Progress in Neuropsychopharmacology & Biological Psychiatry 95 (2019) 109726

Contents lists available at ScienceDirect

Progress in Neuropsychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Repeated maternal separation: Alcohol consumption, anxious behavior and corticosterone were reversed by a non-pharmacological treatment

María Mercedes Odeon[1], Gabriela Beatriz Acosta*

Instituto de Investigaciones Farmacológicas (ININFA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (UBA), Junín 956, 5° floor, Ciudad Autónoma de Buenos Aires C1113AAD, Argentina

ARTICLE INFO

Keywords:
Alcohol
Behavioral parameters
Corticosterone levels
Cold stress
Environmental enrichment
Repeated maternal separation

ABSTRACT

Adverse events in early life have been related to a maladaptive stress response during adulthood, which could predispose individuals to psychiatric and physiological disorders. The purpose of this work was to study the implications of repeated maternal separation (RMS) plus a physical stressor (cold stress), voluntary ethanol consumption and plasmatic levels of corticosterone (Cor) via conflict behavior tests. To this aim, pups were separated daily from their mothers for one hour and subjected to cold stress (4 °C) between postnatal days (PD) 2 and 20. Control groups were left undisturbed with their mothers. Afterwards, all groups were exposed to voluntary ethanol (6%) or dextrose (1%) intake for 7 days. After a 30-day period of environmental enrichment (EE), the animals were again exposed to the voluntary intake protocol for 7 days. At 66 days, they were subjected to different conflict tests. Thereafter, rats were sacrificed by decapitation and blood trunk was collected to determine plasma corticosterone levels. We demonstrated that early RMS increased both voluntary alcohol intake and Cor levels. Moreover, young adult animals showed excessive activity in conflict tests. Whereas in animals exposed to a non-pharmacological treatment, known as environmental enrichment (EE), the effects previously obtained were reversed and/or prevented. In summary, we can conclude that the combination of maternal separation in early life plus cold stress increase both the voluntary exposure to alcohol and disruptive behaviors. This is a risk factor for the development of chronic diseases such as alcoholism and long-term depression. However, we found that an enriched environment may have a beneficial effect with respect to alcohol intake and aggressive behaviors.

1. Introduction

Repeated stressful experiences during childhood are associated with a high incidence of emotional disorders during adulthood. Adverse conditions in the neonatal period may have deleterious effects on neuronal development and cell proliferation, increasing the risks for behavioral disorders and deficits in emotion recognition and understanding. Besides, adverse conditions may also increase drug dependence during adulthood (Chapman et al., 2004; Carr et al., 2013). In rats, daily separation of newborns from their mothers for some hours during the first few weeks of birth is known as maternal separation (MS), and has been suggested as a model of early adversity (Michaels and Holtzman, 2008; Jahng, 2011; Carlyle et al., 2012). This model is associated with alterations in stress reactivity and anxiety-like behaviors. (Gilles et al., 1996; Meaney, 2001; Kalinichev et al., 2002; Dalle Molle et al., 2012; Bendre et al., 2015), and with increased ethanol consumption (Huot et al., 2001; Green et al., 2010; Enoch, 2011; Goldstein and Volkow, 2011).

Alcohol is a recreational drug that can reduce stress, inhibitions, and may produce feelings of well-being (Meaney et al., 2002; Rehm et al., 2009). However, alcohol abuse has become a concerning social problem all around the world, leading to the creation of specific policies to reduce its harmful use and social burden (World Health Organization, and World Health Organization, 2014).

Abbreviations: ANOVA, two-way analysis of variance; CICUAL, Institutional Committee for the Use and Care of Laboratory Animal rules; Cor, Corticosterone; CRF, corticotropin-releasing factor; Dex, Dextrose; EE, environmental enrichment; EPM, Elevated Plus Maze; Et, Ethanol; HPA, hypothalamic-pituitary-adrenal; HPLC, high performance liquid chromatography; IC, impoverished conditions; LDT, Light-Dark transition; MS, maternal separation; OF, Open Field; PD, postnatal day; RMS, Repeated maternal separation; SEM, standard error of the mean; SC, standard group housing; NPY, neuropeptide Y

* Corresponding author at: Gabriela B. Acosta, Instituto de Investigaciones Farmacológicas (ININFA), CONICET-UBA, Junín 956, 5° floor, Buenos Aires C1113AAD, Argentina.

E-mail addresses: odeon.maria@inta.gob.ar (M.M. Odeon), gacosta@ffyb.uba.ar (G.B. Acosta).

[1] Present address: IFAB (INTA-CONICET) EEA, Bariloche, Modesta Victoria 4550.

https://doi.org/10.1016/j.pnpbp.2019.109726
Received 15 June 2019; Received in revised form 25 July 2019; Accepted 28 July 2019
Available online 03 August 2019

Due to innate differences in various systems of neurotransmitters (glutamatergic, GABAergic, glycinergic, dopaminergic and serotoninergic) and neuropeptides (neuropeptide Y, corticotropin releasing factor: CRF) as well as to environmental factors that the individual is exposed to, there will be differences in the initial alcohol sensitivity, and/or in the reinforcing effects of alcohol intake. Evidences indicate that the response of an animal to a drug can be affected by early environmental influences (Ploj et al., 2003; Vengeliene et al., 2008). The physical and social stimulation of environmental enrichment (EE) plays an essential role during early behavioral and brain development. The interaction between these factors seems to have beneficial effects (Bendre et al., 2015), which could be considered protective or used as a treatment in various behavior disorders (Nithianantharajah and Hannan, 2006). However, negative childhood experiences, such as social isolation, increase the vulnerability to behavioral disorders in adulthood such as depression and anxiety (Heinonen et al., 2013). Likewise, it has been observed that positive experiences with the use of an environmental enrichment (EE) would reduce the vulnerability to several of these effects. Numerous researches have shown that an EE stimulation causes changes in cellular, molecular and behavioral studies (Mychasiuk et al., 2012; Baldini et al., 2013). The consequences of adverse experiences in early life on substance abuse have been widely studied (Ploj et al., 2003, Jaworski et al., 2005). Meanwhile, little is known about the effects of EE on voluntary ethanol consumption.

The first biological changes studied as a result of EE in rats were the increase in weight and cortical volume (Rosenzweig et al., 1962; Rosenzweig and Bennett, 1996). The most striking effects that have sparked scientific interest in recent years are those concerning the ability of the EE to increase neurogenesis (Kempermann et al., 1997; Nilsson et al., 1999; Jaworski et al., 2005; Brown et al., 2003), the expression of neurotrophins (Falkenberg et al., 1992; Torasdotter et al., 1998), and the protection against neurodegenerative diseases as well as emotional and behavioral disorders (Laviola et al., 2008). It has also been shown that EE up-regulates the number of granular neurons in the dentate gyrus of rats and mice (Crofton et al., 2015).

The long-term consequences of RMS on alcohol intake in male mice have already been demonstrated suggesting that stress in early life is a risk factor, or a crucial one, for alcohol consumption and abuse (Cruz et al., 2008). Several studies propose the existence of a significant modulation in the activity of the HPA axis depending on the time span of MS (Odeon et al., 2017). Usually, prolonged MS has been associated with an increase in the levels of corticotropin releasing factor (CRF) in the hypothalamic and extra-hypothalamic areas, and in responses to adrenocorticotropic hormone (ACTH) and corticosterone (Cor) (Falkenberg et al., 1992; Torasdotter et al., 1998; Nilsson et al., 1999; Brown et al., 2003; Lippmann et al., 2007; Dalle Molle et al., 2012).

