

Research report

Long-term effects of repeated maternal separation and ethanol intake on HPA axis responsiveness in adult rats

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ABSTRACT

It has been shown that early life manipulations produce behavioral, neural, and hormonal effects. The long term consequences of repeated maternal separation (RMS) plus cold stress and ethanol intake were evaluated during adolescence and adult rats on hypothalamic-pituitary-adrenal (HPA) axis in male adult Wistar rats. RMS+ cold stress was applied from postnatal day (PD) 2 in which the pups were separated from their mothers and exposed to cold stress (4 °C) 1 h per day for 20 days; controls remained with their mothers. Then they were exposed to either voluntary ethanol (6%) or dextrose (1%) intake for 7 days: PD22–29 and PD59–66. Half of the animals were sacrificed, while the others were exposed to acute stress (AS) for 2 h and then they were killed. RMS+ cold stress: a) increased voluntary ethanol intake in adolescent and adult rats; b) reduced protein expression (Western measurements) in corticotropin-releasing hormone (CRH) in hypothalamus (Hyp) and mineralocorticoid receptor (MR) in hippocampus (Hic) while increased glucocorticoid receptor (GR) in Hic; c) decreased plasmatic levels of adrenocorticotrophic hormone (ACTH) and increased corticosterone (COR) levels in HPA axis, d) adult rats exposure a new AS incremented ACTH and COR levels. However, this modification did not alter the HPA axis capacity to respond to a new type of stressor. These results demonstrate the consequences of early life stress on the vulnerability of ethanol consumption and HPA axis responsiveness to a stressor in adult rats.

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1. Introduction

It has been shown that early life stress exposure produces severe and lasting consequences on growth and maturity (Monti et al., 2005; Crews et al., 2007; McCormick and Mathews, 2010). The hypothalamic-pituitary-adrenal (HPA) axis is the main physiological stress response system, a complex molecular pathway including feedback regulatory interactions between the hypothalamus (Hyp), the pituitary, and the adrenal glands (Manian et al., 2014). Its activation starts with the secretion of corticotropin-releasing

hormone (CRH) from the hypothalamic paraventricular nucleus (PVN), which later promotes the release of adrenocorticotrophic hormone (ACTH) from the pituitary which provokes, in turn, the release of glucocorticoids (GCs) from the adrenal cortex. The GCs binds to two types of receptors, mineralocorticoid receptor (MR) and glucocorticoid receptor (GR), thus regulating transcription and repression of genes that lead to adaptive changes and to the HPA axis negative feedback (Sawchenko, 1987; Jacobson and Sapolsky, 1991; Sapolsky et al., 2000; Charmandari et al., 2005; Deppermann et al., 2014). Both MR and GR are part of the nuclear hormone superfamily of ligand activated transcription factors (Datson et al., 2001; Huang et al., 2010). However, excessive or chronic stress may lead to persistent maladaptation of neuronal circuits and may promote the development of psychiatric disorders, such as mood or anxiety disorders (Smith and Vale, 2006; Wada and Breuner, 2008; Lupien et al., 2009; Popoli et al., 2012), that often emerge in adolescence (Kessler et al., 2001).

Alcoholism is a debilitating disorder for the individual and very costly for society (Rehm et al., 2009). The impact of stress on alcohol use and the risk of alcohol use disorders (AUDs) depends on

Abbreviations: ACTH, adrenocorticotrophic hormone; AS, acute stress; AUDs, alcohol use disorders; CNS, central nervous system; COR, corticosterone; CRH, corticotropin-releasing hormone; GC, glucocorticoid; GR, glucocorticoid receptor; Hic, hippocampus; HPA, Hypothalamic-pituitary-adrenal axis; Hyp, hypothalamus; MR, mineralocorticoid receptor; MS, maternal separation; PD, postnatal day; PVN, paraventricular nucleus; RMS, repeated maternal separation.

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various factors such as genetic predisposition, early stress, individual consumption patterns, environmental influences, gender, duration and severity of the stress experienced (Roman and Nylander, 2005; Anacker and Ryabinin, 2010; Keyes et al., 2012; Melchior et al., 2014). The positive reinforcing effects of alcohol are accepted as important motivating factors in alcohol-drinking behavior in the early stages of alcohol use and abuse. Conversely, alcohol's negative reinforcing effects may contribute to alcohol-drinking behavior at this stage in people who suffer psychiatric disorders and use alcohol to self-medicate from these disorders (Fidler et al., 2006; Gilpin and Koob, 2008).

Previous studies investigated the effects of maternal separation (MS) (Zimmerberg and Shartrand, 1993) and found that rat pups were maternally separated at cold temperature, during 6 h from PD2 to PD15 were not maintained the temperature of nest, grew slower, developmentally delayed and were less active in an open-field test. Hofer (1973) showed that 2-week-old rat pups were exhibited a reduced levels of locomotor and exploratory behavior on cold environmental conditions.

On the other hand, Acosta et al. (1993) investigated the function of GABAergic system in definite areas of rat brain after acute and chronic cold stress and showed that the most affected mechanism was the neuronal uptake of GABA which thus appear to be a sensitive marker involve specifically by cold stress. Also, they concluded that the effect GABAA receptors implicated in the stress-induced modifications on endogenous GABA levels and locomotor activity (Acosta and Rubio, 1994). This may be generalized effect of the low temperature since a reduction in the turnover of dopamine (Dunn and File, 1983) and noradrenaline (Stone, 1970) have also been induced by cold, but not by warm stress.

