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Low doses of prenatal ethanol exposure and maternal separation alter HPA axis function and ethanol consumption in adult male rats

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Abstract

Awkward mother behaviors during pregnancy and postnatal unfavorable experiences may be dramatically associated to an increased risk in developing psychiatric disorders as well as a vulnerability to alcohol addiction in adulthood. Here, we examined the effects of ethanol exposure during late pregnancy and postnatal maternal separation (MS) combination on HPA responsiveness, anxiety behavior and preference for alcohol consumption in adult male rats. Animals exposed to both conditions revealed a decrease of allopregnanolone (AP) blood level accompanied with an increased anxiety behavior. In addition, basal blood levels of corticosterone (CTS) were strongly decreased in all experimental groups while foot-shock-induced CTS increase was more pronounced in MS animals. Finally, evaluating the EtOH drinking behavior, MS animals revealed a remarkable EtOH preference even at low doses (0.1-1%). Altogether, these data suggest that unfavourable conditions, alone or in combination, may alter anxiety–like states as well as a modified behavior toward alcohol consumption.

Keywords: HPA axis, Stress, Maternal separation, Prenatal ethanol, Anxiety, Ethanol intake

1. Introduction

In the last two decades, research has been directed to further elucidate the several links between adverse changes occurring during pregnancy and early postnatal stress that, in turn, may contribute to developing chronic diseases such as alcohol abuse later in life (Barker, 1995, 1998). Adverse changes during the prenatal period, childhood and adolescence, affect several mechanisms, including epigenetic factors that may alter the brain function during the lifespan (Andersen and Teicher 2009; Cirulli et al. 2003; Crews 2008; McCrory et al. 2011). These findings suggest that several environmental conditions

early in life, may be pivotal determinants to enhance or reduce the individual vulnerability or resilience to psychopathology as well as substance dependence.

Prenatal ethanol (EtOH) exposure is widely accepted to be a risk factor in child development mostly when associated with other prenatal or environmental risk factors such as stress, poor nutrition and diseases affecting mother's health (Udagawa and Hino, 2016).

The crucial determinant in EtOH-related effects during gestational period concerns mainly the dose used as well as the timing of exposure. The consequent effects of fetal alcohol exposure are collectively referred to a fetal alcohol spectrum disorders (FADS); among these, fetal alcohol syndrome (FAS) is the most severe outcome that results in body as well as mental impairments (Riley et al., 2011). However, epidemiological studies reveal that many women (5-20% in the USA) continue to drink low amounts of EtOH during pregnancy, that may correlate with an estimate very large number of children that are also affected by subtle FASD symptoms (Centers for Disease Control and Prevention, 2012). Thus, an important data is related to how often and when mother consumes EtOH during gestation (Kelly et al., 2000; Sood et al., 2001). Clinical and preclinical studies also suggest a strong correlation between EtOH consumption during pregnancy and the incidence of anxiety-related disorders or anxiety-like phenotype in offspring (Dursun at al., 2006; Hellemans et al., 2008, 2010; Cullen et al., 2013).

Anxiety is often accompanied by a maladaptive stress response, such as increased sensitivity to environmental stimuli, vulnerability to psychopathology and related changes to the hypothalamic-pituitary-adrenal (HPA) axis function, (Serra et al., 2005; Hellemans et al., 2008, 2010; Raineki et al., 2014; Lan et al., 2015).

The endocrine response to stress, via activation of the HPA, leads to a rapid secretion of corticotropin-releasing hormone (CRH), the main secretagogue of adrenocorticotrophic hormone (ACTH) that, in turn, leads to the synthesis and secretion of corticosteroids from

the adrenal cortex with the subsequent activation of glucocorticoid receptors in several brain areas (Biggio et al., 2014a).

The neuroactive steroid 3alpha-hydroxy-5alpha-pregnan-20-one (allopregnanolone, AP), is known to play an important role in stress- and anxiety-related behaviours, modulating the emotional state as a response to acute or chronic stress (Barbaccia et al., 2001). Accordingly, AP, like benzodiazepines (Imaki et al., 1995), exerts a potent inhibitory action on HPA axis activity attenuating the increase of plasma ACTH and corticosterone (CTS) elicited by stress (Bali and Jaggi, 2014; Biggio et al., 2004a; Rupprecht and Holsboer, 1999; Patchev et al. 1996).

Chronic stress, such as maternal separation or social isolation, during early life or adolescence, respectively, are often associated with alterations in the basal amount of both AP and CTS as well as in the abnormal responses to acute stress or EtOH drinking behaviour in adults (Serra et al., 2000; Odeon et al., 2017; Pisu et al., 2016). As reported for prenatal alcohol exposure, change in environmental factors during early life, such as traumatic events (Koss et al., 2003; Wilsnack et al., 1997), may also have serious impact on the pattern of ethanol consumption later in life.

It has been widely reported that, in rats, a stressful event occurred during the first weeks of life, such as separation from dams, is able to affect the pattern of EtOH consumption in adulthood (Gustafsson and Nylander, 2006; Moffett et al., 2007; Roman and Nylander, 2005; Neisewander et al. 2012; Becker et al. 2011). However, the mechanisms by which these stress-related effects may influence the EtOH consumption remain still unclear.

Based on these evidences, the major aim of this work was to investigate whether low amount of ethanol consumption during late pregnancy followed by newborn exposure to a stressful condition induced by daily MS, may alter the offspring sensitivity to acute stress as well as their vulnerability to ethanol consumption in adulthood.

The increasing knowledge of the molecular changes that may occur in adult individuals exposed to those stressful stimuli during perinatal life may help to develop new potential pharmacological strategies to counteract states of alcohol consumption and psychopathology related to early life stress events.

2. Material and methods

2.1. Animals.

The study was performed using males Sprague-Dawley rats (Charles River, Calco, Italy). All animals were housed under an artificial 12h light-dark cycle (8:00 a.m-8:00 p.m.) at controlled temperature (23 ± 2 °C) and humidity (65%). Food and water were available *ad libitum*. Mating occurred in individual cages, using one male and one female of 150 and 120 days of age, respectively, per cage. Coupling was verified by the presence of sperm cap (plug) and this day was considered as gestational day 1 (GD 1). From GD 12 pregnant rats were handled once a day for four days, until GD 16. Handling consists in introducing the intragastric cannula (used for gavage) connected to a 2.5 cc syringe containing a vehicle solution in order to get animals familiar to the treatment. Thus, from GD17 up to GD20, a group of dams (EtOH) was treated intragastrically with a 1g/Kg ethanol (20%) solution (Pueta et al., 2011), diluted in low fat milk; the control vehicle group (VEH) was treated with a solution of low fat milk and water only. On GD 20, each pregnant rat was individually housed in a single cage (40 cm x 60 cm x 20 cm) waiting for delivery. Starting from postnatal day 2 (PND 2), all litters born within 24h period came from dams belonging to the same experimental group were equally distributed in male and female (5-6 for each sex). The whole experimental procedure was in accordance with the Department of Health (685/2015-PR) and approved by the local ethic committee.

2.2. Maternal separation protocol

Pups used in our experiments were randomly assigned to four experimental groups: offspring whose dam was treated with vehicle solution not subjected to maternal separation (VEH-NMS), offspring whose dam was treated with vehicle solution subjected to maternal separation (VEH-MS), offspring whose dam was treated with ethanol solution not subjected to maternal separation (EtOH-NMS) and offspring whose dam was treated with ethanol solution subjected to maternal separation (EtOH-MS). According to Plotsky and Meaney (Plotsky and Meaney, 1993), the separation procedure consists on daily separation of the litter from the dam for 3h (10:30 a.m.–1:30 p.m.) from postnatal day 3 until day 15. During these 3h whole litters (male and female) were transferred to a different room, to prevent vocal communication with the dam. Mothers were left undisturbed in their home cage until litter reunion. Pups were placed in a cage with soft cotton, and the room temperature was maintained at 32–33 °C, consistent with normal nest temperature. Animals not subjected to MS were just handled twice a day (at the same time during MS and their reunion) and left in the home cage with their dam. After PND 15, pups were left undisturbed to a normal housing condition until weaning (PND 21), when males were randomly housed in groups of five per cage and maintaining under standard laboratory conditions until PND 60, when the experiments started.