Taking everything into account, the main objective of this study was to assess whether a non-pharmacological treatment, EE, could reverse or attenuate the effects of stress and anxiety, the increase in the voluntary intake of ethanol, and corticosterone levels in young adult rats.

2. Materials and methods

2.1. Animals

Pregnant Wistar rats were obtained from the School of Pharmacy and Biochemistry, University of Buenos Aires. All the rats were maintained under standard laboratory conditions (12 h light–dark schedule), lights on from 08:00 to 20:00 h, temperature: 21 ± 2 °C, with food and water ad libitum. Dams were housed three per cage during pregnancy. Animals were handled and sacrificed according to the Institutional Committee for the Use and Care of Laboratory Animal rules (CICUAL, School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina). This Committee, under resolution 3195/15, approved the present experimental protocol. The CICUAL adheres to the rules of the "Guide for the Care and Use of Laboratory Animals" (NIH) (2011 revision) and to the EC Directive 86/609/EEC (2010 revision) for animal experiments. All efforts were made to minimize animal suffering and to reduce the number of animals used. Rat weight and general health were evaluated weekly.

2.2. Drugs

We obtained Corticosterone (C2505) from Sigma Chemical Co. St. Louis, MO, USA; and Ethanol (CAS No.: 64-17-5) from Molbase-Chemical Database.

2.3. Experimental procedures

Rat pups subjected to RMS were separated from their mothers and exposed to cold (4 °C) for 1 h from postnatal day (PD) 2 to PD20 (Odeon et al., 2015, 2017). Control pups were left undisturbed with their mothers. The stress treatment usually started at 10:00 h. No mortality was observed in this group. Separation cages were filled with 3 cm of bedding material so that pups could regulate their temperature by burrowing into it and grouping with littermates. Previous studies suggest that, under such conditions, pups can effectively regulate their temperature (Farrell and Alberts, 2007; Odeon et al., 2015, 2017). After 1 h, pups were returned to the home cage with their mothers. At PD22, animals were exposed to voluntary intake of ethanol (6%) or dextrose (1%) for 7 days. The dextrose solution was calorically equivalent to the ethanol solution. In this part of the study, three rats were housed per cage. The animals had free access to ethanol or dextrose, using the two-bottle free choice method (Crabbe et al., 2011). Afterwards, at PD29 animals were exposed to a 30-day wash-out period. Thereafter, animals were again exposed to the two-bottle free choice method with ethanol (6%) or dextrose (1%) from PD59 to PD66. At postnatal day 66, male rats were subjected to the behavioral tests detailed below. After the experiment, the animals were sacrificed by decapitation around 11:00 h (see design in Fig. 1).

Animal behavior was studied by three tests classified as conditional conflict tests: 1- Elevated plus maze; 2- Light-dark transition; 3- Open field test (Costall et al., 1989). The test order was followed considering potentially aversive effects that animals may undergo during the procedure (Crawley, 2007).

All behavioral tests were carried out in a room adequately prepared for this purpose, with adjustable light intensity, soundproof walls, and an air extractor to provide white noise. To minimize olfactory signals, all devices were cleaned with a 70% ethanol solution after each test. The animals were filmed with a video camera. The results were analyzed with the Ethovision XT 7 Noldus program.

2.4. Animals

Pregnant Wistar rats were obtained from the School of Pharmacy and Biochemistry, University of Buenos Aires. All the rats were maintained under standard laboratory conditions (12 h light–dark schedule, lights on from 08:00 to 20:00 h, temperature: 21 ± 2 °C) with food and water ad libitum. Dams were housed three per cage during pregnancy. Animals were handled and sacrificed according to the Institutional Committee for the Use and Care of Laboratory Animal rules (CICUAL, School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina). This Committee, under resolution 3195/15, approved the present experimental protocol. The CICUAL adheres to the rules of the "Guide for the Care and Use of Laboratory Animals" (NIH) (2011 revision) and to the EC Directive 86/609/EEC (2010 revision) for animal experiments. All the efforts were made to minimize animal suffering and to reduce the number of animals used. The weight and general health of rats were assessed weekly.

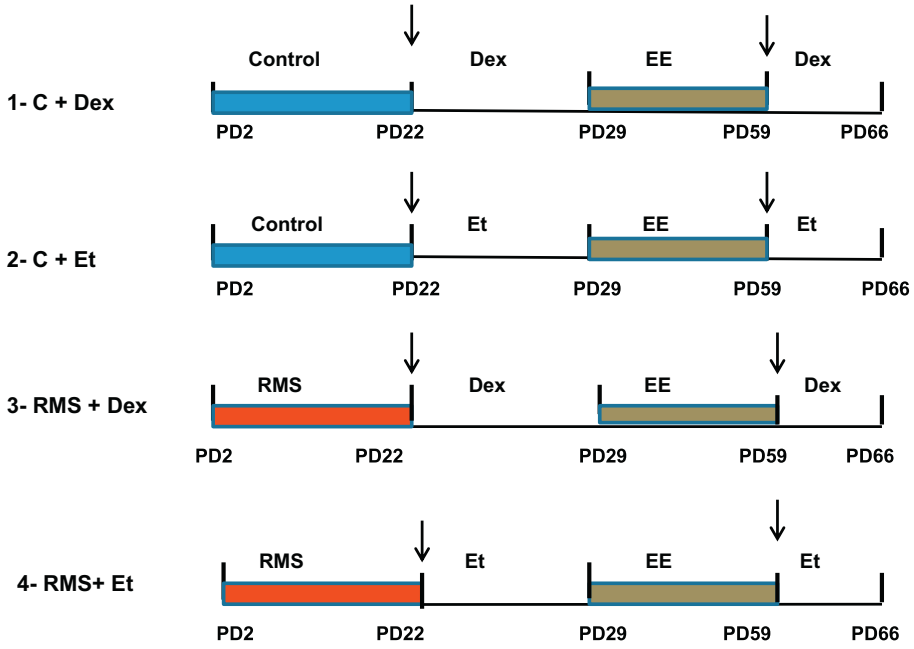

Fig. 1. Schematic representation of the experimental design used in this study. RMS + cold stress or not disturbance (Control group) from postnatal day (PD) 2 to 22. These animals are subjected to the method of choice of two bottles: 1% dextrose or 6% ethanol. Then they are exposed 30 days to an enriched environment. Finally, second intake was from PD59-66 given to drink either water and dextrose: 1% solution (g/100 ml) or water and ethanol: 6% solution (g/100 ml) to the method of choosing two bottles.

2.5. Drugs

We purchased Corticosterone (C2505) from Sigma Chemical Co. St. Louis, MO, USA; Ethanol (CAS No.: 64-17-5) from Molbase-Chemical Database.

2.6. 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 suspended 1 m from the floor. Two arms, enclosed by walls of 40 cm high, were perpendicular to two opposed open arms. To avoid falls, the open arms were surrounded by a wooden wall of 0.5 cm high. Each animal was placed in the center of the EPM, always towards the same open arm. We measured the time that animals spent in both the open and closed arms compared to the total time spent exploring arms. We also measured the number of times the animals entered the open arms. Generally, each animal was observed for 5 min. Once each session finished, the animals were taken to their housing condition. The apparatus was cleaned with a 70% EtOH solution after each test.