The fact that early-life environmental factors can interfere with development of HPA axis function is of importance (Heim et al., 2004; Ladd et al., 2005; Jahng, 2011; Manian et al., 2014) with regard to the use of MS as an experimental model to evaluate consequences of early-life impact on vulnerability to alcohol use disorders (AUD) (Keyes et al., 2012) and on addictive behavior (McEwen, 2006; Moffett et al., 2007; Cruz et al., 2008). There is a close inter-relationship between stress and ethanol consumption (Prendergast

and Little, 2007; Clarke et al., 2008; Miczek et al., 2008; Pautassi et al., 2010; Becker et al., 2011)

We hypothesized that the RMS+ cold stress increase ethanol intake modulating the HPA axis and can affect subsequent brain function during adulthood. The present study investigated whether RMS+ cold stress in combination with voluntary ethanol intake in early adolescence and adults rats induces long-term neurochemical and molecular alterations in adult animals. Hormone concentrations in plasma HPA axis, expression levels of CRH, GR, MR were measured by Western blot analysis in Hyp and Hic adult compared to normal animals. Finally study the susceptibility of the HPA activity induced by exposure to an AS in adulthood in plasma levels of ACTH and COR.

2. Results

Although the repeated treatment, no differences in the weight or other physical parameters (color, abundance of hair) were observed between RMS+ cold stress with control groups in adolescent and adult rats. The general condition of the rats was controlled every week. They showed an overall “good condition”, the skin observed was a good quality appearance. The posture of the experimental animals had no visible modifications.

2.1. Average body weight

Body weights of rats were not significantly different amongst experimental groups or throughout the course of the experiment. (Two-way ANOVA, $F_{3,25} = 0.3477$, $p = 0.7911$). In the first intake, the weight gained at day 29: C + Dex: 167.6 ± 12.89 ; C + Et: 169.1 ± 22.85 ; St + Dex: 175.0 ± 12.64 ; St + Et: 167.8 ± 20.22 g body rat ($n = 7$). In the second intake the weight gained at day 66: C + Dex: 389.7 ± 32.19 ; C + Et: 385.2 ± 30.15 ; St + Dex: 405.0 ± 32.64 ; St + Et: 390.7 ± 28.23 g body ($n = 7$). The total alcohol consumed during the course of the experiment was: Control group: 42.95 ± 10.65 ml ethanol/kg body rat ($t = 4.0357$ $df = 13$, $p = 0.0005$ vs Stressed group: 114.2 ± 28.80 ml ethanol/kg body

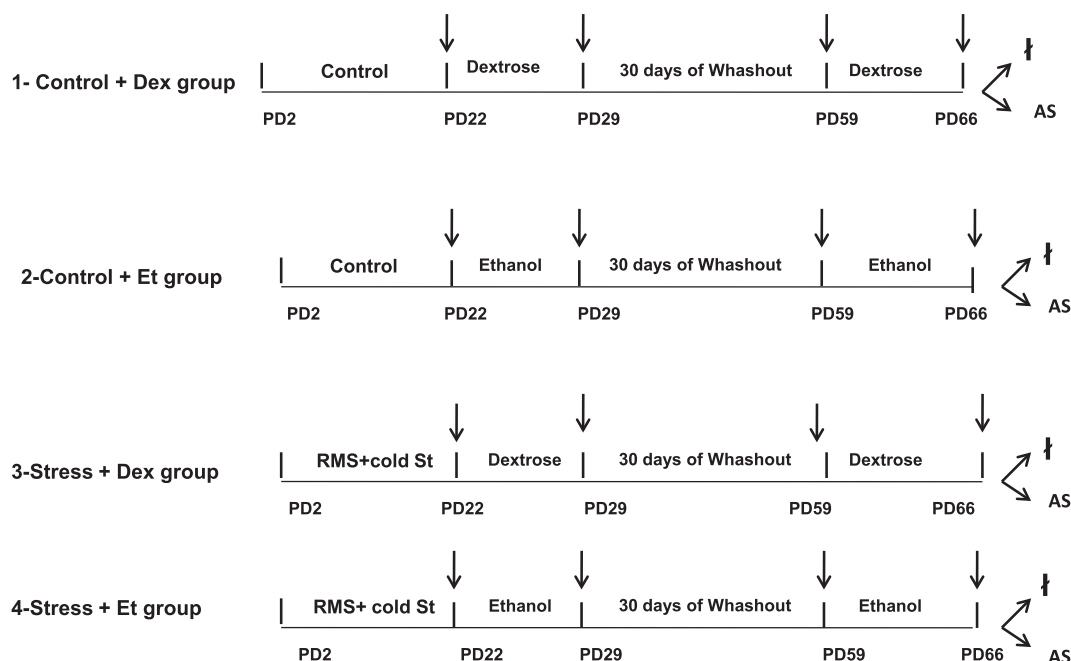


Fig. 1. Schematic representation of experimental design. RMS+ cold stress or not disturbance (Control) from postnatal day (PD) 2. First intake was from PD 22–29 (adolescent rats) and second intake was from PD59–66 (adult rats) given to drink either water and dextrose: 1% solution (g/100 ml) or water and ethanol: 6% solution (g/100 ml).

rat ($t = 3.964$ $df = 13$, $p = 0.0004$). While the amount of dextrose ingested during the experiment was: Control group: 15.80 ± 3.08 ml dextrose/kg body rat ($t = 4.60$ $df = 13$, $p = 0.0005$) vs Stressed group: 65.73 ± 13.83 ml dextrose/kg body rat ($t = 4.754$ $df = 13$, $p = 0.0004$).

