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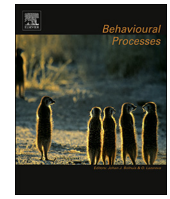

The effects of environmental enrichment and social isolation and their reversion on anxiety and fear conditioning

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ABSTRACT

Animal models of fear and anxiety provide important insight into anxiety-related symptoms in humans. Environmental physical conditions and social contact influence behavior and brain plasticity particularly at early developmental stages and have long lasting effects reaching even adulthood. The potential benefit that a later environmental enrichment may have on rats raised in isolation is however not fully understood. We aim to investigate the effects of housing conditions and their reversion on anxiety and fear-related behaviors in rats. In phase I, we compared the effects of different housing conditions (environmental enrichment, control and social isolation) on anxiety behaviors in the open field test, elevated plus maze and fear conditioning. We found stronger effects of housing on behavioral tests when induced at weaning (phase I), than later in development (phase II). After one month, EE rats showed lower anxiety related behaviors and more freezing in FC. In phase II, we evaluated the effects of the reversion of housing conditions on the same behavioral parameters. We observed a behavioral trend such that the groups started to behave similar to their new housing conditions in OFT mainly. These results suggest that housing conditions at weaning can have long-lasting effects on anxiety and fear-related behaviors. Because the behavioral changes observed after the housing reversal in adulthood were partial, we suggest that more time of social and physical enrichment could be necessary to promote major changes in behavior at this age.

1. Introduction

Fear and anxiety are necessary to ensure survival; however, their persistence can interfere with psychological functioning and manifest as anxiety disorders (Ganella and Kim, 2014). Anxiety disorders are often chronic conditions that affect an individual's quality of life in many aspects including work and social interactions. When it appears early in life, anxiety also disrupts personal and social development (Kheirbek et al., 2012).

In animals, coping with fear and anxiety are influenced by housing conditions. Environmental enrichment (EE) and social isolation (SI) affect different behaviors and brain processes, especially when they start at weaning. EE improves spatial and emotional learning, neurotransmission, neurogenesis, synaptic transmission, increases habituation processes and decreases anxiety behaviors while SI has constantly shown opposing outcomes (Lapiz et al., 2001; Brenes et al., 2008; Lukkes et al., 2009a, 2009b; Mora-Gallegos et al., 2015; Nithianantharajah and Hannan, 2006; Rosenzweig and Bennett, 1996; Soffié et al., 1999). Therefore, the physical and social stimulation of environmental enrichment plays an important role during early behavioral and brain development and the interaction between these factors seem to have beneficial effects (Brenes et al., 2015; van Praag et al., 2000) that can be considered protective or used as a treatment in several behavioral disorders (Nithianantharajah and Hannan, 2006). Even though largest effects of EE are found when applied since weaning, positive behavioral and brain effects are also found when EE starts in adulthood or even at later developmental stages (Rosenzweig and Bennett, 1996; Barbelivien et al., 2006; Beatty et al., 1985; Bell et al., 2009). Although there are some discrepancies between studies since the effects are not that evident or are different as when started at weaning, some studies showed that EE adults or older development stages need more time to acquire learning in different tasks but improved their performance compared to SI rats (Bell et al., 2009; Mora-Gallegos et al., 2015).

A social and environmental enrichment accelerates habituation to novel environments and improves their performance in some learning and memory tests when compared to animals reared in standard or isolated conditions (Bennett et al., 2006; Harburger et al., 2007; see

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review in Mohammed et al., 2002; Mora-Gallegos et al., 2015; Schrijver et al., 2002; Zimmermann et al., 2001). In contrast, animals submitted to an impoverished environment and social isolation (where the competition for resources and social interactions are missing) show hyperactivity in novel environments, decreased performance in learning and memory tasks (Bell et al., 2009; Robbins et al., 1996; Simpson and Kelly, 2011; Weiss et al., 2004; Zimmermann et al., 2001), an impaired inhibitory behavior that enhances their anxiety in response to novelty (Brenes et al., 2008; Lukkes et al., 2009a, 2009b; Schrijver et al., 2002), and hyper-reactivity to stressful situations (Bell et al., 2009; Schrijver et al., 2002; Weiss et al., 2004).

Little is known about the effects of housing conditions like social isolation (SI) and environmental enrichment (EE) on fear conditioning (Lukkes et al., 2009a, 2009b). However, some studies examined the effects of re-socialization of isolated rats and/or reversion of isolation after environmental enrichment (Blanc et al., 1980; Lukkes et al., 2008; Wright et al., 1991). This kind of studies are very relevant to understand and underline the importance that social support has in humans experiencing a traumatic event or anxiety disorders in general (Olff, 2012). Even though that the effects were small, the studies mentioned above showed the reversion of anxiogenic behaviors in re-socialized animals after two to six weeks (Blanc et al., 1980; Lukkes et al., 2009a, 2009b; Wright et al., 1991). According to the literature, we hypothesized that effects of housing that can be kept later in life in continuous EE and ameliorate or reverse the effects of isolation (our condition SI-EE) since the benefits of this kind of environment are seen at different ages through development (Simpson and Kelly, 2011). Additionally, we hypothesized that EE conditions should be maintained to keep the benefits of this environment but this effects can be lost if the rats changed their housing to another with less (or even none) stimulation (condition EE-SI). We expected that two months of reversion were sufficient to induce a total reversion of the previous condition, since other studies used less than two months and found partial reversion of anxiety and fear behaviors (Blanc et al., 1980; Lukkes et al., 2009a, 2009b; Wright et al., 1991).

The first phase of the present study was designed to evaluate the effects of two months of environmental enrichment, social isolation and control housing starting at weaning on fear and anxiety behaviors an. In phase II, we reverted the housing conditions of phase I for two additional months, such that animals that were enriched in the first phase were isolated in the second phase and vice versa. Afterwards, the same behavioral evaluations done in phase I were performed in this phase.

2. Materials and methods

2.1. Phase I

2.1.1. Animals and housing conditions

One hundred eighty male Sprague-Dawley rats obtained from LEBi Laboratories (University of Costa Rica, San José) were transported to the laboratory at post-natal day 21 (PND- 21) and habituated during 1 week into the pre-weaning housing conditions (n = 3 per cage) to minimize the influence of stress following transport from the animal supplier. One week after arrival to the laboratory, animals were randomly distributed into three experimental groups (n = 60 per group, Environmental Enrichment (EE), Control (C) and Social Isolation (SI)) for a period of two months. Socially isolated animals were maintained individually in rectangular stainless steel cages (24 cm length x 17.5 cm height). Control rats were housed in small groups (3 rats per cage) in standard polycarbonate cages (55 cm length x 33 cm width x 19.5 cm height; Alphete, Germany). The enriched rats were housed in specially designed cages (120 cm length × 70 cm width × 100 cm height; 15 animals in each cage) containing non-chewable plastic objects, PVC tubes, food dispensers and water bottles, which were rearranged at least twice a week to create a novel environment and to promote foraging behavior as previously described (Brenes et al., 2006; Mora-Gallegos et al., 2015).

For all groups, bedding, food, and water were changed twice a week. Rats were maintained under 12:12 h light-dark schedule (light on at 6:00 h-light off at 18:00 h), 10 air cycles per hour, room temperature at 25.5 °C ± 1.20 °C and 78–87% of relative humidity and free access to water and food. One hour before behavioral tests, animals were placed in an adjacent dimmed room (one 25 W red bulb) and 5 min prior to test, they were individually placed in a clean cage and transported to the testing room. Animals were tested between 8:00 h and 13:00 h in a predetermined sequence (one rat from each group randomly assigned during all tests). All behavioral tests were videotaped under infrared light (Video camera all view OPCOM BR29 Kit Close Circuit).

All experimental procedures were performed in accordance to the guidelines of the Costa Rican Ministry of Science and Technology for the Care and Use of Laboratory Animals and were approved by the Institutional Committee for Animal Care and Use of the University of Costa Rica. Particular care was taken in order to minimize the number of animals used and to reduce their suffering.