2.7. Light-dark transition

This behavioral procedure exploits the natural tendency of rodents to avoid open and/or brightly lit spaces. This study has been shown to be sensitive to anxiety-like behaviors in mice (Bourin and Hascoët, 2003). The apparatus consisted of a square wooden box (60 × 90 × 90 cm) with two compartments. One compartment was illuminated with a bright light, whereas the other one was dark and had an opaque roof. Both sides were divided by a removable wall. Before beginning the test, rats were always placed in the illuminated side and at the same angle. Then, we removed the slide that divided both compartments and quantified the time that the animals spent in the illuminated side, before crossing to the dark compartment. We counted a "transition" when the animals entered the dark side on all fours. It was recorded the time spent in the dark side of the apparatus, as well as the number of transitions between sides. In general, each animal was observed for a time lapse of 5 min. Under normal conditions rats tend to stay in the illuminated side for short periods of time. Any state of anxiety further decreases the latency time to enter the dark side. The apparatus was cleaned with a 70% EtOH solution after each test. Once each session finished, the animals were taken to their housing condition.

2.8. Open field test

To assess locomotor activity in a novel setting, animals were placed in the center of the box. The apparatus consisted of a delimited square wooden (60 × 90 × 90 cm) with black walls, and a transparent floor divided in 25 equal sections by thin black lines. The arena was illuminated by an incandescent light placed 200 cm above the field. Before beginning the test, rats were placed at the center of the apparatus and at the same angle. Exploratory activity was counted considering the amount of sections crossed by the animal, as well as the number of times the animal entered the central square. The animal movements were recorded by a video camera suspended 1 m above the field, and animals were tested for 30 min. The experiments were always performed at the same time and place, and under similar temperature (23 °C) and light (70 lx) conditions. The apparatus was cleaned with a 70% EtOH solution after each test. Once each session finished, the animals were taken to their housing condition.

3. Environmental enrichment

To evaluate the effects of environmental enrichment on the variables of interest, subjects from each experimental group were housed in EE cages for 30 days during the washout period (Fig. 1). Enriched environments are "a combination of complex inanimate and social stimulation" (Sztainberg and Chen, 2010). EEs were generally constituted by bigger housing cages (60 × 60 × 25 cm) in which animals were hosted in larger groups (6 animals per cage) and into which a variety of objects were introduced: platforms, running wheel plastic, wooden toys, rattles, ropes and food (cheese, quince or apple). Four different combinations were designated, and the objects were changed once a week to avoid habituation and to maintain a novel environmental factor (Witt, 2007; Mora et al., 2007; Simpson and Kelly, 2011). In control groups, animals were housed in groups of 4 in traditional cages (40 × 25 × 17 cm) without the addition of any object. This is known as IC (impoverished conditions) + SC (standard group housing). Both types of cages (enriched and standard) were in the same air-conditioned room (temperature: 20 ± 2 °C) with a 12:12 h light-dark cycle (lights on at 8:00 h a.m.). Both food and water were provided ad libitum.

4. Hormonal measurement

In order to assess the function of the HPA axis in this model we measured plasmatic levels of corticosterone by HPLC.

4.1. Determination of corticosterone

Following decapitation (11:00 h), 15 ml of trunk blood were collected in a conical tube with heparin. Plasma was separated by centrifugation (3000 *g* during 15 min at 4 °C) and frozen at −70 °C. Plasmatic Cor concentration was determined by high performance liquid chromatography (HPLC) (Retana-Márquez et al., 2003; Odeon et al., 2015, 2017). Cor was extracted from 200 µl of plasma by adding 4 ml of diethyl ether-dicloromethane (60:40). We added to each tube 100 µl of internal standard (Phenytoin 1 mg/ml diluted in methanol). The organic phase was evaporated with nitrogen at 37 °C. Samples were resuspended with 150 µl of mobile phase (acetonitrile-water, 40:60), vortexed (15 s) and injected into the HPLC system. The guard column Hypersil GOLD C18, particle size 5 µm, 250 × 4.6 mm (Thermo Fisher Scientific Inc.) was equilibrated using HPLC-grade acetonitrile-water (40:60 v/v) at a flow rate of 1 ml/min. A series of standards (normal human plasma with Cor, covering the range of 0.1–1 µg/ml) were used in daily work. A regression line between the peak heights and the concentration of Cor was calculated and used to determine Cor concentration in the samples.

5. Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). In the experiments of liquid intake behavioral parameters, EE and Cor levels, and the statistical significance were assessed with a two-way analysis of variance (ANOVA). When the interaction was significant, simple effects were reported. Also, a post hoc Bonferroni test was performed. In the rest of the experiments, one-way ANOVA and post hoc Tukey test were used to analyze the significance of the differences. Values of $p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = *** were considered statistically significant.

It was used the Infostat statistical program (developed by the InfoStat Group), teacher-researchers of Statistics and Biometry and Experimental Design of the National University of Córdoba (FCA- NC). It is available online: http://www.infostat.com.ar/

6. Results

6.1. Ethanol consumption

Fig. 2 shows ethanol consumption during the second intake as g ethanol/kg rat body weight/day. During the second two-bottle free choice procedure (PD66) in adult rats, stressed animals consumed ethanol by > 292% (t = 2.804 df = 12; **p < 0.01) compared with their respective control groups. Volume (ml) of 2nd voluntary intake of ethanol per rat in 7 days, with a 30-day period of EE between intakes (n = 6). Two-way ANOVA, RMSX EE. ($F_{1,\ 20}$ = 80.17); C + EE t = 1.874 gl = 10; RMS (t = 11.73 gl = 10); RMS + EE (t = 11.53 gl = 10).

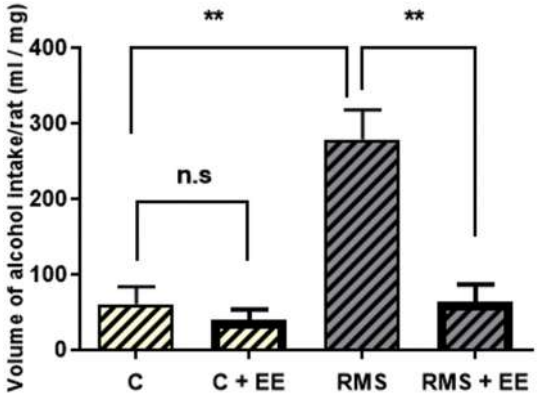

Fig. 2. Second voluntary ethanol intake expressed in Volume (ml) of ethanol (Et) consumed/kg body weight/day for 7 days, with a 30-day period of environmental enrichment between intakes.

Apart from that, when we studied water consumption, we obtained similar intake values than those of dextrose. Hence, the increase in liquid consumption is specific to EtOH (Fig. 2).

6.2. Behavioral studies

To study possible long-term behavioral changes on stressed animals, we performed a set of conflict tests. The tests were performed in the following order, as recommended by Crawley (2007): A- Elevated Plus Maze (Fig. 3A); B- Light-Dark Transition (Fig. 3B); C– Open Field Test (Fig. 3C).