2.3. Measurement of hormone levels

Animals were sacrificed between 10:00 a.m. and 12:00 p.m. with a guillotine and blood was rapidly collected into K3-EDTA tubes, centrifuged at 900 x g for 10 minutes at 4°C and stored at -80°C until use. Levels AP and CTS were assayed in the same rats. AP was extracted from plasma as previously described (Serra et al., 2000). The combined organic phases were dried under vacuum. The recovery (70-80%) of AP through the extraction procedure was monitored by addition of a trace amount (6000 to 8000 cmp; 20-80 Ci/mmol) of [3H] AP (Perkin Elmer Italia, Monza) to the plasma samples. AP levels were

quantified by radioimmunoassay with a specific antibody generated in sheep as previously described (Purdy et al., 1991; Serra et al., 2000). The enzyme immunoassay was used to quantify plasma levels of CTS. ELISA was performed according to the manufacturer's instruction (Corticosterone ELISA, IBL International, Germany) using a 96-well plate that was pre-coated with a polyclonal antibody against an antigenic site on the CTS molecule. The kit also provided a seven-point standard curve using two-fold serial dilutions. Each sample was run in duplicate.

2.4. Foot-shock stress

The foot-shock (FS) stress consists in a series of electrical impulses delivered in individual boxes with floor made of 18 brass rods positioned 2 cm apart. Shocks (0.2 mA for 500ms) were delivered every second over a period of 5 min. All experimental groups had a paired group of non-shocked rats for comparison. Animals were killed 25 min after the end of the foot-shock exposure for plasma AP and CTS detection.

2.5. Elevated Plus Maze

Elevated plus maze (EPM) was used to test anxiety-like behavior of animals. The apparatus consists in a black polyvinyl chloride cross maze that contained two open arms and two closed arms (12 x 60 x 3 cm) elevated 50 cm above the floor. The arms were connected by a central square (12 x 12 x 3 cm). The maze was located in a quiet and dimly light room. Each rat was tested only once. The animal was placed on the central square facing towards to one of open arms and was allowed to freely explore the maze for 5 min. The number of entries into and time spent in the open arms were recorded considering the presence of all four feet of the animal as entrie. The maze was cleaned after each trial with a 20% methanol solution

2.6. Ascending voluntary ethanol consumption paradigm

According to preview report (Goodwing and Amit, 2000; Martinetti et al., 2006), experimental procedure was conducted in small cages (42.5 x 26.6 x 15.5 cm^2) in which the animals were put for the 1h daily drinking session occurring in the dark period of the inverted cycle. Before starting the experiment, rats were given free access to tap water. After a 2-weeks of habituation period in a reverse dark/light cycle, EtOH-access protocol with a free-choice paradigm (Wise, 1973) begins and was conducted every day starting at PND 30 or PND 90. EtOH solutions (v/v) were prepared fresh daily with 99.8% (v/v) EtOH (Fluka. Sigma-Aldrich) and tap water. The daily 2h drinking session started during dark cycle at 10 a.m. when the animals had free access to two different bottles (switched every day to avoid position preference); containing tap water and EtOH solution, respectively. For the ascending procedure, the daily concentration of the EtOH solution followed an increasing trend during the treatment period (55 days) according to the following schedule: 0,01%, 0,02% to 0,1% by 0,02%, 0,1% to 1% by 0,1%, 1% to 5% by 0,2%, 5% to 10% by 0,5% and 10% to 20% by 1% (Goodwing and Amit, 2000; Martinetti et al., 2006). The weight of the bottles was daily recorded before and after each drinking session.

2.7. Statistical analysis

Quantitative data are presented as means ± SEM and were compared by analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test (Statistica 6.0, StatSoft Inc.) or t-Test. A $p< 0.05$ value was considered statistically significant.

3. Results

3.1. Effects of prenatal EtOH exposure on plasma AP and CTS levels in male rats subjected to maternal separation.

We first evaluated the effect of prenatal EtOH exposure, MS as well as their association, on basal plasma levels of AP and CTS. When estimated alone in adult rats, neither prenatal EtOH exposure nor MS were able to induce changes in plasma levels of AP when compared with control animals (VEH-NMS, VEH-NMS) (Fig 1A). The combination of both events induced a significant ($p<0.05$) decrease in plasma levels of AP when compared to VEH-MS and EtOH-NMS groups (Fig 1A). ANOVA revealed a significant effect of alcohol prenatal treatment [$F(1, 28) = 10.0756$, $p = 0,0036$] and MS [$F(1, 28) = 4.7862$, $p = 0.0371$] and no significant interaction between factors [$F(1, 28) = 2.5098$, $p = 0.1243$]. In contrast to what observed for AP levels, prenatal EtOH exposure and MS themselves induced a consistent ($p<0.05$) decrease in plasma CTS content compared to control group (VEH-NMS) (Fig. 1B). The association of both treatments failed to further reduce the plasma levels of CTS. ANOVA showed a significant effect of ethanol prenatal treatment [$F(1, 29) = 4.8394$, $p = 0,03593$] and MS [$F(1, 29) = 9.0664$, $p = 0.0053$, but no significant interaction between factors [$F(1, 29) = 0.9550$, $p = 0. 3365$].

3.2. Effects of prenatal EtOH exposure and maternal separation on HPA responsiveness to acute stress.

Different unfavorable conditions affect the HPA axis responsiveness towards an experimentally-induced acute stress (Biggio et al., 2014; Kosten et al., 2006; Pisu et al., 2013). Here, we evaluated whether prenatal EtOH exposure, MS or their association may alter the FS stress-induced changes in plasma levels of AP and CTS.

FS stress increased significantly ($p < 0.01$) the AP plasma levels in all experimental groups, (Fig. 2A). The increase in AP induced by FS is much greater in EtOH-MS group compared to EtOH-NMS as well as VEH-MS ($p < 0.001$). ANOVA revealed a significant effect of EtOH prenatal treatment [$F(1, 27) = 22.4608$, $p=0,0000$] and MS [$F(1, 27) = 6.9229$, $p = 0,0138$], with a significant interaction between factors [$F(1, 27) = 18.5052$, $p = 0,0001$] (Fig. 2A). FS increased CTS blood levels in all experimental groups compared to not-

shocked rats ($p < 0.001$). This effect was more consistent in animal exposed to both prenatal treatment with EtOH and to subsequent MS, compared to that observed in animals not subjected to both stress ($p < 0.001$) (Fig 2B). ANOVA revealed a significant effect of alcohol prenatal treatment [$F(1, 32) = 41.1047$, $p = 0.000$] and MS [$F(1, 32) = 98.0813$, $p = 0.000$] with no significant interaction between factors [$F(1, 32) = 1.6435$, $p = 0.2090$]. Interestingly, the a significant increase ($p < 0.05$, t-Test) of CTS observed in VEH-MS was more pronounced (315.4 ± 44.13 %) respect to what founded in EtOH-MS animals (142.3 ± 18.14 %) (Fig. 2B).

3.3. The combination of prenatal EtOH exposure and maternal separation impaired anxiety state in male rats.

Given that stress-related changes in steroid levels are often parallel to modifications in the emotional state, we evaluated, using the EPM test, whether prenatal EtOH exposure, MS or their combination may impair the anxiety levels in rats. Only the combination of prenatal EtOH exposure and subsequent MS significantly altered the emotional state of adult animals. As shown in Fig. 3A, EtOH-MS rats showed a significant decrease in the number of entries into the open arms respect to close arms when compared with EtOH-NMS group ($p < 0.05$). ANOVA revealed no significant effect of alcohol prenatal treatment [$F(1,66) = 0,4829$, $p = 0.4895$], a significant effect of maternal separation [$F(1, 66) = 5.8061$, $p = 0.0187$], and no significant interaction between factors [$F(1, 66) = 3,6306$, $p = 0.061$]. However, arms total entries did not change in all experimental groups (Fig. 3B).