Additionally, we demonstrated that RMS+ cold stress and alcohol intake did not reduce food intake, which would indicate absence of toxicity by ethanol consumption.

Fig. 1 provides an experimental model used to evaluate the long term consequences of RMS+ cold stress and ethanol intake.

2.2. Ethanol intake

Fig. 2A (PD29) adolescent rats shows g ethanol/kg rat body weight/day as well as plain water for 7 days. In first intake ethanol consumption (ml)/cage/day, RMS+ Et group increased by 79% of ethanol intake volume per cage of three rats ($t = 6.58$; $df = 12$; $^{**}p < 0.01$) compared with non-stressed controls. Fig. 2B (PD 66) adults rats shows as g ethanol/kg rat body weight/day as well as plain water for 7 days in second intake. Stressed adults animals consumed 292% more Et ($t = 2.804$ $df = 12$; $^{**}p < 0.01$) compared with control group.

2.3. Dextrose intake

Fig. 2C (PD29) young rat observes as g dextrose/kg rat body weight/day as well as plain water for 7 days. In first intake dextrose consumption (ml)/cage/day, Stressed + Dex group drinking 114% volume of dextrose per cage of three rats compared with non-stressed controls ($t = 6.5$; $df = 12$; $^{***}p < 0.001$). Fig. 2D (PD66) adult rats shows as g dextrose/kg rat body weight/day as well as plain water for 7 days in second intake. Stressed adult rats

increased 146% of dextrose intake volume per cage of three rats vs non-stressed controls ($t = 2.119$; $df = 12$; $^{**}p < 0.01$).

2.4. Western blot analysis of CRH, GR and MR in hypothalamus and hippocampus

We analyzed CRH, GR and MR proteins expression by western blot in Hyp and Hic. These blots reveal that the homogenates include CRH, GR and MR proteins (Figs. 3, 4A and B). Single bands appeared in homogenates at 25, 86 and 102 kDa for CRH, GR and MR, respectively.

Measurements were performed using a protein extract of hypothalamic and hippocampal homogenates. The levels of expression of CRH were reduced in the hypothalamus in both groups: St + Et vs Control ($^{*}p < 0.05$) and St + Et vs Control + Et ($^{*}p < 0.05$) (Fig. 3). Two-way ANOVA, Interaction StressXEthanol not significant (NS) ($F_{1,19} = 0.1432$). Main effects: Stress ($F_{1,19} = 4.954$, $p < 0.05$), Ethanol ($F_{1,19} = 0.215$, NS). Blots are representative of 3 different experiments. Data are quoted as the mean \pm SEM ($n = 5$ animals per group). (Two-way ANOVA, Interaction StressXEthanol not significant (NS) ($F_{1,19} = 0.1432$) Main effects: Stress ($F_{1,19} = 4.954$, $p < 0.05$), Ethanol ($F_{1,19} = 0.215$, NS). Blots are representative of 3 different experiments.

On the other hand, GR expression increased in the hippocampus in three groups; C + Et ($^{*}p < 0.05$), St ($^{*}p < 0.05$) and St + Et ($^{*}p < 0.05$) compared with control group (GR, Two-way ANOVA, Interaction StressXEthanol ($F_{1,19} = 4.101$; $p < 0.05$). Significant simple effects: test t: C vs St $p = 0.0002$; C vs St + Et $p = 0.0032$ C + Et vs S $p = 0.0111$) (Fig. 4A). However, the levels of expression of MR in the hippocampus was increased in stressed group versus control ($^{*}p < 0.05$). While, levels of MR was reduced in Stressed group consumed ethanol vs stress group ($^{*}p < 0.05$) (Fig. 4B). Data are quoted as the mean \pm SEM ($n = 5$ animals per group). (MR; Two-way