2.1.2. Open Field Test (OFT)

After the habituation week, an open field test was performed (PND-28 baseline test; see Fig. 1 for the design of experiment 1). The open field arena consisted of one black, square, wooden chamber (55 cm × 55 cm × 40 cm) virtually divided in four identical quadrants. In the central area an additional smaller square was also virtually drawn (25% of the total surface). Single animals were placed in the center of the arena and during a 10-min session behavioral variables were scored. The testing room was painted black, illuminated with dimmed red light, with 10 air cycles per hour and with white noise from the air extractor. Between tests, the maze was cleaned with a 70% EtOH solution to remove odor traces from the previous animal. The behaviors registered were the following: 1) rearing as a measure of exploratory behavior (elevation of the paws rising more than 45° over the floor), 2) locomotion (measured as total distance traveled and automatically scored using the video tracking system ANY-maze, version 4.72, Stoelting Co., USA), 3) grooming (washing or mouthing of forelimbs, hind paws, face, body and genitals) and time spent in 4) central and 5) peripheral area (automatically scored using the video tracking system ANY-maze). Frequency and time of rearing and grooming behavior (posture sustained with hind paws on the floor) was manually scored off-line from video recordings using the Etholog 2.25 software by a trained observer (Ottoni, 1997).

Two days after the first OFT (OFT-1), rats were assigned to the different housing conditions (PND- 30), where they were kept for two months without further manipulation except for the bedding change twice a week. Animals were tested again one month later under the same conditions in the OFT (PND-58 OFT-2), to evaluate the effects of the different housing conditions. At another two months (PND- 88), a third OFT (OFT-3) was conducted (Fig. 1).

2.1.3. Elevated plus maze (EPM)

The EPM consisted of four arms of equal dimensions (50 cm long × 10 cm width), connected by a central area (100 cm^2) and raised 50 cm above the floor. Two arms, enclosed by walls 40 cm high, were perpendicular to two opposed open arms. To avoid falls, the open arms were surrounded by a formica rim 0.5 cm high. Each animal was placed in the center of the maze always towards the same open arm. Sessions lasted 5 min and the following behavioral parameters were scored: frequency of stretch-attend posture (SAP) and head-dipping (HD), time spent in open and closed arms (entries were counted when all four paws were placed on any arm), total locomotion and locomotion in open and closed arms (measured as distance traveled with Anymaze 4.3 software, Stoelting Co.). The apparatus was cleaned with a 70% EtOH solution after each test. Once each session finished, the animals were taken to their housing condition.

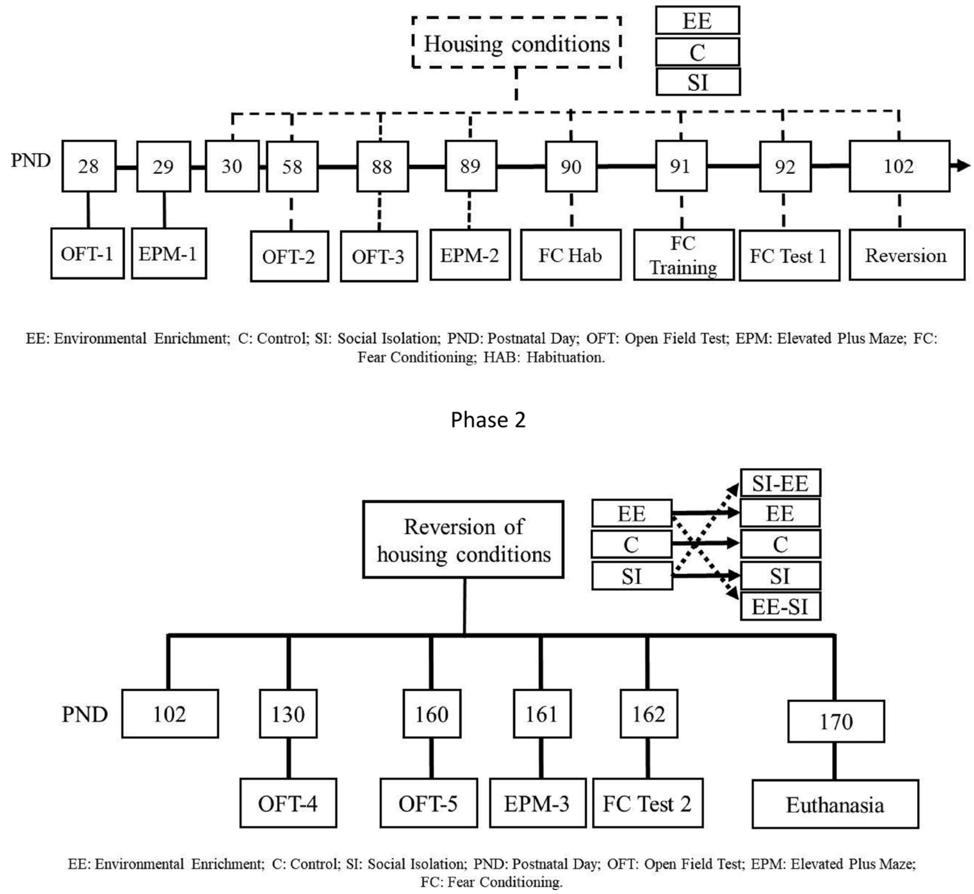

Fig. 1. Design of phase 1 and 2.

In phase I, two EPM tests were carried out. The first EPM was performed one day after OFT-1, prior to the beginning of housing conditions (PND- 29; EPM-1). The second EPM was performed one day after the last OFT (PND- 88; EPM-2) and one day before fear conditioning (Fig. 1).

2.1.4. Fear conditioning (FC)

One day after EPM-2, fear conditioning was carried out over three consecutive days (one session each day). Each day between tests, the fear box was cleaned with a 70% EtOH solution to remove odor traces from the previous animal.

In the habituation session (PND- 90), rats were individually exposed to the fear box where all the stimuli and conditions where the same as during fear conditioning but without the foot shock. In the training session (PND- 91) the FC protocol was applied. The protocol lasted 510 s (8:30 min) and was divided as follows: 5 min of habituation, immediately followed by a sound (CS) of 80 dB for 5 s, paired in the last second with a single foot shock (US) of 0.8 mA. This sequence was repeated 5 times at 30 s intertrial intervals and followed by one final minute with no stimuli before removing the animal from the box. In the test session (Test 1: context + tone; PND - 92), rats were exposed to the fear box without the foot shock in order to analyze the freezing response to the context (the same sequence but only with tone and without shock). The total freezing duration was analyzed and compared between training and test session. Freezing behavior was automatically measured with the software (Panlab) as activity lower than 5% for at least 2 s below that percentage of activity. We visually checked also for piloerection or for crouching posture to discard other behavioral bias like immobility in this measure and to avoid software bias. Additionally, we analyzed freezing minute by minute in each session to evaluate the kinetics of this behavior within tests. A video camera was placed inside the test chamber to measure rearing and grooming behaviors as previously described.

2.2. Phase II: reversion of housing conditions

2.2.1. Animals and housing conditions

One week after concluding the three sessions of fear conditioning (habituation, training and test 1), 30 animals of each housing group (EE, C and SI) were euthanized for gene expression (not included in this paper). Half of the remaining animals were subjected to a housing condition reversion, meaning that half of the enriched animals were placed in social isolation (EE—SI; n = 15) and the other half was maintained in environmental enrichment (EE; n = 15). On the other side, half of the isolated animals were switched to enrichment cages (SI—EE; n = 15) and the other half was maintained in the isolated condition (SI; n = 15). For the control group (C) some rats remained in the same condition (n = 15) and the other half was randomly excluded to maintain the number of rats equal to the other housing groups (n = 15) for further statistical analysis. Housing conditions were the same as described in phase I but in this phase we had five groups: EE, EE—SI, C, SI, and SI—EE. Two OFT were performed one month and two months after reversion, (PND-130 and PND-160 respectively), and at the end of the second month, rats were again exposed to EPM (PND-161) and one day later to a second test session (Test 2: context + tone; PND-162). One week later all animals were euthanized (PND-170; Fig. 1).