3.4. Effects of prenatal EtOH exposure and MS on EtOH drinking behavior.

In order to test the vulnerability to EtOH consumption in adulthood, the ascending EtOH consumption paradigm proposed by Goodwin and Amit, 2000 and Martinetti et al., 2006 has been performed in all experimental groups. As shown in Fig. 4A there is not

significant difference between groups in terms of volume of EtOH consumption during the whole treatment. Furthermore, accordingly with previous data (Martinetti et al., 2006), a slight decrease on consumption has been observed with increasing ethanol doses. With a more detailed comparison, we thus averaged the values for EtOH intake and preference in 3 different concentration ranges: 0.1-1%, 3-5%, and 10-15%. Interestingly, only VEH-MS animals revealed a greater consumption (ml/Kg) of EtOH even at the lower range, when compared with other experimental groups. In the range 0.1-1% (Fig. 4B), ANOVA revealed no significant effect of alcohol prenatal treatment [$F(1, 396) = 3.1636$, $p = 0,0760$] and maternal separation [$F(1, 396) = 0.4259$, $p=0.5143$] and significant interaction between factors [$F(1, 396) = 8.9268$, $p = 0.0029$]. Moreover, EtOH-MS group showed a significant less consumption of EtOH at the range 3-5%, ANOVA revealed a no significant effect of alcohol prenatal treatment [$F(1, 436) = 2.278$, $p = 0,1319$], significant effect of maternal separation [$F(1, 436) = 11.443$, $p = 0.0007$] and significant interaction between factors [$F(1, 436) = 12.513$, $p = 0.0004$]. No difference between groups was evident at the highest range (Fig. 4A-B). We then calculated the g/kg of EtOH consumed (Fig. 4C-D). VEH-MS animals revealed a greater amount of EtOH assumed only at lowest range (Fig. 4D). For the range 0.1-1% ANOVA revealed no significant effect of alcohol prenatal treatment [$F(1, 375) = 0.9719$, $p = 0,3248$] and maternal separation [$F(1, 375) = 0.7767$, $p = 0.3787$] and significant interaction between factors [$F(1, 375) = 7.7455$, $p = 0.0056$]. In the 3-5% range EtOH-MS (Fig. 4D) showed a less consumption of EtOH compared to other groups. ANOVA revealed a no significant effect of alcohol prenatal treatment [$F(1, 435) = 1.631$, $p = 0,2022$], significant effect of maternal separation [$F(1, 435) = 12.103$, $p = 0.0005$] and significant interaction between factors [$F(1, 435) = 10.424$, $p = 0.0013$]. Even in this case, no difference between groups was evident at the highest range (Fig. 4C-D). In order to better clarify the ethanol consumption results, we calculated the preference as percentage of EtOH or water volume consumed, during the drinking session, by each rat, compared to the total fluid intake (Fig.

5). In line with the latest results, only VEH-MS showed a preference at lowest range of dose (0.1-1%) of EtOH ($p < 0.05$ vs. 50%) compared to the other experimental groups (Fig. 5A-B). ANOVA revealed a significant effect of alcohol prenatal treatment [$F(1, 396) = 5.080$, $p = 0,0247$], MS [$F(1, 396) = 19.270$, $p = 0.000$] and significant interaction between factors [$F(1, 396) = 5.124$, $p = 0.0241$]. A slight decrease in EtOH preference has been reported in EtOH-MS group when compared with other experimental groups at middle range while the same group showed a faint increase of preference was reported at the highest range where almost no preference for EtOH consumption has been reported for other experimental groups (Fig. 5B).

Finally, we also evaluated the same parameters in offspring that started to drink EtOH during youth (PND 30). While the average of EtOH consumed by adults was between 5-6 ml/kg, adolescents consumed a greater averaged amount of EtOH (14 ml/kg). Adolescents drastically reduced their consumption at high EtOH concentrations (Fig. 6A-B). EtOH-MS animals showed a significant reduction in the amount of EtOH consumed both in term of ml/kg (Fig. 6A-B) or gr/kg (Fig. 6C-D) when tested at lowest range of doses. For ml/Kg, in the range 0.1-1%, ANOVA revealed a significant effect of alcohol prenatal treatment [$F(1, 388) = 5.225$, $p = 0,0228$], maternal separation [$F(1, 388) = 5.044$ $p=0.0252$] and significant interaction between factors [$F(1, 388) = 4.594$, $p= 0.0327$]. For gr/Kg, in the range 0.1-1%, ANOVA revealed a significant effect of alcohol prenatal treatment [$F(1, 373) = 6.1212$, $p = 0,0138$], no significant effect of maternal separation [$F(1, 373) = 3.3058$ $p = 0.0698$] and no significant interaction between factors [$F(1, 373) = 1.3859$, $p= 0.2398$]. Both groups, VEH-MS and EtOH-NMS, had a significant reduction in the amount of EtOH consumption at the 3-5% range while in the group of EtOH-MS we found a dramatic reduction of EtOH consumption when compared with their controls, VEH-MS and EtOH-NMS (Fig. 6A-D). For ml/Kg, in the range 3-5%, ANOVA revealed a significant effect of alcohol prenatal treatment [$F(1, 410) = 45.534$, $p = 0,0000$], maternal separation [$F(1, 410) = 64.366$ $p = 0.0000$] and

no significant interaction between factors [F(1, 410) = 0.714, p = 0.3986]. For gr/Kg, in the range 3-5%, ANOVA revealed a significant effect of alcohol prenatal treatment [F(1, 410) = 45.969, p = 0,0000], maternal separation [F(1, 410) = 65.629 p = 0.0000] and no significant interaction between factors [F(1, 410) = 0.910, p = 0.3424]. Therefore, we evaluate the preference for EtOH consumption also during youth. As reported by scatter plots (Fig. 7A) and bar graph (Fig 7B), the preference was slightly evident only in VEH-NMS and VEH-MS groups at the 0.1-1% range of EtOH concentration (P<0.05 vs 50%). Interestingly, all groups show a dislike for EtOH consumption at the highest range (10-15%) tested. In the range 3-5%, ANOVA revealed a significant effect of alcohol prenatal treatment [F(1, 410) = 77.476, p = 0,0000], maternal separation [F(1, 410) = 22.730, p = 0.0000] and no significant interaction between factors [F(1, 410) = 3.826, p = 0.0511].

4. Discussion

Here we demonstrate that two different discomfort conditions, such as prenatal EtOH exposure and MS, produced profound and long lasting changes in the neuroendocrine response to acute stress as well as on the emotional state and vulnerability to EtOH consumption when male offspring become adults.

Our results strengthen the previews reports describing the long-lasting negative effects of perinatal stress events on many biochemical, functional and behavioral parameters that reflect the activity of HPA axis (Girardi et al., 2014, Maccari and Morley-Fletcher, 2007) associated to behavior towards EtOH consumption (Odeon et al., 2017; Romano-López et al., 2015; Roman and Nylander, 2005). Accordingly, acute, as well as chronic stress, may produce a fast and long lasting responsiveness of the HPA axis (Herman and Cullinan, 1997; Serra et al., 2005; Biggio et al., 2014a, Manian et al., 2014). These conditions may lead to persistent functional changes in neuronal circuits in different brain areas promoting an increase in the potential vulnerability to develop several behavioral

disorders such as anxiety states, depression, and propensity to EtOH drinking (Pisu et al., 2011; Barbaccia et al., 2001; Biggio et al., 2007; Lupien et al., 2009; Butler et al., 2016).

Many authors have shown that during early life the function of HPA axis is modified by several environmental and experimental conditions (Heim et al., 2004, Heim and Binder, 2012, Ladd et al., 2005, Jahng, 2011, Manian et al., 2014).

