant ANOVA effects. #  $p < 0.037$  (Duncan’s multiple range test; one-tailed, since we had the directed hypothesis that RHA-C rats should explore less the “social hole” than RLA-C rats, based on previous studies).

ANOVA on the expression levels of *Homer1* revealed strain ( $F(1,55) = 12.69$   $p = 0.001$ ), age ( $F(1,55) = 59.67$   $p < 0.001$ ) and “strain x NH x age” ( $F(1,55) = 7.00$   $p = 0.011$ ) effects (Fig. 8B). *Posthoc* Duncan’s tests revealed that adult RHA-C rats overexpressed *Homer1* compared to RLA-C rats. Moreover, adolescent RHA-NH had higher expression levels than RHA-C rats. Finally, adult RHA-C rats show an overexpression compared to adolescent RHA-C rats (Fig. 8B).

ANOVA of *Bdnf* expression revealed strain ( $F(1,52) = 4.96$   $p = 0.031$ ), NH, ( $F(1,52) = 7.09$   $p = 0.011$ ), and “strain x NH x Age” ( $F(1,52) = 4.92$   $p = 0.032$ ) effects (Fig. 9). *Posthoc* Duncan’s tests showed that adult RHA-C exhibit higher expression than RLA-C rats, NH led to



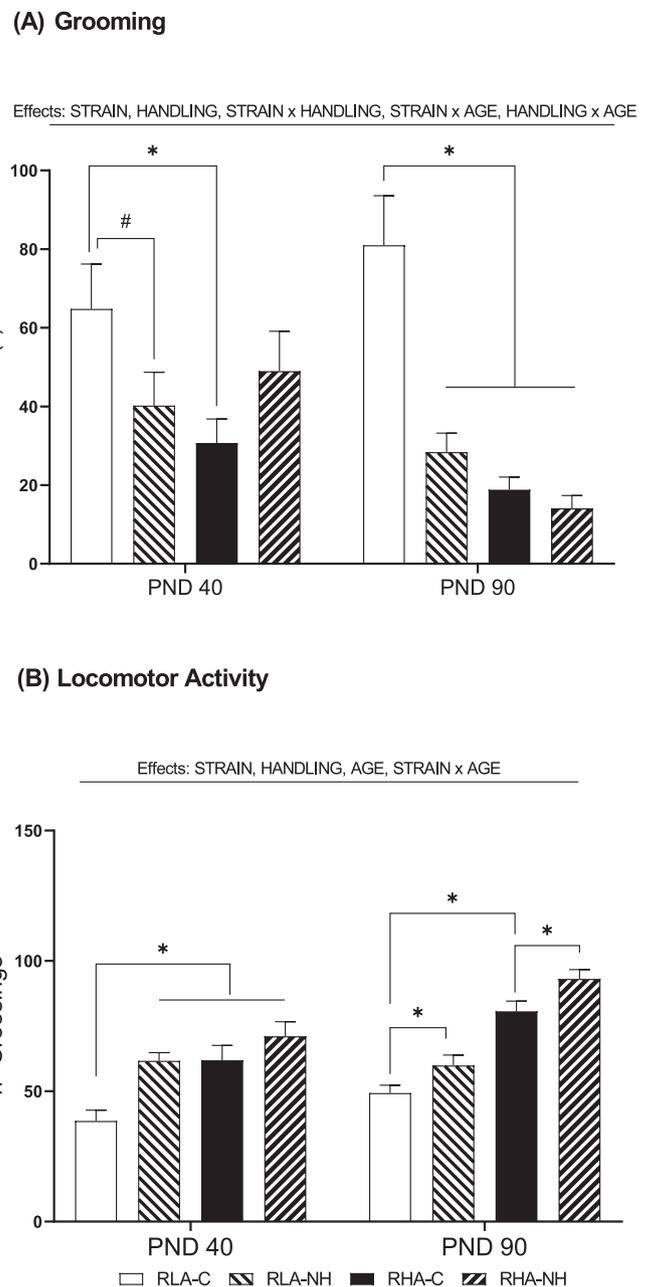
**Fig. 4.** Means ( $\pm$  SEM) for the social interaction (SI). A) Total Non-Social Time: time spent exploring the “non-social hole” during the 15-min test. B) Non-social Interaction intervals: time spent exploring the “non-social hole” during each 5-min interval of the test. Group symbols: RLA-C, RHA-C; control, non-handled groups. RLA-NH, RHA-NH; groups receiving neonatal handling. “0-5 MIN, 5-10 MIN, 10-15 MIN”, are the three 5-min intervals of the SI test. The number of rats per group was,  $n = 10$  for adolescents (PND40), and  $n = 18$  for adults (PND90). “AGE” effect refers to statistically significant effect from factorial ANOVA  $* p < 0.05$ , Duncan’s multiple range test following significant ANOVA.

increased expression in RLA-NH vs. RLA-C rats and, adolescent RHA-NH showed higher expression than control RHA-C rats. Furthermore, there is overexpression in adult RHA-C rats compared to adolescent RHA-C rats (Fig. 9).