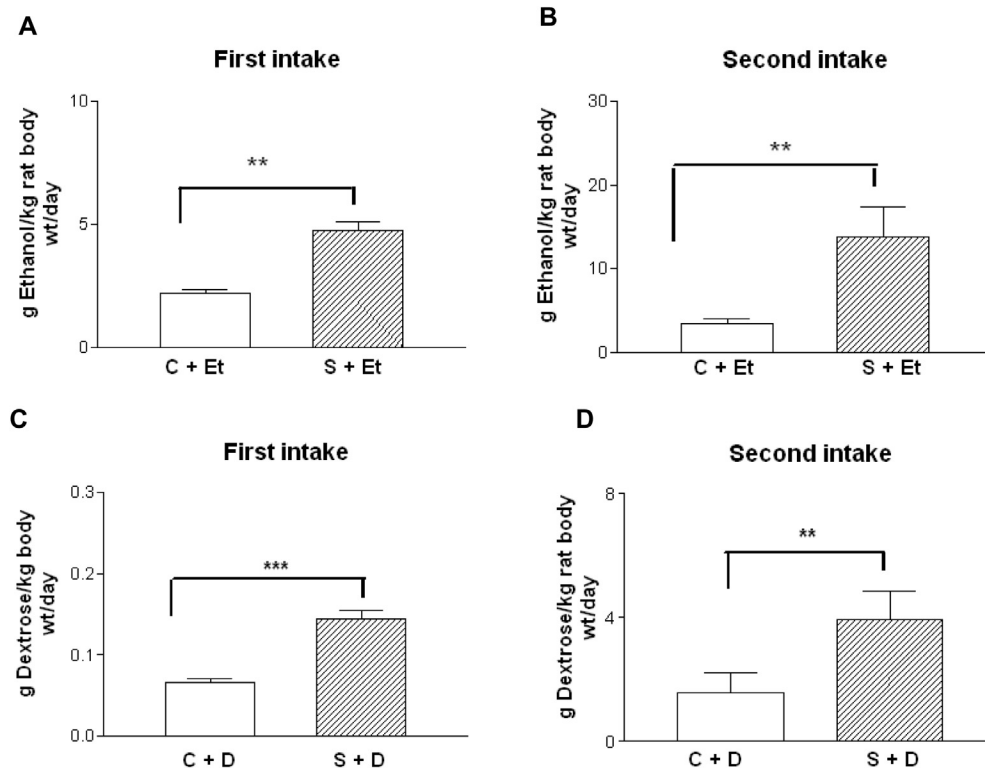


Fig. 2. A: First voluntary ethanol intake expressed in grams of ethanol (Et) consumed/kg body weight/day. B: Second voluntary ethanol intake expressed in grams of ethanol (Et) consumed/kg body weight/day. C: First voluntary dextrose intake expressed in grams of dextrose (D) consumed/kg body weight/day. D: Second voluntary dextrose intake expressed in grams of dextrose (D) consumed/kg body weight/day. Values are the mean \pm in 7 animals. Test de t, $^{**}p < 0.01$.

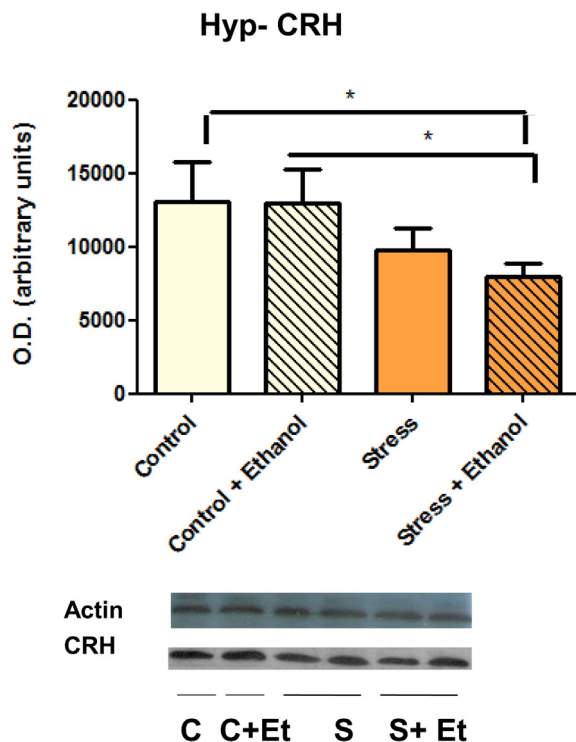


Fig. 3. Western blot analysis for hypothalamic CRH (MW ~ 25 kDa). Measurements were performed using a protein extract of hypothalamic homogenates. The expression level corresponds to the number of black pixels of each band counted using Image J. Actin was used as loading control. O.D: optical density (arbitrary units).

ANOVA, Interaction StressXEthanol ($F_{1,19} = 5.909$; $p < 0.05$). Significant simple effects: test t: C vs St $p = 0.0252$; St vs St + Et $p = 0.0059$). In all samples run Actin (42 kDa) was found and there were no significant differences among the different groups (data not shown). For each band, optic density (OD) values were relativized to OD values of Actin. The results are presented as the ratio between OD values of RMS and RMS+ Ethanol group versus OD values of Control group.

2.5. HPA function: regulation of ACTH and COR

To assess HPA neuroendocrine activity in the RMS group, we measured the plasmatic levels of ACTH and COR in adult rats. The study of basal ACTH plasma levels showed a significant reduction in all groups: C vs St ($*p < 0.05$); C + Et vs St ($*p < 0.05$); C + Et vs St + Et ($**p < 0.01$); C vs St + Et ($**p < 0.01$). Two-way ANOVA, Interaction StressXEthanol not significant (NS) ($F_{1,19} = 0.4498$). Main effects: Stress ($F_{1,19} = 30.88$, $p < 0.001$), Ethanol ($F_{1,19} = 0.4825$, NS; $n = 5$) (Fig. 5A).