Evaluating the levels of endogenous steroids in response to such kind of stress, here, we show that both prenatal exposure to moderate doses of EtOH, administered during the late pregnancy or MS during the first three weeks of birth, did not alter the basal AP plasma content in adult rats. Conversely, as stated before, the combination of both treatments led to a significant reduction in AP. In addition, foot shock stress increased by a greater extent the plasma content of AP in adult rats that received the combination of stressful stimuli (EtOH prenatal exposure and MS) respect to animals receiving only one single stress. These findings suggest that MS-EtOH rats developed a greater sensitivity to stress when become adults. Our data are in agreement with those reported by Zimmerberg and Brown (1998) who have shown that in Long-Evans rats prenatal alcohol exposure combined with early MS (during the first week of life) were associated with an increase in the endogenous levels of AP in the prefrontal cortex and hippocampus of adult offspring in response to an acute stress. Interestingly, this effect was much pronounced in females suggesting that these changes are not probably dependent by the animal strain used but that gender may be crucial to increase the sensitivity to such stress.

The decrease in AP basal level and the greater sensitivity of HPA axis to stress, in EtOH-MS group, is consistent with the increase in anxiety behavior, demonstrated by a significant reduction, shown by these animals, in the open arms entries in the Elevated Plus Maze test. Furthermore, the lack of difference in the total entries between all experimental groups suggests that there are no significant changes in locomotion and exploratory activity. The finding that such early stressful treatment results later in negative emotional states is in

perfect agreement with our recent behavioral finding showing that the intake of EtOH during pregnancy and MS significantly reduce the quality of maternal care in these animals (unpublished data). In addition profound change in maternal care behavior (reduction in total nursing but not licking and grooming behavior) as a consequence of EtOH consumption during pregnancy was also recently reported by other authors (Workmann et al., 2015). Altogether these data are consistent with previous reports from our and other laboratories showing that in rats chronically stressed by the social isolation, the reduction of AP basal levels is associated with an increased state of conflict behavior, a reduction of maternal behavior and a greater sensitivity to the steroidogenic effect induced by stress (Pisu et al., 2017; Ramos-Ortolaza et al., 2017; Serra et al., 2000). These data are in line with an exacerbate HPA axis response associated to a significant increase of CTS levels in animals subjected to both stress.

In maternally separated adult rats, some studies reported either an increase in basal CTS (Marais et al., 2008) or decrease in ACTH and CTS levels (Odeon et al., 2017; Faure et al., 2006). These discrepancies may depend by differences in the MS paradigm protocol, time points examined, or animal strain.

We showed previously that socially isolated animals are more sensitive to the foot shock stress-induced increase in CTS blood levels (Serra et al, 2000), when compared to their control counterparts; conversely, animals subjected to both MS and social isolation are less responsive to an acute stress (Biggio et al, 2014b) suggesting that MS might activate some protective mechanisms. These data are consistent with the “mismatch hypothesis” in which early aversive experiences may predict some adaptive processes, rendering subject more resilient and able to better tolerate uncomfortable events occurring in adulthood (Schmidt, 2011; Nederhof and Schmidt, 2012; Scharf and Schmidt, 2012). Moreover, in agreement with recent reports (Odeon et al., 2017), our data showed that MS caused a marked decrease in CTS blood levels in adult rats with no different extent from that observed

in prenatal EtOH exposure group. Interestingly, combination of the two stressful conditions failed to cause a further reduction of CTS. In addition, foot-shock stress alone induced a different pattern of increase in CTS blood levels observed in VEH-MS and ETOH-MS groups when compared to respective control group (VEH-NMS and ETOH-NMS. Still in agreement to the report by Odeon and colleagues (Odeon et a., 2017), FS elicited a more pronounced increase in CTS blood levels in MS animals respect to that observed in EtOH-MS, when compared with their controls (VEH-MS and EtOH-NMS, respectively). This data is very interesting because suggests that some mechanisms involved in stress responses, when occurred before MS (e.g. dam's exposed to intragastric EtOH treatment), may change the hormonal pattern and the stress-evoked HPA responsiveness in adulthood.

Finally, differently from our preview finding (Biggio et al., 2014b) the basal CTS levels in animals subjected to MS are significantly decreased respect to controls. This discrepancy may be due to the treatment occurred during pregnancy that probably may produce some, even slight, changes in offspring during grow and development. In fact, recent experiments (Popoola et al., 2015), conducted in different rat strain, concluded that the gavage procedure itself had an impact on dams behavior that may be then transposed to offspring.

Altogether, our results are in agreement with previous reports suggesting that adverse events experienced during prenatal period and immediately after birth have long-lasting term consequences in response to cumulative stressful experiences during adult life (Zimmemberg and Weston, 2002) including behavior towards ethanol consumption (Odeon et al., 2017). Accordingly, stress exposure may markedly contribute to promoting ethanol drinking (Brady and Sonne, 1999) and a significant correlation with higher levels of CTS and greater EtOH intake and preference (Butler et al., 2013).

Although MS in rodents has been reported to be a model to study the consequence of early life stress on vulnerability to alcohol use in adulthood (Odeon et al., 2017; McEwen 2006, Moffet et al., 2007, Cruz et al., 2008), there are many conflicting reports concerning

the tendency toward alcohol consumption in animal exposed to MS. Some authors report an increase in alcohol consumption after MS exposure (Huot et al., 2001), while others showed a lack of effect (Roman et al., 2004). The reasons for the presence of such conflicting data might be due to the different separation protocols, as well as to the different rodent strains used (Nylander and Roman, 2013). Sprague Dawley rats that do not usually drink easily compared to other strain of rats (Martinetti et al., 2006), may represent a good model to study the effect of stress on vulnerability in alcohol consumption.

The main finding of this work is related to the marked EtOH preference for concentrations, as low as 0.04%, observed in animals exposed to MS. This aspect seems correlated with the CTS hormonal pattern as well as the higher responsiveness to acute stress show by VEH-MS compared to ETOH-MS. Our data are consistent with the notion that stress occurred during pregnancy or early in life may impact the HPA axis response. In particular, we suggest that changes in AP blood levels observed in adults exposed to both uncomfortable events may affect mainly the emotional state both in basal condition and after acute stress exposition. On the other hand, impairments in CTS concentrations seem not related to such stress summation and may mainly depend on MS effects. In addition, according to Odeon et al. (2017), changes in CTS plasma levels observed in animals exposed to MS, prenatal EtOH, or combination of both, seem strongly correlated to EtOH preference, rather than anxiety state. As observed in our results testing adult Sprague Dowley, Wistar rats exposed to MS during the first stage of life, show a low basal levels of CTS when compared to controls as well as an exacerbate response in increased levels of CTS after an acute stress. All these data are accompanied by an increase of EtOH intake.

Finally, since adolescents result more sensible to ethanol action (see Novier et al., 2015 for review), we also evaluated whether prenatal EtOH exposure as well as MS or their combination may affect the EtOH consumption in a different extent to what observed in adults. Adolescent rats showed a similar trend of consumption to moderate EtOH

concentrations but failed to exhibit any preference for EtOH except for the VEH-NMS. This limited EtOH consumption observed in adolescent animals, prenatally exposed to EtOH and subjected to early MS, may be due to an aversive reaction to the substance, developed during the ethanol intragastric administration in dams in the last stages of pregnancy. These data, in agreement with other authors, suggest that early experience with dams exposed to EtOH may predict the appetitive conditioned responses toward alcohol in young rats (Pepino et al., 2001; Molina et al., 2000). Further studies will be crucial to understand the neurobiological mechanisms behind these age-specific differences.

Conclusions

Studying the mechanisms underlying the alterations in the adult brain caused by prenatal EtOH exposure and early maternal separation, may help to find new pharmacological approaches for the treatment of psychopathologies caused by enhance ethanol intake during pregnancy and/or a stress experience occurred in perinatal period.

Our neurochemical and behavioral findings suggest that in rats two different discomfort events occuring during the perinatal period, are able to produce, although with different extent, long lasting changes in the emotional state. These results are consistent with an altered neuroendocrine response (changes in AP and CTS levels), vulnerability to acute stressful events as well as a higher need toward EtOH consumption observed in adulthood.