Regarding the levels of gene expression for receptors, ANOVA for *Grin2b* showed strain ( $F(1,58) = 7.83 p = 0.007$ ) and age ( $F(1,58) = 39.69 p < 0.001$ ) effects. *Posthoc* Duncan’s tests showed that adolescent RHA-NH rats have higher expression levels than RHA-C (Fig. 10A). As for *Drd1* expression, ANOVA showed age ( $F(1,63) = 21.41 p < 0.001$ ) and “strain x NH x age” ( $F(1,63) = 5.56 p = 0.022$ ) effects. *Posthoc* Duncan’s tests revealed that adolescent RHA-NH exhibited higher expression levels than RHA-C rats, whereas adult RHA-C showed increased expression relative to adolescent RHA-C rats (Fig. 10B).

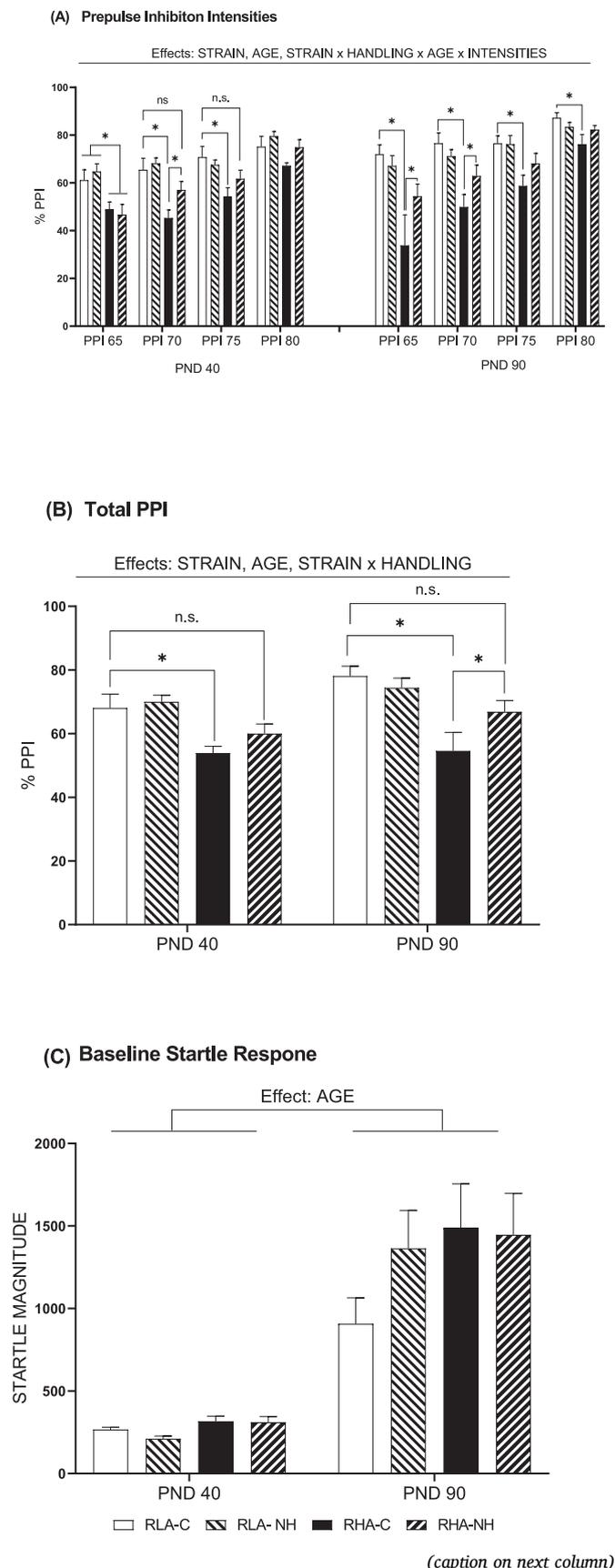
### 3.5. Correlations among gene expression levels and between gene expression and behaviour

Pearson’s correlations are shown in Supplementary Table 1-2.



**Fig. 5.** Means ( $\pm$  SEM) for A) Grooming (time spent self-grooming), and B) Locomotor activity (number of crossings) during the SI test. The number of rats per group was,  $n = 10$  for adolescents (PND40), and  $n = 18$  for adults (PND90). Group symbols: RLA-C, RHA-C; control, non-handled groups. RLA-NH, RHA-NH; groups receiving neonatal handling. “STRAIN”, “HANDLING”, “AGE”, “STRAIN x HANDLING”, “STRAIN x AGE”, and “HANDLING x AGE” effects refer to statistically significant main or interaction effects from factorial ANOVA.  $* p < 0.05$ , Duncan’s multiple range test following significant ANOVA.  $\# p < 0.035$  (Duncan’s multiple range test; one-tailed, as we had a directed hypothesis on that difference based on previous studies).