The plasmatic levels of COR were notably lower in both group St compared to Control ($**p < 0.01$) and St vs Control + Et ($**p < 0.01$) while St + Et increased vs St ($**p < 0.001$). Also, we can observe that St + Et group reached value of C + Et group. (Two-way ANOVA, Interaction StressXEthanol ($F_{1,23} = 5.256$, $p = 0.0318$). Significant simple effects: test t: C vs S $p = 0.0027$, C + Et vs S $p = 0.0031$, S vs S + Et $p = 0.0007$; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $n = 6$) (Fig. 5B).

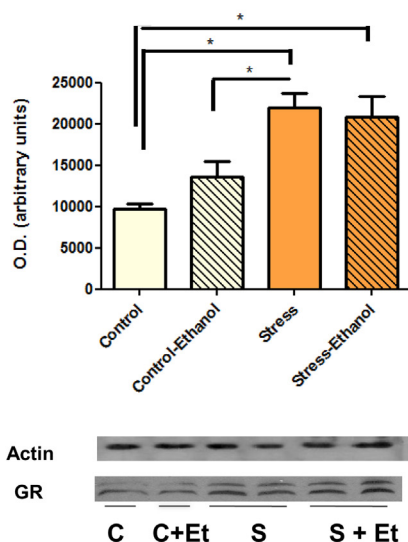
2.6. HPA axis activity in plasmatic levels of COR and ACTH in maternally separated rats following novel acute stress

To assess HPA axis function in the maternally separated rats plus cold stress, we measured plasma ACTH and COR concentrations following immobilization stress. The immobilization stress consisted of placing the animals in a cylindrical plastic tube (15 cm in length \times 3 cm in diameter) with holes for fresh air supply for 2 h in the morning. After acute stress, the rats were sacrificed by decapitation.

Following immobilization stress, there was a significant increase of ACTH concentrations (expresses as pg/ml) either in Control-AS versus Control group ($*p < 0.05$) or Stress-AS versus St group ($**p < 0.01$). We showed a reduction in St + Et-AS vs Control-AS group ($***p < 0.001$) (Fig. 6A) (Two-way ANOVA, stress + Ethanol X acute stress (AS). Interaction $F_{2,29} = 10.10$, $p = 0.0006$: Significant simple effects: test t: C vs C + AS $p = 0.0108$, S vs S + AS $p = 0.0032$, C + AS vs (S + Et) + AS $p = 0.0007$; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $n = 5$).

Plasma COR concentrations (expresses as $\mu\text{g/ml}$) were significantly increased in Control-AS vs Control group ($***p < 0.001$), St-AS vs St group ($**p < 0.01$). However St + Et-AS decreased vs

A- Hic-GR



B- Hic-MR

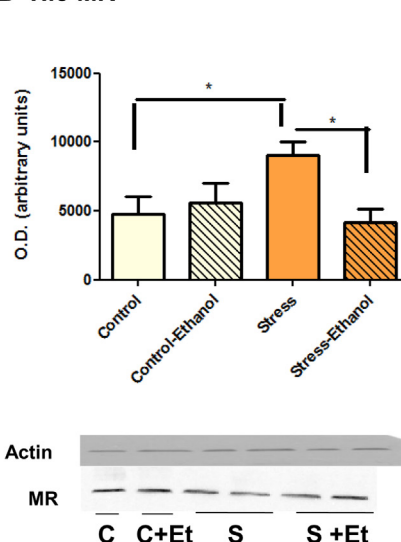


Fig. 4. Western blot analysis for A. GR (MW ~ 86 kDa) and B. MR (MW ~ 102 kDa) in homogenates of hippocampus. Band densities were quantified using Image J and are shown as a bar chart (OD: optical density). Data are quoted as the mean \pm SEM. The expression level corresponds to the number of black pixels of each band counted using Image J. Actin (MW ~ 42 kDa) was used as a loading control. Blots are representative of 3 different experiments.

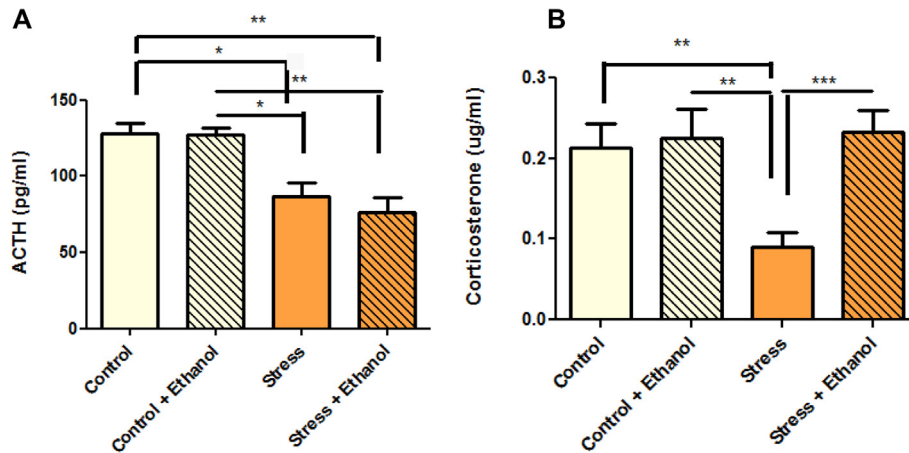


Fig. 5. HPA function axis A. Determination of plasma levels of ACTH by sequential immunometric assay, $n = 5$. B. Determination of plasma concentration of corticosterone by HPLC, $n = 6$.