In particular MS correlates with a decrease of CTS basal levels, a higher response to acute foot-shock stress-induced increase in CTS and a remarkable preference for low doses of EtOH without affecting the anxiety-like state of rats. On the other hand, prenatal EtOH, like MS, exposure induced a decrease in CTS basal levels affecting the response of an acute stress but, in contrast to MS, failed to change the preference for EtOH consumption as well as anxiety levels.

Finally, the combination of both unfavorable conditions strongly increase the anxiety-like behavior revealed by the elevated plus maze and by the gain in AP levels both at basal condition and after foot-shock stress, but did not alter the sensitivity to EtOH preference.

Understanding perinatal stress-related brain alterations results usually complex depending on animal age, sex, stress protocol used as well as how and when they may occur during life.

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Figure legends

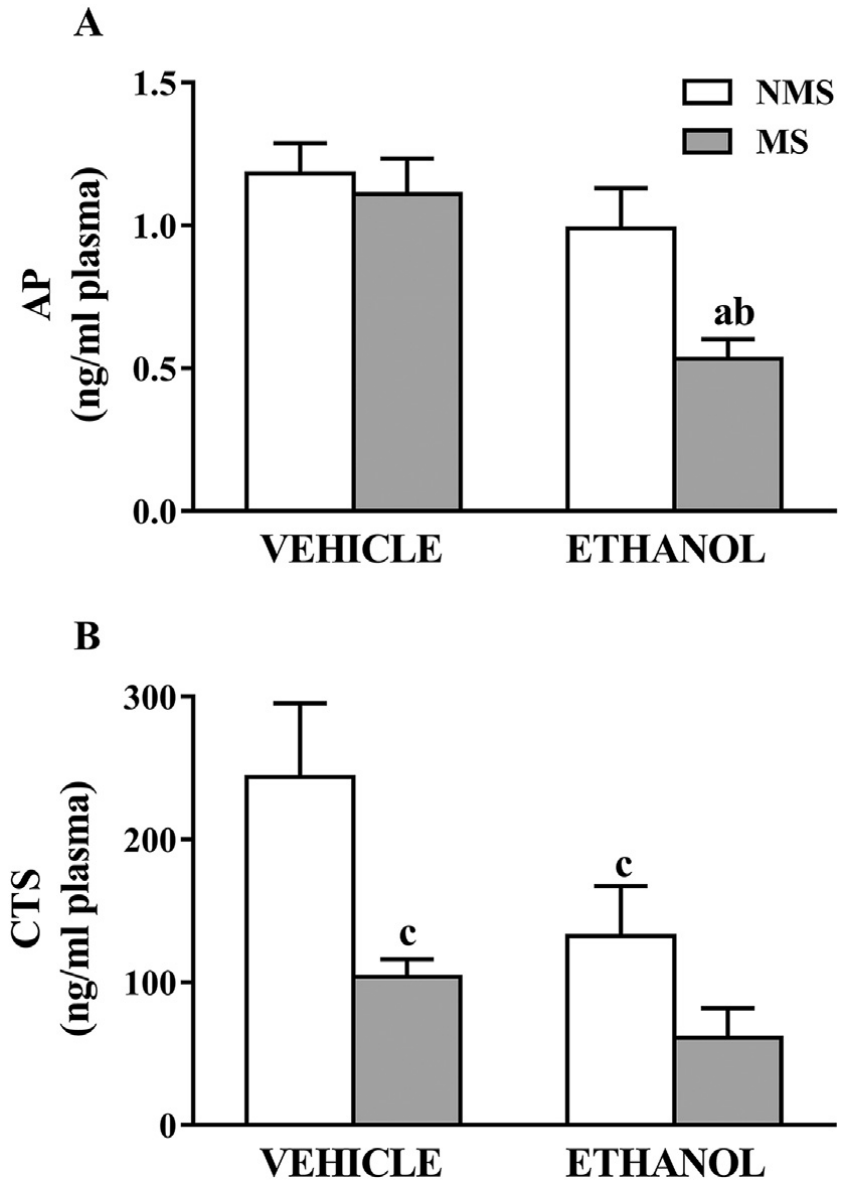

Figure 1. Two month-old rats were divided into 4 experimental groups: non prenatally ethanol exposed not subjected to MS (VEH-NMS), non prenatally ethanol exposed subjected to MS (VEH-MS), prenatally ethanol exposed not subjected to MS (EtOH-NMS) and prenatally ethanol exposed subjected to MS (EtOH-MS). (A) Effects of prenatal EtOH exposure and MS on plasma allopregnanolone (AP) levels in adult male rats. Data are expressed as means ± SEM of values from 6-9 rats of each experimental group, and were analyzed with two way ANOVA followed by Newman–Keuls post-hoc test. *[a]p<0.05 vs VEH-MS, [b]p<0.05 vs ETOH-NMS.* (B) Effects of prenatal EtOH exposure and maternal separation

on plasma corticosterone (CTS) levels in adult male rats. Data are expressed as means ± SEM of values from six to nine rats of each experimental group, and were analyzed with two way ANOVA followed by Newman–Keuls post-hoc test. *[c]p<0.01 vs VEH-NMS.*

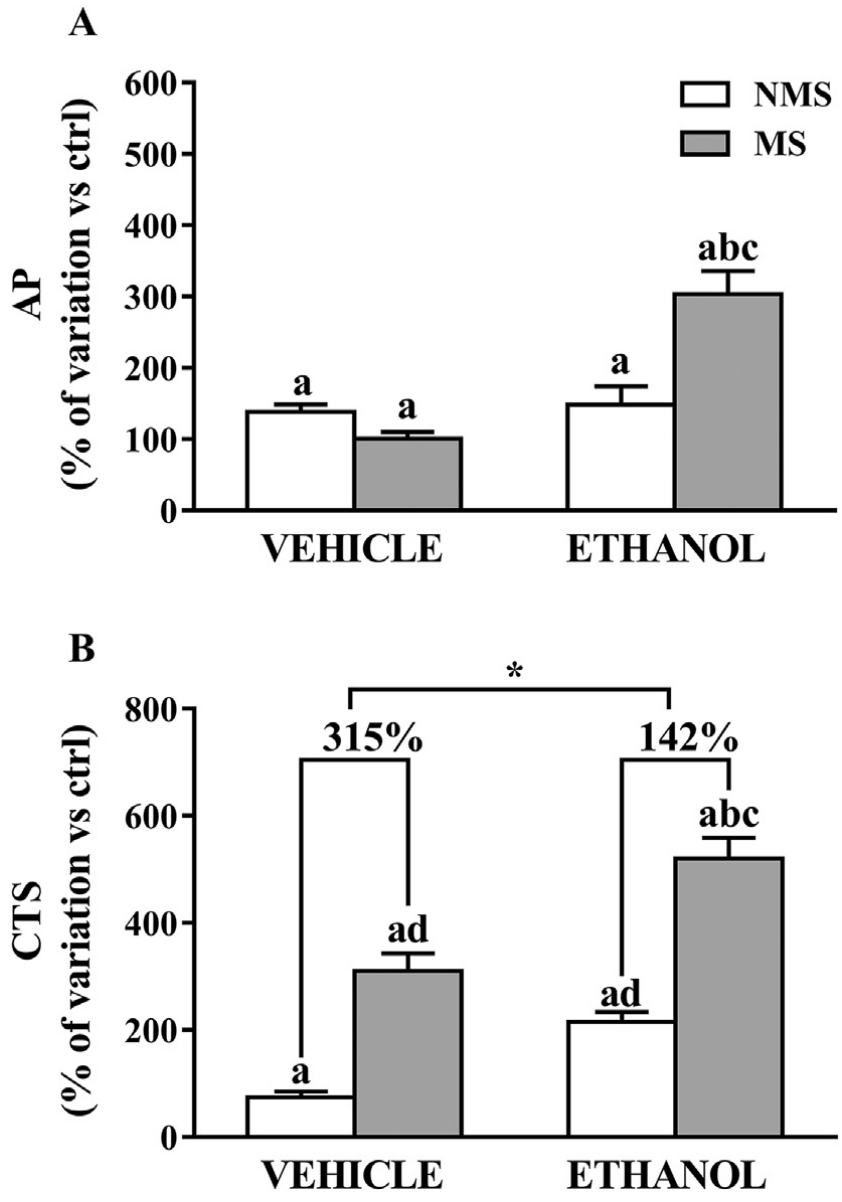

Figure 2. Two-months-old rats were divided into 4 experimental groups: non prenatally ethanol exposed not subjected to MS subjected to foot-shock (VEH-NMS-FS), non prenatally ethanol exposed and subjected to MS and subjected to foot-shock (VEH-MS-FS), prenatally ethanol exposed and not subjected to MS but subjected to foot-shock (EtOH-NMS-FS) and prenatally ethanol exposed, and subjected to MS and subjected to foot-shock