2.3. Data analysis

2.3.1. Phase I

For OFT data, a repeated measures MANOVA was carried out with housing condition as a between subjects factor (EE, C and SI) and OFT was used as a within-subjects factor (OFT-1 PND-28, OFT-2 PND-58 and OFT-3 PND-88). We used locomotion (as total distance traveled), the frequency and time of rearing and grooming and time in central and peripheral areas as dependent variables. Tukey post-hoc tests were used to evaluate significant differences between groups.

For the two EPM tests, another repeated measures MANOVA was carried out with housing condition as a between subjects factor (EE, C and SI) and EPM tests (EPM-1 PND-29 and EPM-2 PND-89) were used as a within-subjects factor. The frequency of SAP and HD, frequency and time of rearing and grooming, total locomotion and locomotion in open and closed arms were used as dependent variables. Tukey post-hoc tests were used to evaluate significant differences between groups.

For the FC procedure, another repeated measures MANOVA was carried out with housing condition as a between subjects factor (EE, C and SI) and the training and test 1 sessions as a within-subjects factor. The frequency and time of rearing and grooming and the percentage of freezing were used as dependent variables. Tukey post-hoc tests were used to evaluate significant differences between groups. Additionally, for FC we conducted a repeated measures ANOVA from minute 1 to 8 for freezing behavior to evaluate the kinetics of this behavior between groups. We used confidence intervals to evaluate significant differences between groups.

Statistical significance was defined at $p < 0.05$ in all cases. Marginally significant differences were defined as values of significance between 0.05 and 0.07.

2.3.2. Phase II

As in phase I, for OFT a repeated measures MANOVA was conducted with housing condition as between subjects factor (EE, EE-SI, C, SI and SI-EE) and OFT (OFT-4 PND-130 and OFT-5 PND-160 respectively) was used as a within-subjects factor. Dependent variables were the same used in phase I.

A MANOVA for each independently: EPM (PND-161) and Test 2 (PND-162) were performed over the same variables described for phase I and using housing conditions as between subjects factor (EE, EE-SI, C, SI and SI-EE). Tukey post-hoc tests were used to evaluate significant differences between groups in all tests. Statistical significance was defined as $p < 0.05$.

We also conducted a repeated measures ANOVA for minute by minute analysis in the second exposure to the context + tone (Test 2) for freezing behavior as described in phase I (housing condition as between subject factor). We used confidence intervals to evaluate significant differences between groups. Marginally significant differences were defined as values of significance between 0.05 and 0.07.

3. Results

3.1. Phase I

3.1.1. Open field test

The repeated measures MANOVA revealed a main effect of housing in multivariate contrasts: F (14, 340) = 9.15, $p < .001$, $\eta^2 = .19$, a main effect of OFT: F (14, 162) = 8.25, $p < .001$, $\eta^2 = .41$) and, importantly, an interaction between housing conditions and OFT (F (28, 326) = 3.91, $p < .001$, $\eta^2 = .25$. Prior to the start of differential housing (PND-28), no differences were observed between groups for any of the variables measured.

All groups showed a decrease in locomotion (distance) along OFT (F (2, 350) = 25.92, $p < .001$, $\eta^2 = .13$). Furthermore, a difference of distance traveled was also observed between housing conditions (F (2, 175) = 25.69, $p < .001$, $\eta^2 = .22$) and also an interaction between OFT and housing (F (4, 350) = 9.74, $p = .001$, $\eta^2 = .010$) showing that EE rats traveled less distance than the other groups at PND-58 (C $p = .001$, SI $p = .001$) and PND-88 (C $p = .001$, SI $p = .001$) but no differences were found between SI and C rats (Fig. 2a).

Similar to locomotion, differences were observed in rearing behavior where all groups decreased their frequency and time comparing OFT (frequency F (2, 350) = 12.16, $p < .001$, $\eta^2 = .065$ and time F (2, 350) = 17.43, $p < .001$, $\eta^2 = .091$). By housing conditions, we observed that EE rats showed the lowest frequency and time of rearing and the SI rats the highest (frequency F (2, 175) = 3.60, $p = .029$, $\eta^2 = .040$ and time F (2, 175) = 3.61, $p = .029$, $\eta^2 = .040$)). Nevertheless, this result was only significant at PND-58 as revealed by Tukey post-hoc test (frequency: EE compared to C $p = .001$, and SI $p = .001$ and time: EE compared to C $p = .003$, and SI $p = .001$). The EE rats showed a pronounced decrease in the frequency and time (data not shown) of rearing from PND-28 to PND-58, while for C and SI rats this behavior remained almost constant over the same period and then showed a pronounced decrease in this behavior from PND-58 to PND-88 (frequency F (4, 350) = 3.342, $p = .011$, $\eta^2 = .037$; time F (4, 350 = 5.60, $p < .001$, $\eta^2 = .06$) for this behavior. At PND-88 no differences were found between groups (Fig. 2b) but all groups showed lower values than on day 28.

We found an effect of OFT on grooming frequency and time (frequency F (2, 350) = 4.511, $p = .012$, $\eta^2 = .025$ and time F (2, 350) = 11.158, $p = .001$, $\eta^2 = .060$). C and SI rats showed a decrease in both parameters and no differences were found between them. In contrast, EE rats increased grooming from PND-28 to PND-58 and this higher grooming was maintained at PND-88 (Fig. 2c). Differences by housing conditions also showed statistical differences both in frequency and time of grooming (frequency F (2, 175) = 26.54, $p = .001$, $\eta^2 = .23$; time F (2, 175) = 64.80, $p < .001$, $\eta^2 = .42$). EE rats showed higher levels of grooming than the C and SI rats (housing conditions*OFT: frequency F (4, 350) = 9.121, $p = .001$, $\eta^2 = .09$; time F (4, 350) = 26.45, $p < .001$, $\eta^2 = .23$) at PND-58 (frequency: EE compared to C $p = .001$, and SI $p = .001$ and time: EE compared to C $p = .003$, and SI $p = .001$) and PND-88 (frequency: EE compared to C $p = .001$, and SI $p = .001$ and time: EE compared to C $p = .001$, and SI $p = .001$). Note that SI and C groups showed similar trends in all behaviors measured in the OFT (Fig. 2c).

Time spent in central area significantly increased while the time in peripheral area decreased with time for all groups (main effect of OFT: central area F (2, 350) = 5.481, $p = .005$, $\eta^2 = .03$, peripheral area F (2, 350) = 5.137, $p = .006$, $\eta^2 = .03$) but no differences were found between housing conditions in these measures of the OFT (effect of housing: central area p = 0.623, peripheral area p = .651; interaction OFT*housing: central area p = 0.244, peripheral area p = .319).

3.1.2. Elevated plus maze

At PND-29 no differences were found between housing conditions for any behavioral parameter analyzed: frequency of SAP and HD, the frequency and time of rearing and grooming, time spent in the open and closed arms and locomotion (total, open or closed arms).

We found significant differences between EPM (PND-29 and PND-89) (F (11, 163) = 13.57, $p < .001$, $\eta^2 = .47$) for all behaviors except for frequency of SAP ($p = .396$), frequency and time of rearing ($p = .325$ and $p = .228$ respectively) and grooming frequency ($p = .090$) and time (marginally significant $p = .053$). For the frequency of HD (F (1, 173) = 49.02, $p < .001$, $\eta^2 = .22$), time spent in open arms (F (1, 173) = 9.10, $p = .003$, $\eta^2 = .05$), total distance traveled (F (1, 173) = 16.83, $p < .001$, $\eta^2 = .08$), distance in open arms (F (1, 173) = 19.16, $p < .001$, $\eta^2 = .10$) and distance in closed arms (F (1, 173) = 20.77, $p < .001$, $\eta^2 = .10$) a decrease in behaviors was observed from PND-29 to PND-89 except for time spent in closed arms that showed a significant increase in all groups (F (1, 173) = 17.98, $p < .001$, $\eta^2 = .09$) (Fig. 3a–d).