Elevated Plus Maze (Fig. 3A) shows the percentage of time spent exploring the open arms. The animals of both RMS + Dex and RMS + Et groups spent significantly more time exploring the open arms. Controls were performed considering the total distance traveled and the number of entries to both arms. One-way ANOVA ($F_{2;38}$ = 6678, p = 0,0034) showed that RMS + Et animals spent significantly more time exploring the open arms of the maze (t = 5.421, ***p < 0,0001) than their respective control groups.

Fig. 3B exhibits how much time the animals spent in the illuminated side during an LDT test. We observed similar values in the time spent in the illuminated side within the two control groups (C + Dex; C + Et) and within the stressed groups (RSM + Dex; RMS + ET). One-way ANOVA (F2;34 = 20.18, *** p < 0.0001) revealed significant differences between controls and stressed groups, since the latter spent significantly more time walking around the illuminated side than their respective controls.

Fig. 3C shows the percentage of time that the animals spent in the center of the arena in the Open Field (OF) test. Stressed animals spent a significantly greater time exploring the center of the arena than their control counterparts. One-way ANOVA, (F2;35 = 6886, p = 0,0032).

As described in the introduction, EE plays a key role in the determination of behavioral phenotypes. In this sense, we were interested in evaluating if EE may possible reverse the effects of RMS + cold stress in some of the variables studied.

We aimed to assess the potential of EE to revert the effects of RMS and ethanol consumption. Behavioral reversal was confirmed given that the RMS + EE group drank the same levels of ethanol as the control group.

In order to study the possible effect of the enriched environment on the behavior of stressed animals, we repeated the set of conflict tests. They were carried out in the same order as established previously (Crawley, 2007): A- Elevated Plus Maze (Fig. 4A); B- Light-Dark Transition (Fig. 4B); C– Open Field Test (Fig. 4C).

Fig. 4A (EPM) demonstrates the percentage of time spent exploring the open arms, either by rats reared in standard cages or in EE cages, during the wash-out period. Control “t = 3.248 gl = 27”, RMS “t = 5.421 gl = 18”, RMS + Dex “t = 4.703 gl = 20”, RMS + Et “t = 6.703 gl = 21”.

As Fig. 4B illustrates, both C + Dex + EE and C + Et + EE increased the time spent exploring the illuminated sides, but when together, such as RMS + Dex + EE or RMS + Et + EE, the time spent exploring the illuminated sides decreased to control levels. Controls ““t = 3.112 gl = 27 ““, RMS ““t = 2.977 gl = 21 ““, RMS + Dex “t = 4.703 gl = 20” RMS + Ethanol ““t = 3.279 gl = 22 ““. This may indicate that EE and RMS increase the exploration of the aversive environment in different ways, possibly by opposite mechanisms. For instance, by an increase in the impulsive behavior due to RMS, with the

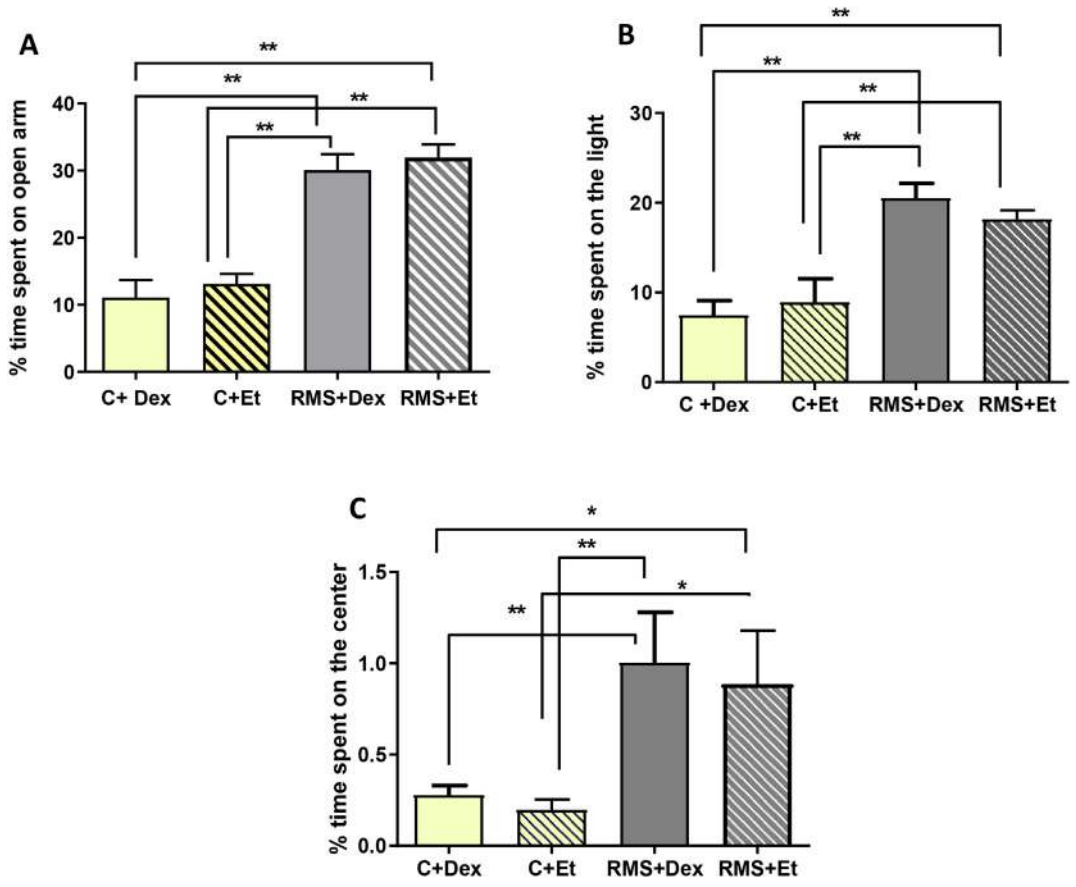

Fig. 3. Conflict tests. A) Elevated plus maze (EPM), percentage of time (% time) spent in the open arm. Controls corresponding to the test were performed (total distance traveled and number of entries to both arms). B) Light/dark transition test (LDT), percentage of time (% time) spent on the light side. The controls corresponding to the test were performed (total distance traveled and number of entries to both arms). C) Open field (OF), percentage of time (% time) spent on the center. The controls corresponding to the test were performed (total distance traveled).

consequent decrease in anxiety caused by EE which leads to an increase in exploratory behavior.

In Fig. 4C, we found that this effect was successfully reverted by EE, both in ethanol and dextrose RMS + EE groups ($t = 6703$, $^{***}p < 0{,}0001$). It was reverted up to control levels ($^{**}p < .01$). Controls ""$t = 3.112$ $gl = 27$ "", RMS ""$t = 2.977$ $gl = 21$ "", RMS + Ethanol ""$t = 3.279$ $gl = 22$ "".

Behavioral reversal was confirmed in terms of ethanol intake, since the RMS+ EE group drank the same levels of ethanol as the control group. We observed an anxiolytic effect in control groups through the behavioral tests. This effect has been described by other authors (Simpson and Kelly, 2011) (Figs. 4A, B, C).

Given that behavioral studies have shown that chronic stress in adults produces high levels of anxiety and that the type of tests used serves to assess exploratory activity- aversive environment, our hypothesis is that these animals may have altered levels of impulsivity. This would explain the behavioral response observed in diverse studies as well as the increase in ethanol intake.