First, it is noteworthy that correlations of expression levels among the different genes reveal 23 significant coefficients in adolescent rats (Suppl. Table 1) compared to 11 significant correlation coefficients in adult rats (Suppl. Table 2), i.e., variations in gene expression levels are more “synchronized” during neurodevelopment than in adulthood. Importantly, some significant correlations with the same sign are present at both ages: *Cables1* with *Cdk5*, *Nrg1*, *Homer1* and *Bdnf* (ranging from  $r = 0.335$  to  $r = 0.703$ ); *Cdk5* with *Nrg1* (from  $r = 0.385$  to  $r = 0.701$ ); and *Nrg1* with *Homer1* and *Bdnf* (from  $r = 0.456$  to  $r = 0.653$ ).



**Fig. 6.** Means ( $\pm$  SEM) of prepulse inhibition levels (%PPI) in adolescent (PND40) and adult (PND90) male Roman rats, and effects of the neonatal handling treatment on, A) %PPI across prepulse intensities, B) the total %PPI (averaged for the 4 prepulse intensities), and C) Baseline Startle Response. PPI65-80; prepulse intensities of 65-80 dB. Group symbols: RLA-C, RHA-C; control, non-handled groups. RLA-NH, RHA-NH; groups receiving neonatal handling.  $n = 10/$  group. “STRAIN”, “AGE”, “STRAIN x HANDLING”, and “STRAIN x HANDLING x AGE x INTENSITY” effects refer to statistically significant main or interaction effects from factorial ANOVA. \*  $p < 0.05$ , Duncan’s multiple range test following significant ANOVA.

(Suppl. Table 1 and 2).

Second, there were 5 significant correlations among gene expression levels and behavioural measures in adolescent rats, compared to 16 significant coefficients in adult rats (Suppl. Table 1 and 2), i.e., in the mature FC, gene function is better correlated with behaviour. It is noteworthy that these correlations were mostly between genes and exploratory behaviour (NOE and crossings) and grooming (Suppl. Table 1 and 2). Conversely, only *Snap25* in adolescent rats, and *Cables1* in adult rats, correlated with %PPI ( $r = -0.371$  and  $r = -0.402$ , respectively; Suppl. Table 1 and 2), and only *Cdk5* expression in adult rats correlated with “Social time” ( $r = 0.388$ ) in the SI test (Suppl. Table 2).

#### 4. Discussion

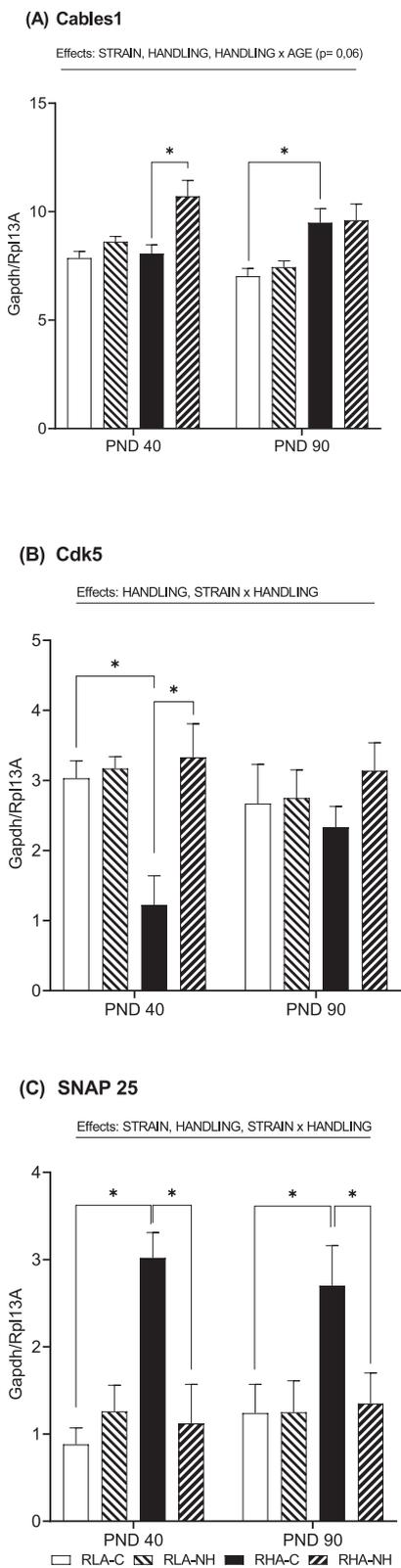
The present study showed for the first time, by use of the Roman rat model, a maturational effect, as shown in adolescent rats, and long-term effect, as shown in adult rats, of neonatal handling on behaviours related to novelty-induced inhibition/exploration (NOE), anxiety (grooming), locomotor activity (SI test), social interaction (SI test) and attention/sensorimotor gating (PPI test). In addition, we found differential gene expression of targets involved in neurodevelopment, neuroplasticity, and synaptic processes as a function of strain, neonatal treatment, and age.