At PND-89 we found a main effect of housing condition (F (22, 328) = 1.69, $p = .028$, $\eta^2 = .10$), with EE rats showing a lower frequency of SAP (F (2, 173) = 6.39, $p = .002$, $\eta^2 = .06$) compared to C rats ($p < .001$) and SI rats ($p = .024$) who also differed between each other ($p = .037$) (Fig. 3a). EE rats showed significantly more HD (F (2,

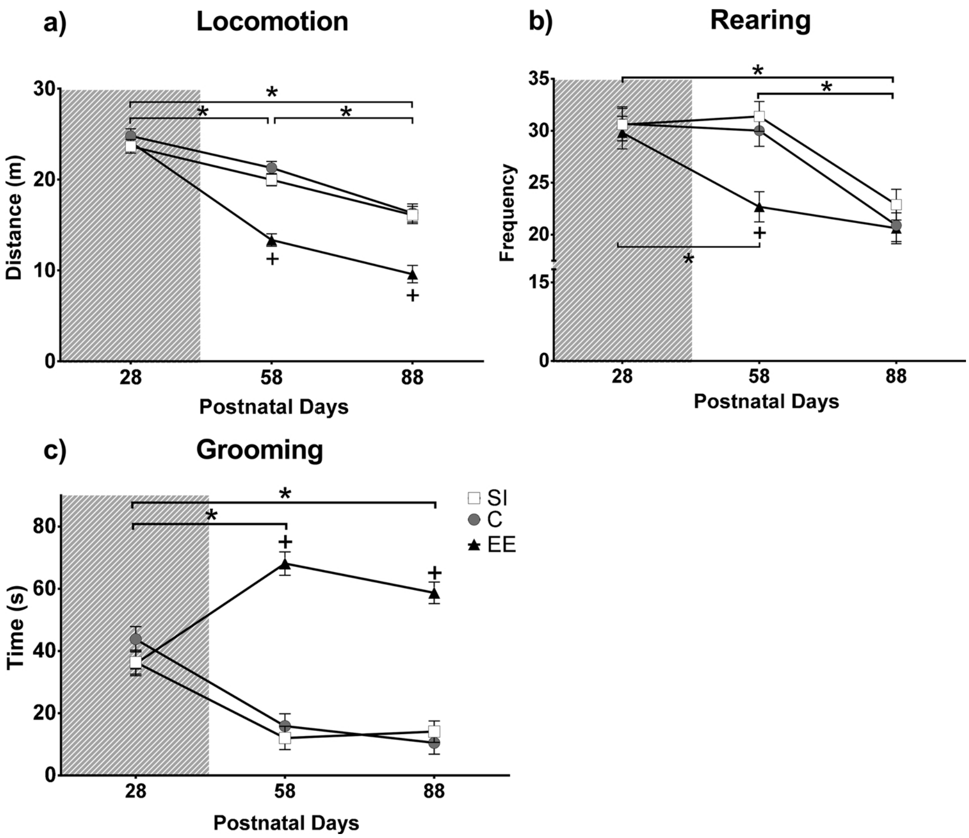

Fig. 2. Locomotion (a; distance), frequency of rearing (b), time (s) of grooming (c) of the three groups in three open field tests (OFT): before housing conditions (PND-28), at one month of housing (PND-58) and two months of housing (PND-88) (* = significant differences within groups, $p < 0.05$; + = significant differences between groups, $p < 0.05$).

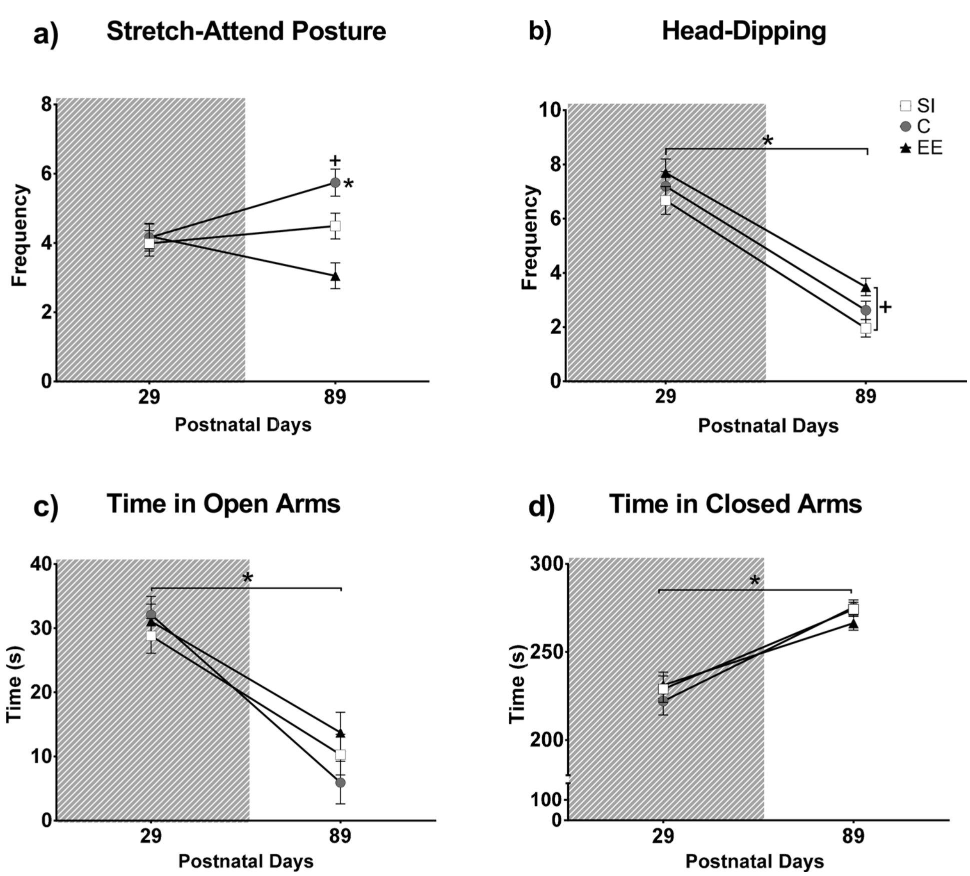

Fig. 3. Frequency of Stretch-Attend Posture (a), Head-Dipping (b); time spend in open (c) and closed (d) arms in the first two elevated plus maze tests (PND-29 and 89) (* = significant differences within groups, $p < 0.05$; + = significant differences between groups, $p < 0.05$.

173) = 4.07, p = .019, η^2 = .04) than SI rats (p = .005) but no differences were found compared to C rats (p = .132) or between SI and C rats (p = .483) (Fig. 3b). We also found significant differences for time of grooming (F (2, 173) = 3.35, p = .037, η^2 = .03) where EE rats showed higher levels than the other groups (EE compared to C $p < .001$, and SI p = .002). No differences were found between SI and C rats (p = .417). We did not find significant differences between groups for the other behaviors.

Taken together, the interaction of housing conditions and the repeated measures of EPM (F (22, 328) = 2.83, $p < .001$, η^2 = .16), we found differences in rearing frequency (F (2, 173) = 7.08, $p < .001$, η^2 = .07) and rearing time (F (2, 173) = 14.74, $p < .001$, η^2 = .14) (data not shown). EE rats showed less rearing than the other groups for frequency (C p = .020 and SI p = .017) and time (C p = .002 and SI $p < .001$) at PND-89. The interaction was also significant for grooming frequency (F (2, 173) = 3.74, p = .026, η^2 = .04) (data not shown), grooming time (F (2, 173) = 6.85, $p < .001$, η^2 = .07), and SAP frequency (F (2, 173) = 6.26, p = .002, η^2 = .06) (Fig. 3).

3.1.3. Fear conditioning

We found a main effect of housing (F (10, 256) = 14.36, $p < .001$, η^2 = .36), sessions (F (10, 122) = 34.93, $p < .001$, η^2 = .74), and their interaction (F (20, 246) = 9.02, $p < .001$, η^2 = .40). All groups showed a decrease in rearing and grooming behaviors and an increase in freezing across sessions.