Fig. 5 illustrates the plasmatic concentration of Cor across the different groups, as measured by HPLC. We performed a two-way ANOVA, RMSXEE ($F2;34 = 5348$, $p = 0{,}0096$). Significant simple effects: test t: RMS + Dex vs RMS + Dex + EE "$t = 3776$ $gl = 12$". Stressed animals had a significantly lower plasmatic Cor concentration. The decrease in Cor was reversed by RMS + EE, + Ethanol or the combination of both RMS + Et and RMS + Et + EE until reaching the levels of the control group.

7. Discussion

The present work shows the behavioral and neurochemical changes induced by RMS + cold stress in young adult rats.

RMS increases the voluntary intake of ethanol. This treatment showed alterations in the HPA axis by decreased corticosterone levels.

Some of the following effects were reversed using a non-pharmacological treatment, such as the enriched environment: ethanol intake, the behavioral response in conflict tests, and the levels of hormones related to stress.

Many animal models have been developed to observe not only the effects of alcohol intake on specific social situations, but also to assess the participation of genetic factors (Anacker and Ryabinin, 2010). We have found an animal model to study the voluntary intake of ethanol in rats. The most used animal models propose that in adulthood, a stress factor should be simultaneous with the offer of the liquid or drug to be studied. The postnatal period and the bond between mother and child are particularly important in the development and conformation of the response to normal stress and emotional behavior (Ellenbroek and Cools, 2000; Pryce et al., 2005). This model allows us to study the long-term effects of adverse stimuli in early life and to link them with substance abuse vulnerability.

Lodge and Lawrence (2003) demonstrated an interaction between the dysregulation of the HPA axis induced by isolation and alcohol. This study indicated that the activation of the HPA axis was important for the preference towards ethanol observed in socially isolated adult rats. We have demonstrated a decrease in basal levels of Corticosterone, one of the stress hormones of the HPA axis induced by RMS + cold stress. Besides, plasma corticosterone levels decreased in the treated groups.

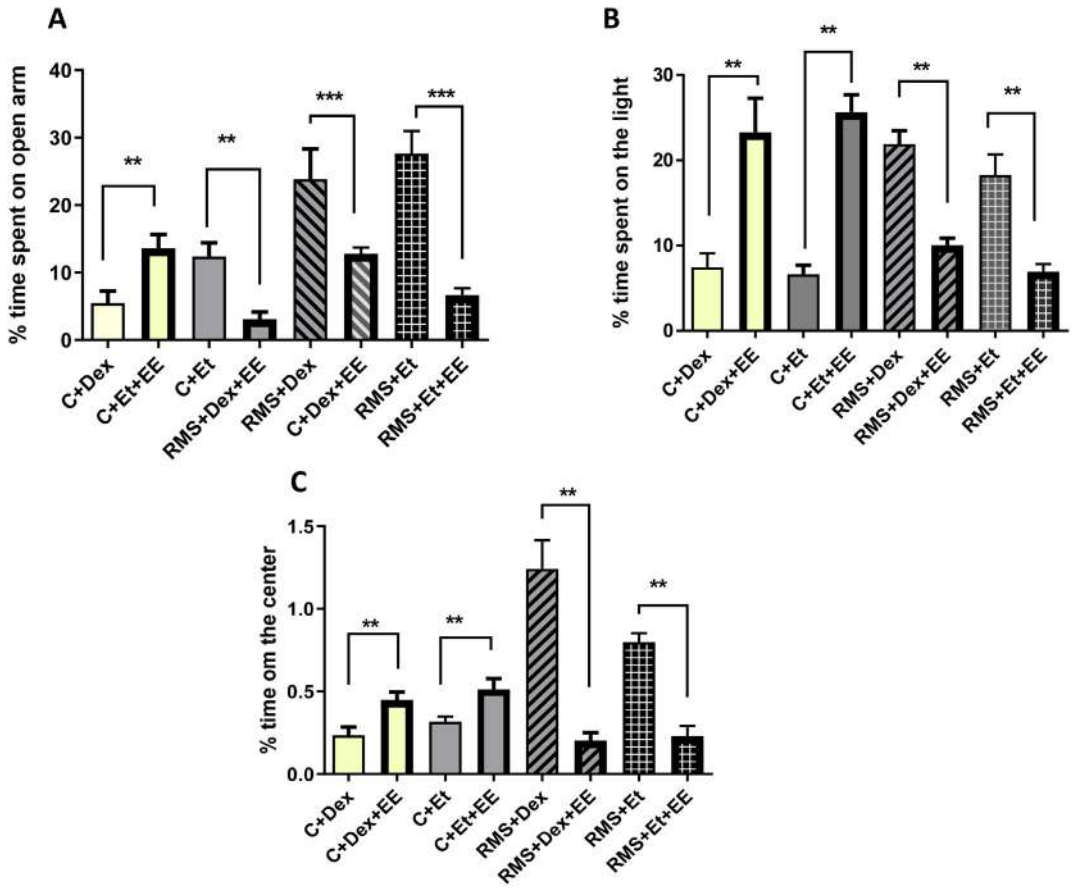

Fig. 4. Environmental enrichment. A) Elevated Pluz Maze: Percentage of time in the open arm in rats with standard treatment and rats raised in an enriched environment during the inter-intake period. B) Light Dark Transicion: Percentage of time on the lit side in rats with standard treatment and rats raised in an enriched environment during the period between intakes. C) Open Field: Percentage of time on the lit side in rats with standard treatment and rats reared in an enriched environment during the period between ingests.

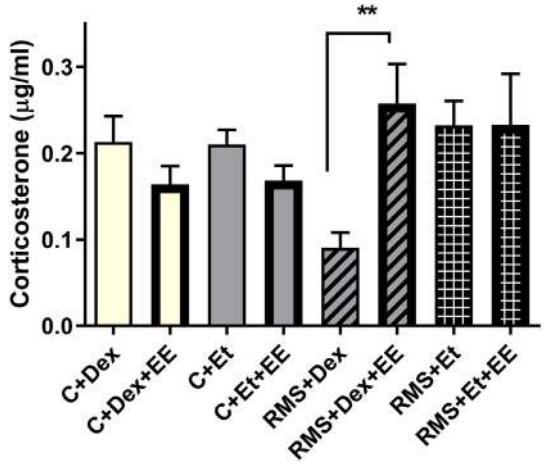

Fig. 5. Plasma corticosterone levels measured by HPLC Rats subjected to chronic postnatal stress, the two voluntary intakes of ethanol with a period (30 days) of environment enriched between intakes.

These results point to a lower basal hormone level of the axis that could be due to the increase in the hippocampal receptors MR and GR. These receivers are responsible for the regulation of the axis negative feedback.

Masini et al., 2012 demonstrated the adaptive capacity of the HPA axis before the repetition of a stressor. Our model not only shows habituation through decreased levels of the hormones involved in the repetition of the stimulus, but also that this is a long-term effect. We found these levels decreased even in adulthood, 60 days after starting the treatment. The animals generate a response, but the hormonal peak is still lower than the controls.

These results show the great effect that RMS + cold stress has on the developing CNS, how RMS + cold stress can exert long-term effects and alter the response to stress throughout an individual's life.