(EtOH-MS FS). (A) Combined effects of prenatal ethanol treatment and MS on allopregnanolone (AP) plasma levels of adult male rats and subjected to foot-shock stress. Data are expressed as a percentage of increase in allopregnanolone content compared to control (not shocked) animals and are expressed as means ± SEM of values from 8-10 rats of each experimental group, and were analyzed with two way ANOVA followed by Newman–Keuls post-hoc test. [a]$p<0.001$ vs. respective control NFS, [b]$p<0.001$ vs. VEH-MS-FS, [c]$p<0.001$ vs. EtOH-NMS-FS. Value express in ng/ml: 1,18±0,10 (VEH-NMS-NFS), 2,81±0,12 (VEH-NMS-FS), 1,10±0,12 (VEH-MS-NFS), 2,22±0,10 (VEH-MS-FS), 0,98±0,14 (EtOH-NMS-NFS), 2,45±0,25 (EtOH-NMS-FS), 0,53±0,17 (EtOH-MS-NFS), 2,14±0,49 (EtOH-MS-FS). (B) Combined effect of prenatal ethanol treatment and MS on corticosterone (CTS) plasma levels of adult male rats subjected to foot-shock stress. Data are expressed as percentage of increase of corticosterone levels above control (not shocked) animals and are means ± SEM of values from 8-10 rats. Data were analyzed with two way ANOVA followed by Newman–Keuls post-hoc test. [a]$p<0.001$ vs. respective control NFS, [b]$p<0.001$ vs. VEH-MS-FS, [c]$p<0.001$ EtOH-MS-FS, [d]$p<0.001$ vs. VEH-NMS-FS. *$p<0.05$, t–Test. Value expressed in ng/ml: 243,81±51,67 (VEH-NMS-NFS), 425,85±25,87 (VEH-NMS-FS), 103,92±12,20 (VEH-MS-NFS), 426,20±34,23 (VEH-MS-FS), 132,37±34,99 (EtOH-NMS-NFS), 416,64±24,61 (EtOH-NMS-FS), 61,03±20,85 (EtOH-MS-NFS), 378,56±23,77 (EtOH-MS-FS).

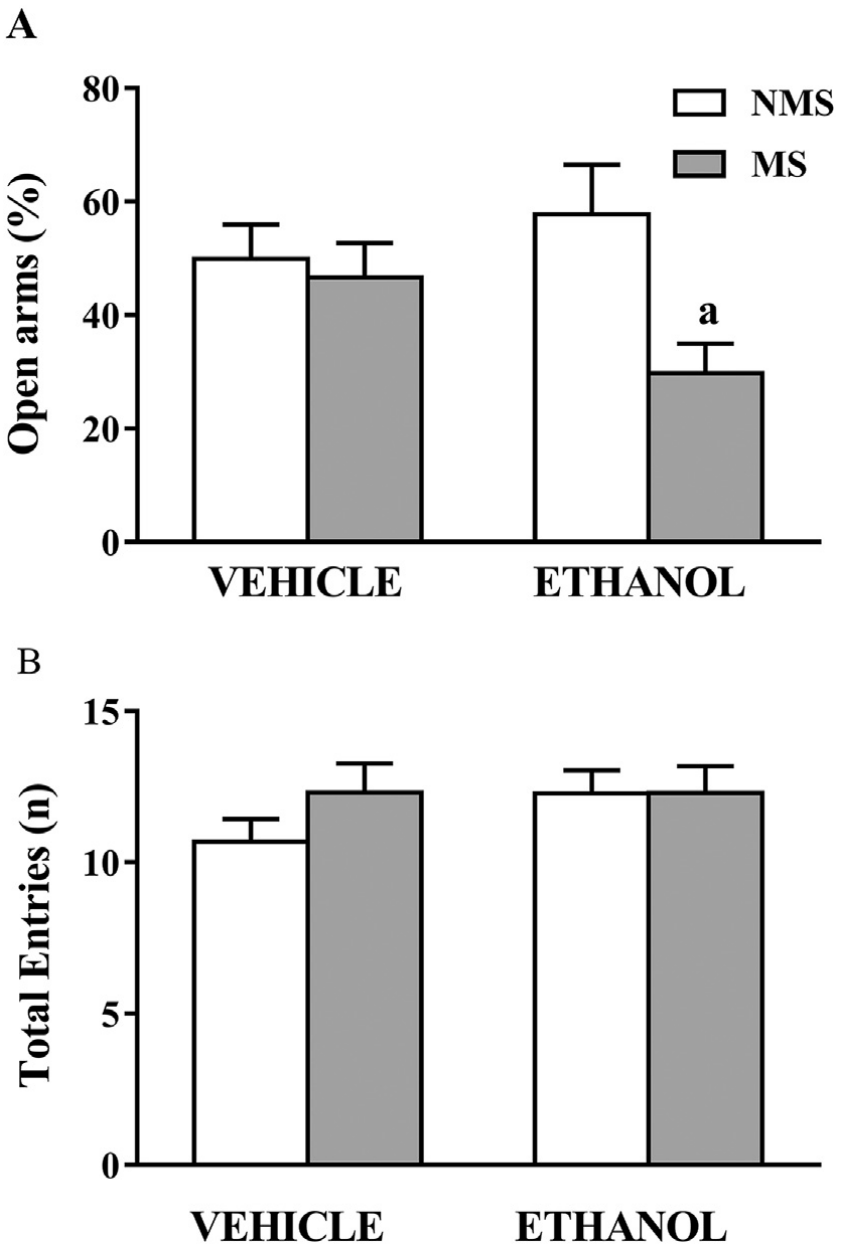

Figure 3. Combined effects of prenatal ethanol treatment and MS on emotional status evaluated with the elevated plus maze using adult male rats. Two-months-old rats were divided into 4 experimental groups: non prenatally ethanol exposed and not subjected to MS (VEH-NMS), non prenatally ethanol exposed and subjected to MS (VEH-MS), prenatally ethanol exposed not subjected to MS (EtOH-NMS), and prenatally ethanol exposed and subjected to MS (EtOH-MS). (A) Bar graph expressing the % of time spent in open arms.

Data are expressed as the mean ± SEM of values from 14-20 rats for each experimental group, analyzed with two-way ANOVA followed by Newman–Keuls post-hoc test. [a]p<0.05 vs EtOH-NMS. (Two-way ANOVA followed by Newman–Keuls post-hoc test). (B) Bar graph expressing the % of number of total entries in closed as well as open arms.