For rearing behavior, we found differences between housing conditions (frequency F (2, 131) = 41.54, $p < .001$, η^2 = .38 and time F (2, 131) = 21.56, $p < .001$, η^2 = .24), between sessions (frequency F (2, 262) = 52.93, $p < .001$, η^2 = .28 and time F (2, 262) = 29.07, $p < .001$, η^2 = .18), and an interaction between housing conditions and sessions (frequency F (4, 262) = 18.73, $p < .001$, η^2 = .22 and time F (4, 262) = 12.49, $p < .001$, η^2 = .16). SI rats showed higher levels of rearing compared to C and EE rats. EE rats showed the lowest values in all sessions and all groups showed a decrease in frequency (Habituation: EE compared to SI $p < .001$ and C $p < .001$ and SI compared to C $p < .001$; training: EE compared to SI $p < .001$ and C $p < .001$ and SI compared to C p = .049, test 1: EE compared to SI p = .004 and C p = .952 and SI compared to C p = .015) and time of this behavior (Habituation: EE compared to SI $p < .001$ and C p = .507 and SI compared to C $p < .001$; training: EE compared to SI $p < .001$ and C p = .001 and SI compared to C p = .023, test 1: EE compared to SI p = .582 and C p = .557 and SI compared to C p = .997) (Fig. 4a).

For grooming behavior, we found significant differences between housing conditions (frequency F (2, 131) = 17.18, $p < .001$, η^2 = .20 and time F (2, 131) = 31.88, $p < .001$, η^2 = .32), sessions (frequency F (2, 262) = 39.91, $p < .001$, η^2 = . 09 and time F (2, 262) = 18.03, $p < .001$, η^2 = .12) and an interaction between housing conditions and session (frequency F (4, 262) = 9.43, $p < .001$, η^2 = .12 and time F (4, 262) = 13.96, $p < .001$, η^2 = .17). EE rats showed more frequent grooming in the habituation session than SI (p = .002) and C rats (p = .001) who also showed differences between them (p = .002) (data not shown). The same trend was found for time of grooming in the habituation (EE compared to SI $p < .001$, and C $p < .001$ and SI compared to C p = .017). In the training session the time spent grooming showed differences between EE and C rats ($p < .001$) but not between EE and SI ($p < .087$) or SI and C ($p < .089$). This behavior was almost absent in test 1 session in all groups (Fig. 4b).

For freezing behavior, we observed differences between housing conditions (F (2, 131) = 22.39, $p < .001$, η^2 = .25) and between sessions (F (1, 131) = 574.73, $p < .001$, η^2 = .81) but no interaction. All groups increased their freezing time from training to test 1. In both sessions SI rats showed less freezing time than the other groups (training and test 1: SI and EE $p < .001$) but not between EE and C.

When minute by minute analysis was conducted, significant differences were found between EE and SI groups (between housing conditions F (2, 133) = 14.69, $p < .001$, η^2 = .18), between minutes (F (7, 931) = 19.53, $p < .001$, η^2 = .12), and by the interaction of housing conditions and minutes (F (14, 931) = 2.03, $p < .013$, η^2 = .030). In the training session differences were found from minute 2 to 6 and in minute 8 with EE rats showing more freezing than SI rats. In test 1, the observed significant differences between groups were found from minute 1 to minute 5 (when no CS was presented) where EE rats showed more freezing than SI rats (Fig. 4d).

3.2. Phase II

3.2.1. Open field test

After reversion of housing conditions, analyses showed a main effect of housing (F (28, 248) = 1.87, p = .006, η^2 = .17) in grooming behavior (frequency F (4, 65) = 2.56, p = .046, η^2 = .13 and time F (4, 65) = 4.06, p = .005, η^2 = .20) but not for the other variables measured. In spite of this, in univariate test we observed that all groups significantly increased locomotion from PND-130 to PND-160 (F (1, 67) = 5.98, p = .017, η^2 = .08) (Fig. 5a). On day 130 the group EE-SI showed the highest frequency of rearing, while the EE group showed the lowest. However, differences between groups were not significant (Fig. 5b).

At PND-130 EE rats showed higher grooming than C rats for frequency (p = .005). The time spent grooming was also different between EE and C (p = .001) and EE and EE-SI (p = .016) (Fig. 5c). At PND-160 we only observed significantly more grooming time in the SI-EE group compared to the C group (p = .034). No differences were found for time spent in the central and peripheral area (data not shown) and the interaction of housing conditions and OFT for any of the behavioral variables.

3.2.2. Elevated plus maze

Differences were observed between housing conditions (F (48, 252) = 1.49, p = .027, η^2 = .22). The Control group showed a significantly higher frequency of SAP (F (4, 77) = 4.14, p = .005, η^2 = .18) than all the other housing groups (EE p = .031, EE-SI p = .002, SI-EE p = .024, and SI p = .008) (Fig. 6a). EE rats showed a higher frequency of HD (F (4, 71) = 2.88, p = .028, η^2 = .14) compared to SI rats (p = .025) (Fig. 6b), and more time spent in the open arms (F (4, 71) = 3.14, p = .019, η^2 = .15) compared to EE-SI (p = .037) and marginally significant compared to SI rats (p = .070) (Fig. 6c). There was a marginally significant difference in time spent in closed arms (F (4, 71) = 2.26, p = .071, η^2 = .11) between EE and SI (p = .048) (Fig. 6d). Additionally, EE rats traveled a greater distance in the open arms (F (4, 71) = 4.04, p = .005, η^2 = .18) compared to the C (p = .017), EE-SI (p = .009) and SI (p = .007) rats. No differences were found in any other behavioral parameter measured.

3.2.3. Fear conditioning test 2 (context + tone)

There was no significant effect of housing condition on frequency and time of rearing and grooming. For freezing behavior, there was a marginally significant effect between groups (F (4, 72) = 4.19, $p < .004$, η^2 = .19) SI-EE rats showed significantly lower levels of freezing than the other groups (EE p = .003, C p = .027, EE-SI p = .027, SI p = .050) (Fig. 7a to 7c). Analysis by minute revealed differences from minute 2 to 5 between the SI-EE group and the other groups (Fig. 7d).

4. Discussion

We aimed to study the effects of different housing conditions on fear and anxiety and to evaluate the effects of the reversion of those housing conditions on behavioral parameters. Our main findings show consistent behavioral effects of EE and SI conditions when housing started at early developmental stages supporting the notion that this is the most sensitive period to find effects of differential housing. These initial housing effects that we observed in our results, were partially reverted