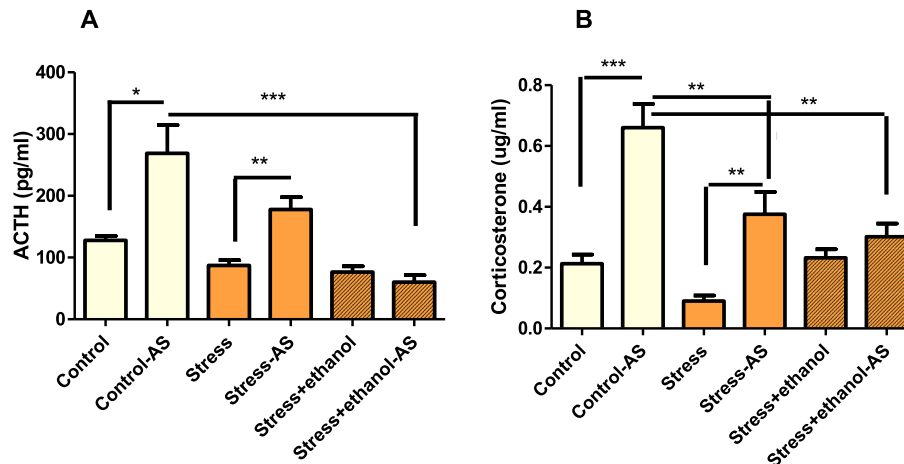


Fig. 6. RMS rats subjected to chronic stress, voluntary ethanol intake and PD66 were exposed to acute immobilization stress for 2 h. After the stress session were sacrificed by decapitation. A. Determination of plasma levels of ACTH by sequential immunometric assay. B. Measurement of plasma corticosterone levels by HPLC, $n = 6$.

Control-AS (** $p < 0.01$). Plasma levels of ACTH manifested the similar pattern of COR (Fig. 6B), but in COR concentrations, the stressed group than COR groups (St + Et and St + Et + AS). (Two-way ANOVA, Stress + Ethanol X Acute Stress: $F_{2,34} = 8.073$, $p = 0.0014$: Significant simple effects: test t : C vs C + AS $p = 0.0005$ S vs S + AS $p = 0.0007$, C + AS vs St + AS $p = 0.0043$ C + AS vs (St + Et) + AS $p = 0.0035$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 6$).

The levels of ACTH and COR remained unchanged in St + Et and St + Et + AS groups compare with control groups.

3. Discussion

The present results give evidences that RMS+ cold stress alter ethanol intake, activity and protein expression of CRH, MG, GR and the function of HPA axis. The main findings of this study are: 1. RMS+ cold stress increased voluntary ethanol intake in adolescent and adult rats. 2. RMS+ Et intake decreased ACTH levels in both St and St + Et groups. 3. Conversely, plasma COR levels notably lower in Stress group compared to Control group although St + Et group reached value of C + St group. 4. The levels of expression of both CRH and MR were decreased in Hyp and Hic if GR expression increased in Hic. 5. A new AS, applied after RMS produces a

significant increase in plasmatic levels of ACTH and COR in HPA axis.

These animals did not reduce food intake. Therefore the treatment applied did not affect average body weight gain healthy. It is well known that alcohol exposure/consumption during adolescence may induce important long-term effects on brain function.

Our experimental animal model exhibited an increment on both voluntary alcohol and dextrose intake in adolescent and adult male rats. We have established an experimental animal protocol using RMS+ cold stress as a model to study early environmental influence on adolescent and adult behavior.

Psychological stress activates the PVN primarily via limbic pathways whereas physical stress stimulates the PVN via brain-stem nuclei limbic brain circuitry (Emmert and Herman, 1999).

Our experimental protocol provides an appropriate model for further about the relationship between HPA axis and ethanol consumption. We demonstrated a decrease in basal hormone levels of HPA axis induced by RMS+ cold stress. Plasmatic levels of ACTH and COR decreased in RMS in Hic, whereas both CRH in Hyp and MR in Hic showed a significant decrease. These data would indicate a lower basal level due to an increase in the hippocampal GR protein expression. The GR certainly increased and known to be responsible for the regulation of the HPA axis negative feedback. A small alteration in the concentration of CRH generates greater variation in levels of corticosterone. During adolescence RMS

produces a profound long term effect on HPA axis sensitivity (Rivarola and Suárez, 2009).

Evidence suggests that adolescent rats may be more sensitive to stress than adults (Stone and Quartermain, 1997). Risk of stress-related disorders in adulthood, suggesting that alcohol may interfere with development of the stress system (Karanikas et al., 2013). In adulthood, the neuroendocrine response to stressors such as RMS plus cold stress is diminished after repeated or chronic exposure (Girotti et al., 2006). Our model not only shows a decrease of hormones involved in the repetition of the stimulus, but also that this effect is produced in the long-term. We found that GR levels increased whereas MR expression did not. It is known that at different doses, ethanol increases plasma COR levels in both adolescents and adults rodents (Willey et al., 2012); though little is known about the mechanisms involved.