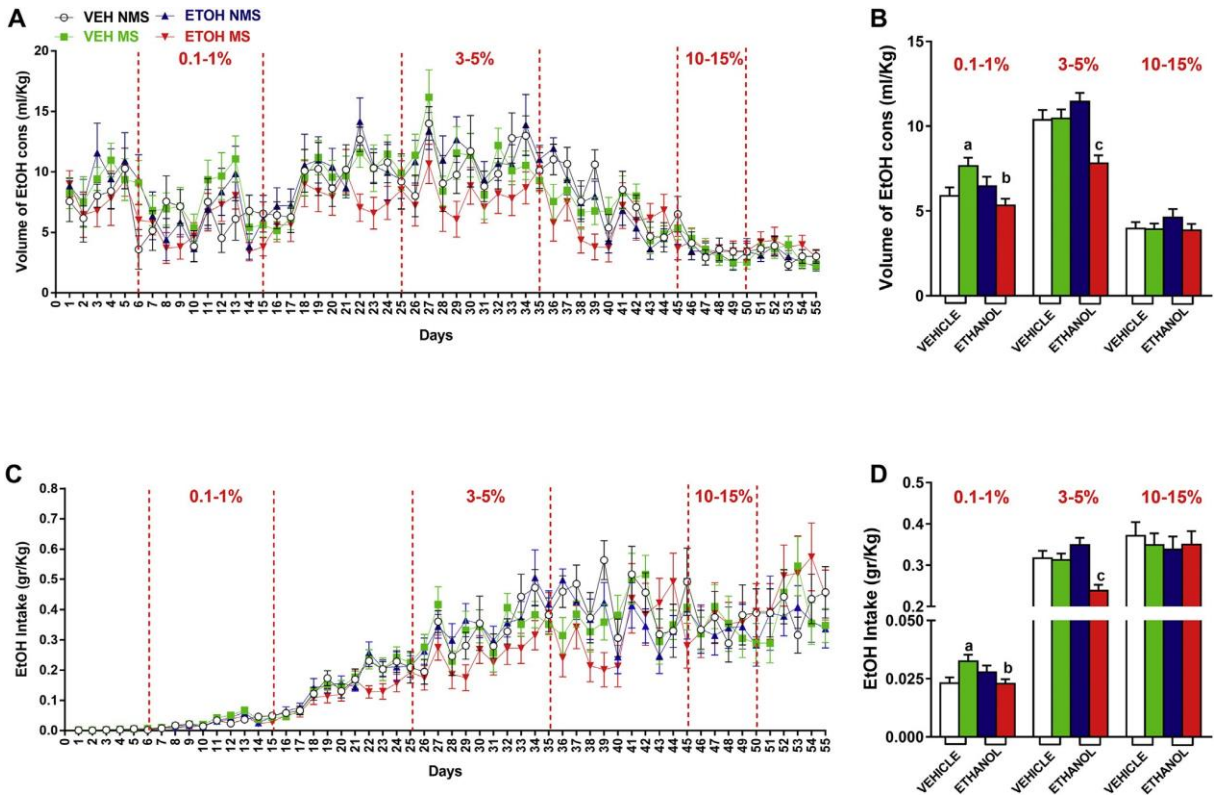

Figure 4. Effect of prenatal ethanol treatment and MS on ethanol consumption (ml/Kg) (A) and (g/Kg) (C) during the ascending voluntary consumption treatment paradigm in adult rats that began the protocol at PND90. (B, D) Three different ranges of EtOH are reported (0.1-1%, 3-5%, 10-15%) and the mean volume of ethanol solution consumed (ml/Kg) (B) and ethanol intake (gr/Kg) (D) were calculated. Data are expressed as means ± SEM of values from 10 rats for each experimental group, and were analyzed with two way ANOVA followed by Newman–Keuls post-hoc test. [a]p<0.05 vs VEH-NMS [b]p<0.05 vs VEH-MS and [c]p<0.05 vs VEH-MS and EtOH-NMS.

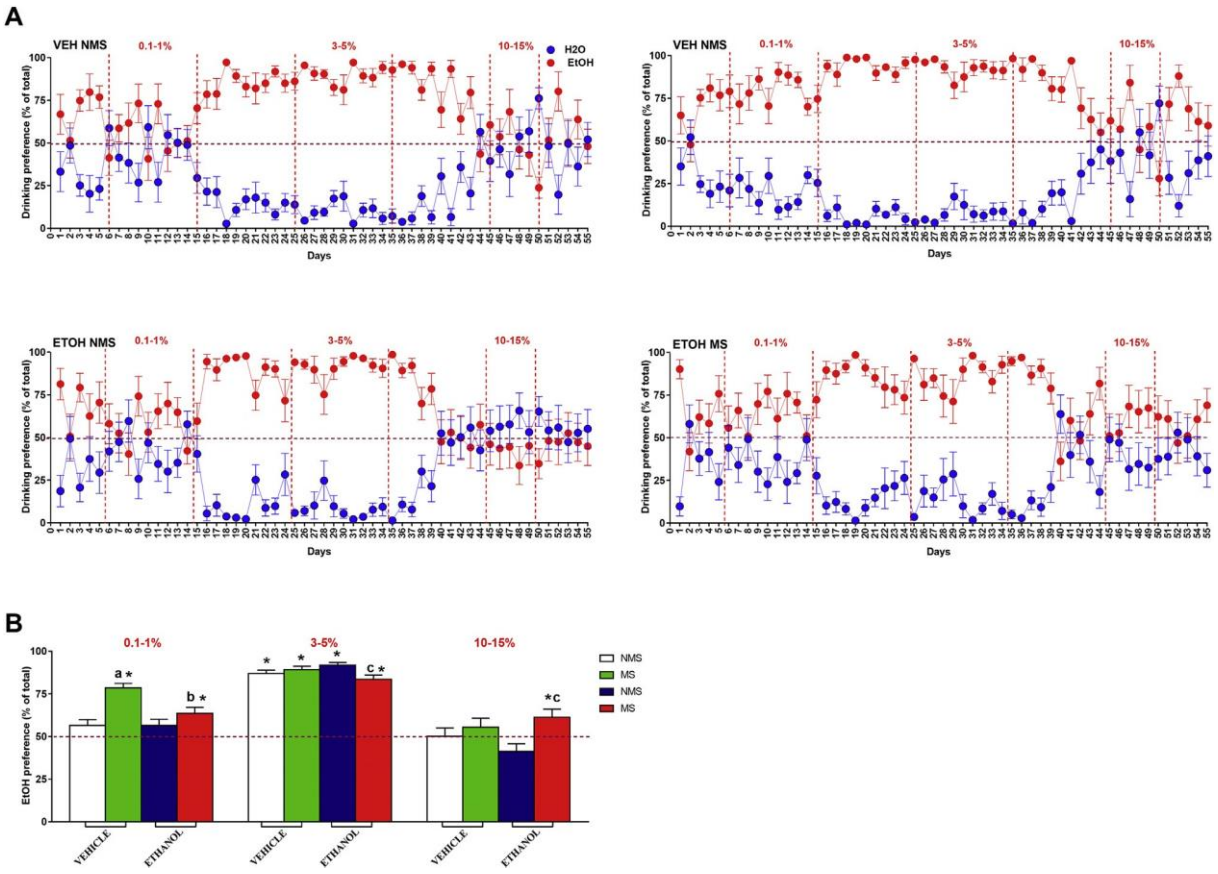

Figure 5. Effect of prenatal ethanol treatment and MS on preference ratio (ethanol/total fluid consumed during the 1-hour access period) calculated during the treatment (A) and averaged at different EtOH dose range (B) as reported in Fig 4B, in the ascending voluntary consumption paradigm in adult rats that began the protocol at PND90. (B) Bar graph representing the average of three different ethanol concentration ranges (0.1-1%, 3-5%, 10-15%). Data are expressed as means ± SEM of values from 10 rats for each experimental group, and were analyzed with two way ANOVA followed by Newman–Keuls post-hoc test. $^{a}p<0.0001$ vs VEH-NMS $^{b}p<0.05$ vs VEH-MS and $^{c}p<0.05$ vs EtOH-NMS. t-Test was used to calculate the ethanol preference versus 50% of total fluid, *p<0.01.