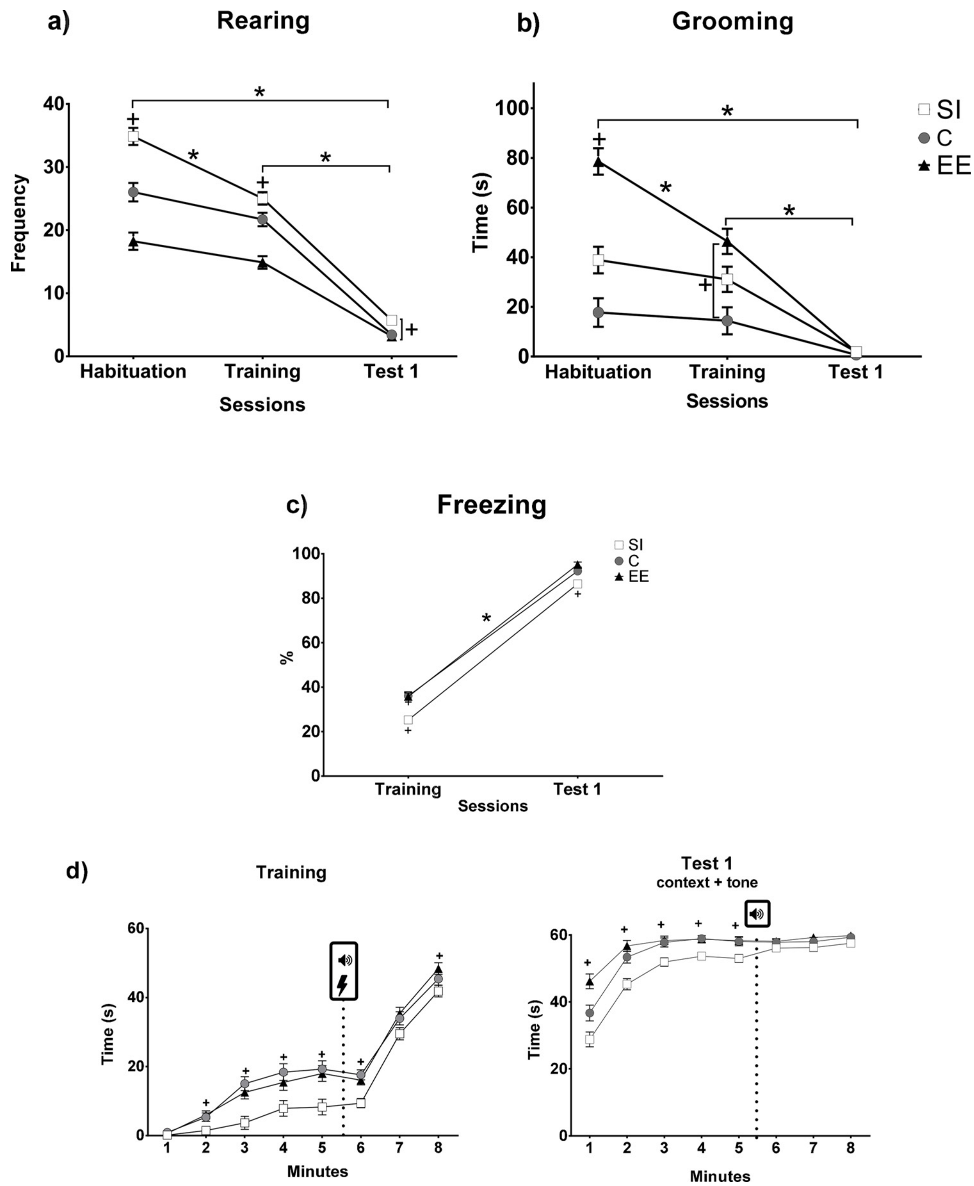

Fig. 4. Frequency of rearing (a), time of grooming (b), freezing percentage (c) and time of freezing (s) min by min (d) of the three groups in the three sessions of fear conditioning procedure (Habituation PND-90, Training PND-91, Context + Tone PND-92) (* = significant differences within groups, $p < 0.05$; + = significant differences between groups, $p < 0.05$. Analysis by minute showed differences between SI and the other groups when marked). The speaker + electricity symbol represents the beginning of the presentation of both stimulus (5 times). The speaker represents the beginning of tone presentations (5 times).

(as some trends were observed) when they started in adulthood (phase II), probably because at this age more than two months are needed to make evident the effects of EE (in the case of SI-EE). It is noteworthy that the change from EE to SI showed a slight behavior worsening, suggesting that EE housing should be maintained in time in order to keep the benefits of this kind of housing.

4.1. Effects of different housing conditions at weaning

Previous to the start of differential housing conditions, we analyzed the baseline behavior for all animals in the OFT and the EPM (PND-28). No differences between groups were found for any behavioral parameter. One month after the onset of housing conditions (PND-58), differences began to appear in some anxiety-related behaviors that remained at PND-88 (except for rearing). In general EE animals showed faster habituation represented by a decrease in rearing behavior, and locomotion, a consequent reduction in time spent in central area and finally a clear increase in grooming behavior. This is in line with other studies, where EE rats showed an accelerated habituation to novelty compared to isolated and/or control rats (Schrijver et al., 2002). Zimmermann et al. (2001) relate this faster habituation to improved spatial abilities that these animals acquired after exposure to a more complex housing environment (Hellemans et al., 2004; Mora-Gallegos et al., 2015). It is important to note that the other two groups did not differ between each other in any analyzed behavioral parameters in the OFT. Our results are also in agreement with studies in which SI rats showed locomotor hyperactivity and higher levels of rearing in an OFT compared to rats living in groups. It is suggested that these behavioral characteristics result from a deficit in behavioral inhibitory control and from inadequate acquisition of spatial information (Barbelivien et al., 2006; Fone and Porkess, 2008; Heidbreder et al., 2000; Schrijver et al., 2002, reviewed in Hall, 1998; Zimmermann et al., 2001).

The EPM test is frequently used to evaluate rodents' innate response to avoid open spaces and risk-assessment behaviors. We did not observe differences in the time spent in open and closed arms between animals weaned in different housing conditions, but we found a trend of EE rats

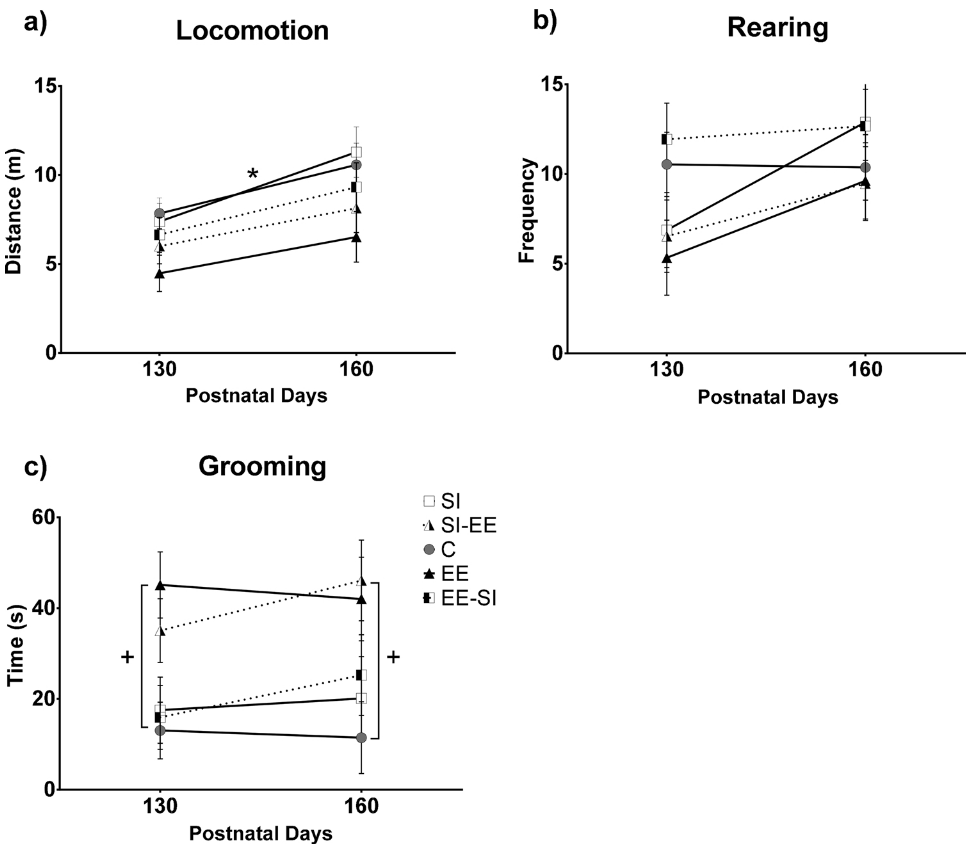

Fig. 5. Locomotion (a), frequency of rearing (b) and time of grooming (c) of the five groups in open field tests (OFT) one month after reversion of housing conditions (PND-130) and two months after reversion of housing conditions (PND-160) (*+ =significant differences within groups, $p < 0.05$; *= significant differences between groups, $p < 0.05$).