##### 4.1. Strain differences and neonatal handling effects on behaviour as a function of age

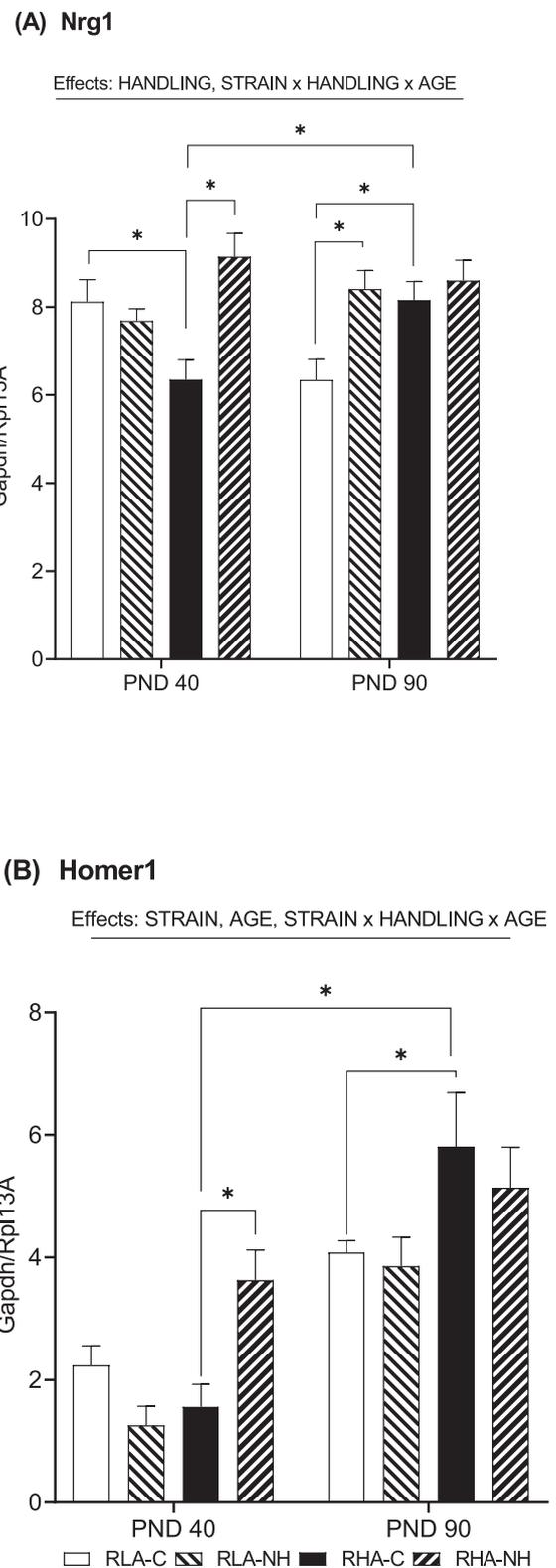
We found increased novelty exploration in adolescent RHA rats, consistent with previous findings on adult RHA/RLA rats (Cuenya et al., 2016; Río-Álamos et al., 2015, 2017a, 2017b, 2019; Sampedro-Viana et al., 2021). This, together with the other differences in horizontal exploratory behaviour (locomotion), grooming, and PPI performance, indicate that these strain differences are already present early during development. Further, these strain differences become more evident in adulthood, agreeing with previous studies from our group (Oliveras et al., 2015, 2022a; Tapias-Espinosa et al., 2018, Tapias-Espinosa et al., 2019). Conversely, strain differences in social interaction (social time) were only manifested in adult rats.

Neonatal handling had a positive effect on novelty exploration, locomotor activity and anxiety-related behaviour (grooming) in adolescent RLA rats. In the RHA strain, the treatment had primarily a positive effect on the PPI performance, as the treatment increased %PPI levels at 70-75 dB prepulse intensities in the adolescent RHA rats, but not in the RLA strain. Of interest, the handled RLA exhibited behaviours equal to the untreated RHAs (see NOE test, grooming and locomotor activity), suggesting the early intervention had a regulating effect on the further development of coping-linked and anxiety-related responses (i.e., grooming, exploring a novel object; Fernández-Teruel and Estanislau, 2016). This may involve early neurodevelopmental changes in neural circuits, neurotransmitters, and neuroendocrine factors known to play relevant roles in shaping those stress/anxiety-coping behavioural responses (e.g., Antoniazzi et al., 2014; Meaney et al., 1988, 1989; Monroy et al., 2010; Papaioannou et al., 2002; Park et al., 2003).