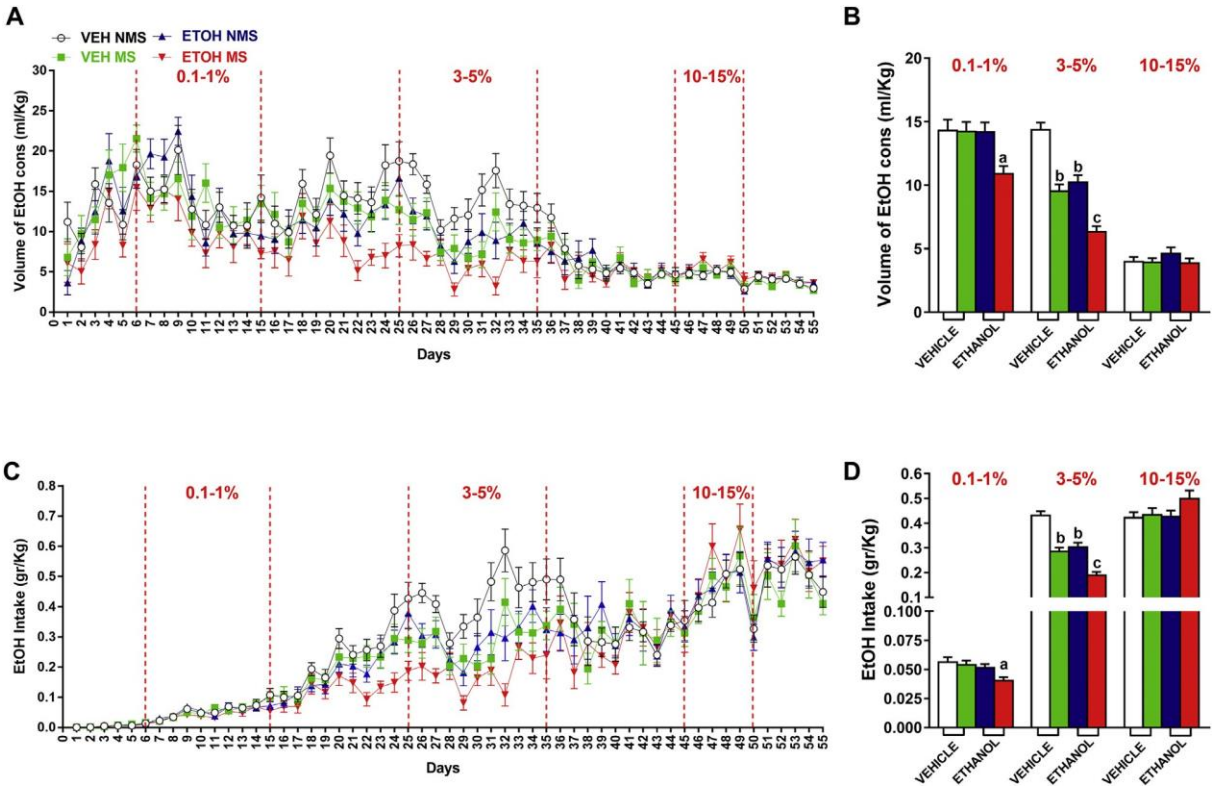

Figure 6. Effect of prenatal ethanol treatment and MS on ethanol consumption (ml/Kg) (A) and (g/Kg) (C) during the treatment of the ascending voluntary consumption paradigm in adult rats that began the protocol at PND30. (B, D) Three different ranges of EtOH are reported (0.1-1%, 3-5%, 10-15%) and the mean of volume of ethanol consumed (ml/Kg) (B) and ethanol intake (gr/Kg) (D) were calculated. Data are expressed as means ± SEM of values from 10 rats for each experimental group, and were analyzed with two way ANOVA followed by Newman–Keuls post-hoc test. $^{a}p<0.05$ vs VEH-MS and EtOH-NMS, $^{b}p<0.001$ vs VEH-NMS, $^{c}p<0.001$ vs VEH-MS and EtOH-NMS.

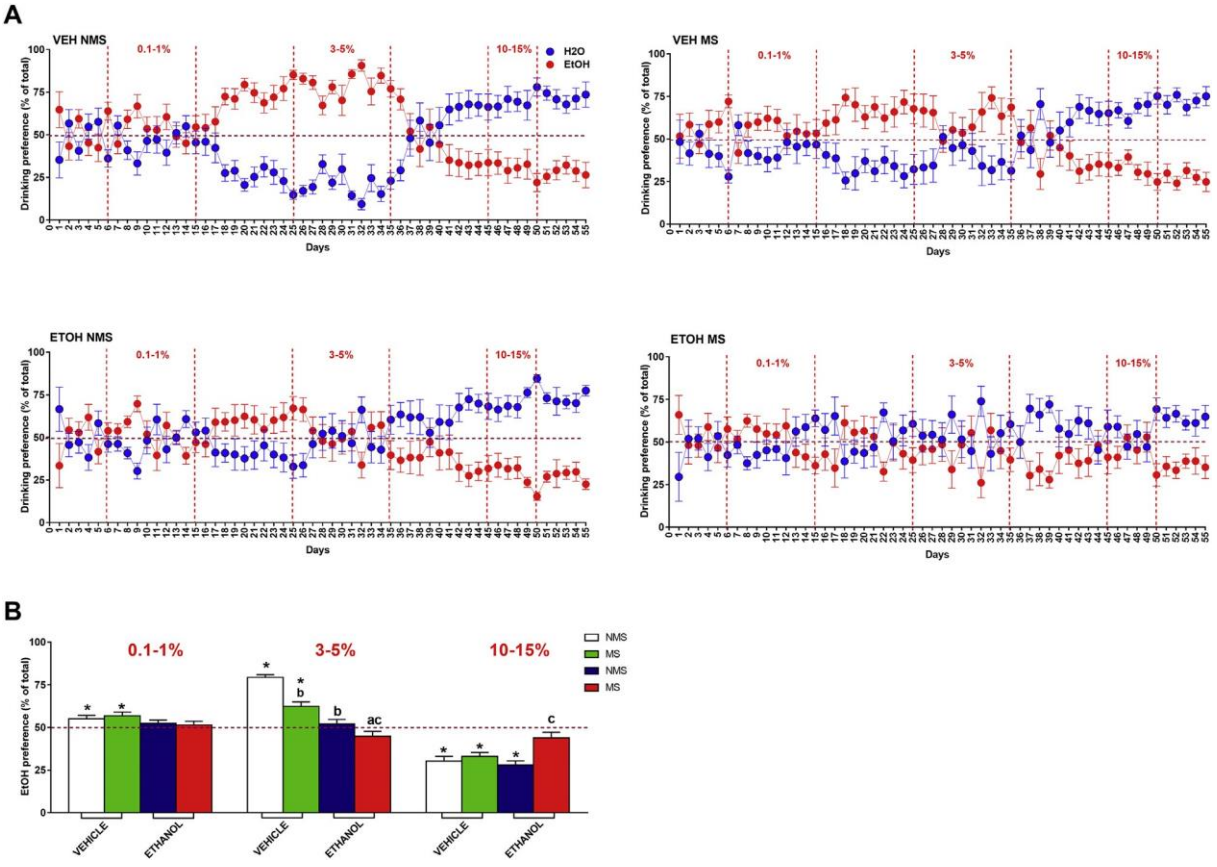

Figure 7. Effect of prenatal ethanol treatment and MS on preference ratio (ethanol/total fluid consumed during the 1-hour access period) calculated during the treatment (A) and averaged at different EtOH dose range (B) as reported in Fig 4B, in the ascending voluntary consumption paradigm in adult rats that began the protocol at PND30. (B) Bar graph representing the average of three different ethanol concentration ranges (0.1-1%, 3-5%, 10-15%). Data are expressed as means ± SEM of values from 10 rats for each experimental group, and were analyzed with two way ANOVA followed by Newman–Keuls post-hoc test. $^{a}p<0.05$ vs VEH-MS and EtOH-NMS, $^{b}p<0.001$ vs VEH-NMS, $^{c}p<0.001$ vs VEH-MS and EtOH-NMS. t-test was used to calculate the ethanol preference versus 50% of total fluid, *p<0.01.

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