We analyzed the relationships between the neurochemical and hormonal parameters of RMS + cold stress, alcohol consumption, and animal behavior. To this aim, we studied the possible alterations of anxiety levels in rodents for which we performed three conflict tests: EPM, LDT and OF. We observed in all tests the same animal behavior: increased exploration time in aversive environments. This would indicate a decrease in anxiety levels. The elevated cross EPM and the light-dark transition box are some of the most widely used models for the study of unconditioned anxiety and have been well characterized and validated both ethologically and pharmacologically (Pellow and File, 1986; Rodgers and Dalvi, 1997; Crawley, 2007). It is considered that the behavior in the open arms of the EPM indicates lower levels of anxiety. Anxiolytics, such as benzodiazepines and low doses of alcohol, increase the behavior in open arms just as anxiogenic drugs decrease it (Pellow and File, 1986).

Other behavioral studies show that both acute stress in adolescents and adults, and chronic stress in adults cause an increase in the levels of anxiety (Pryce et al., 2005; Spencer et al., 2015). Apart from that, the tests we have used to evaluate the relation exploratory activity-aversive environment, allow as to deduce that the levels of impulsivity in these animals could be altered. This may explain the behavioral response

observed among the different tests as well as the increase in ethanol consumption.

It has been observed that EE produces a reversal in alcohol consumption due to RMS. This is the main result of these experiments, since a reversal in alcohol intake will allow us to delve into the relationship between stress and alcohol consumption.

Some studies indicate that EE could play a protective role in the self-administration of other psychostimulant substances such as amphetamine (Bardo et al., 2001; Green et al., 2002). However, these data are not consistent with the increase in alcohol and cocaine consumption observed by Fernández-Teruel et al. (2002), and Hill and Powell (1976), or with the increase in conditioned place preference before the administration of opioid k-agonists (Smith et al., 2003). In general, results in favor of reduced self-administration in animals in enriched environments are only obtained when low drug doses are used. Anxyolitics, such as benzodiazepine and ethanol in low doses increment the exploration of open arms, and anxiogenic drugs decrease it (Pellow and File, 1986; Rodgers and Dalvi, 1997; Crawley, 2007).

When we studied the hormonal response, we observed that the EE produced a significant increase in the basal levels of corticosterone in the stressed group, although a decrease is seen in the control group.

Regarding the influences of EE on the basal levels of the HPA axis hormones, the results are not consistent, although in general, they seem to indicate a decrease in emotional reactivity and a greater adaptation to stressful stimuli (Fox et al., 2006).

There is no consensus regarding the effects of RMS on basal corticosterone levels. There are works in which no differences in basal corticosterone levels were found (Pham et al., 1999; Roy et al., 2001; Francis et al., 2002). Other reports suggest that EE decreases the activity of the HPA axis in basal conditions (Mohammed et al., 1993; Larsson et al., 2002) or through the exposure to stressful situations (Roy et al., 2001; Francis et al., 2002; Welberg et al., 2006). It has also been pointed that enriched animals have higher baseline corticosterone levels than controls (Benaroya-Milshtein et al., 2004; Mohammed et al., 1993; Moncek et al., 2004). Clearly, the results are very variable and difficult to interpret. In our investigation, the groups that experienced RMS and received an EE showed an increase in corticosterone compared with the non-enriched groups. In this case, the EE counteracted the negative effects of RMS on the HPA axis. The reversal produced by the EE with regard to the effect of RMS is more important than the increase or decrease of HPA hormones and its possible implications, for which we should carry out a deeper study.

On the other hand, the EE also reversed the behavioral effects in the conflict tests. In the control group, the treatment increased the percentage of time in the aversive sites, which we interpreted as an anxiolytic effect. Conflict tests are widely used to evaluate the anxious behavior of rodents, thus the increased time in aversive places is indicative of lower levels of emotion or anxiety (Pellow and File, 1986). This result shows a general reduction of emotion, which is consistent with other works (Fernández-Teruel et al., 2002; Benaroya-Milshtein et al., 2004; Friske and Gammie, 2005; Schneider et al., 2006). It has been suggested that in classical anxiety tests the decrease in emotionality frequently perceived in animals reared in enriched environments could be explained in terms of a reduction in phobia of novelty, which in turn, increases the motivation to explore new spaces (Fernández-Teruel et al., 2002; Roy et al., 2001).

The experimental protocols of EE vary among different laboratories. For example, there can be differences in time duration, the age at which the EE is applied, the number of animals per cage, the size of the cages, the objects used for the treatment, etc. On the whole, it is not well-defined which component of EE causes the changes observed.

It can be seen, that the novelty achieved by periodically changing objects, or their position in space, provides additional stimulation that can improve the formation of cognitive maps. All these factors allow an increase in stimulation, physical activity, and in the ways of interacting with the individuals that are part of the environment.

8. Conclusion

We have demonstrated the susceptibility and vulnerability during the early life of an individual, the ability to adapt to adversity, and the great plasticity of the central nervous system (CNS). We found that while negative experiences during childhood could induce drug use as well as neurochemical and behavioral disorders in the long term, positive experiences such as growing up in an enriched environment can reverse these effects.

Ethical statement

Animals were handled and sacrificed according to the Institutional Committee for the Use and Care of Laboratory Animal rules (CICUAL, School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina). This Committee, under resolution 3195/15, approved the present experimental protocol. The CICUAL adheres to the rules of the “Guide for the Care and Use of Laboratory Animals” (NIH) (2011 revision) and to the EC Directive 86/609/EEC (2010 revision) for animal experiments. All the efforts were made to minimize animal suffering. The rats were weighed, and general health assessed weekly.

These authors declare that they have no conflicts of interest, and they alone are responsible for the content and writing of the manuscript.

Acknowledgements

This work has been supported by grant PIP No 0409 from National Scientific and Technologic Research Council (CONICET) to GBA. GBA and MMO are members of CONICET. We want to thank Lic. Cynthia Silberman for helping us to correct this manuscript. These authors declare that they have no conflicts of interest, and they alone are responsible for the content and writing of the manuscript.

References

Anacker, A.M.J., Ryabinin, A.E., 2010. Biological contribution to social influences on alcohol drinking: evidence from animal models. Int. J. Environ. Res. Public Health 7 (2), 473–493.

Baldini, S., Restani, L., Baroncelli, L., Coltelli, M., Franco, R., Cenni, M.C., Maffei, L., Berardi, N., 2013. Enriched early life experiences reduce adult anxiety-like behavior in rats: a role for insulin-like growth factor 1. J. Neurosci. 33 (28) (117152).

Bardo, M.T., Klebaur, J.E., Valone, J.M., Deaton, C., 2001. Environmental enrichment decreases intravenous self-administration of amphetamine in female and male rats. Psycho Pharmacol. (Berl) (3), 278–284.

Benaroya-Milshtein, N., Hollander, N., Apter, A., Kukulansky, T., Raz, N., Wilf, A., Pick, C.G., 2004. Environmental enrichment in mice decreases anxiety, attenuates stress responses and enhances natural killer cell activity. Eur. J. Neurosci. 20 (5), 1341–1347.

Bendre, M., Comasco, E., Nylander, I., Nilsson, K.W., 2015. Effect of voluntary alcohol consumption on Maoa expression in the mesocorticolimbic brain of adult male rats previously exposed to prolonged maternal separation. Translational Psychiatry 5, e690.

Bourin, M., Hascoët, M., 2003. The mouse light/dark box test. Eur. J. Pharmacol. 463 (1–3), 55–65.