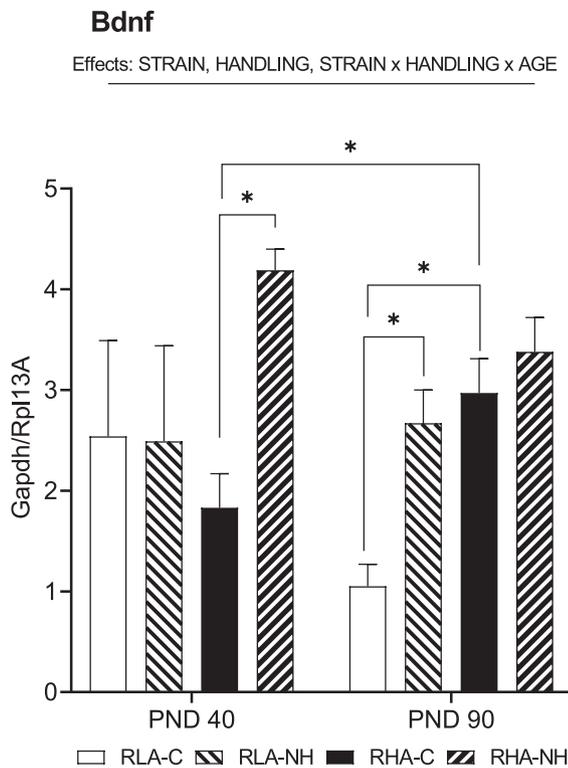
When comparing adult rats, and thereby the long-term effect of the neonatal handling, we observed an effect on novelty-induced locomotor



**Fig. 7.** Means ( $\pm$  SEM) for gene expression levels of presynaptic markers in the FC of adolescent and adult male Roman rats. (A) *Cables1* gene. (B) *Cdk5* gene. (C) *Snap25* gene.  $n = 5-8/$  group. Group symbols: RLA-C, RHA-C; control, non-handled groups. RLA-NH, RHA-NH; groups receiving neonatal handling. Gapdh/Rpl13A: Relative transcription level of the target genes was normalized to that of average for housekeeping genes (*GAPDH* and *RPL13A*). “STRAIN”, “HANDLING”, “STRAIN x HANDLING” and “HANDLING x AGE” effects refer to statistically significant main or interaction effects from factorial ANOVA. \* $p < 0.05$  Duncan’s multiple range test following significant ANOVA.



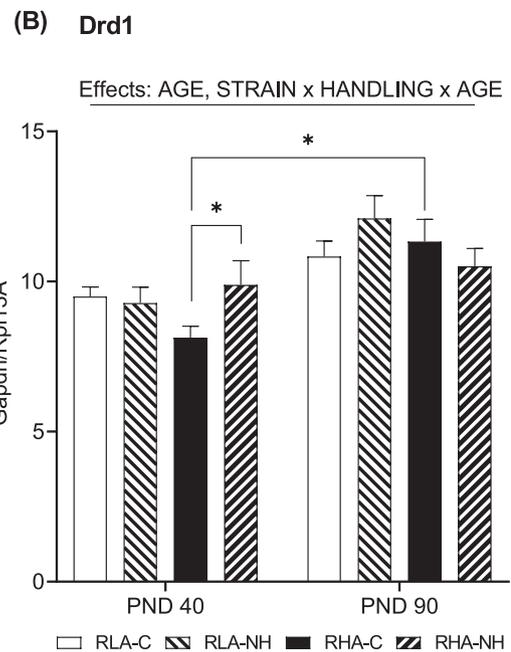
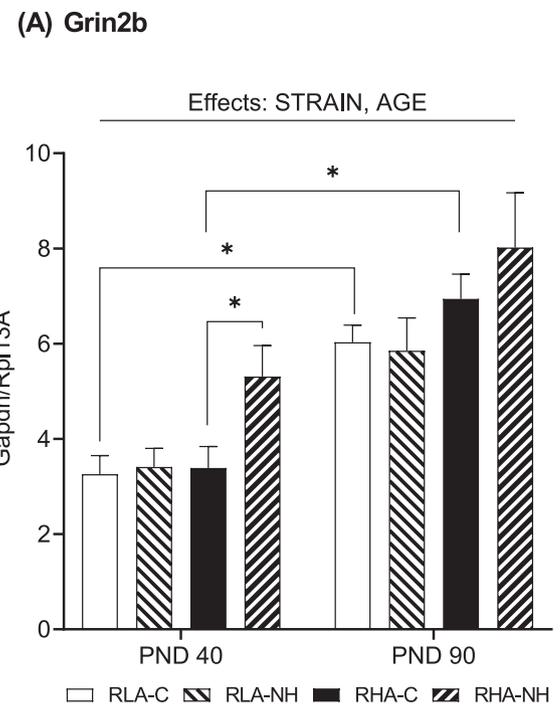
**Fig. 8.** Means ( $\pm$  SEM) for gene expression levels of postsynaptic markers in the FC of adolescent (PND40) and adult (PND90) male Roman rats. (A) *Nrg1* gene. (B) *Homer1* gene.  $n = 5-8/$  group. Gapdh/Rpl13A: Relative transcription level of the target genes was normalized to that of average for housekeeping genes (*GAPDH* and *RPL13A*). Group symbols: RLA-C, RHA-C; control, non-handled groups. RLA-NH, RHA-NH; groups receiving neonatal handling. “STRAIN”, “HANDLING”, “AGE” and “STRAIN x HANDLING x AGE” effects refer to statistically significant main or interaction effects from factorial ANOVA. \* $p < 0.05$  Duncan’s multiple range test following significant ANOVA.