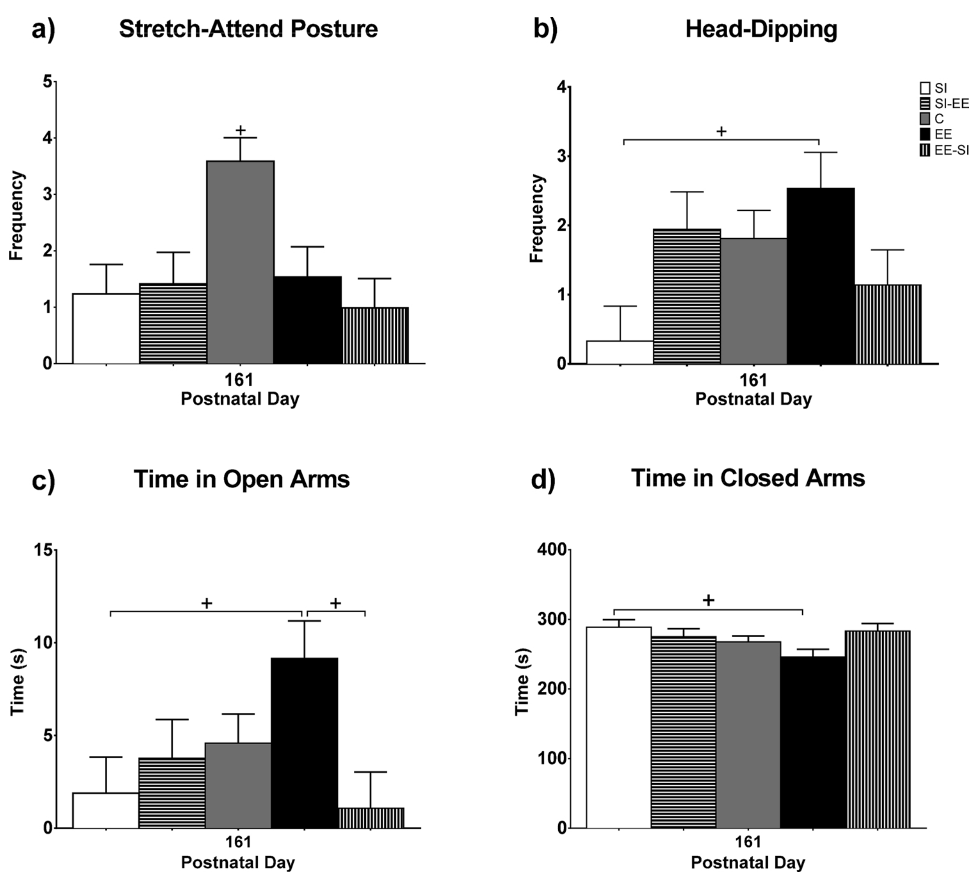

Fig. 6. Frequency of Stretch-Attend Posture (a), Head-Dipping (b), time spend in open (c) and closed (d) arms in elevated plus maze test (EPM) (DPN-161) (+ = significant differences between groups, $p < 0.05$).

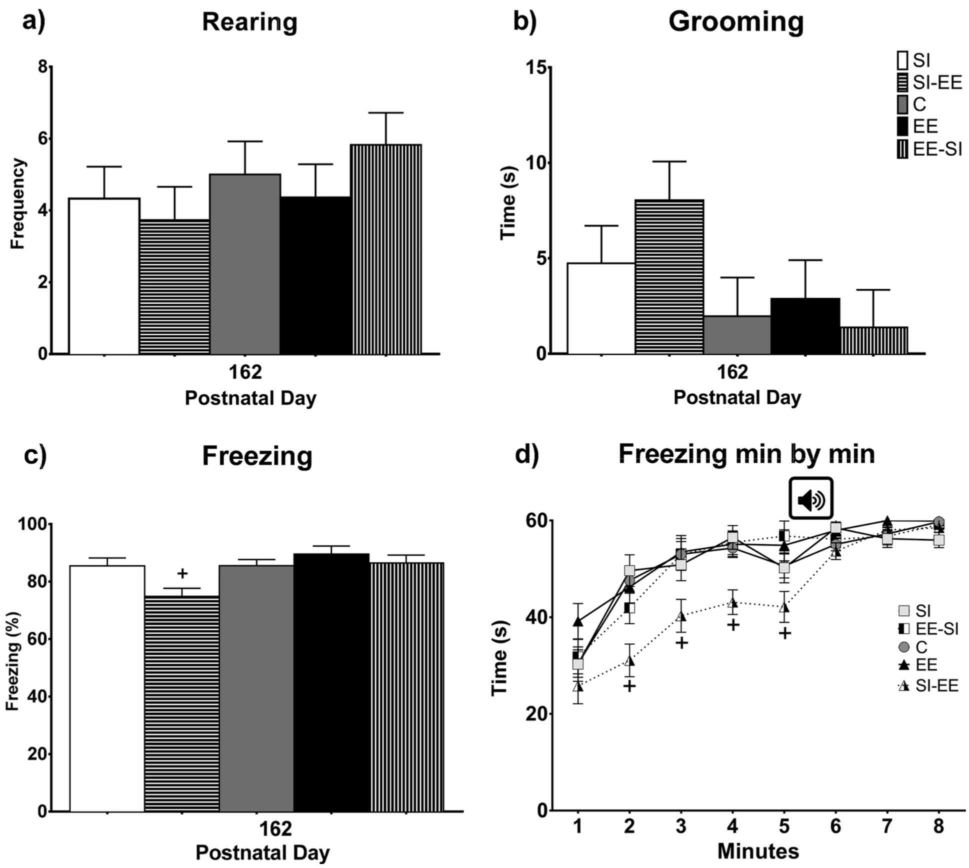

Fig. 7. Frequency of rearing (a), time of grooming (b), freezing percentage (c) and freezing min by min of the five groups in the second exposure to the context + tone of fear conditioning (Test 2), two months after reversion of housing conditions (PND-162) (+ = significant differences between groups, $p < 0.05$). The speaker represents the beginning of tone presentations (5 times).

to show more time in open spaces that becomes a significant difference in phase II (EE significantly different from SI and SI-EE). In this test, it is expected that animals with less anxiety will show more risk behaviors and spend more time in open spaces (Rodgers, 1997). However, as in previous studies (Griebel et al., 1996; Rodgers, 1997; Rodgers et al., 1997; Wall and Messier, 2001,; Roy and Chapillon, 2004) we also included the evaluation of behavioral parameters (besides time in open/closed arms) as risk assessment behaviors to have a detailed and more complete picture of the anxiety profile in EPM. However, behaviors like SAP and head-dipping are barely reported (Carobrez and Bertoglio, 2005; Litvin et al., 2011; Rodgers, 1997; Wall and Messier, 2001). Consistent with our results, Weiss et al. (2004) also found lower risk assessment behaviors in SI rats when compared to social grouped rats. Additionally, some researchers have made efforts to demonstrate the utility of including behaviors like SAP and head-dipping to demonstrate the effects of anxiolytic drugs like benzodiazepines (Griebel et al., 1996). Benzodiazepines enhance time spent in open arms and head-dipping behavior (Rodgers et al., 1997; Silva and Brandao, 2000) and decrease behaviors like SAP (Griebel et al., 1996; Carobrez and Bertoglio, 2005). This is the same trend that we and others have found in EE rats compared to SI and C rats, suggesting an anxiolytic effect of this housing condition (Peña et al., 2006).

In order to assess the effects of housing condition on FC, as traditionally done, we analyzed freezing duration. We decided to analyze additional behavioral parameters like rearing and grooming to obtain more information about the anxiety profile in this test. In the habituation session, we observed differences between groups in this novel environment in behaviors related to anxiety (rearing and grooming) but not in fear (freezing). EE rats showed a similar behavioral outcomes in the FC task as in the OFT and EPM with less rearing and more grooming compared to the other groups (Brenes et al., 2006; Weiss et al., 2004; Zimmermann et al., 2001). In the training session, differences between groups for both rearing and grooming showed a similar pattern. EE animals showed the smallest values, while the SI rats showed the highest values. For freezing, the EE group showed slightly higher values compared with the other two groups. In the test 1 session, the three groups increased their freezing behavior and decreased rearing and grooming. These behavioral results are expected because freezing is the more adaptive behavior that shows that the animals learned to predict the possible danger (Blanchard et al., 2008; Bruchey and Jones, 2010; Cain and LeDoux, 2008; Maren, 2001). All groups showed a tendency to decrease anxiety-related behaviors and to increase fear responses across the three sessions. Previous studies found that isolated and group reared rats show normal learning of the CS-US association training and in contrast reduced freezing to the context in SI rats (Lukkes et al., 2009a, 2009b; Weiss et al., 2004). Taken together, the results of the behavioral variables measured in FC, showed that the SI rats had a more anxious and reactive response (more rearing and less grooming) to the new environment which could interfere with their learning process about the context evidenced through less freezing behavior in the test 1 session. This is in accordance with the literature showing that SI impairs attention and learning and memory processes and increases emotionality compared to EE (Fone and Porkess, 2008; Hellemans et al., 2004; Simpson and Kelly, 2011; Weiss et al., 2004).