Almeida et al. (2000) demonstrated that repeated stress leads to similar expression patterns in adult hippocampus (GR rises, MR decreases) and to a consequent reduction in the number of neurons. The same occurs in our RMS model. This is reflected in the large increase of GR, which would indicate a compensatory mechanism.

COR exerts its effects via GR and MR, which are in Hic and amygdala. GR and MR activation influence neuronal excitability and regulate the expression of genes that are involved in membrane maintenance properties (de Kloet et al., 2005), cell metabolism (Joels and Baram, 2009), neuronal plasticity (Mirescu and Gould, 2006; Alfarez et al., 2009) and synaptic transmission (Venero and Borrell, 1999; Lee et al., 2003).

When the animals were exposed to immobilization stress for 2 h (acute stress), we found an increment of COR and ACTH levels (Herman and Cullinan, 1997; Weinstock et al., 1998; Jankord and Herman, 2008).

This study the notion that early life stress exposure (maternal separation) during the first 2 weeks of birth may induce alterations in the feedback regulatory processes (synthesis and release of CRF, corticotropin, corticosterone and/or glucocorticoid receptor function).

The present results allow us to obtain an animal model of voluntary alcohol intake. We detected a basal stress-related hormones levels and a lower reactivity of the HPA axis. The reduced response to repeated stressors to protect the animal from high levels of GCs yet does not reduce the capacity of the HPA axis to respond to a new type stressor (Armario et al., 2004). We demonstrated that re-exposure to stressful-like stimuli during adulthood significantly increased the vulnerability to ACTH and COR in rats. It has also been determined that the alterations of the HPA activity of the molecular mechanisms underlying this effect. The HPA axis plays a vital role in adaptation of the organism to homeostatic.

Several animal models have been developed to make that study possible; not only to observe the effects of specific social circumstances on alcohol intake (McBride and Li, 1998) but also to evaluate the participation of neural or genetic factors (Anacker and Ryabinin, 2010).

4. Experimental procedures

4.1. Animals

Pregnant Wistar rats were obtained from School of Pharmacy and Biochemistry, University of Buenos Aires. All animals were maintained under standard laboratory conditions (12 h light–dark schedule; lights on from 08:00 to 20:00 h; temperature: $21 \pm 2^\circ\text{C}$) with food and water *ad libitum*. Pregnant dams were housed three per cage. We used 6 different litters in this study. The number of animals per experimental group was seven. The “unit” considered

for statistical analyses, was the total number of rats ($n = 7$). All animals procedures were performed according to Guide on Care and Use of Laboratory Animals, US National Research Council, 1996 and approved (RS CD 1456-16) by the Institutional Animal Ethics Committee for the Care and Use of Laboratory Animals (CICUAL) School of Pharmacy and Biochemistry, University of Buenos Aires. All efforts were made to minimize animal suffering.

4.2. RMS+ cold stress

In our experimental animal model, RMS psychologically stressor and cold stress (physical stress) (Odeon et al., 2013, 2015). Stressors can vary in the degree to which psychologically and physically stressful studied in animals (Herman and Cullinan, 1997; Grissom and Bhatnagar, 2009).

The RMS+ cold stress procedure was based on a standardized protocol (Ogawa et al., 1994; Odeon et al., 2015). Litters were randomly assigned to one of two rearing conditions: prolonged maternal separation + cold stress or non-maternal separation (control). MS litters, dams were removed from the home cage and placed in an adjacent cage. The whole litter was left in the home cage with the light on for 1 h during 20 days from postnatal (PD) 2 (Ladd et al., 1996; Odeon et al., 2013, 2015) (day of birth was designated PD1). No mortality was observed with this procedure. The separation cages were lined with 3 cm deep bedding material so that pups could further thermoregulate by huddling with littermates and burrowing into the bedding. Studies have shown that, under similar conditions, pup core temperature is maintained, indicating effective behavioral and physiological thermoregulation (Farrell and Alberts, 2007). After 1 h the mother was returned to the home cage. Separations were carried out between 10:00 h and 11:00 h. The control litters remained with the dams undisturbed until weaning age at PD22, except for routine cage cleaning. These groups are considered the most appropriate controls (Lehmann and Feldon, 2000). After weaning, rats were housed in same sex groups under standard conditions. All rats were handled daily by the same investigator to minimize stress reactions to manipulation. Unrelated rats were used to avoid confounding litter effects (each experimental group was made up of rats from at least three litters).

Stress treatment 20 days began at PD2. We used in this study 7 rats per experimental group. The experimental animal model describes drink either: water and dextrose, 1% solution (g/100 ml); or water and ethanol, 6% solution (g/100 ml) as well as plain water for 7 days. The animals had free access to drinking, using the two-bottle free choice method (Crabbe et al., 2011). With free access ethanol or dextrose, the animals entered a 30 days period of washout, in this instance we offered only water. At PD59, the animals were exposed to voluntary consumption with free access Et or dextrose for 7 more days. Half of the studied animals were sacrificed at PD66, while the other of them were exposed to a new AS (acute stress) for 2 h (immobilization stress) (see Fig. 1).