**Fig. 9.** Means ( $\pm$  SEM) for *Bdnf* gene expression levels in the FC of adolescent (PND40) and adult (PND90) male Roman rats.  $n = 6-8/$  group. Gapdh/Rpl13A: Relative transcription level of the target genes was normalized to that of average for housekeeping genes (*GAPDH* and *RPL13A*). Group symbols: RLA-C, RHA-C; control, non-handled groups. RLA-NH, RHA-NH; groups receiving neonatal handling. “STRAIN”, “HANDLING” and “STRAIN x HANDLING x AGE” effects refer to statistically significant main or interaction effects from factorial ANOVA. \* $p < 0.05$  Duncan’s multiple range test following significant ANOVA.

activity for both strains, while reduced anxiety-related behaviour (i.e., grooming) in RLA rats, and increased social interaction and PPI in RHA rats. Such long-term effects of neonatal handling on adult rats are in line with other studies using different rat strains, supporting the belief that neonatal handling might enduringly improve cognition/attention-related processes particularly in the RHA rats (e.g., Aguilar et al., 2002; Fernández-Teruel et al., 1997, 2002; Fu and Depue, 2019; Peralta-Vallejo et al., 2024; Rainekei et al., 2014; Río-Álamos et al., 2015, 2017b, 2019; Sampedro-Viana et al., 2021, 2023a, 2023b; Steimer et al., 1998).

As said earlier, neonatal handling produces long-lasting neurodevelopmental changes in neuronal circuits, neurotransmitters and neuroendocrine processes (e.g., Antoniazzi et al., 2014; Meaney et al., 1988, 1989; Monroy et al., 2010; Papaioannou et al., 2002; Park et al., 2003). Although the “neonatal handling” intervention presents variations among studies, it most commonly involves a brief daily separation of the pups from the mother and the litter during the first two or three weeks of life, and (in most studies) daily placing each pup individually in a container (or novel cage) for periods ranging 3-15 min (e.g., see Levine, 1956; Meaney et al., 1988; Escorihuela et al., 1994). It is known that maternal separation (MS) induces changes in maternal care in rodents (e.g., Baracz et al., 2020; Macri and Würbel, 2006; Rombaut et al., 2023), and this has led to studies exploring whether variations in maternal behaviour due to the brief MS involved in the neonatal handling procedure might explain the effects of the latter intervention. For example, both neonatal handling and brief MS (of equivalent time to that of the “neonatal handling” intervention, but not involving isolation or stroking of the pups), have been found to elicit similar increases of maternal care in rats (Macri et al., 2004). However, in spite that neonatal handling and brief MS led to similar amounts of maternal



**Fig. 10.** Means ( $\pm$  SEM) for gene expression levels for receptors in the FC of adolescent (PND40) and adult (PND90) male Roman rats. (A) *Grin2b* gene. (B) *Drd1* gene.  $n = 6-8/$  group. Gapdh/Rpl13A: Relative transcription level of the target genes was normalized to that of average for housekeeping genes (*GAPDH* and *RPL13A*). Group symbols: RLA-C, RHA-C; control, non-handled groups. RLA-NH, RHA-NH; groups receiving neonatal handling. “STRAIN”, “AGE” and “STRAIN x HANDLING x AGE” effects refer to statistically significant main or interaction effects from factorial ANOVA. \* $p < 0.05$  Duncan’s multiple range test following significant ANOVA.

behaviour, only the former produced enduring reductions of stress-induced hormone responses and fear/anxiety-related behaviour in the adult offspring (Macrì et al., 2004). This indicates that increased maternal care (due to MS) cannot be the sole mediator of the long-lasting effects of neonatal handling (Macrì and Würbel, 2006; Macrì et al., 2004; Macrì et al., 2008), and that other components of this intervention (e.g., gentle stroking of pups, brief pup isolation) likely contribute to its enduring neurobehavioral effects (e.g., Fernández-Teruel, 2022; Guzzetta et al., 2009). It is worth to mention that our present neonatal handling procedure, involving two daily 8-min periods of individual separation of the pups from the mother and the litter, and gently stroking each pup (with the hands) for 9–12 s during each 8-min period, has successfully been used since the 1990s to produce long-lasting cognitive effects and reductions of anxiety-related and stress hormone responses in RLA and RHA rats (e.g., Aguilar et al., 2002; Escorihuela et al., 1995; Fernández-Teruel et al., 1991, 1992, 1997, 2002; Río-Álamos et al., 2017b, 2019; Sampedro-Viana et al., 2021; Steimer et al., 1998).