4.2. Reversion of housing conditions

Many studies have examined the effects of housing conditions on anxiety and/or fear; however, only a few studies have focused on the effects of housing conditions reversion (Lukkes et al., 2009a, 2009b; Wright et al., 1991). Two to six weeks of housing condition reversion of SI (using re-socialization from SI to a condition with some conspecifics) did not produce any effects. This is one of the reasons we did not just re-socialize SI animals for a period of two months (the same time before and after fear conditioning). Similar to Hellemans et al., in 2004, we also added social and non-social physical stimuli as environmental enrichment for the SI-EE rats and deprived the EE-SI rats of social and physical contact (like the original SI condition). In the OFT conducted at PND-130 and PND-160 we did not find significant differences between groups for any of the variables measured; however, we observed some interesting trends in our data. The EE and SI-EE rats showed the lowest levels of rearing behavior and locomotion at PND-130 and PND-160, consistent with our phase I data in which the EE animals showed the lowest levels of both behaviors. Also similar to phase I, the EE and SI-EE showed higher levels of grooming behavior at PND-130 and PND-160 than the other groups. These results suggest on one hand, that the reversion situation was acting differently on rearing frequency and locomotion, and time spent with grooming on the other hand, one and two months after reversion. In other words, the changes we observed are behavior and time-dependent. In the same way different dynamics have been reported for rearing and locomotion in some works (Thiel et al., 1999; Görisch and Schwarting, 2006) and for grooming behavior in other (Millan, 2003). We expected to find an increase in rearing and locomotion and decrease in grooming suggesting an anxiogenic profile in SI rats and the opposite trends in the EE rats suggesting reduced anxiety (Hellemans et al., 2004; Peña et al., 2006; Wright et al., 1991; Weiss et al., 2004). Our results show the expected trend for SI and EE groups and for the reverted groups SI-EE and EE-SI, suggesting that reversion of housing modified the behavioral effects of the initial housing condition. It is also important to note that from PND-130 to PND-160 we did not observe an effect as pronounced as for housing in phase I, perhaps because of the repeated exposures to the tasks and/or because the reversion was conducted in adulthood. The latter is important to consider because the main effects of housing conditions have been reported at early stages but the effects in adulthood or in aged animals are scarcely reported (Leggio et al., 2005; Mora et al., 2007; Mora-Gallegos et al., 2015; Schrijver et al., 2002). It is important to note that for this test, we observed the strongest effects of housing at PND-58 which reflect the housing effects shortly after weaning.

In the reversion phase, we observed similar tendencies at PND-161 for the EE and SI groups in the EPM. C rats showed higher levels of SAP behavior than the other groups at PND-89 (Fig. 3). For the variables of head-dipping and time spent in the open and closed arms, the tendencies observed in the EPM conducted at PND-89 remained the same at PND-161. The EE group showed higher levels of head-dipping and spent more time in the open arms and less time in the closed arms than other groups and similar values of SAP than C group, similar to the tendencies observed in Phase I. There is strong evidence in different paradigms suggesting that an increase in the time spent in the closed arms and a decrease in time spent in the open arms represents increased anxiety (Fone and Porkess, 2008; Wright et al., 1991; Weiss et al., 2004). Similar to our results in EPM (more anxious behaviors of SI than EE), while other studies found that SI rats were more anxious in the EPM characterized by a reduction of entries and time spent in the open arms (Hellemans et al., 2004; Wright et al., 1991; Weiss et al., 2004).

Additionally, although there were no significant differences, there was a trend in the reverted conditions to behave similar to their “new” housing condition (SI similar to EE-SI and EE similar to SI-EE). These results are in accordance with our phase I results and with previous literature on EE and SI profiles (Brenes et al., 2006; Hellemans et al., 2004; Peña et al., 2006; Wright et al., 1991; Weiss et al., 2004). The repeated exposure to EPM did not reduce the anxiety-related behaviors. One plausible explanation is that submitting the animals to OFT (PND-160) potentiated the effects in the EPM (Carobrez and Bertoglio, 2005).

There are also contradictory results related to reversion of housing conditions. In agreement with our results, Hellemans et al. (2004) found that isolated conditions increase locomotor activity in the open field and increase anxiety in the EPM with opposite tendencies for the EE group. According to Hellemans et al. (2004), switching housing conditions partially reversed the effects of the original conditions 6 weeks after switching. Hyperlocomotion persisted in isolated-to-enriched animals and in isolated-to-standard animals, while locomotion was partially reversed in the enriched-to-isolated group. The authors proposed that the enriched-to-isolated group showed higher anxiety than the isolated-to-enriched group because the enrichment reduced anxiety levels (Hellemans et al., 2004). However, a study conducted by Wright et al. (1991) found that re-socialization of rats isolated from weaning in adulthood did not reverse the anxiogenic profile observed in the isolated rats (hyperactive behavior in novel environments and decreased time spent in the open arms of the EPM). Lukkes et al. (2009a, 2009b) also found that re-socialization of rats after 3 weeks of isolated housing did not revoke the anxiogenic profile of these animals. The observed differences could be due to the fact that Hellemans et al. (2004) used both social and physical stimulation in their enrichment conditions (Hellemans et al., 2004). Both social and physical EE could be necessary to revert the neural and behavioral effects of isolation housing (Brenes et al., 2015; Hellemans et al., 2004) especially when housing conditions are maintained for two months (based on our results).

In the second exposure to the fear context + tone (Test 2; PND-162), animals spent the majority of the time freezing. However, we saw an interesting trend in the SI-EE rats which showed significantly lower levels of freezing than the other groups and a tendency of increased grooming. One possible explanation is that SI-EE rats are regulating their emotional arousal induced by the context through their grooming behavior (Millan, 2003; Spruijt et al., 1992). This possibility is supported by our minute by minute analysis. Also in this session, although the SI-EE rats showed significantly less total freezing compared to the other groups (Fig. 7c), the SI-EE rats reached the same level of freezing as the other groups when the tone (CS) appeared. This indicates that they had less fear of the context than the other groups but the response to the tone was unaffected, they learned the association between sound and shock. A better understanding of this hypothesis has to be demonstrated with further research.

5. Conclusions

The behavioral effects induced by differential housing at early developmental stages can be long-lasting and can be kept throughout life. We observed partial effects when reversion was conducted at late stages. Since they were already adults at reversion phase, probably their brain plasticity could have been reduced compared to early developmental stages (Kempermann et al., 2002). Based on the slight behavioral effects we observed after 2 months of reversion, and knowing the relevance of environmental enrichment described elsewhere, it could be interesting in further studies to increase the reversion period (more than two months) to detect the plausible behavioral changes or benefits that occur in adulthood.

It is important to note the relevance of environmental enrichment as both social and physical stimulation together because of the high similarity to the use of exercise and social support to treat anxiety disorders and promote brain plasticity in humans (Olff, 2012). In this sense, environmental enrichment effects are important to be maintained after two months of housing but it seems that if this environment changes to one with lower physical and social stimulation (from EE to SI) anxiety and fear related behaviors can remain or prevail in time. Therefore, we speculate that environments with different levels of stimulation could influence the way people perceive and cope facing different situations. Such non-invasive treatments like environmental stimulation could have positive long-term effects in anxiety-related symptoms.

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