4.3. Hormonal measurement

In order to assess the function of HPA axis in this model (RMS+ cold stress and voluntary ethanol consumption), the following measurements were performed: 1. expression of CRH levels in Hyp and GR and MR in Hic by western blot; 2. plasma levels of ACTH by a sequential immunometric assay; 3. plasmatic levels of COR by HPLC.

4.3.1. Determination of ACTH

After decapitation (11:00 h), blood was collected from the trunk in a 15 ml conical tube containing EDTA. Plasma was separated by centrifugation (3000g, during 15 min, at 4°C) and frozen at -70°C .

Plasmatic ACTH concentration was determined by a solid-phase, two-site sequential chemiluminiscent immunometric assay, IMMULITE® 1000 system, SIEMENS Healthcare.

4.3.2. Determination of corticosterone

Following decapitation (11:00 h), blood was collected from the trunk in a 15 ml conical tube with heparin. Plasma was separated by centrifugation (3000g, during 15 min, at 4 °C) and frozen at –70 °C. Plasmatic corticosterone (COR) concentration was determined by high performance liquid chromatography (HPLC) (Retana-Márquez et al., 2003).

Corticosterone was extracted from 200 µl of plasma by adding 4 ml of diethyl ether-dichloromethane (60:40). We added each tube 100 µl of internal standard (Phenytoin 1 mg/ml diluted in methanol). The organic phase was evaporated at 37 °C under nitrogen. 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 corticosterone, 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 corticosterone was calculated and used to determine corticosterone concentration in the samples.

4.3.3. Western blotting

This analysis was used to measure levels of CRH, GR and MR protein expression in Hyp and Hic homogenates. Both areas were dissected on a Petri dish at 0 °C, according to Glowinski and Iversen (1966) with glass-PTFE homogenizer in lysis buffer (Tris Base 50 mM; NaCl 150 mM; EDTA 2 mM; sodium dodecyl sulfate (SDS) 0.05%; Triton-X100 1%; phenylmethanesulfonyl fluoride PMSF 100 g/ml; Leupeptin 1 µg/ml). Protein concentration in the samples was analyzed by Lowry et al. (1951) technique. Aliquots (total proteins 20 µg) were separated on 8% Sodium Dodecyl Sulfate-Polyacrylamide Gel (SDS-PAGE) at 130 V and transferred to a nitrocellulose membrane using a blot system (Transblot, BioRad). The hypothalamus and hippocampus from each rat was run separately. The membranes were incubated in blocking buffer (1× tris-buffered saline (TBS) and 5% non-fat dry milk) for 1 h at Room Temperature (RT) and afterwards incubated overnight at 4 °C with one of the following specific primary antibodies: α-CRF (1:500, Santa Cruz Biotechnology, INC), anti-GR (1:1000, Millipore Chemicon) or anti-MR (1:200, Santa Cruz Biotechnology, INC) antibodies, and anti-actin antibodies (1:1000, Sigma Chemical Co).

They were then washed three times for 10 min in 1× TBS-T 0.1% at RT with membranes were incubated with secondary antibody (1:2000) for 1 h at RT. Immunodetection of bands was accomplished using the Pierce Super Signal Chemiluminescence Kit (Kit-ECL, Western blotting substrate from Pierce, Thermo Scientific, IL, USA) and further exposure on X-ray film. For quantification of band intensity, blots were scanned and analyzed using Image J PC software analysis. The expression level corresponds to the number of black pixels of each band. The results were expressed as Optical Density (OD) in arbitrary units. Actin was used as a loading control. The amount of protein was analyzed as a ratio between the protein and actin.

4.4. Acute stress

At PD66 the animals were exposed to stress by immobilization for 2 h, which consisted of taking rats from the home cage and placing them in adjustable length (15.52 cm long and 6.3 cm diameter). Plexiglass tubes with air holes in the front, top and back. This

stressor is considered to be primarily psychological because it does not produce pain or direct physical insult (Herman and Cullinan, 1997). Immobilization stress took place in a separate room adjacent to the home cage room. Immediately after AS exposure, plasma stress-related hormone levels were evaluated: ACTH and COR.

4.5. Sources of materials

Corticosterone (C2505) from Sigma Chemical Co. St. Louis, MO, USA, Kit -ECL Western blotting substrate from Pierce, Thermo Scientific IL-USA. MR (1:200) (sc-11412 from Santa Cruz Biotechnology, INC, CA, USA), α-GR (1:1000) (AB9918, Millipore Chemicon, USA), α-CRF (1:500) (sc-10718 de Santa Cruz Biotechnology, INC, CA, USA). Actin, rabbit (A2066) from Sigma Chemical Co. St. Louis, MO, USA. Secondary antibodies: α-Rabbit IG (A6154) from Sigma Chemical Co., St. Louis, MO, USA.

4.6. Statistical analysis

All data are as mean ± standard error of the mean (S.E.M.). In the experiments of RMS and cold stress, there are two factors, alcohol and stress, so the statistical significance was assessed with two-way analysis of variance (ANOVA). When the interaction was significant, simple effects were performed when there was no interaction, the post hoc Bonferroni test was used (Grafen and Hails, 2002). Alcohol intake data (Fig. 2) have only two so result was analyzed by Student's *t*-test. Significance was set at *p* < 0.05.

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Approval of animal experiments

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.

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