#### 4.2. Strain and neonatal handling effects on gene expression as a function of age: associations between gene expression and behaviour

We found *Cables 1* expression was upregulated by the neonatal treatment in the adolescent RHA rats. We also found an increased expression of this gene in untreated adult RHA rats compared to RLAs. This is contrary to previous findings from Sønderstrup et al. (2023). This could be explained by the different age of the rats, that were younger here, or to the fact that the rats of this study underwent behavioural testing whereas in Sønderstrup et al. the rats were experimentally naive.

*Cables 1* is an activator of *Cdk5* (cyclin-dependent kinase 5), and indeed neonatal handling increased the expression of *Cdk5* in adolescent RHA in correlation with *Cables 1* (Suppl. Table 1), supporting a joined action on brain development. *Cdk5* is an important modulator of synaptogenesis and synaptic plasticity by regulating NMDA2B receptor subunit degradation as well as by stabilizing dendritic spines into maturational states (Huang et al., 2017; Hawasli et al., 2007; Easley-Neal et al., 2013). The lower constitutive expression of *Cdk5* in the adolescent RHA strain may contribute to the impaired synaptic maturation characterizing this strain (Sanchez-Gonzalez et al., 2021), and this may be reversed by the neonatal handling, as suggested by the normalization of altered *Snap25* and *Cdk5* expression levels, and the effect on *Grin2b* expression in the handled adolescent RHA rats. In fact, alterations of *Cdk5* and upregulation of presynaptic SNARE components, such as *Snap25*, have been related to schizophrenia and schizophrenia-linked behavioural phenotypes (Jeans et al., 2007; Engmann et al., 2011; McKee et al., 2010; Oliver and Davies, 2009; Ramos-Miguel et al., 2015). Thus, it is noteworthy that the markedly increased expression of *Snap25* in RHA rats of both ages is normalized by neonatal handling, which in parallel improves PPI (considered an endophenotype related to schizophrenia) in this rat strain.

Further consistent with the notion that aberrant (or delayed) synaptic maturation of the FC is a characteristic of RHA rats (Elfving et al., 2019; Sánchez-González et al., 2021; Sønderstrup et al., 2023), and that such neurodevelopmental alteration/s might be normalized by the neonatal handling is our findings of altered and reversed *Bdnf*, *Homer1* and *Nrg1* expression in the RHA strain. The neurotrophic factor BDNF has long been associated with the cellular, cytoarchitectural and volumetric alterations in schizophrenia (Angelucci et al., 2005; Nieto et al., 2013), with human postmortem studies reporting significant alterations in BDNF mRNA and protein levels in patients with schizophrenia in both cortical and hippocampal regions (Durany et al., 2001; Reinhart et al., 2015; Takahashi et al., 2000). The *Bdnf* gene and protein alterations observed in the adult RHA rats here and in previous studies (Elfving et al., 2019; Sanna et al., 2019; Serra et al., 2022, 2023; Sønderstrup et al., 2023) align well with the frontocortical and hippocampal volumetric alterations (Río-Álamos et al., 2017b; Río-Álamos et al., 2019;

Tapias-Espinosa et al., 2019) and sensorimotor gating deficits (Notaras et al., 2016; Oliveras et al., 2015; Peralta-Vallejo et al., 2024; Río-Álamos et al., 2019; Esnal et al., 2016), characterizing this strain. Other genes involved in synaptic plasticity and communication are *Nrg1* and the postsynaptic density (PSD) protein *Homer1*, both reduced in adolescent RHA rats and, in agreement with previous findings (Elfving et al., 2019; Sønderstrup et al., 2023), increased in adult RHA rats relative to RLAs. Similar to *Snap25*, neonatal handling affected *Homer1*, *Nrg1* and *Bdnf*, as well as *Drd1* and *Grin2b* expression in adolescent RHAs, increasing their expression levels. *Homer1* polymorphisms are associated with differential treatment response in schizophrenia (Föcking et al., 2015; Spellmann et al., 2011), whereas *Nrg1* has a role in regulating excitatory/inhibitory neurotransmission at the PSD and has been linked to schizophrenia risk (Mostaid et al., 2016) and modulation of sensorimotor gating (Hong et al., 2008; Karl et al., 2011; Rhein et al., 2013; Roussos et al., 2011).