Brown, J., Cooper-Kuhn, C.M., Kempermann, G., Praag, H., Winkler, J., Gage, F.H., Kuhn, H.G., 2003. Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. Eur. J. Neurosci. 17 (10), 2042–2046.

Carlyle, B.C., Duque, A., Kitchen, R.R., Bordner, K.A., Coman, D., Doolittle, E., Papademetris, X., Hyder, F., Taylor, J.R., Simen, A.A., 2012. Maternal separation with early weaning: a rodent model providing novel insights into neglect associated developmental deficits. Dev. Psychopathol. 24 (4), 1401–1416.

Carr, C.P., Martins, C.M.S., Stingel, A.M., Lemgruber, V.B., Juruena, M.F., et al., 2013. The role of early life stress in adult psychiatric disorders: a systematic review according to childhood trauma subtypes. J. Nerv. Ment. Dis. 201 (12), 1007–1020.

Chapman, D.P., Whitfield, C.L., Felitti, V.J., Dube, S.R., Edwards, V.J., Anda, R.F., 2004. Adverse childhood experiences and the risk of depressive disorders in adulthood. J. Affect. Disord. 82 (2), 217–225.

Costall, B., Jones, B.J., Kelly, M.E., Naylor, R., Tomkins, D.M., 1989. Exploration of mice in a black and white test box: validation as a model of anxiety. Pharmacol. Biochem. Behav. 32 (3), 777–785.

Crabbe, J.C., Harris, R.A., Koob, G.F., 2011. Preclinical studies of alcohol binge drinking. Ann. N. Y. Acad. Sci. 1216, 24–40.

Crawley, J.N., 2007. What’s Wrong with my Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice. John Wiley & Sons.

Crofton, E.J., Zhang, Y., Green, T.A., 2015. Inoculation stress hypothesis of environmental enrichment. Neurosci. Biobehav. Rev. 49, 19–31.
Cruz, F.C., Quadros, I.M., Planeta Cda, S., Miczek, K.A., 2008. Maternal separation stress in male mice: long-term increases in alcohol intake. Psychopharmacology 201 (3), 459–468.
Dalle Molle, R., Portella, A.K., Goldani, M.Z., Kapczinski, F.P., Leistner-Segal, S., Salum, G.A., Manfro, G.G., Silveira, P.P., 2012. Associations between parenting behavior and anxiety in a rodent model and a clinical sample: relationship to peripheral BDNF levels. Transl. Psychiatry 2, e195.
Ellenbroek, B.A., Cools, A.R., 2000. The long-term effects of maternal deprivation depend on the genetic background. Neuropsychopharmacology 23 (1), 99–106.
Enoch, M., 2011. The role of early life stress as a predictor for alcohol and drug dependence. Psychopharmacology 214, 17–31.
Falkenberg, T., Mohammed, A.K., Henriksson, B., Persson, H., Winblad, B., Lindefors, N., 1992. Increased expression of brain-derived neurotrophic factor mRNA in rat hippocampus is associated with improved spatial memory and enriched environment. Neurosci. Lett. 138 (1), 153–156.
Farrell, W.J., Alberts, J.R., 2007. Rat behavioral thermoregulation integrates with nonshivering thermogenesis during postnatal development. Behav. Neurosci. 121 (6), 1333.
Fernández-Teruel, A., Giménez-Llort, L., Escorihuela, R.M., Gil, L., Aguilar, R., Steimer, T., Tobeña, A., 2002. Early-life handling stimulation and environmental enrichment: are some of their effects mediated by similar neural mechanisms? Pharmacol. Biochem. Behav. 73 (1), 233–245.
Fox, C., Merali, Z., Harrison, C., 2006. Therapeutic and protective effect of environmental enrichment against psychogenic and neurogenic stress. Behav. Brain Res. 175 (1), 1–8.
Francis, D.D., Diorio, J., Plotsky, P.M., Meaney, M.J., 2002. Environmental enrichment reverses the effects of maternal separation on stress reactivity. J. Neurosci. 22 (18), 7840–7843.
Friske, J.E., Gammie, S.C., 2005. Environmental enrichment alters plus maze, but not maternal defense performance in mice. Physiol. Behav. 85 (2), 187–194.
Gilles, E.E., Schultz, L., Baram, T.Z., 1996. Abnormal corticosterone regulation in an immature rat model of continuous chronic stress. Pediatr. Neurol. 15 (2), 114–119.
Goldstein, R.Z., Volkow, N.D., 2011. Dysfunction of the prefrontal cortex in addiction: neuroimaging findings and clinical implications. Nat. Rev. Neurosci. 12, 652–669.
Green, T.A., Gehrke, B.J., Bardo, M.T., 2002. Environmental enrichment decreases intravenous amphetamine self-administration in rats: dose-response functions for fixedand progressive- ratio schedules. Psychopharmacology 162 (4), 373–378.
Green, T.A., Alibhai, I.N., Roybal, C.N., Winstanley, C.A., Theobald, D.E., Birnbaum, S.G., Graham, A.R., Unterberg, S., Graham, D.L., Vialou, V., Bass, C.E., Terwilliger, E.F., Bardo, M.T., Nestler, E.J., 2010. Environmental enrichment produces a behavioral phenotype mediated by low cyclic adenosine monophosphate response element binding (CREB) activity in the nucleus accumbens. Biol. Psychiatry 67 (1), 28–35.
Heinonen, E., Knekt, P., Härkänen, T., Virtala, E., Lindfors, O., 2018. Associations of early childhood adversities with mental disorders, psychological functioning, and suitability for psychotherapy in adulthood. Psychiatry Res. 264, 366–373.
Hill, S.Y., Powell, B.J., 1976. Cocaine and morphine self-administration: effects of differential rearing. Pharmacol. Biochem. Behav. 5 (6), 701–704.
Huot, R.L., Thrivikraman, K.V., Meaney, M.J., Plotsky, P.M., 2001. Development of adult ethanol preference and anxiety as a consequence of neonatal maternal separation in Long Evans rats and reversal with antidepressant treatment. Psychopharmacology (Berl) 158 (4), 366–373.
Jahng, J.W., 2011. An animal model of eating disorders associated with stressful experience in early life. Horm. Behav. 59 (2), 213–220.
Jaworski, J.N., Francis, D.D., Brommer, C.L., Morgan, E.T., Kuhar, M.J., 2005. Effects of early maternal separation on ethanol intake, GABA receptors and metabolizing enzymes in adult rats. Psychopharmacology (Berl) 181 (1), 8–15.
Kalinichev, M., Easterling, K.W., Plotsky, P.M., Holtzman, S.G., 2002. Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long-Evans rats. Pharmacol. Biochem. Behav. 73 (1), 131–140.
Kempermann, G., Kuhn, H.G., Gage, F.H., 1997. More hippocampal neurons in adult mice living in an enriched environment. Nature 386 (6624), 493.
Larsson, F., Winblad, B., Mohammed, A.H., 2002. Psychological stress and environmental adaptation in enriched vs. impoverished housed rats. Pharmacology, Biochemistry, and Behavior 73 (1), 193–207.
Laviola, G., Hannan, A.J., Macr, S., Solinas, M., Jaber, M., 2008. Effects of enriched environment on animal models of neurodegenerative diseases and psychiatric disorders. Neurobiol. Dis. 31 (2), 159–168.
Lippmann, M., Bress, A., Nemeroff, C.B., Plotsky, P.M., Monteggia, L.M., 2007. (2007). Long-term behavioural and molecular alterations associated with maternal separation in rats. Eur. J. Neurosci. 25 (10), 3091–3098.
Lodge, D., Lawrence, A., 2003. The CRF1 receptor antagonist antalarmin reduces volitional ethanol consumption in isolation-reared fawn-hooded rats. Neuroscience 117 (2), 243–247.
Meaney, M.J., 2001. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu. Rev. Neurosci. 24 (1), 1161–1192.
Meaney, M.J., Brake, W., Gratton, A., 2002. Environmental regulation of the development of mesolimbic dopamine systems: a neurobiological mechanism for vulnerability to drug abuse? Psychoneuroendo-crinology 27 (1–2), 127–138.
Michaels, C.C., Holtzman, S.G., 2008. Early postnatal stress alters place conditioning to both muand kappa-opioid agonists. J. Pharmacol. Exp. Ther. 325, 313–318.
Moncek, F., Duncko, R., Johansson, B.B., Jezova, D., 2004. Effect of environmental enrichment on stress related systems in rats. J. Neuroendocrinol. 16 (5), 423–431.
Mohammed, A.H., Henriksson, B.G., Söderström, S., Ebendal, T., Olsson, T., Seckl, J.R., 1993. Environmental influences on the central nervous system and their implications for the aging rat. Behav. Brain Res. 57 (2), 183–191.
Mora, F., Segovia, G., del Arco, A., 2007. Aging, plasticity and environmental enrichment: structural changes and neurotransmitter dynamics in several areas of the brain. Brain Res. Rev. 55 (1), 78–88.
Masini, C.V., Day, H.E.W., Gray, T., Crema, L.M., Nyhuis, T.J., Babb, J.A., Campeau, S., 2012. Evidence for a lack of phasic inhibitory properties of habituated stressors on HPA axis responses in rats. Physiology & Behavior 105 (2), 568–575.
Mychasiuk, R., Zahir, S., Schmold, N., Ilnytskyy, S., Kovalchuk, O., Gibb, R., 2012. Parental enrichment and offspring development: modifications to brain, behavior and the epigenome. Behav. Brain Res. 228 (2), 294–298.
Nilsson, M., Perfilieva, E., Johansson, U., Orwar, O., Eriksson, P.S., 1999. Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. J. Neurobiol. 39 (4), 569–578.
Nithianantharajah, J., Hannan, A.J., 2006. Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nat. Rev. Neurosci. 7 (9), 697–709.
Odeon, M.M., Andreu, M., Yamauchi, L., Grosman, M., Acosta, G.B., 2015. Chronic postnatal stress induces voluntary alcohol intake and modifies glutamate transporters in adolescent rats. Stress 18 (4), 427–434.
Odeon, M.M., Yamauchi, L., Grosman, M., Acosta, G.B., 2017. Long-term effects of repeated maternal separation and ethanol intake on HPA axis responsiveness in adult rats. Brain Res. 1657, 193–201.
Pellow, S., File, S.E., 1986. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. Pharmacol. Biochem. Behav. 24 (3), 525–529.
Pham, T.M., Ickes, B., Albeck, D., Söderström, S., Granholm, A.C., Mohammed, A.H., 1999. Changes in brain nerve growth factor levels and nerve growth factor receptors in rats exposed to environmental enrichment for one year. Neurosci. 94 (1), 279–286.
Ploj, K., Roman, E., Nylander, I., 2003. Long-term effects of maternal separation on ethanol intake and brain opioid and dopamine receptors in male Wistar rats. Neuroscience 121 (3), 787–799.
Pryce, C.R., Rüedi-Bettschen, D., Dettling, A.C., Weston, A., Russig, H., Ferger, B., Feldon, J., 2005. Long-term effects of early-life environmental manipulations in rodents and primates: potential animal models in depression research. Neurosci. Biobehav. Rev. 29 (4–5), 649–674.
Rehm, J., Mathers, C., Popova, S., Thavorncharoensap, M., Teerawattananon, Y., Patra, J., 2009. Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders. Lancet 373, 2223–2233.
Retana-Márquez, S., Bonilla-Jaime, H., Vazquez-Palacios, G., Martinez-Garcia, R., Velazquez-Moctezuma, J., 2003. Changes in masculine sexual behavior, corticosterone and testosterone in response to acute and chronic stress in male rats. Horm. Behav. 44 (4), 327–337.
Rodgers, R.J., Dalvi, A., 1997. Anxiety, defence and the elevated plus-maze. Neurosci. Biobehav. Rev. 21 (6), 801–810.
Rosenzweig, M.R., Bennett, E.L., 1996. Psychobiology of plasticity: effects of training and experience on brain and behavior. Behav. Brain Res. 78, 57–65.
Rosenzweig, M.R., Krech, D., Bennett, E.L., Zolman, J.F., 1962. Variation in environmental complexity and brain measures. J. Comp. Physiol. Psychol. 55, 1092–1095.
Roy, V., Belzung, C., Delarue, C., Chapillon, P., 2001. Environmental enrichment in BALB/c mice: effects in classical tests of anxiety and exposure to a predatory odor. Physiol. Behav. 74 (3), 313–320.
Schneider, T., Turczak, J., Przewłocki, R., 2006. Environmental enrichment reverses behavioral alterations in rats prenatally exposed to valproic acid: issues for a therapeutic approach in autism. Neuropsychopharmacology 31 (1), 36.
Simpson, J., Kelly, J.P., 2011. The impact of environmental enrichment in laboratory rats behavioural and neurochemical aspects. Beh Brain Research 222 (1), 246–264.
Smith, M.A., Bryant, P.A., McClean, J.M., 2003. Social and environmental enrichment enhances sensitivity to the effects of kappa opioids: studies on antinociception, diuresis and conditioned place preference. Pharmacol. Biochem. Behav. 76 (1), 93–101.
Spencer, S.J., Emmerzaal, T.L., Kozicz, T., Andrews, Z.B., 2015. Ghrelin's role in the hypothalamic- pituitary-adrenal Axis stress response: implications for mood disorders. Biol. Psychiatry 78 (1), 19–27.
Sztainberg, Y., Chen, A., 2010. An environmental enrichment model for mice. Nat. Protoc. 5 (9), 1535–1539.
Torasdotter, M., Metsis, M., Henriksson, B.G., Winblad, B., Mohammed, A.H., 1998. Environmental enrichment results in higher levels of nerve growth factor mRNA in the rat visual cortex and hippocampus. Behav. Brain Res. 93 (1-2), 83–90.
Vengeliene, V., Bilbao, A., Molander, A., Spanagel, R., 2008. Neuropharmacology of alcohol addiction. Br. J. Pharmacol. 154 (2), 299–315.
Welberg, L., Thrivikraman, K.V., Plotsky, P.M., 2006. Combined pre- and postnatal environmental enrichment programs the HPA axis differentially in male and female rats. Psychoneuroendocrinology 31 (5), 553–564.
Witt, E.D., 2007. Puberty, hormones, and sex differences in alcohol abuse and dependence. Neurotoxicol. Teratol. 29 (1), 81–95.
World Health Organization, & World Health Organization, 2014. Management of Substance Abuse Unit. 2014 World Health Organization, Global status report on alcohol and health.