The lasting effect of the NH intervention in reversing the constitutively higher *Snap25* expression in the RHA is of particular interest as this gene is involved in the maturation of synaptic connections and brain networks regulating attention (Hayashi et al., 2021; Söderqvist et al., 2010). Attention deficit/hyperactivity disorder (ADHD), considered a highly heritable neurodevelopmental disorder, has been associated with specific polymorphisms in *Snap25* (Wang et al., 2018). This aligns well with the observed reversal of the inherent attention (PPI) deficit in the adult RHA strain by the neonatal handling, suggesting a positive impact of the early intervention by priming brain maturation through the regulation of *Snap25* expression. SNAP25 is a tightly controlled intrinsically disordered and aggregation-prone protein (Kraichely et al., 2025), therefore the higher *Snap25* expression in the RHA may not necessarily be related with increased protein expression but with a compensatory response to an increased SNAP25 degradation or turnover. From previous studies we know that increased *Snap25* expression in FC does not translate to higher protein levels (Elfving et al., 2019). Nevertheless, the potential behind reversing the attentional deficit by regulating *Snap25* during brain maturation should be considered for future research.

In RLAs, most genes were not affected by the neonatal handling during adolescence, but the treatment led to increased *Nrg1* and *Bdnf* levels in adulthood. The significant negative correlations between *Nrg1* and *Bdnf* expression levels and NOE latency and grooming in adult rats (Suppl. Table 2) may suggest a modulatory role of these genes on these anxiety-linked behaviours. In fact, neonatal handling treatment has a well-known and enduring anti-anxiety (and anti-stress) effect, represented here by the increase of both NOE performance and locomotion under novelty conditions, as well as by the reduction of novelty-induced self-grooming (Fernández-Teruel and Estanislau, 2016; Fernández-Teruel et al., 2002; Fu and Depue, 2019; Raineki et al., 2014; Río-Álamos et al., 2017b; Sampedro-Viana et al., 2021, 2023a).

In summary, we report the first time that neonatal handling has a general positive effect on gene expression of synaptic-related genes specifically in adolescent RHA rats, the strain exhibiting abnormal schizophrenia-linked behavioural phenotypes. Gene expression changes in *Cdk5*, *Bdnf*, *Homer1*, *Nrg1*, *Drd1*, *Grin2b* and *Snap25* accompany for the most part the neonatal handling-induced effects on PPI and novelty exploration (NOE test) in adolescent RHA rats. Conversely, neonatal handling did not manifest in changes on gene expression in adult RHAs, except for the normalization of *Snap25* expression levels, which could be linked to the treatment effects on PPI levels. Further, the treatment increased *Nrg1* and *Bdnf* expression in adult RLA rats, in parallel to the decrease of anxiety-related behaviours. While the present findings are novel and interesting, some caution is advised in their interpretation, as variations in gene expression do not necessarily always correspond to variations in the corresponding proteins. Thus, determining protein levels would be necessary in the future to draw more robust conclusions about the observed effects.

The non-inclusion of female rats in the present study is an obvious

limitation to the generalizability of the findings. The narrow time window involved in performing the behavioural tests and brain sampling within the rats' adolescent period, prevented the inclusion of eight more groups of females (two strains, two treatment conditions, two ages) to be evaluated in parallel to the eight groups of males. It is worth to mention, however, that we initiated independent studies on adult females (from both rat strains, receiving neonatal handling or no treatment), from which we sampled and stored their FC for future gene expression studies, which show both some similar and some different effects of strain or neonatal handling (relative to the effects found here in males) in several behavioural/cognitive tests (Peralta-Vallejo et al., 2024). Hence, it is undoubtedly recommendable to include females of both rat strains in future studies to explore possible sex-, strain- and treatment-related effects, as well as their interactions, on the parameters measured here.

To conclude, we report for the first time that a "positive" early environmental treatment, such as neonatal handling, may have the capacity to modulate the endophenotypic synaptic/behavioural alterations in RHA rats. This supports previous evidence of positive long-lasting effects of neonatal handling on many emotional responses, social behaviour, attention-related processes and cognition (e.g., Aguilar et al., 2002; Fernández-Teruel et al., 2002; Peralta-Vallejo et al., 2024; Raineke et al., 2014; Río-Álamos et al., 2017b, 2019; Sampedro-Viana et al., 2021, 2023a). Our findings add support to the notion of an aberrant neurodevelopment and immature frontal cortex in RHA rats, consistent with their profile of schizophrenia-relevant behavioural phenotypes, and the importance of this model in contributing to defining the most important time-window for intervention studies.

#### CRedit authorship contribution statement

**Natalia Peralta-Vallejo:** Writing – original draft, Investigation, Data curation. **Toni Cañete:** Supervision, Methodology, Investigation, Conceptualization. **Daniel Sampedro-Viana:** Supervision, Methodology, Investigation, Data curation, Conceptualization. **Pau Güell-Falgueras:** Investigation, Data curation. **Cristóbal Río-Álamos:** Methodology, Investigation. **Ignasi Oliveras:** Software, Methodology, Data curation. **Adolf Tobeña:** Supervision, Methodology, Formal analysis, Conceptualization. **Susana Aznar:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Alberto Fernández-Teruel:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

#### Ethical statement

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

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#### Declaration of competing interest

The authors declare that they have no conflict of interest.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pnpbp.2025.111364>.

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